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Communication

Effect of 2,5-Norbornadiene upon Ethylene Biosynthesis in Midclimacteric Carnation Flowers

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ABSTRACT

The climacteric increase in ethylene production in carnation (*Dianthus caryophyllus* L. cv White Sim) flowers is known to be accompanied by an increase in 1-aminocyclopropane-1-carboxylate (ACC) synthase and ethylene forming enzyme (EFE) activities. When midclimacteric flowers were exposed to 2,5-norbornadiene, which blocks ethylene action, ethylene production began to decrease after 2 to 3 hours. ACC synthase activity was markedly reduced after 4 hours and the increase in EFE activity was blocked indicating that the autocatalytic signal associated with ethylene action stimulates both enzyme activities.

Carnation flowers exhibit a sharp increase in respiration and ethylene production which is very similar to the climacteric of many fruit (19). This climacteric ethylene production in carnations is closely associated with the senescence of the flowers and wilting (in-rolling) of the petals occurs near the peak of ethylene production (12). Climacteric ethylene production in carnation flowers (10) and in fruits (1, 5) is autocatalytic, and inhibitors which block ethylene action such as Ag^+ and NDE[1] will block this autocatalytic ethylene production (15, 17, 18). This indicates that some signal, as yet unidentified, associated with ethylene action feeds back to increase ethylene biosynthesis. NDE competes with ethylene for binding and counters its action in pea epicotyl elongation (16) and carnation flower senescence (15). NDE vaporizes readily at room temperature and as a gas rapidly penetrates into tissues.

In essentially all cases where inhibitors have been used to block autocatalytic ethylene production, they were applied prior to the increase in ethylene production; before the autocatalytic signal was produced (2, 6, 15, 17, 19). In this study, NDE was applied after autocatalytic ethylene production had begun to examine the importance of the autocatalytic signal in maintaining autocatalytic ethylene production, obtain information about its stability, and to determine which step(s) of the ethylene biosynthesis pathway is controlled by the signal.

[1] Abbreviations: NDE, 2,5-norbornadiene; ACC, 1-aminocyclopropane-1-carboxylic acid; EFE, ethylene forming enzyme which catalyzes the conversion of ACC to ethylene; EPPS, *N*-(2-hydroxyethyl)-piperazine-*N'*-3-propane sulfonic acid; MACC, 1-(malonylamino)cyclopropane-1-carboxylic acid.

A preliminary report of this study has been presented in abstract form (13).

MATERIALS AND METHODS

Plant Material

Cut carnation flowers (*Dianthus caryophyllus* L. cv White Sim) were purchased from a wholesale florist the day of harvest at which stage the petals were extended about 2 cm beyond the sepals. Stems were placed in deionized water containing 100 μg/mL Physan 20 and kept in the laboratory at room temperature (22–25°C).

Flowers for all experiments were selected 5 to 6 d after harvest when ethylene production had started to increase and was approximately one-half that of the maximum rate reached at the climacteric peak, at which stage they were considered midclimacteric. The maximum rate of ethylene production varied seasonally and ranged from about 70 nL/g·h to 140 nL/g·h.

NDE Treatments

The effect of NDE on ethylene production in flower petals over time was determined. Petals from a single flower were divided into two equal-weight groups to provide a control (air) and a NDE-treated sample. Petals were placed in 50 mL syringes and for the first 2 h both samples were connected to an air flow system (flow rate 11 mL/min) with flow provided by a peristaltic pump. Ethylene production was measured during this time to establish a baseline production rate before the NDE treatment began. After 2 h one sample was connected to an air-NDE flow with NDE concentration of 3,000 μL/L. NDE was provided from a gas bag. At 20 min intervals throughout the experiment gas samples for ethylene determination were taken from the gas effluent.

To determine the effect of NDE upon ACC synthase activity, ethylene production was followed on the flowers until the production rate was about one-half that reached at the climacteric peak. Ethylene production was determined each morning by placing each flower in a 500 mL jar, sealed for 30 min at room temperature (22–25°C), at the end of which time a 1 mL gas sample was taken for ethylene measurement. These midclimacteric flowers were separated into three groups with each group having approximately equal ethylene production rates. One group was immediately homogenized for the determination of ACC synthase activity and ACC level.

