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Cytokinins in cut carnation flowers. II. Relationship between endogenous ethylene and cytokinin levels in the petals

J. VAN STADEN*, B.C. FEATONBY-SMITH*, S. MAYAK**, H. SPIEGELSTEIN**, and A.H. HALEVY**
*UN/CSIR Research Unit for Plant Growth and Development, Department of Botany, University of Natal, Pietermaritzburg 3200, Republic of South Africa and **Department of Ornamental Horticulture, The Hebrew University of Jerusalem, Rehovot 76–100, Israel

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Abstract. Tentative identification using HPLC and RIA techniques indicated the presence of zeatin-O-glucoside, zeatin, ribosylzeatin, dihydrozeatin, *iso*-pentenyladenine and *iso*-pentenyladenosine in the petals of carnation flowers. Dihydrozeatin is apparently responsible for most of the biological activity. Within the petals most activity was detected in the basal parts which also senesced much slower than the upper parts of the petals. Treatment with AOA extended petal longevity and reduced ethylene production. This was associated with higher cytokinin-like activity in the basal parts of the petals.

These higher levels of cytokinins were not observed in the petals of ACC treated flowers or in the detached control flowers. It is suggested that cytokinin transport and/or metabolism may play an important role in regulating ethylene production in cut carnations.

Introduction

It is generally accepted that plant hormones are involved in the regulation of flower senescence and that changes in the levels of these compounds act as regulating signals for the commencement or discontinuation of specific reactions. In carnations ethylene and cytokinins apparently act as regulating signals with respect to the onset and delay of senescence [2, 6, 11]. The dramatic rise in ethylene, largely produced in the basal portions of the petals [11, 13], is associated with increases in membrane permeability which is symptomatic of irreversible inrolling and wilting of the petals [9, 18]. This onset and acceleration of petal senescence can be delayed or counteracted by cytokinin application [5, 12]. While the exact relationship between ethylene and cytokinins is still unclear there is now considerable evidence that cytokinins reduce both ethylene synthesis and the sensitivity of carnation flowers to ethylene [2, 4, 11]. Benzyladenine appears to be operative at more than one site. It effectively prevented ACC production and also reduced the capacity of the flowers to convert ACC to ethylene [2, 12]. It would appear that the cytokinin acted within the petals, as both isolated and attached petals responded similarly to cytokinin application, particularly the basal portion which produced the largest amounts of ethylene [12]. It was suggested that this regulatory effect of benzyladenine on ethylene evolution might be due to membrane stabilization by the cytokinin [12].

While not yet established there are indications that endogenous cytokinins may participate in the natural senescence of carnations, especially as these hormones decline in the petals as they age and senesce [17].

In this study the evolution of ethylene was modified in carnation flowers by treating them with either ACC, or with α-aminooxyacetic acid (AOA), an inhibitor of ACC-synthetase. The effect of these treatments on the endogenous cytokinins of both upper and basal portions of the petals was monitored.

Materials and methods

Plant material

Carnation flowers (*Dianthus caryophllus* L. cv. White Sim) were grown in raised beds in a fibreglass greenhouse as described previously [14]. For experimentation flowers were cut at the commercial stage of development; fully open with a yellowish tinted center. The flower stems were cut to 5 cm, placed in test tubes and the level of water or test solution maintained at a level 2 cm below the calyx. Flowers were treated with water (control), 0.1 mM ACC or 2 mM AOA for the duration of the experiment which was determined by the physical appearance and ethylene evolution of the flowers. In parallel, flowers were tagged and allowed to develop on the plant, they were designated as attached flowers. The treated cut flowers were maintained at 22°C, RH of 60–70% and a light irradiance of 3 Wm^{-2} for 12 h a day provided by cool white fluorescent tubes. At certain times the flowers were sampled with respect to ethylene evolution and cytokinin content. For cytokinin analysis the petals were divided into the upper wide portions and the lower, narrow green part. These samples were frozen with liquid air and kept at −18°C until processed.

At the commencement of the experiment petals were collected from flowers, freeze dried and the dried material analysed so as to establish the tentative identity of the cytokinins in carnation petals.

Ethylene measurements

The stems of treated cut flowers and the flowers left attached to plants to serve as untreated controls were trimmed to 5 cm and placed individually in specially constructed 300 ml glass containers. The ethylene which accumulated in the headspace over a 3 h period was determined by gas chromatography as described previously [14].

