Gartenbauwissenschaft, **66** (1). S. 34–38, 2001, ISSN 0016–478X. © Verlag Eugen Ulmer GmbH & Co., Stuttgart

Cultivar Differences in Regulation of Ethylene Production in Miniature Rose Flowers (*Rosa hybrida* L.)

Sortenspezifische Unterschiede bei der Regulierung der Ethylenproduktion in Blüten von *Rosa hybrida* L.

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Summary

In *Rosa hybrida* 'Bronze' ethylene exposure resulted in an increased ethylene production of flowers, presumably as a result of an upregulation of genes for ACC (1-aminocyclopropane-1-carboxylic acid) oxidase and ACC synthase. At the same time ethylene exposure caused a considerable increase in ACC content, indicating that ACC synthase activity was enhanced by a larger factor than ACC oxidase activity. In the cultivar 'Bronze' ethylene production of flowers also increased as flowers senesced, even in absence of exogenous ethylene. The ethylene production in response to exogenous ethylene was totally inhibited by treatment with the ethylene action inhibitor 1-MCP (1-methylcyclopropene), while the effectiveness of 1-MCP was less efficient in senescing flowers. Flowers of the cultivar 'Vanilla' only produced low amounts of ethylene and did not exhibit a substantial increase in ethylene production after ethylene exposure.

Zusammenfassung

Bei *Rosa hybrida* 'Bronze' verursachte die Behandlung mit Ethylen eine erhöhte Ethylenproduktion der Blüten, offenbar aufgrund einer erhöhten Expression der Gene für ACC-Oxidase und ACC-Synthase. Gleichzeitig resultierte die Behandlung mit Ethylen in einem beträchtlichen Anstieg des ACC-Gehalts, was darauf schließen läßt, daß die Aktivität von ACC-Synthase um einen größeren Faktor erhöht war als die von ACC-Oxidase. In der Sorte 'Bronze' stieg die Ethylenproduktion der Blüten im Laufe der Seneszenz an, auch wenn kein Ethylen in der Umgebungsluft vorhanden war. Die Behandlung der Pflanzen mit 1-MCP, einem Inhibitor der Ethylenwirkung, verhinderte die durch exogene Ethylenapplikation ausgelöste Ethylenproduktion vollständig. In seneszenten Blüten verringerte sich die Effektivität von 1-MCP. Blüten der Sorte 'Vanilla' produzierten lediglich geringe Mengen Ethylen und Ethylenbehandlung resultierte nicht in einer wesentlichen Erhöhung der Ethylenproduktion.

Introduction

The use of silver has become common practice in flower industry in order to reduce ethylene induced postharvest losses (SEREK, 1993). For carnation flowers, it was shown that pre-treatment with silver thiosulfate (STS) completely prevented the autocatalytic rise in ethylene production (BUFLER et al., 1980) However, being a heavy metal the use of STS raises environmental concern and should be replaced by the use of less harmful chemicals. One of these is the inhibitor of ethylene responses 1-methylcyclopropene (1-MCP), which significantly improved postharvest characteristics in *R. hybrida* by competing with ethylene for binding sites (SEREK et al., 1994). The positive effect of ethylene inhibitors on the postharvest performance of miniature roses was less clear in ethylene free environment (MÜLLER et al., 2000), even though several miniature rose cultivars showed a climacteric rise in ethylene production during flower senescence (MÜLLER et al., 1998).

The following experiments were carried out to examine miniature rose flowers' autocatalytic ethylene production at different developmental stages, and to investigate the effect of 1-MCP on ethylene production and 1-aminocyclopropane-1-carboxylic acid (ACC) content. Additionally, we studied expression of biosynthesis genes in rose flowers after treatment with ethylene. The correlation among ethylene production, ACC content and expression of biosynthetic genes may contribute to understanding of regulation of ethylene biosynthesis after ethylene exposure.