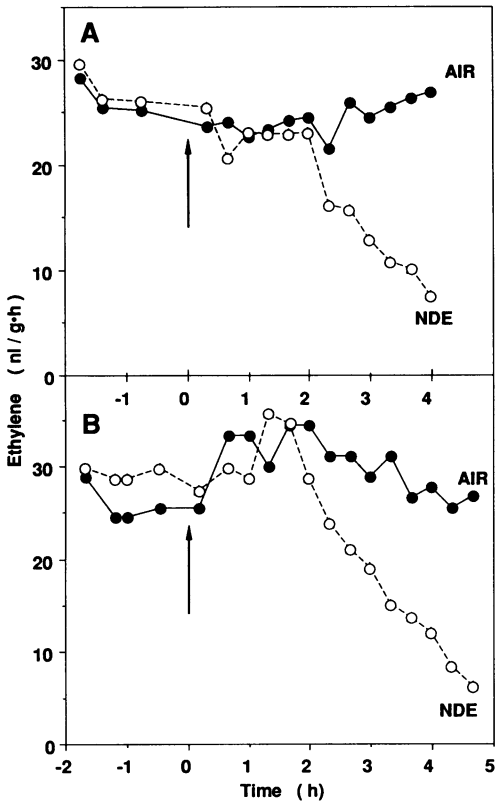

Figure 1. Ethylene production rate from midclimacteric carnation petals removed from a flower reaching the climacteric on the 6th d after harvest (A) and on the 5th d after harvest (B). Petals were held in a gas flow (11 mL/min) of air (●——●) or air plus 3000 μL/L NDE (O---O). Petals from a single flower were divided equally by weight for the air and the NDE samples. The arrow indicates the time when NDE exposure began.

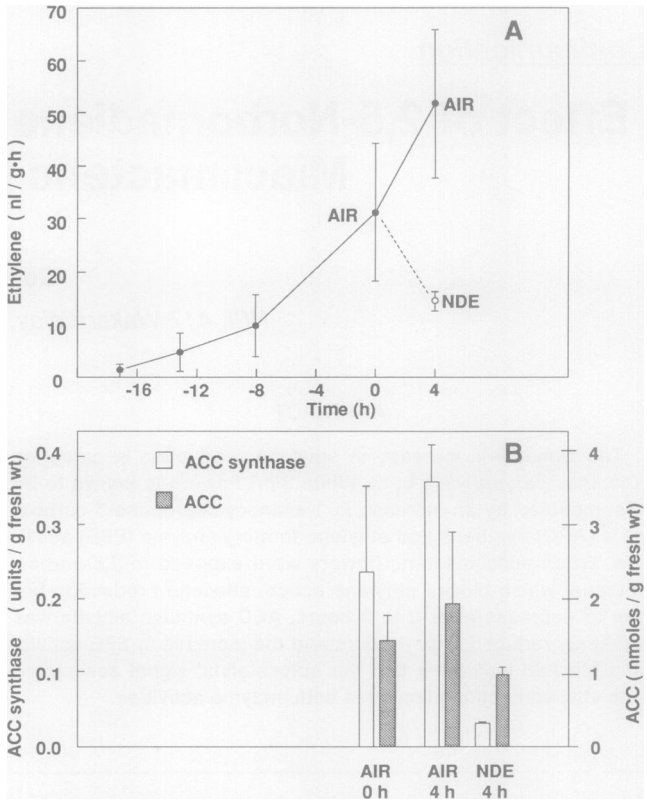

Figure 2. Effect of NDE upon ethylene production (A) and ACC synthase activity and ACC levels (B) in midclimacteric carnation flowers. The values for ethylene production (A) and for ACC synthase activity and ACC level (B) were measured on the same flowers. The NDE treatment began at 0 h. Each value for ethylene production from −17 to −8 h is the average of nine flowers; following this period, each value is the average from three flowers. Error bars indicate standard deviation.

The second group, kept in air, was placed in 1.1 L jars, one flower/jar, and sealed. The third group, the NDE treatment, was likewise placed in 1.1 L jars, NDE added to give 3,000 μL/L, and sealed. At 2 h all jars were flushed with air to prevent ethylene from accumulating too high (not above 0.4 μL/L), the NDE replenished in the NDE-treated flowers, and the jars resealed. At 4 h ethylene was measured, then the flowers from the air and NDE treatments were homogenized for the determination of ACC synthase activity and ACC level.