Estimation and tentative identification of cytokinins in petals

For the tentative identification of the cytokinins 400 g of petals were homogenised with 80% ethanol and extracted at room temperature for 24 h. The

material was then filtered and purified with a Dowex 50 cation exchange resin as described earlier [16]. The cytokinins were removed from the cation exchange resin with 5N NH_4OH and the extract reduced to a small volume *in vacuo* at 40°C. The concentrated extract was strip-loaded onto Whatman No. 1 chromatography paper and the constituents in the extract separated with *iso*-propanol: 25% NH_4OH: water (10:1:1 v/v). After drying the chromatograms were divided into 10 equal zones and the equivalent of 20 g fresh material assayed for cytokinin-like activity using the soybean callus bioassay [10]. The biologically active fractions were recorded and the remaining portions of the chromatograms were divided into a slow-moving fraction A (Rf 0–0.5) and a fast-moving fraction B (Rf 0.5–1.0). The compounds associated with these fractions were eluted from the paper with 80% ethanol, the extracts were filtered, concentrated to 1 ml and the constituents then fractionated on a Sephadex LH-20 column (2.5 × 93 cm) using 35% ethanol as eluant [1]. Forty ml fractions were collected and 2 ml of each fraction removed for the detection of biological activity using the soybean callus bioassay. The remainder of each fraction was taken to dryness in a stream of air and the fractions stored until the positions of the biologically active peaks were known. Once the flasks containing the biological activity were identified the compounds in them were resuspended in 100 μl methanol and this extract then fractionated by means of HPLC using a Varian 5000 instrument. A 250 × 4 mm i.d. Hypersil 5 ODS, C18 column was used for all separations. The flow rate was 1.5 ml min^{-1} and the UV absorbance was recorded at 265 nm. For those biologically active peaks which co-eluted on Sephadex with zeatin, ribosylzeatin and their respective dihydroderivatives the solvent was as follows: 10% acetonitrile isocratically for 10 min then to 18% acetonitrile over 5 min, isocratically for 5 min then to 20% acetonitrile over 20 min. Where the biological activity on Sephadex co-chromatrographed with *iso*-pentenyladenine and *iso*-pentenyladenosine HPLC separation was achieved by eluting with 50% methanol for 3 min then to 30% methanol over 7 min. In all instances fractions (1.5 ml) were collected every min. These were divided in half. One half was used for the detection of biological activity [10]. For verification of the nature of the cytokinins present the remainder of the fractions were subjected to RIA analysis. The residues were redissolved in 1 ml methanol and duplicate 100 μl aliquotes analysed. For *iso*-pentenyladenine and *iso*-pentenyladenosine the technique of Cutting et al. [3] was used. Dihydrozeatin was detected by the technique of Hofman et al. [8] and zeatin and ribosylzeatin by that Hofman et al. [7].

To determine the effect of various treatments on the endogenous cytokinin levels in senescing petals the upper and lower halves were extracted as described previously and the obtained Dowex 50 extracts fractionated on paper chromatograms [16]. The respective Rf zones were then assayed for cytokinin-like activity using the soybean callus assay [10]. Bioassays were performed in duplicate and the activity expressed as kinetin equivalents. The aqueous extracts did not yield any cytokinin-like activity and were therefore discarded.

Results

Tentative identification of cytokinins in carnation petals

Four peaks of cytokinin-like activity were detected in the Dowex 50 extracts of the petals following separation by paper chromatography (Figure 1). Most of this activity co-chromatographed with zeatin, ribosylzeatin, *iso*-pentenyladenine and *iso*-pentenyladenosine. Some activity was however, also associated with a peak which co-chromatographed with zeatin-O-glucoside. Elution of the remaining constituents from paper chromatograms and fractionation on Sephadex LH-20 with 35% ethanol resulted in the detection of a number of biologically active peaks (Figure 2). In the polar fraction (Rf 0–0.5 on paper chromatograms) most of the cytokinin-like activity co-eluted with zeatin-O-