Materials and Methods

Plant material

Rosa hybrida L. 'Bronze' of the Parade breeding line (Poulsen Roser, Fredensborg, Denmark) and 'Vanilla' of the Kordana breeding line (Rosen Kordes, Sparrieshoop, Germany) were used. Plants were produced in commercial nurseries in Denmark. Production time for all cultivars was 11–12 weeks; the plants were grown in 10 cm pots under standard commercial growing conditions at

19–20°/19 °C (day/night temperature) and a relative humidity (RH) of 60–85 %. Natural daylight was supplemented with 230 µmol·m^{-2}·s^{-1} PAR from SON-T lamps during a 22-h photoperiod. As retardant, Paclobutrazol was used at a concentration of 0.5 %. At normal commercial maturity (1–3 open flowers per plant), the plants were transported within 24 h to the University.

1-MCP and ethylene treatment

Immediately after receipt, *R. hybrida* 'Bronze' and 'Vanilla' were placed in sealed glass containers, at 20 °C ± 1 °C and a light intensity of 20 µmol·m^{-2}·s^{-1} PAR. The plants were treated with 1-MCP by releasing it from the commercial powdered formulation (EthylBloc Biotechnologies for Horticulture, South Carolina, USA), 10 mL water was added to 0.02 g to release 100 nL · L^{-1} 1-MCP in the 160 L container. After this pre-treatment, the potted roses were exposed for 3 days to 1.2 µL·L^{-1} ethylene in a 160 L container, in which the ethylene concentration was maintained by a simple diffusion system (SALTVEIT, 1978). Control plants were kept in identical chambers under the same conditions without ethylene, ventilated with ethylene free air for the same time period.

After 1-MCP treatment and ethylene exposure, the plants were placed in the controlled environment, at 20 °C ± 1 °C and 60–70 % RH. The plants were exposed to 12 h light from fluorescent lamps (Thorn) providing 20 µmol·m^{-2}·s^{-1} PAR, irrigated from above with tap water as needed. On day 4 after treatment, the three flower stages: bud (A), flower (B) and senescent flower (C) were harvested, 0.4 g of the petal tissue was immediately used for ethylene measurement, and 0.4 g frozen with liquid nitrogen and stored in a −80 °C freezer for later ACC measurement.

Ethylene measurement

Ethylene measurement was conducted by sealing 0.4 g petals in glass vials (9 mL) with serum caps which were held for 1 h at 20 °C. A 1-mL aliquot of the air in the vial was withdrawn for measurement of ethylene, using a gas chromatograph (Photovac 10S plus, New York, USA) fitted with a photoionization detector.

Extraction and analysis of ACC

Frozen petal tissue (0.4 g fresh weight) was homogenized in 2 mL 80 % ethanol, and centrifuged at 10,000 g for 10 min. The supernatant was saved and the resulting pellet reextracted with 2 mL of 80 % ethanol. Following centrifugation, the supernatants were combined and evaporated to dryness. The resulting residue was resuspended in 1 mL water. ACC was assayed essentially as described by LIZADA and YANG (1979). Briefly, 0.6 mL 2µM $HgCl_2$ was added to 0.2 mL of the extract in a 9 mL vial, which was sealed with a serum cap and held at 0 °C. Approximately 0.2 mL of a cool mixture of 10 % 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 of RNA and northern blot hybridization after ethylene treatment

Polysomal RNA was extracted as described by SCHULTZ et al. (1994) from open, not senescing flowers of *R. hybrida* 'Vanilla' and 'Bronze' with or without previous ethylene treatment. Ethylene treatment was conducted as described under 1-MCP and ethylene treatment but ethylene concentration was only 0.25 µL · L^{-1}.

Identical amounts (8 µg per sample) of total RNA were electrophoresed on 1.2 % agarose gel containing 2.2 M formaldehyde. The gel was EtBr stained to check the intactness of the RNA before it was transferred to positively charged nylon membranes (Böhringer, Mannheim) in 10 x SSC. RNA was fixed to the membranes by exposing to 0.6 J · cm^{-2} UV light. Transcript size was estimated by the use of RNA markers covering the range 0.28–6.58 kb (Promega).

