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Activity of Ageing Carnation Flower Parts and the Effects of 1-(Malonylamino)cyclopropane-1-Carboxylic Acid-Induced Ethylene[1]

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ABSTRACT

Peak levels of 1-aminocyclopropane-l-carboxylic acid (ACC) in flower parts of ageing carnations (*Dianthus caryophyllus* L. cv Scanea 3C) were detected 6 to 9 days after flower opening. The ethylene climacteric and the first visible sign of wilting was observed 7 days after opening. The concentration of conjugated ACC in these same tissues peaked at day three with reduction of 70% by day 4. From day 5 to day 9 all parts followed a diurnal pattern of increasing in conjugate levels 1 day and decreasing the next. Concentrations of conjugated ACC were significantly higher than those of ACC in all ageing parts. Preclimacteric petals treated with ACC or 1-(malonylamino)-cycloprane-1-carboxylic acid (MACC), started to senesce 30 to 36 hours after treatment. When petals were treated with MACC plus by 0.1 millimolar aminoethoxyvinylglycine, premature senescence was induced, while ethylene production was suppressed relative to MACC-treated petals. Petals treated with MACC and silver complex produced ethylene, but did not senesce. The MACC-induced ethylene was inhibited by the addition of 1.0 millimolar $CoCl_2$. These results demonstrate MACC-induced senescence in preclimacteric petals. The patterns of ACC and MACC detected in the flower parts support the view that an individual part probably does not export an ethylene precursor to the remainder of the flower inducing senescence.

Petal wilting is a consequence of senescence in carnation flowers. Production of a burst of ethylene is coincident with this first visible sign of senescence (14). The immediate signal responsible for the increased ethylene is not yet known. It has been proposed that a stimulus produced in one part of the flower moves into the petals causing the climacteric rise of ethylene (7, 16), although removal of the ovary or the styles in the presenescent flower does not affect petal senescence (13, 17). It has been observed that detached petals senesce similarly to those still attached to the intact flower (12, 13).

ACC[4] is the immediate precursor to ethylene biosynthesis in higher plants (1). An increase of endogenous ACC levels precedes ethylene production in all parts of the flower (2). When petals detached from flowers producing considerable amounts of ethylene were treated with aminoxyacetic acid, an inhibitor of ACC synthase, a marked decrease in ethylene production and a fivefold decrease in ACC concentration was observed (12). This suggested that petals have the ability to synthesize their own ACC.

MACC is well established as a primary conjugate form of ACC (6) In some systems MACC is the inactive endproduct of ACC not being oxidized to ethylene (5,6), the MACC then serving as poor ethylene precursor (5). Significant amounts of MACC have been detected in different plant tissues (5, 9, 11, 18), but a definitive role of the conjugate in ethylene biosynthesis has not yet been established. Jiao *et al.* (8) demonstrated that exogenous application of MACC to various vegetative tissues could induce some ethylene production. They detected MACC-hydrolase activity in the cell-free extract of watercress stems. It was concluded that several hours of incubation in high concentrations of MACC were necessary to induce the hydrolytic enzyme and maintain its continued activity.

The present study was initiated to study the senescence of ageing carnation flower parts differing in longevitity, *i.e.* petals, styles, and ovary senesce prior to the receptacle and green tissues. It was our intent to determine whether all parts behaved in synch or if one or more flower parts acted singularily to possibly mobilize the ethylene precursor(s) triggering senescence. In addition, we have investigated the role of MACC in the senescence phenomena and whether exogenous MACC could be utilized as an effective ethylene precursor in petals, resulting in ethylene-mediated senescence.