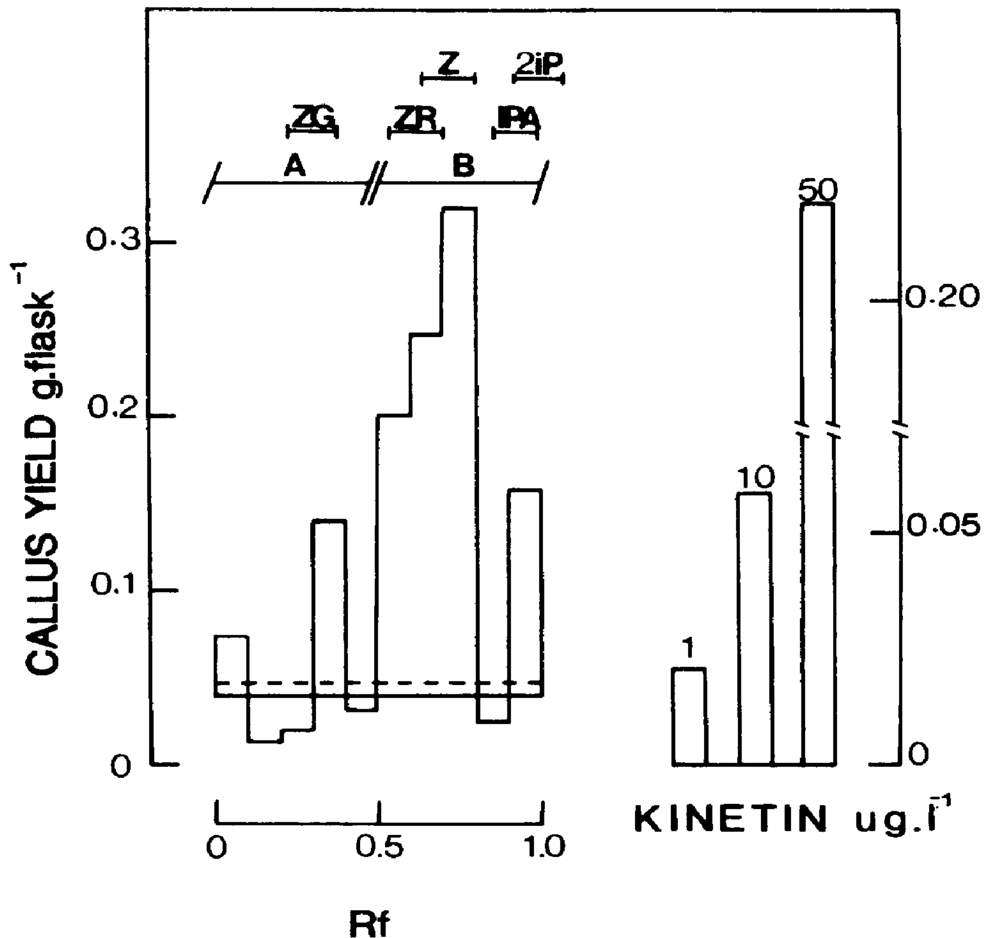


Fig. 1. Cytokinin-like activity detected in the equivalent of 20 g fresh carnation petals following paper chromatography. A and B represent zone subsequently eluted and subjected to Sephadex LH-20 fractionation. ZG = zeatin-O-glucoside; ZR = ribosylzeatin; Z = zeatin; IPA = *iso*-pentenyladenosine; 2iP = *iso*-pentenyladenine.