Hybridization and washing conditions for northern blots: The membranes were prehybridized for 1 h in prehybridization solution (Standard buffer + 50 % formamide, Böhringer Mannheim) at a temperature of 50 °C for ACC oxidase and 60 °C for ACC synthase transcripts. Hybridization was carried out overnight at the same temperatures in 6 mL hybridization solution (Standard buffer + 50 % formamide) containing 100 ng/mL DIG-labeled probe (ACC oxidase: GACO2, GenBank acc. no. U19856; ACC-synthase: GACS2, GenBank acc. no. U88971, labeled with Böhringer DIG RNA Labeling Kit, and denaturated for 15 min at 68 °C. After hybridization, nylon filters were washed 2 times for 15 min in 2 x SSC at room temperature followed by 2 times for 15 min in 0.5 x SSC at 68 °C. After hybridization and washing, the membranes were exposed to a chemiluminescent detection using the reagent CSPD® (Böhringer Mannheim), and exposed to Lumi-film for 45 min (GACO2, GACS2).

Results

Ethylene production and ACC content after 1-MCP and ethylene treatment

'Bronze' stage A

In *R. hybrida* 'Bronze', ethylene treatment resulted in severe bud drop after 4 days. Because of this bud drop it was not possible to measure ethylene production or ACC content on day 4 after treatment. However, bud drop after 1-MCP treatment was insignificant on day 4, and ethylene production of the buds (stage A) after this pre-treatment was very low (<0.2 nL · L^{-1}·h^{-1}). Buds held in air, pre-treated with 1-MCP or non treated, did not produce noticeable amounts of ethylene either (Abb. 1a).

'Bronze' stage B

Open flowers (stage B) of 'Bronze' without preceding ethylene exposure did not produce substantial amounts of ethylene and ACC, neither with nor without 1-MCP pre-treatment (Abb. 1a, Abb. 2). Treatment of open 'Bronze' flowers (stage B) with 1.2 µL · L^{-1} ethylene increased ethylene production and ACC content signifcantly (Abb. 1a, Abb. 2). In contrary, flowers treated with 1-MCP before ethylene exposure only had a very