MATERIALS METHODS

Plant Material

Carnation flowers (*Dianthus caryophyllus* L. cv Scanea 3C) were grown under normal greenhouse conditions. An accurate assessment of flower development was achieved by tagging flowers just prior to flower opening. A preliminary study determined days to flower opening after following bud length of closed carnation buds to first fully open flowers. Flowers were then harvested when outer petals first reflexed at right angles to the stem. Stems were cut to 30 cm, held in 200 mg L^{-1} Physan (a microbicide) solution, and kept in a growth chamber at 22°C under continuous fluorescent lighting for 0 to 9 d. On each sampling day individual flowers were retrieved

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[4] Abbreviations: ACC, l-aminocyclopropane-l-carboxylic acid; MACC, l-(malonylamino)-cyclopropane-l-carboxylic acid; AVG, aminoethoxyvinylglycine; STS, silver thiosulfate complex.

from the growth chamber and floral parts excised (styles, ovary, five petals from the outermost whorl, receptacle, and green tissue including the calyx, two leaves, and 12 cm of stem). ACC and conjugated ACC analyses for each of the floral parts on each of the 0 to 9 sampling days were replicated four times, a replicate being a repeat of an entire experiment (replications occurred in series from September through June) For conjugated ACC metabolism studies petals were removed from the outermost whorl of flowers 1 d after flower opening. All excised petal experiments were replicated three times.

Chemicals

All solvents were of analytical grade and purchased commercially. ACC was purchased from Sigma. [2,3-^{14}C]ACC (80 μCi/μM) was from Research Products International (Mount Prospect, IL). MACC and AVG were generous gifts of Drs. S. F. Yang and A. K. Mattoo, respectively.

ACC and Conjugated ACC Extraction and Determination

At each sampling day, floral parts were excised, weighed, frozen, and lyophilized. Dried tissues were ground in a Wiley mill (20 mesh screen) or a mortar and pestle. Tissue samples of 200 mg were extracted in 10 mL of 80% (v/v) ethanol at 4°C for 24 h. Samples were centrifuged at 5000*g* for 15 min and the pellet resuspended and recentrifuged. Ethanolic fractions were combined and evaporated under vacuum and the residue was taken up in 5 mL of distilled water. ACC was determined by a slightly modified version of Lizada and Yang (10). Briefly, a 250 μL aliquot of the aqueous extract was combined with 100 μL of 10 mM $HgCl_2$ and 550 μl distilled water. Each tube was sealed with a rubber septum and 100 μL commercial bleach (5 NaOCl) and saturated NaOH (2:1, v/v) was added with a 1.0 mL syringe. The tubes were vortexed 20 s and kept on ice. After 5 min a 1 mL gas sample was withdrawn and analyzed for ethylene using a Shimadzu GC-8A gas chromatograph and GC integrator. Efficiency of conversion of authentic ACC added to a replicate tube was generally between 80 and 90%. Conjugated ACC was hydrolyzed to ACC units following the method of Hoffman *et al.* (6) and analyzed as indicated above. Authentic MACC was also added to a replicate tube to determine efficiency of the assay and was determined to be approximately 75%.

Identification of ACC and Conjugated ACC Fractions

Radiolabeled [2,3-^{14}C]ACC (0.54 μCi/10 μL) added to crude petal extracts was recovered after separation using a cation exchange column and TLC (10). Endogenous ACC from a replicate petal extract was separated similarily and activity assayed as conversion to ethylene. Endogenous conjugated ACC was separated from petal extracts as described by Hoffman *et al.* (6) and co-chromatographed with authentic MACC. The purity of the authentic MACC was estimated after column separation steps and was determined to contain 0.045% free ACC. This amount of ACC contamination was very low and was not enough to induce premature senescence in excised carnation petals. Exogenous application of 0.1 mM ACC did not induce premature wilting in preclimacteric carnation petals.

Excised Petal Experiments

One petal per treatment was incubated on filter paper in 20 mL vials containing 1 mL feeding solution. After 24 h most of the solution had been absorbed. After addition of 0.5 mL distilled water, an aliquot was taken to measure ACC and MACC uptake. All tissues were kept in continuous fluorescent lighting at 22°C. Incubation media included various concentrations and combinations of ACC (1.0 mM), MACC (0.25–1.0 mM), AVG (0.1 mM), $CoCl_2$ (0.1–1.0 mM), and 4 mM thiosulfate complex (STS) (19) as indicated. Control petals were treated with distilled water. To determine the effects of exogenous ACC and MACC, ethylene was measured every 6 h, starting at 24 h, until petals began inrolling, and on each subsequent day until control petals inrolled. In the MACC-plus-inhibitors experiment ethylene was analyzed every 3 h from 24 to 48 h. At each sampling time a 1 mL gas sample was withdrawn from the headspace and analyzed as stated above. After sampling, vials were uncapped, flushed with air, and returned to the growth chamber. Vials were capped again 1 h prior to next sampling time.

