Reversible Inhibition of Ethylene Action and Interruption of Petal Senescence in Carnation Flowers by Norbornadiene

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ABSTRACT

The inhibitory effects of the cyclic olefin 2,5-norbornadiene (NBD) on ethylene action were tested in carnation (Dianthus caryophyllus L. cv White Sim) flowers. Treatment of flowers at anthesis with ethylene in the presence of 500 microliters per liter NBD increased the concentration of ethylene required to elicit a response (petal senescence), indicating that NBD behaves as a competitive inhibitor of ethylene action. Transfer of flowers producing autocatalytic ethylene and exhibiting evidence of senescence (petal in-rolling) to an atmosphere of NBD resulted in a rapid reduction in ethylene production, petal 1-aminocyclopropane-1-carboxylic acid synthase activity, 1-aminocyclopropane-1-carboxylic acid content, and ethylene forming enzyme activity. Removal of NBD resulted in recovery of ethylene biosynthesis. These results support the autocatalytic regulation of ethylene production during the climacteric stage of petal senescence and suggest that continued perception of ethylene is required for maintenance of ethylene biosynthesis. The inhibition of ethylene action by NBD after the flowers had reached the climacteric peak was associated with interruption of petal senescence as evidenced by reversal of senescence symptoms. This result is in contrast to the widely held belief that the rate of petal senescence is fixed and irreversible once petals enter into the ethylene climacteric.

Senescence of carnation petals is associated with a climacteric-like increase in ethylene production (5, 8, 19). Precociously opening flowers produce a low, constant rate of ethylene until a critical stage is reached at which time there is a coordinate increase in the activities of ACC synthase and EFE (10, 19). ACC synthase and EFE convert SAM to ACC and oxidize ACC to ethylene, respectively. The ethylene climacteric in carnation petals appears to be under autocatalytic regulation since exposure to ethylene induces ethylene biosynthesis (8). The transition to autocatalytic ethylene production during the aging of petals occurs as a result of a change in tissue responsiveness to ethylene (8, 22). Aging petals increase in their capacity to respond to exogenous ethylene by the induction of autocatalytic ethylene production (2, 22), indicating a gradual release of the ethylene biosynthetic pathway from restriction. It is generally believed that this change in responsiveness is the result of the decline of a hypothetical inhibitor of ethylene biosynthesis or accumulation of another affecter in the petals.

It is clear that increased ethylene production plays a critical role in the regulation of carnation petal senescence. Application of inhibitors of ethylene biosynthesis or action delays petal senescence (1, 5, 14), while exposure of preclimacteric petals to ethylene accelerates senescence (2, 8, 22). Responses of plants to ethylene are believed to involve the interaction of ethylene with a metalloprotein receptor (6). Ethylene binding has been demonstrated in carnation petals (14). This binding fulfills normal ligand-receptor interaction in that it is saturable and reversible (4). Furthermore, the dissociation constant is in the range of physiologically active ethylene concentrations. At present, little is known about the sequence of events linking ethylene binding to senescence in petals. The availability of ethylene action inhibitors should prove useful in the elucidation of this signal transduction pathway.

Sisler and Pian (12) first described the competitive inhibition of ethylene action by the cyclic olefin NBD. More recent studies confirm the ability of NBD to act as an antagonist of ethylene action in many plants (3, 13, 14). It is generally accepted that NBD blocks ethylene action by competing with ethylene for binding sites. Indeed, NBD was found to inhibit ethylene binding in carnation petals (15).

In this paper we characterize the competitive nature of NBD as an inhibitor of ethylene action in carnation petals. Using NBD we show that inhibition of ethylene action subsequent to the induction of autocatalytic ethylene is associated with a rapid decline in the rate of ethylene biosynthesis and interruption of petal senescence.

MATERIALS AND METHODS

Plant Material

Carnation (Dianthus caryophyllus L. cv White Sim) flowers were harvested from plants grown under greenhouse conditions as previously described (20). Flowers were harvested at anthesis when outer petals were reflexed at 90° angles to the axis of the calyx. Stems were cut to 10 cm, placed in distilled water, and held in the laboratory. Experiments were repeated with similar results each time. Results are from individual experiments.

Chemical Treatment

Individual flowers were placed in 2.5 L jars with their stems in water and ethylene and/or NBD injected into the jar yielding the required gaseous concentration. Control flowers were held in jars without added ethylene or NBD. The jars...
were purged with air for 10 min every 12 h, resealed, and treatments reestablished.

Ethylene Measurement

Prior to, or at the end of the incubation period, flowers were enclosed in 1 L jars for 0.5 h after which a gas sample was removed and analyzed for ethylene by gas chromatography using an activated alumina column and a flame ionization detector (23).