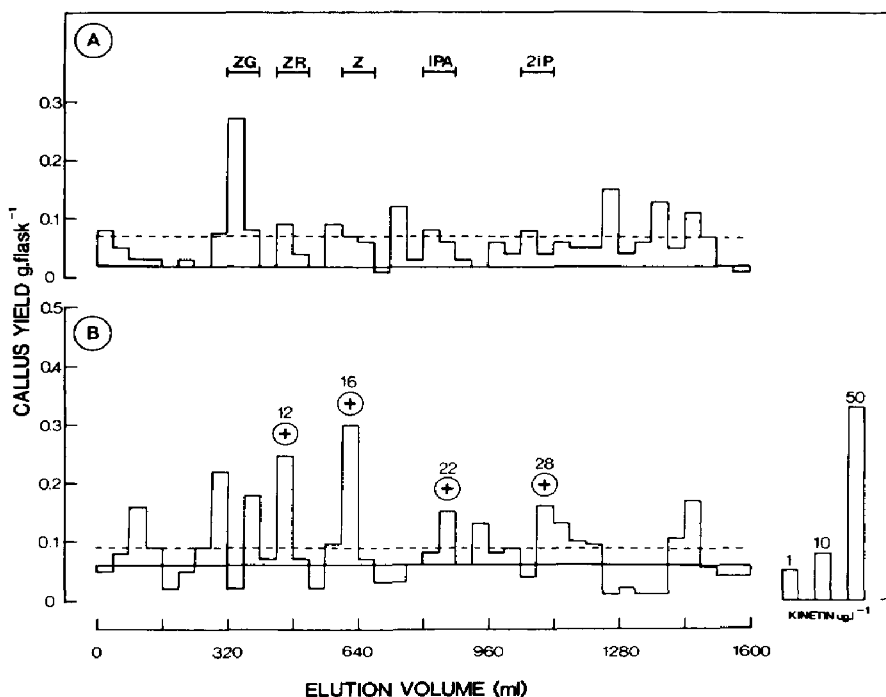
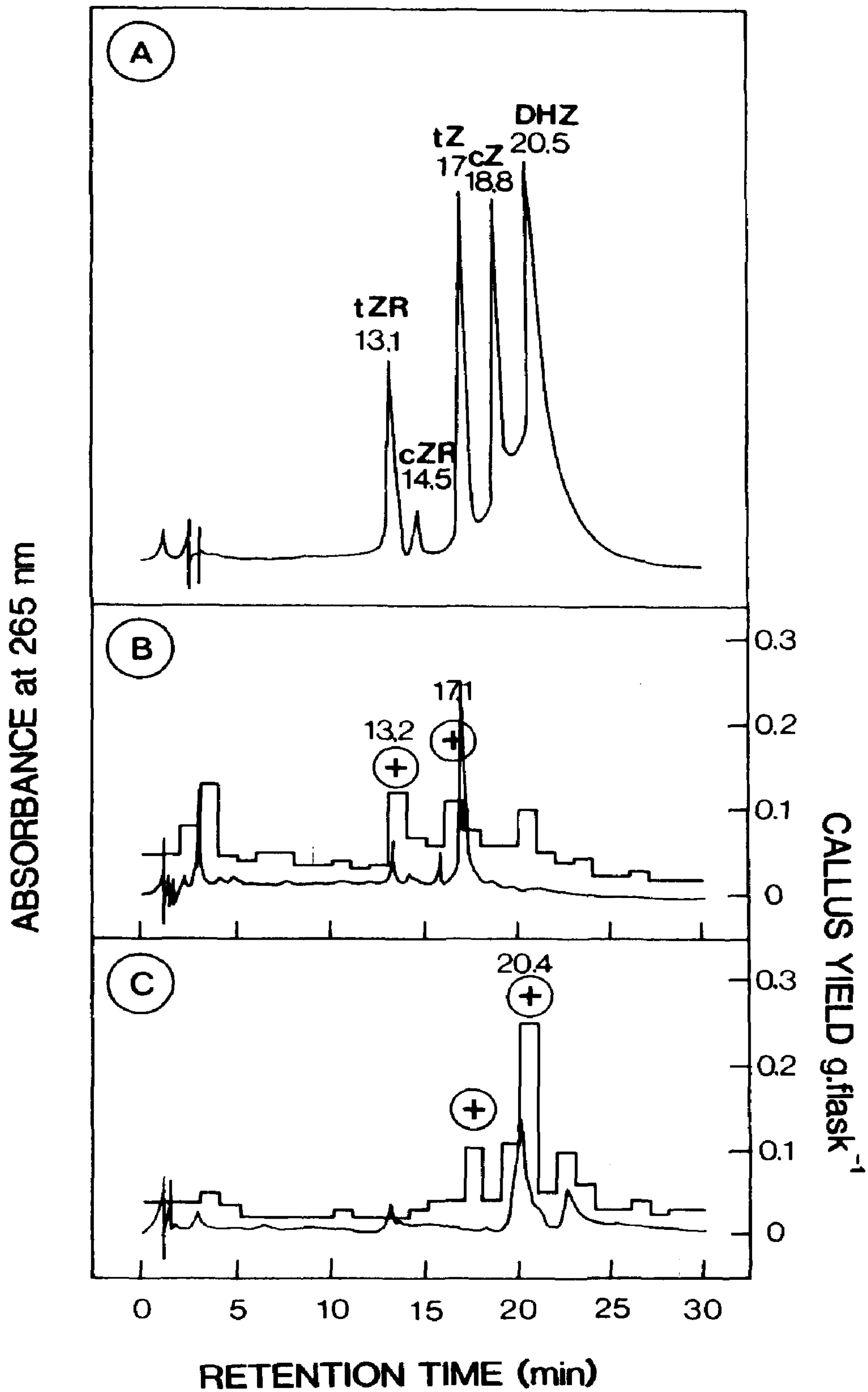


Fig. 2. Cytokinin-like activity detected in petal extracts following fractionation on Sephadex LH-20. A, represents the polar compounds eluted from paper chromatograms (Rf 0–0.5; Figure 1). B, represents the non-polar compounds eluted from paper chromatograms (Rf 0.5–1.0; Figure 1). Abbrebviations as for Figure 1. Plus signs indicate that the relevant fractions (numbers) gave a positive response with respect to RIA assay.

glucoside (Figure 2A). Overall most cytokinin-like activity was however, associated with the non-polar fraction (Figure 2B). Nine peaks of activity were recorded. of these, four co-eluted with authentic ribosylzeatin, zeatin, *iso*-pentenyladenosine and *iso*-pentenyladenine. Aliquots of these four peaks were subsequently subject to HPLC and RIA analysis in an attempt to learn more about their identity. The peak which co-eluted with ribosylzeatin and its dihydro derivative (Figure 2B) gave four biologically active peaks following HPLC separation (Figure 3B). One peak had a retention time of 4 min and did not co-elute with any markers. The other three peaks co-chromatographed with *trans*-zeatin and dihydrozeatin respectively. When subjected to RIA using the appropriate antisera the eluants which co-eluted with *trans*-ribosylzeatin and *trans*-zeatin showed significant ability to displace the relevant tracer from the antibody, thus confirming the presence of the N^6-side chain. Following Sephadex fractionation most activity was associated with the peak which co-eluted with zeatin and dihydrozeatin. HPLC fractionation and RIA analysis confirmed the presence of both *trans*-zeatin and dihydrozeatin (Figure 3C). Most biological activity was associated with the fraction co-eluting with dihydrozeatin. The biologically active peaks which co-eluted with *iso*-pentenyladenosine and *iso*-pentenyladenine following Sephadex LH-20 fractionation (Figure 2B) co-chromatographed with authentic markers of these compounds following HPLC