RESULTS

ACC and Conjugated-ACC Levels in Ageing Flower Parts

The pattern of ACC concentrations detected in all flower parts was similar to previously reported studies (2, 7, 14). A peak of ACC was observed 6 to 9 d after flower opening (Fig. 1, a and b). The ethylene climacteric peak was observed at 7 d (data not shown). Both the receptacle and the petals produced a transient peak at 3 d after opening, which lasted 1 to 2 d, respectively (Fig. 1b). With the exception of the styles, levels of ACC remained high in all parts through 9 d. The pattern of ACC-conjugate accumulation was very different then what has been observed in other senescing or stressed plant tissue. Levels of the conjugate changed with time in a synchronous pattern in all flower parts (Fig. 2). An increase of conjugated-ACC was detected after 1 d, with a very large peak occurring at 3 d. At 4 d the conjugate had fallen to 70% of the 3 d peak except for the green tissue, which had decreased to almost zero. Another large peak was detected again at 7 d coinciding with ACC accumulation and ethylene production. After a decrease of conjugated ACC at 8 d, levels increased again by day 9.

Excised Petal Experiments

Exogenous applications of ACC and MACC both induced ethylene production in preclimacteric carnation flower petals (Fig. 3). In the ACC-treated petals increasing ethylene production occurred after 24 h and wilting was detected at about 30 h. When petals were treated with exogenous MACC, increasing ethylene production was detected after 30 h, followed by irreversible wilting after 33 h. Approximately 87% of the applied material was taken up for both ACC and MACC (data not shown). Control petals did not start producing

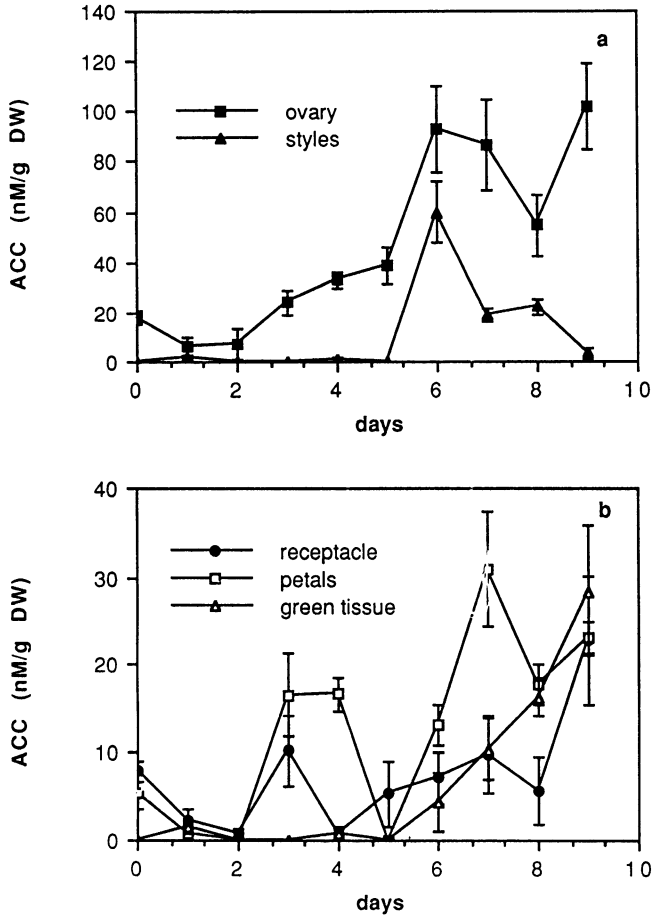

Figure 1. Endogenous ACC concentrations/g dry weight (DW) in the ovary and styles (a) and the receptacle, petals, and green tissue (b) in ageing carnation flowers 0 to 9 d after opening.