Extraction and Analysis of ACC

Petal tissue (2 g fresh weight) was homogenized in 10 mL of 80% ethanol using a polytron homogenizer. The homogenate was centrifuged at 10,000 g for 10 min, the supernatant saved, and the resulting pellet reextracted with 10 mL of 80% ethanol. Following centrifugation, the supernatants were combined and evaporated to dryness. The resulting residue was resuspended in 2 mL of water. ACC was assayed essentially as described by Lizada and Yang (9). Briefly, 8 μmol of HgCl2 was added to 0.5 mL of the extract in a 25 mL vial which was sealed with a serum cap and held at 0°C. Approximately 0.2 mL of a cold mixture of 5.5% NaOCl and saturated NaOH (2:1, v/v) was injected into the vial through the serum cap. The mixture was vortexed and incubated at 0°C for 2 min. Following a second vortexing, a 1 mL gas sample was removed for ethylene determination by gas chromatography.

Extraction and Assay of ACC Synthase

Carnation petals (5 gm) were powdered under liquid N2 and extracted with 20 mL of 100 mm EPPS buffer (pH 8.0) containing 8 mM DTT and 10 μM pyridoxal phosphate. The homogenate was filtered through four layers of cheesecloth and centrifuged at 10,000 g for 10 min. MgCl2 was added to the supernatant (2 mg/mL) which was then fractionated with (NH4)2SO4. The pellet obtained from the 30 to 90% (NH4)2SO4 saturation fraction was resuspended in 5 mL of 10 mm EPPS (pH 8.0) buffer containing 0.2 mM DTT and 2.0 μM pyridoxal phosphate. The extract was dialyzed overnight in 2 L of the same buffer with one change of buffer. All steps were carried out at 0 to 4°C. ACC synthase activity was assayed by incubating 0.4 mL extract with 0.1 mL of 65 μM SAM at 30°C for 1 h. The ACC produced was determined as described above.

EFE Assay

Petal tissue (0.3 g fresh weight) was placed in a 50 mL side-arm flask containing 1 mM ACC. A vacuum (30 mm Hg) was applied to the flask for 1 to 2 min and released. Infiltrated tissue was placed on moistened filter paper for 30 min then transferred to a 25 mL vial and sealed with a serum cap for 0.5 h, after which a gas sample was removed for ethylene determination. In preliminary experiments, this concentration of ACC was determined to saturate the ethylene producing system.

RESULTS

Effect of NBD on Ethylene Induced Petal Senescence

Exposure of carnation flowers to ethylene resulted in decreased petal longevity as indicated by irreversible petal rolling and wilting. The dose-response curves obtained in the presence or absence of 500 μL/L NBD are shown in Figure 1. In the absence of NBD, the ethylene response (petal senescence) was saturated at 10 μL/L. In contrast, treatment of flowers with ethylene in the presence of NBD shifted the dose-response curve such that a higher concentration of ethylene was required to elicit the response.

Inhibition of Autocatalytic Ethylene by NBD

The senescence of carnation petals is associated with a climacteric-like increase in ethylene production (8). We determined the effect of NBD on the ethylene biosynthesis in senescing carnation petals following the commencement of the ethylene climacteric. Flowers exhibiting evidence of petal senescence (in-rolling) and producing autocatalytic ethylene were transferred to atmospheres containing 0 to 2,000 μL/L NBD. Following 12 h of incubation, flowers held in air were producing 47 nL/g fresh weight/h ethylene (Fig. 2). In contrast, flowers incubated in NBD were inhibited in their autocatalytic ethylene production. The reduction in ethylene production rate was dependent on NBD concentration. Treatment of flowers with 250 μL/L NBD reduced the rate of ethylene production by 53% in comparison to untreated flowers. Maximal inhibition of autocatalytic ethylene production was obtained with NBD concentrations of 500 μL/L or greater. At these concentrations, ethylene production was inhibited by greater than 90%.

Both the synthesis of ACC and the oxidation of ACC to ethylene increase during the autocatalytic phase of ethylene production associated with carnation petal senescence (10, 19). Therefore, it was of interest to determine the effects of NBD on these steps in the ethylene biosynthetic pathway. The activity of ACC synthase and the accumulation of free ACC in senescing petals was reduced following 12 h incuba-
tion in NBD (Fig. 2). In addition, the capacity of petals to covert ACC to ethylene (EFE activity) was inhibited by NBD (Fig. 2). As with ethylene production, close to 90% inhibition of both ACC synthase and EFE was obtained at 500 \( \mu \text{L/L} \) NBD.

**Kinetics and Reversibility of NBD Inhibition of Autocatalytic Ethylene Production**

In another experiment we determined the kinetics of NBD inhibition of autocatalytic ethylene production in climacteric carnation flowers. Flowers were transferred to an atmosphere of 500 \( \mu \text{L/L} \) NBD at 0800 h early in their climacteric rise. The rate of ethylene production by flowers incubated in air continued to increase for 9 h, then decreased for approximately 9 h, after which it began to increase again (Fig. 3). We reproducibly observe a diurnal trend in ethylene production in this tissue during the climacteric. In contrast, flowers treated with NBD exhibited reduced rates of ethylene production following 3 h of incubation. Ethylene production by these flowers continued to decrease through 12 h and remained low through the 24 h of incubation in the NBD atmosphere. This inhibition of climacteric ethylene production by NBD was reversible since returning treated flowers to air following 12 h in NBD resulted in increased ethylene production within 6 h.