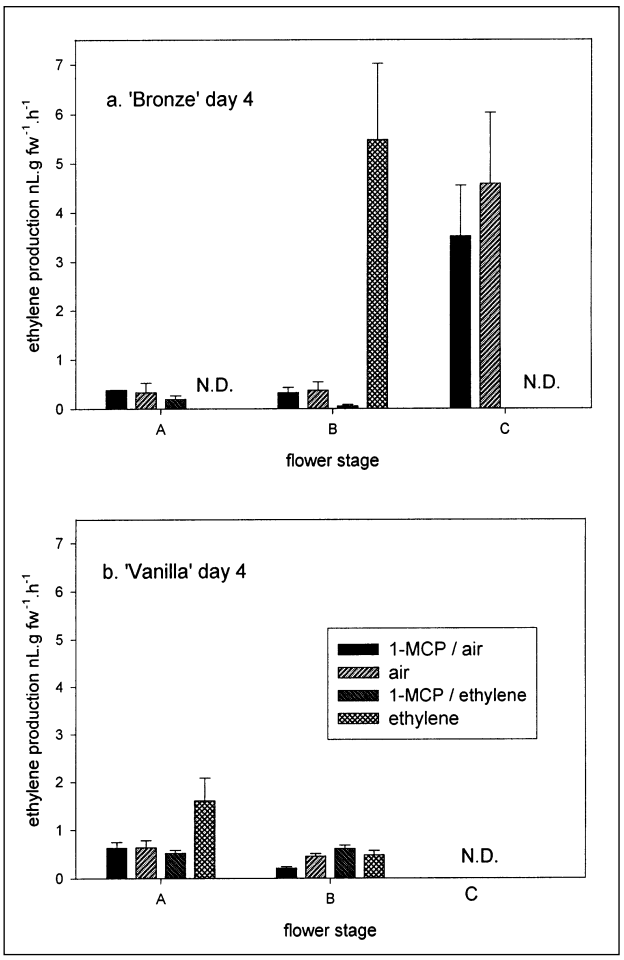


Abb. 1. Ethylene production of *Rosa hybrida* a. 'Bronze' buds (stage A), open flowers (stage B) and senescing flower (stage C), and b. 'Vanilla' buds (stage A) and open flowers (stage B) in response to 1.2 µL·L^{-1} ethylene for 3 days, without pre-treatment or after pre-treatment with 100 nL·L^{-1} 1-MCP (N.D. = not determined).
Ethylenproduktion von Rosa hybrida *a. 'Bronze' Knospen (Stadium A), offene Blüten (Stadium B) und seneszente Blüten (Stadium C), und b. 'Vanilla' Knospen (Stadium A) und offene Blüten (Stadium B) nach Behandlung mit 1.2 µL·L^{-1} Ethylen für einen Zeitraum von 3 Tagen, mit oder ohne Vorbehandlung mit 100 nL·L^{-1} 1-MCP.*

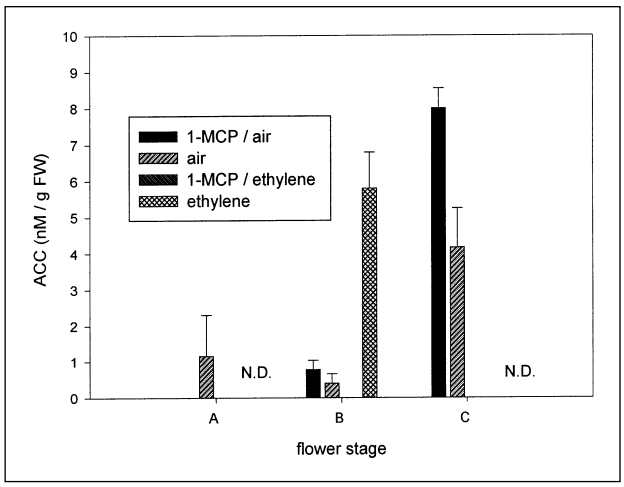


Abb. 2 ACC concentration (nmol·gfw^{-1}) by open and senescent flowers (stage B or C) in *R. hybrida* 'Bronze' in response to 1.2 µL·L^{-1} ethylene for 3 days, without pre-treatment or after pre-treatment with 100 nL·L^{-1} 1-MCP (N.D. = not determined)
ACC Konzentration (nmol·gfw^{-1}) von offenen und seneszenten Blüten (stadium B oder C) von R. hybrida *'Bronze' nach Behandlung mit 1.2 µL·L^{-1} Ethylen für einen Zeitraum von 3 Tagen, mit oder ohne Vorbehandlung mit 100 nL·L^{-1} 1-MCP.*

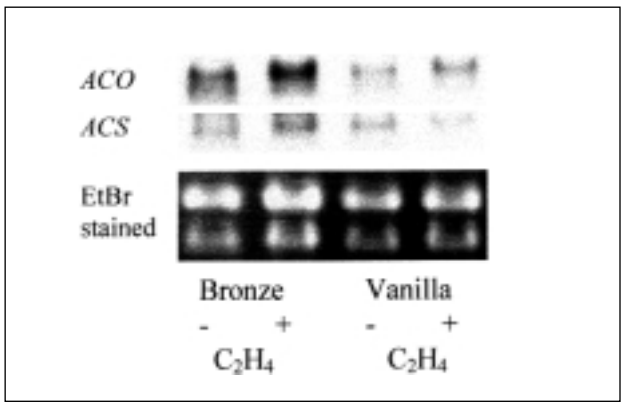


Abb. 3 Northern blot analysis of the expression of ACC oxidase and ACC synthase in flowers (stage B) of the two cultivars *Rosa hybrida* 'Bronze' and 'Vanilla'. Intact miniature rose plants were treated with 0.25 µL·L^{-1} ethylene or held in air for 3 days. The Ethidiumbromid (EtBr) stained ribosomal bands are shown as a reference.
Northern Analyse der Expression von ACC-Oxidase und ACC-Synthase in Blüten (Stadium B) in den zwei Sorten Rosa hybrida *'Bronze' und 'Vanilla'. Intakte Topfrosen wurden für einen Zeitraum von 3 Tagen mit 0.25 µL·L^{-1} Ethylen behandelt, Kontrollpflanzen wurden für 3 Tage in ethylenfreier Luft gehalten. Die Ethidiumbromid (EtBr) gefärbten ribosomalen Bänder sind als Referenz gezeigt.*