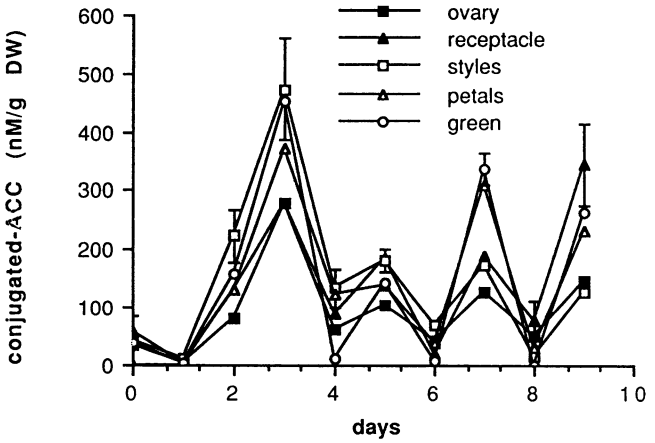

Figure 2. Endogenous MACC concentrations/g dry weight (DW) in ageing carnation flower parts 0 to 9 d after opening. Bars represent the pooled SE for each day of four replications.

measurable amounts of ethylene until after 6 d, with wilting commencing at 7 d. The response time for MACC-induced ethylene production was concentration-dependent (Fig. 3). As the concentration of MACC increased, days to onset of wilting

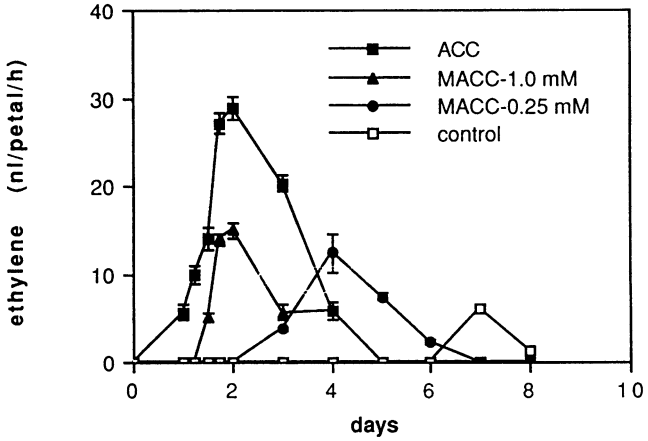

Figure 3. Ethylene production by preclimacteric carnation petals after treatment with 1.0 mM ACC or 0.25 to 1.0 mM MACC. Control petals treated with distilled water. The SE bars of three replications are indicated.

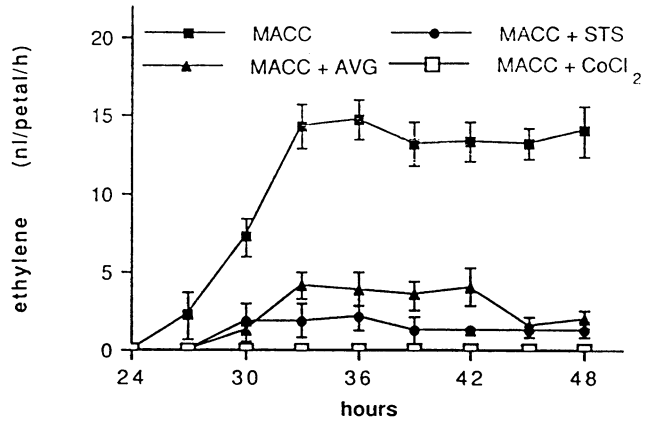

Figure 4. Ethylene production by preclimacteric petals after treatment with 1.0 mM MACC + 0.1 mM AVG, 1.0 mM $CoCl_2$, or STS. The SE bars of three replications are indicated.

decreased. At 0.25 mM MACC, days to wilting occurred 3 days prior to control petals.

Treating excised petals with ethylene inhibitors combined with 1.0 mM MACC resulted in a response similar to that observed by Jiao *et al.* (8) in vegetative tissue. After petals were treated with MACC + AVG, an inhibitor of ACC synthase, ethylene production was observed and petal inrolling was detected at 36 h (Fig. 4). When petals were treated with STS, an inhibitor of ethylene action (19), and then fed MACC, measurable ethylene production was detected, but premature senescence was not observed (Fig. 4). When MACC was combined with 1.0 mM $CoC1_2$, the petals did not produce any measurable ethylene and premature senescence was not observed (Fig. 4). No visible signs of senescence were detected in MACC-treated petals plus STS or $CoC1_2$ even after 9 d (data not shown).