A
DHZ
20.5
tZ
17
cZ
18.8
tZR
13.1
cZR
14.5
B
17.1
13.2
C
20.4
ABSORBANCE at 265 nm
CALLUS YIELD g.flask⁻¹
0.3
0.2
0.1
0
0
5
10
15
20
25
30
RETENTION TIME (min)

separation. The eluants gave positive responses with the soybean callus and RIA techniques employed (Figure 4B and C). These results indicate that both the zeatin and *iso*-pentenyladenine groups of cytokinins are present in carnation petals.

Flower longevity, ethylene production and endogenous cytokinin levels

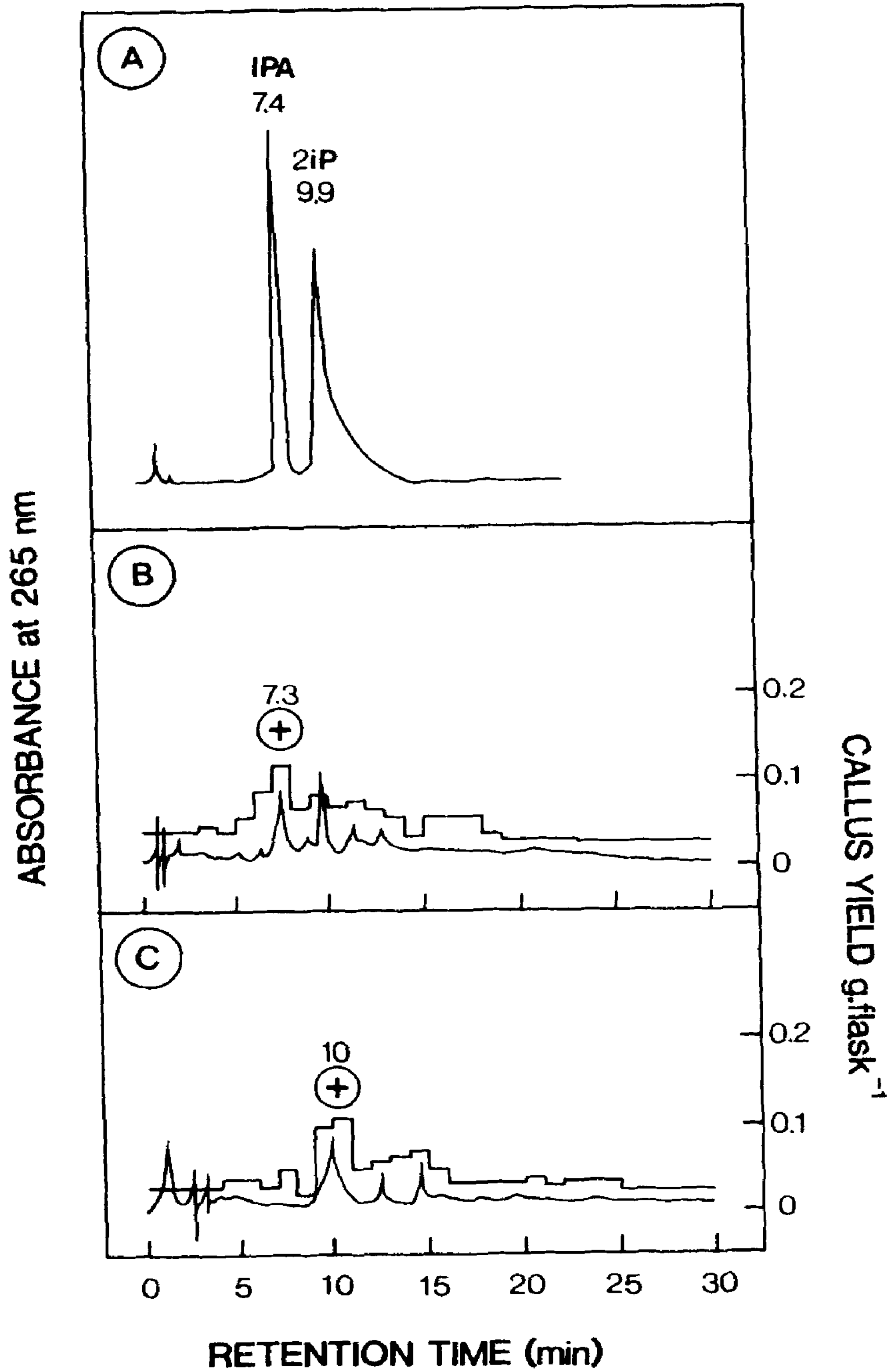
Cut flower longevity was the greatest in attached flowers followed by AOA-treatment, control cut flowers and ACC-treatment in decreasing order. Ethylene evolution was greatest in ACC-treated and lowest in AOA-treated flowers. In the "attached" controls which senesced slowly an increase in ethylene was only observed on day 9 whereas in cut flowers this occurred by day 6. The level of ethylene evolution in attached flowers was much lower than that recorded for the cut flowers (Figure 5).