low ethylene production. Pre-treatment with 1-MCP apparently prohibited autocatalytic ethylene production in open flowers of 'Bronze'. The treatment of flowers with only 100 nL · L^{-1} 1-MCP reduced the rate of ethylene production by 91 % (Abb. 1a).

Bronze stage C

Senescent flowers (stage C) of 'Bronze' exhibited a clear rise in ethylene production (4.5 nL·L^{-1}·h^{-1}) (Abb. 1a) and ACC content (Abb. 2) compared to earlier flower stages. Additionally, endogenous ethylene in 1-MCP treated senescent flowers was lower (3.5 nL · L^{-1} h^{-1}) than in flowers without pre-treatment with the ethylene action inhibitor (Abb. 1a). 1-MCP pre-treatment decreases ethylene production by 20 % and doubles ACC content. Ethylene and ACC measurements in senescent flowers (stage C) after ethylene treatment was not possible because all flowers at this stage were totally wilted on day 4 of the observation period.

'Vanilla' stage A

In buds (stage A) of *R. hybrida* 'Vanilla' treatment with 1.2 µL·L^{-1} ethylene resulted in a weak autocatalytic ethylene production (ca. 1.5 nL·L^{-1} ·h^{-1}) while buds pretreated with 1-MCP did not exhibit a rise in endogenous ethylene. Buds of 'Vanilla' held in air, pre-treated

with 1-MCP or non treated, only produced negligible amounts (<0.7 $nL \cdot L^{-1} \cdot h^{-1}$) of ethylene (Abb. 1b).

'Vanilla' stage B

Contrary to the observed autocatalytic rise in ethylene production in 'Bronze', open flowers (stage B) of 'Vanilla' did not produce substantial amounts of ethylene. The ethylene production was very low in all treatments (Abb. 1b). On day 4 of the experiment no flowers of 'Vanilla' were senescent, thus a measurement of ethylene production and ACC content at stage C was not feasible.

Expression of ethylene biosynthetic genes in ethylene treated rose flowers

Northern blotting under high stringency conditions with an ACC oxidase probe from *Pelargonium zonale* identified two bands on the gel. Their position was consistent with the expected size of ACC oxidase mRNA (Abb. 3). The abundance of both ACC oxidase transcripts increased in ethylene treated flowers of the two investigated rose cultivars, but transcript abundance was only slightly regulated in 'Vanilla'. The maximum signal strength was higher in 'Bronze' than in 'Vanilla'.

An ACC synthase probe from *Pelargonium zonale* identified a single band with the expected size of ACC synthase on the gel (Abb. 3). The signal strength was slightly higher in 'Bronze' than in 'Vanilla'. In 'Bronze' ACC synthase transcript level increased after ethylene treatment, in 'Vanilla' the abundance of ACC synthase transcript was not regulated.

Discussion

In 'Bronze' flowers, ethylene presumably increases the activity of both biosynthetic enzymes, since the ethylene production increased significantly after ethylene exposure. Northern blot analysis of ACC synthase and ACC oxidase confirmed increased abundance of both transcripts (Abb. 3). Ethylene enhanced both ACC oxidase and ACC synthase activity, but enhanced ACC synthase by a larger factor than ACC oxidase, as indicated by the observed increase in ACC.