DISCUSSION

The increasing ACC levels just prior to or coincident with the ethylene climacteric in our ageing carnation flower parts,

corresponds with previous results for carnations (2, 15) and climacteric fruits (9, 11). The small transient peak of ACC detected in both the petals and the receptacle at 3 d, did not result in increased ethylene production at this time. Hsieh and Sacalis (7) also reported a transient peak of ACC in the receptacle at this stage of development in carnation flowers, and suggested that this early peak may be a source of ACC for other parts of the flower. However, their data showed that concentrations of ACC in the petal bases and tops were higher than that found in the receptacle at 3 d, which is not consistent with their suggestion. The transient peaks of ACC we observed in the preclimacteric petals and receptacle may have been mobilized elsewhere in the inflorescence, but movement did not result in detectable free ACC or ethylene accumulation elsewhere in the flower. We did detect significant levels of conjugated ACC in all parts at this stage, however. Perhaps the malonyltransferase enzyme responsible for conjugating ACC was saturated in the receptacle and petals at this stage of development. We also found synchrony among all ageing flower parts. Therefore, it is unlikely that mobilization of an ethylene precursor from one part of the flower to another is necessary for the synchronous production of ethylene observed throughout the flower.

Although the patterns of conjugated ACC detected throughout the flower were very different from previously reported data, we believe the results are highly significant. Confidence in our results not only rely on adequate replication of the experiment, but also in the method of harvest for the plant material used. Flowers grown in our greenhouses availed us the opportunity to harvest at a more precise stage of development than had we relied on commercially grown carnations.

The large peak of conjugated ACC that was detected in the preclimacteric flower suggested to us that bound ACC may have a role in the senescence phenomena. It was necessary to determine whether carnation flower petals had the enzymatic ability to hydrolyze exogenous MACC and produce ethylene. Jiao *et al.* (8) observed MACC-induced ethylene production in vegetative tissues, but we are unaware of any previous report of it in reproductive tissue. MACC fed to 1-d-old petals induced abundant premature ethylene production with the onset of irreversible wilting. Through the use of specific ethylene inhibitors, Jiao *et al.* (8) established that the MACC-induced ethylene in vegetative tissue was derived from the MACC and not *de novo* ACC synthesis. We have now shown this to be true for petal tissue also. When the MACC feeding solution was amended with AVG, an inhibitor of ACC synthase, the petals still produced ethylene and senesced prematurely. The concentration of ethylene was reduced in the MACC-AVG treatment relative to MACC alone. This suppression probably reflects the inhibition of autocatalytic ethylene produced after MACC treatment, but not after the inhibition of ACC-synthase following AVG treatment. When the MACC solution was amended with 1.0 mM $CoCl_2$, which inhibits ACC conversion to ethylene, petals did not produce any measurable ethylene. When we applied STS, an inhibitor of ethylene binding, as a dip prior to MACC treatment, the petals produced ethylene, but did not wilt. Wilting is thought to be a consequence of ethylene binding (19).

Many different plant tissues have the ability to conjugate ACC to the malonylated form (4–6, 9, 18). Generally, it has been regarded as an inactive end product of ACC synthesis (5). We found that all parts of the flower contained peak levels of conjugated ACC 3 d after opening. This did not coincide with increased levels of either ACC or ethylene. In the preclimacteric apple fruit, Mansour *et al.* (11) found levels of MACC about 10 times greater than levels of ACC. MACC also accumulated in developing cocklebur seeds, before any increase in ACC or ethylene (18). Perhaps the accumulation of conjugated ACC is a common phenonmena in developing reproductive tissue.

The synchronous changes in concentrations of this metabolite during ageing (Fig. 2) may have physiological significance. Bufler and Romani (3) observed synchronous changes in polysome populations in aging carnation flower parts. They noted this as an interesting observation since the different flower parts are at very different stages of development during this time. The reduction of conjugated ACC that we measured at 4 d did not coincide with an increase of ethylene. The restricted capacity to produce ethylene may lead to alternative utilization of ACC in the presenescent flower.

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