Cytokinin-like activity was detected in all petal extracts. The lower portions of the petals yielded much higher activity than upper portions. Cytokinin changes measured in intact petals were largely determined by the levels in the lower portions of the petals (Figure 6A and C). Initially the cytokinin-like activity increased in the petals of all treatments. In the cut controls, the attached flowers, and the AOA-treatments the levels subsequently decreased. While the level remained low in the control petals it again increased significantly in the attached and AOA-treated petals (Figure 6A). This was largely due to an increase in the lower portions of the petals (Figure 6C). Analysis of the upper portions of the petals, which largely reflect flower longevity, indicated that the cytokinin-like activity in this portion is very low (Figure 6B). However, after 4 days relatively high levels of activity were detected in the upper portions of the attached and AOA-treated flowers which showed the longest longevity. This increase in cytokinin-like activity was transient and was no longer detectable after 6 days.

Discussion

Cytokinin-like activity which co-chromatographed with zeatin-O-glucoside,ribosylzeatin, zeatin, *iso*-pentenyladenosine and *iso*-pentenyladenine have been

Fig. 3. Separation of authentic cytokinins (A) and aliquots of biologically active peaks (B and C) by means of HPLC. The mobile phase was 10% acetonitrile for 10 min, then to 18% acetonitrile over 5 min, isocratic for 5 min, then to 20% acetonitrile over 20 min. The solid line represents the UV-trace recorded and the histograms the biological acitivity detected using the soybean callus bioassay. Plus signs indicate that the fractions (numbers) gave a positive response with respect to RIA assay. B, represents the biological activity detected after fraction 12 (elution volume 440–480 ml; Figure 2B) was separated by HPLC. C, represents the biological activity detected after fraction 16 (elution volume 600–640 ml; Figure 2B) was separated by HPLC. tZR = *trans*-ribosylzeatin; cZR = *cis*-ribosylzeatin; tZ = *trans*-zeatin; cZ = *cis*-zeatin; DHZ = dihydrozeatin.



A
IPA
7.4
2iP
9.9
B
7.3
+
C
10
+
ABSORBANCE at 265 nm
CALLUS YIELD g.flask^{-1}
0.2
0.1
0
0
5
10
15
20
25
30
RETENTION TIME (min)

reported to be present in carnation petals [17]. The current experiments, where more sophisticated HPLC and RIA techniques were used, confirmed the earlier data and indicated that dihydrozeatin was also present in young petals. This compound was in fact responsible for most of the biological activity detected. In all treatments the cytokinin-like activity was much greater in the lower or basal portions of the petals.

Previous studies have indicated that ACC-synthetase and EFE activity are higher in the basal than in the upper portions of carnation petals [11, 15]. As a result it was suggested that ethylene production by the upper portion, and hence senescence, is controlled by ACC synthesis in the basal portion of the petals [11]. The effect of cytokinins as an anti-senescence factor appears to be related to an ability to reduce ethylene synthesis [2, 4, 11], apparently by controlling both ACC synthesis and the conversion of ACC to ethylene [12]. Results obtained with applied benzyladenine indicated that this cytokinin exerts its effect within the petals, probably by controlling the enzymes responsible for ethylene production. The data obtained in this study suggests that the endogenous cytokinins may act in a similar way. Treatment of carnations with AOA or leaving them attached to the plants eliminated or delayed and reduced ethylene production. This was paralleled by high levels of endogenous cytokinins in the basal portions of the petals where the highest level of ACC production has been recorded. In the ACC-treated and control cut flowers which senesced faster this increase was not observed. The small transient peak observed in untreated (control) petals observed in an earlier study [17], was again detected. AOA-treatment and leaving the flowers attached to the parent plant also affected the cytokinin-like activity in the upper portions of the petals. After 4 days a transient peak of activity was detected in this part of the petals. The effect of this activity on the longevity of the petals is currently not known. It has been shown that benzyladenine application to upper portions of petals reduced the conversion of ACC to ethylene. The endogenous cytokinins may fulfill a similar function and reduce ethylene production which is harmful with respect to membrane integrity and petal wilting. In attached flowers it would be expected that there would be a gradual increase in the import of cytokinins from the rest of the plant. The decline in cytokinin activity in the upper part of the petals may result in part from metabolism gradually increasing with the progression of senescence, and in part from re-mobilization of cytokinins or their metabolites to the lower parts or petals, or the rest of the plant. Thus, the observed sustained increase in

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Fig. 4. Separation of authentic cytokinins (A) and aliquots of biologically active peaks (B and C) by means of HPLC using a Hypersil 5 ODS (25 cm, C18) column at an elution rate of 1.5 ml min^{-1}.