To determine the effect of NDE upon EFE activity in midclimacteric flowers, EFE activity was determined by measuring ethylene production from flowers given saturating levels of ACC. The saturating level of ACC was determined by measuring ACC to ethylene conversion in midclimacteric flowers given 1, 3, 6, 10, and 30 mM ACC. No increase in conversion was observed beyond 10 mM ACC. To ensure that the internal pool was saturated, 30 mM ACC was used. With saturating levels of ACC the ethylene production of the flowers is dependent only upon EFE activity. Stems from midclimacteric flowers were cut to 1.5 cm and placed in buffer (50 mM Mes, pH 6.0) containing 30 mM ACC and the flowers left in open air in the laboratory for 30 min to encourage transpiration and uptake of the ACC. Then flowers were placed singly in 250 mL Mason jars and one group (three flowers) treated with air and another group (three flowers) treated with air plus NDE (5,000 μL/L) in a flow system with flow rate of 20 mL/min. NDE was administered by pumping 50,000 μL/L NDE from a gas bag into the air flow at a rate one-tenth that of the air flow rate. Ethylene in the gas effluent was measured every hour.

Experiments to determine ACC synthase activity, ACC levels, and EFE activity were conducted three times with similar results obtained as those presented.

Ethylene Measurement

Ethylene was measured using a gas chromatograph with Poropak Q/N (20/80%, w/w) column and flame ionization detector.

ACC Synthase Activity

Flowers with stem removed were first rapidly cut into small pieces with a razor blade, then homogenized in buffer (5 mL/g fresh weight) plus insoluble PVP (5%, w/w, of fresh weight). Buffer contained 150 mM EPPS (pH 8.0), 4 mM DTT, and 5 μM pyridoxal phosphate. After centrifugation a portion of the

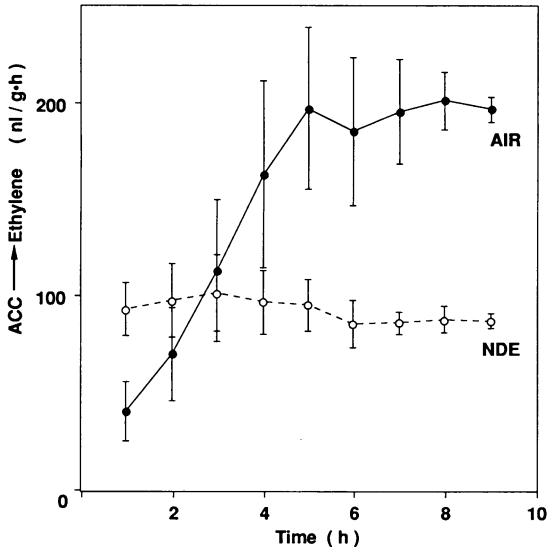

Figure 3. EFE activity from midclimacteric whole flowers held in a flow of air (●——●) and 5000 μL/L NDE (○---○) with a flow rate of 20 mL/min; three flowers in each treatment. EFE activity was measured as ACC to ethylene conversion from flowers held in 30 mM ACC. NDE exposure began at 0 h and flowers were placed in ACC solution 30 min prior to this time. Error bars indicate standard deviation.

supernatant was dialyzed overnight with two changes of buffer containing 10 mM EPPS (pH 8.0), 0.1 mM DTT, and 5 μM pyridoxal phosphate. ACC synthase activity was measured on the dialyzed extract according to Yu *et al.* (21) except 50 mM EPPS (pH 8.0), and 5 μM pyridoxal phosphate were used. The undialyzed extract was used for determination of ACC (9).

RESULTS

Ethylene production of petals freshly removed from midclimacteric flowers began to decrease during exposure to NDE (Fig. 1). This drop in ethylene production started 2 to 3 h after the start of the NDE exposure in petals from seven different flowers examined. NDE-Treated petals from some flowers showed a small increase in ethylene production just preceding the drop in ethylene production (Fig. 1B). This appeared to be related to the age of the flower, *e.g.* time relative to the harvest date that it started the climacteric ethylene production. Petals from flowers starting to produce climacteric ethylene early (5th d after harvest) generally showed this small increase in ethylene production, while those flowers starting climacteric ethylene production later (6th or 7th d after harvest) did not. Midclimacteric whole flowers treated with NDE also exhibited a decrease in their ethylene production 2 to 3 h after the start of the NDE treatment similar to that of the petals (data now shown).