In carnation, ethylene had a promoting effect on ACC oxidase activity. Already at very low concentrations (0.1 $\mu L \cdot L^{-1}$) the ACC oxidase activity was stimulated over 10 times, thereby preparing the tissue to convert ACC to ethylene (OVERBEEK and WOLTERING, 1990). This observation is in accordance with MANNING (1985) who showed that ACC oxidase activity in carnation was markedly stimulated by treatment with ethylene. In petals from STS pre-treated flowers no increase in ACC oxidase activity was observed, which further supports the notion that ethylene induces ACC oxidase activity. In carnation, TENHAVE and WOLTERING (1997) found that both ACC synthase and ACC oxidase gene expression were induced by ethylene in all flower parts, starting in the ovary. The expression of ethylene biosynthetic genes generally preceded the production of ethylene. Recently, it was found that in *etr1-1* transgenic carnations ACO specific mRNA levels were strongly reduced relative to control plants (BOVY et al., 1999). This suggests that in these plants the autocatalytic production of ethylene biosynthesis genes is blocked preventing the burst of ethylene production required for flower senescence.

It was expected that 1-MCP eliminates the observed effects of exogenous ethylene, i.e. autocatalytic ethylene production and increased ACC content. The present study confirmed this hypothesis, 1-MCP pre-treatment resulted in low ethylene production and at the same time low ACC content.

The finding that the autocatalytic ethylene production in flowers of the cultivar 'Bronze' can be inhibited by 1-MCP (Abb.1a) is analogous to the response of carnations to pre-treatment with ethylene action inhibitor 2,5-norbornadiene (NBD). Treatment with NBD also prevented the autocatalytic rise in ethylene production after ethylene exposure (WANG and WOODSON, 1989). Pre-treatment of carnation flowers with STS completely prevented the autocatalytic rise in ethylene production, without affecting the basal ethylene production by flowers, or the basal level of ACC in the petals (BUFLER *et al.*, 1980).

ACC content of senescing 'Bronze' flowers (especially at stage C) pre-treated with 1-MCP was clearly higher than in the untreated control. The accumulation of ACC in 1-MCP treated flowers may indicate that 1-MCP treatment mainly reduces ACC oxidase activity and less ACC synthase. However, no accumulation of ACC was observed after 1-MCP treatment and subsequent ethylene exposure, suggesting that not only ACC oxidase but also ACC synthase was inhibited by 1-MCP.

LELIEVRE et al. (1996) showed that treatment with 1-MCP resulted in reduced accumulation of ACC oxidase and ACC synthase transcript, and ethylene production during chilling of pear fruits. In mung beans hypocotyls, ethylene induced an increase in the level of ACC oxidase mRNA, but suppressed the expression of ACC synthase (KIM et al., 1996).

On the contrary, flowers of the cultivar 'Vanilla' produced only moderate amounts of ethylene as flowers senesced. In a previous study it was shown that this cultivar is almost insensitive to exogenous ethylene (MÜLLER et al., 1998), while the present experiments demonstrate that 'Vanilla' flowers do not exhibit a substantially increased ethylene production after ethylene exposure. This observation may partly explain the insensitivity of flowers to exogenous ethylene. Anyway, regulation of autocatalytic ethylene production may be different in 'Vanilla' flowers in response to higher ethylene concentrations as applicated in this experiment. It has previously been shown that in this cultivar higher ethylene concentrations are necessary to induce an ethylene response (unpublished results), though when a threshold is passed the autocatalytic regulation of ethylene production may be different.

However, in 'Vanilla' buds ethylene exposure resulted in a distinct increase in endogenous ethylene. 1-MCP pre-treatment inhibited this autocatalytic rise in ethylene production in buds. Further investigations are necessary to elucidate whether 'Vanilla' buds are more sensitive to ethylene exposure than open flowers.

The authors would like to thank Prof. Arne S. Andersen for fruitful discussions during this research and for crit-

ical review of the manuscript, the rose grower Erik Moes (Bent Halby Nursery, Denmark) for kindly suplying the plants, and Dr. Kathleen Brown, The Pennsylvania State University, USA for providing the *Pelargonium zonale* cDNA clones (GACO2 and GACS2). This research was supported by a grant to R. Müller from The Danish Agricultural and Veterinary Research Council.

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Eingegangen: 25. 5. 2000 / 4. 8. 2000

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