The mobile phase was 50% methanol for 3 min, then to 30% over seven minutes. The solid line represents the UV-trace recorded and the histograms the biological activity detected using the soybean callus bioassay. Plus signs indicate that the fractions gave a positive response with respect to RIA assay. B, represents the biological activity detected after fraction 22 (elution volume 840–880 ml; Figure 2B) was separated by HPLC. C, represents the biological activity detected after fraction 28 (elution volume 1080–1120 ml; Figure 2B) was separated by HPLC. IPA = *iso*-pentenyladenosine; 2iP = *iso*-pententyladenine.

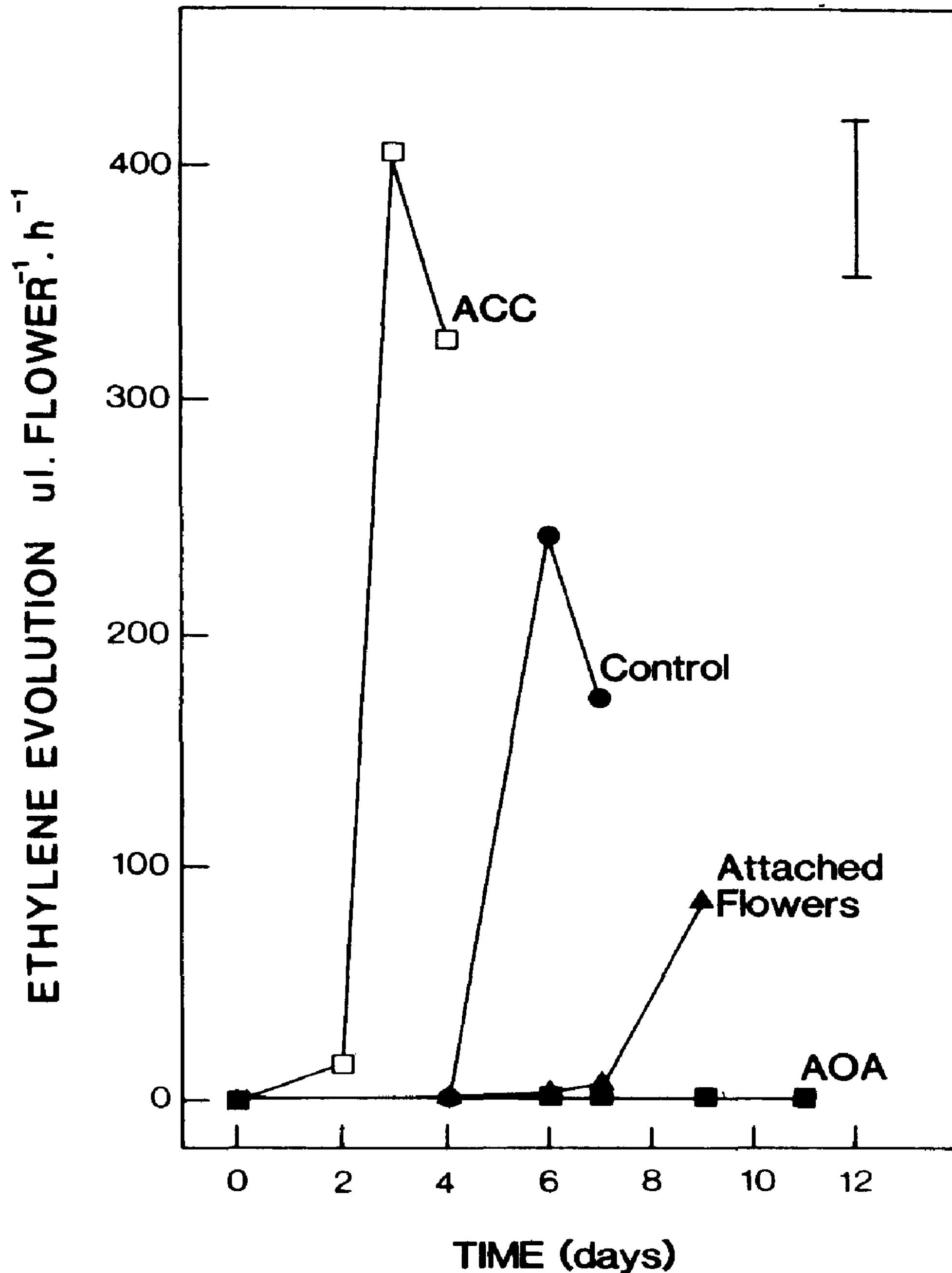

Fig. 5. Ethylene evolution of carnation cut flowers after different treatments. Control (●); attached flowers (▲);treated with 0.1 mM ACC (□); treated with 2 mM AOA (■). Bar represents LSD P = 0.05.