The reduction in ethylene production caused by NDE presumably occurs through a reduction in ACC synthase and/or EFE activity. To examine this, first the effect of NDE upon ACC synthase activity was determined. Midclimacteric whole flowers were used and after 4 h of NDE exposure (3000 μL/L), ethylene production was about 30% of that of the flowers held in air for 4 h (Fig. 2A). However, at this same time ACC synthase activity in the NDE-treated flowers had dropped to about 10% of that from flowers held in air (Fig. 2B). ACC levels were also lower in the NDE-treated flowers compared to the flowers kept in air (Fig. 2B). These results indicate that blocking the autocatalytic signal with NDE leads to a rather sharp drop in ACC synthase activity.

EFE activity was measured in midclimacteric whole flowers to determine if it also declines when the autocatalytic signal is blocked with NDE. For determination of EFE activity, the conversion of ACC to ethylene was measured from flowers given a saturating level of ACC. EFE activity was measured *in vivo* since its activity has not been demonstrated in a cell-free system (19). EFE activity from flowers exposed to NDE did not show any decrease in activity over a 9 h exposure (Fig. 3). EFE activity from flowers held in air increased for 4 to 5 h before reaching a plateau near 200 nL/g h (Fig. 3). EFE activity is known to increase in climacteric carnation flowers (11) and tomato fruit (19) compared with their preclimacteric levels. It is important to note that though EFE activity in the NDE-treated flowers did not decline over the 9 h period, it did not increase as observed in the flowers held in air. This indicates that blocking the autocatalytic signal with NDE did block an increase in EFE activity, presumably by blocking new synthesis of EFE. That actual synthesis of ACC synthase and EFE could potentially be blocked by NDE is supported by a recent study showing that specific messages are induced by ethylene in carnation petals and these induced messages are reduced or blocked by NDE (18).

In the experiment presented in Figure 3, the NDE-treated flowers were a little further along than the air controls as indicated by the 1 h values for ACC to ethylene conversion, though 24 h prior to placing the flowers in ACC solution, both groups had just begun the climacteric with similar ethylene production rates, 4.4 ± 1.0 and 1.5 ± 0.4 for the NDE flowers and the air controls, respectively. In another experiment, the air controls were a little further along than the NDE flowers with similar results obtained as in Figure 3 in that ACC to ethylene conversion increased in the air controls while that of the NDE flowers remained essentially constant.

DISCUSSION

The results indicate that when NDE blocks ethylene binding and action, the drop in ethylene production observed in Figure 1 results primarily, if not solely, from a drop in ACC synthase activity leading to lower ACC levels (Fig. 2). No reduction in EFE activity occurred over the 9 h experimental period (Fig. 3). One step of the ethylene biosynthesis pathway not examined in this study which may affect ethylene production is MACC formation (19). However, present evidence (8) suggests that MACC formation would also decline when autocatalysis is blocked and, therefore, not contribute to the decrease in ethylene production observed in this study.

These results indicate that the autocatalytic signal is necessary for presumably the synthesis of both ACC synthase and EFE since ACC synthase activity decreased and EFE activity did not increase during the NDE exposure (Figs. 2 and 3). This is similar to results in apple which indicated that ethylene must be continuously present to maintain ACC synthase and EFE activities (3, 4). The lack of decline in EFE activity compared with that of ACC synthase probably results from

the difference in half-life for the two enzymes. The half-life for ACC synthase and EFE has been estimated to be 30 min (7, 20) and 6 h (8), respectively. With an estimated half-life of 6 h for EFE, we would have expected to see a drop in activity during the latter part of the 9 h NDE-treatment. That this did not occur suggests that the half-life is longer than previously estimated. A longer maintenance of EFE activity than expected from its half-life was also observed in a study where EFE activity was examined in preclimacteric tomato fruit (8). EFE activity increased when these tomato fruit were treated with ethylene, though when the ethylene was removed, EFE activity remained the same for 24 h and then gradually declined during the next 100 h experimental period. To explain this result these authors suggested that the message encoding EFE may be long lived.

Though ethylene production dropped approximately 2 h after treatment to NDE and continued to decrease for the duration of the experiment (Fig. 1), it has been reported that ethylene production can increase in vegetative tissue after long exposures, 24 h or longer, to NDE (14). The different responses of ethylene production to NDE treatment between the present and this earlier study may result from different durations of NDE treatment, short *versus* long, and/or using different tissues, reproductive *versus* vegetative.

The nature of the autocatalytic signal is unknown. An estimate of the lifetime of this signal is difficult to make since it cannot be directly measured, yet the results of Figure 1 offer some information about its stability. As ethylene production began to decline after 2 h of NDE exposure and had dropped by 50% after 3 h, this indicates that the activity of the autocatalytic signal begins to decline before 2 h and its half-life is less than 3 h.

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