**Interruption of Petal Senescence by NBD**

It has been shown previously that treatment with the ethylene action inhibitor STS (18) or NBD (14) prior to the onset of the ethylene climacteric delays petal senescence and prevents increased ethylene. The question remains as to what effect inhibition of ethylene action would have on the progression of senescence once autocatalytic ethylene production has commenced and symptoms of the onset of petal senescence are apparent. To test this, flowers exhibiting visual symptoms of the onset of senescence (petal in-rolling and...
slight wilting) were transferred to an atmosphere of 2000 μL/L NBD and their rate of senescence followed for an additional 3 d (Fig. 4). Within 24 h of transfer to the NBD atmosphere, flowers exhibited evidence of recovery from senescence symptoms. These flowers displayed no evidence of petal senescence following 3 d in the NBD atmosphere. In contrast, those flowers enclosed in jars without added NBD continued to senesce and the petals appeared water soaked following 24 h after the initiation of treatments indicating a loss of cellular compartmentalization.

**DISCUSSION**

Exposure of preclimacteric carnation flowers to ethylene induces the developmental program of petal senescence (8). This response has been shown to be dependent on both the concentration and duration of ethylene exposure (2). The presented results indicate that in the presence of NBD, the threshold level of ethylene required to elicit a response (petal senescence) increased, suggesting a competitive interaction between ethylene and NBD, perhaps at the receptor. Sisler et al. (14) have previously shown that NBD is capable of inhibiting ethylene action in carnations. They reported that flowers held in an atmosphere of 500 μL/L NBD exhibited a delayed ethylene climacteric and petal senescence. Furthermore, NBD was found to compete with ethylene for binding sites in carnation petals (15).

The onset of petal senescence in carnation is associated with a climacteric increase in ethylene production (8). The increase in ethylene is a result of the concomitant increase in the activities of both ACC synthase and EFE, which convert SAM to ACC and oxidize ACC to ethylene, respectively (10, 19). Exposure of preclimacteric petals to ethylene results in increased ethylene production (8). Thus, ethylene production by this tissue appears to be under autocatalytic regulation. Furthermore, the ethylene climacteric during petal senescence appears to be autocatalytic in nature since pretreatment of flowers with an ethylene action inhibitor (STS) prevents the increased production of ethylene and accumulation of ACC (18). In this report we have shown that treatment of flowers with NBD following the natural rise in ethylene results in a rapid reduction in ethylene production by the flower (Fig. 3). NBD was effective in reducing ethylene production at a low concentration of 250 μL/L (Fig. 2). Furthermore, the effects of NBD were not likely a result of a general toxicity since removal of the volatile resulted in recovery of ethylene production. This finding is consistent with the autocatalytic regulation of ethylene synthesis. The reduction of autocatalytic ethylene production following treatment with NBD was associated with decreased levels of ACC, a reduction of ACC synthase activity, and decreased capacity to convert ACC to ethylene (Fig. 2). This indicates the positive feedback mechanism of ethylene action functions at both ACC synthase and EFE activities.

![Figure 4](https://www.plantphysiol.org) Interruption of petal senescence by NBD. Flowers in the climacteric stage of senescence (petal-in-rolling) were transferred to air (left) or 2000 μL/L NBD (right) and photographed at various times. (A, B, C, D) Photographed 0, 24, 48, and 72 h, respectively.
In many cases the responses of plants to hormones requires the continuous presence of the hormone for a sustained effect. Indeed, Riou and Yang (11) showed that exposure of citrus leaves to ethylene resulted in autocatalytic ethylene synthesis which rapidly declined when exogenous ethylene was removed. However, it is thought that in climacteric fruits and flowers the autocatalytic effect is irreversible once the climacteric has been initiated (24). Our data suggest that in carnation flowers the continued perception of ethylene is necessary for the maintenance of the high rate of ethylene production.

The senescence of carnation flower petals has been characterized as a phasic phenomenon with the ethylene surge signaling the onset of the final phase (7, 16). In most cases, treatments which delay senescence such as low temperature storage or vase solutions containing carbohydrates appear to act by extending the first phase (7, 8). The implication of these studies is that once the petals have entered into the ethylene climacteric senescence proceeds at a fixed rate. Our results show that this is not the case, and that inhibition of ethylene action after the petals have entered the climacteric stage results in reversal of senescence symptoms and extends the life of the petals. Given the rapid reduction in ethylene biosynthesis and the interruption of senescence by NBD, it is clear that ethylene plays a very active role in regulating the progression of senescence throughout the climacteric period. A recent study suggests that inhibition of ethylene action by silver is capable of interrupting the ripening program of tomatoes once initiated (17). Tomatoes are regarded as a climacteric fruit.

Senescence is a very active developmental process which requires active gene expression and protein synthesis (20, 21). The climacteric stage of carnation petal senescence is associated with the accumulation of new mRNAs and proteins (20). Furthermore, exposure of preclimacteric petals to ethylene results in the accumulation of several of these senescence-related mRNAs (22). One possibility is that ethylene is required for the expression of the senescence program at the molecular level and that the continued presence of ethylene is essential for this expression. Clearly, a great deal of research is needed to characterize the action of ethylene at the molecular level in relation to the physiology of petal senescence.

LITERATURE CITED