cytokinin activity in the lower part may stem partly from transport into the petals and partly from export from the upper parts of the petals. The much smaller increase, followed by a decline, occurring in detached flowers, obviously not attached to a cytokinin source supports this suggestion. In detached flowers

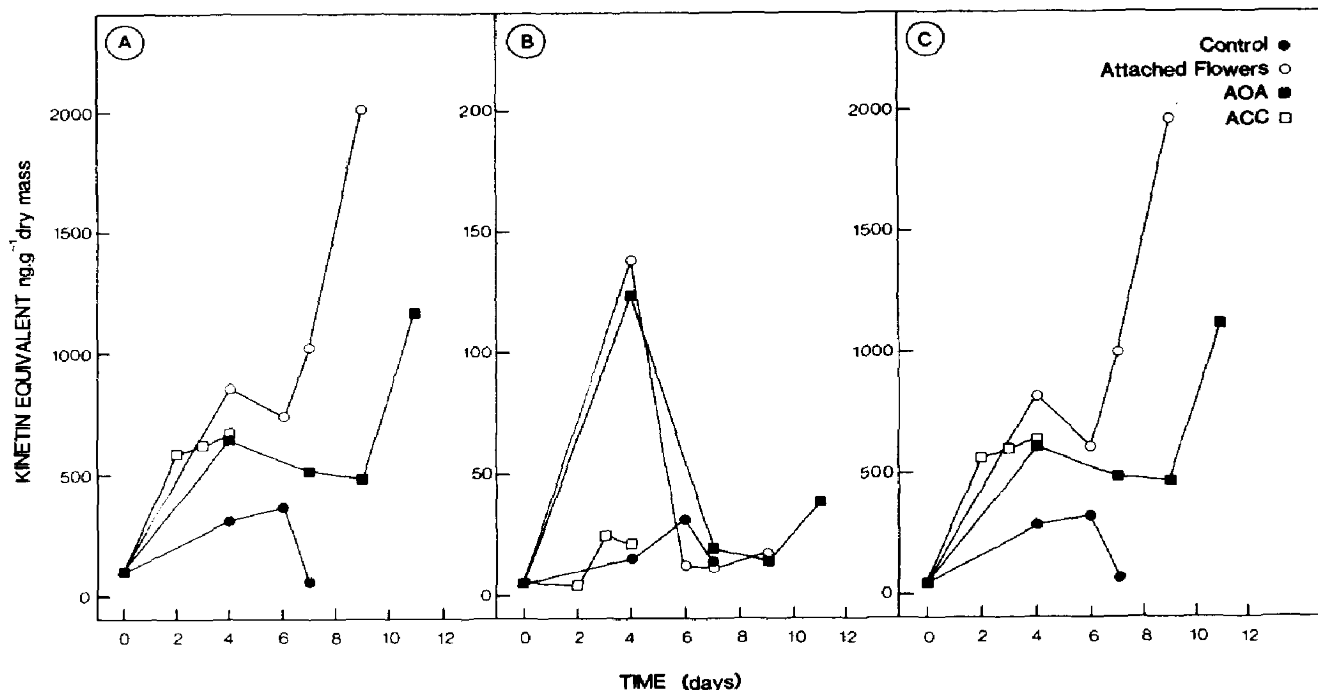

Fig. 6. Cytokinin-like activity (expressed as kinetin equivalents ng g^{-1} dry weight) detected in intact (A), upper portions (B) and lower portions (C) of petals collected from cut flowers subjected to various treatments accelerating or retarding ethylene production. Dowex 50 extracts were chromatographed on paper and the cytokinin-like activity assessed using the soybean callus bioassay. Activity above the controls is expressed as kinetin equivalents. Control (cut untreated) (●); attached control flowers (○); treated with 0.1 mM ACC (□); treated with 2 mM AOA (■).

metabolism would probably be the dominant event. No information is currently available on cytokinin transport in intact cut flowers or detached petals.

AOA has been shown to delay senescence of cut carnation flowers. It is suggested that the metabolism of cytokinins, an event associated with senescence, is being reduced in AOA treated flowers. The higher cytokinin-like activity detected throughout the development of AOA treated flowers as compared with control cut flowers is in line with this explanation.

Acknowledgement

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