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Influence of ethylene on cytokinin metabolism in relation to *Petunia* corolla senescence

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Abstract

Exogenous ethylene induced, and cytokinin suppressed, *Petunia* corolla senescence. 6-Benzylaminopurine and zeatin riboside, the principal cytokinin in corollas at the time of corolla opening, were effective senescence retardants but *O*-glucosyldihydrozeatin riboside was considerably less effective. Corolla senescence was preceded by a rise in cytokinin *O*-glucoside level, *O*-glucosyldihydrozeatin being the principal glucoside formed. Exogenous ethylene promoted conversion of dihydrozeatin to *O*-glucosides, but not 7-glucoside, and of zeatin riboside to adenosine and AMP. Hence it appears that ethylene production which induces corolla senescence also promotes inactivation of cytokinins by *O*-glucosylation and degradation and this may facilitate the senescence process. The use of cellulose TLC to separate cytokinins is discussed.

Keywords: *Petunia hybrida*; Solanaceae; Cytokinin levels; Cytokinin metabolism; Corolla senescence; Ethylene; Cellulose TLC

1. Introduction

The senescence of some flowers, e.g. carnation, morning glory and *Petunia*, is induced by ethylene (Whitehead, Halevy, & Reid, 1984; Reid, 1995) while exogenous cytokinin suppresses the senescence of cut carnation flowers apparently by inhibiting ethylene biosynthesis and action (Cook, & Van Staden, 1988). To further elucidate the interaction between cytokinin and ethylene in flower senescence, we have now: (a) shown that exogenous cytokinin delays the senescence of *Petunia* corollas; (b) defined the changes in endogenous cytokinin level associated with onset of *Petunia* corolla senescence; (c) determined the effect of ethylene on metabolism of exogenous radioactive cytokinins in the corollas. In these studies, the methods used for cytokinin separation included cellulose TLC and R_f data for a range of natural cytokinins on this layer is reported.

2. Results

2.1. Corolla senescence and cytokinin levels

In the senescence assay system based on *Petunia* corolla explants (corolla plus attached stamen with stigma and style), the ethylene-releasing compound 2-chloroethylphosphonic acid (ethrel, 0.7 mM) reduced the time to onset of marked wilting from 9 to 5 d. Senescence of detached but intact *Petunia* flowers and of excised corollas was suppressed by immersion for 1 h in a solution (1 mM) of silver thiosulphate, which inhibits ethylene action. The cytokinin 6-benzylaminopurine (BAP) retarded the senescence of the *Petunia* corolla explants in two assay systems: (i) explants were allowed to take up water or BAP solution for 5–6 d and senescence was then compared; (ii) explants

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received water or BAP for 24 h and were then supplied with ethrel. BAP at 0.2 mM almost completely prevented senescence during the period of both assays, but a clear retardation of senescence was also evident at 2 and 20 μM. Zeatin riboside ([9*R*]Z) also retarded senescence at 0.2 mM, but *O*-glucosyldihydrozeatin riboside, (OG)[9*R*]DZ, a prominent *O*-glucoside in the corolla (see below), was considerably less effective. These results confirm the importance of ethylene in *Petunia* corolla senescence and demonstrate the senescence-retarding activity of cytokinins. Reduction and glucosylation of the isoprenoid sidechain of [9*R*]Z reduced this activity.

Prior to determination of cytokinin levels in corollas by immunoassay, extracts of corollas (harvested at the initiation of senescence) were freed of pigment with PVP columns (Wang, Letham, Taverner, Badenoch-Jones, & Hocart, 1995) and then separated into anionic and basic fractions using cellulose phosphate columns (Badenoch-Jones, Letham, Parker, & Rolfe, 1984); these fractions were subjected to TLC on cellulose (solvent C). Cytokinin activity (*Amaranthus* betacyanin bioassay) in the basic fraction was largely concentrated in one zone which cochromatographed with zeatin (Z) and dihydrozeatin (DZ) and their ribosides; with the anionic fraction, the principal zone of activity cochromatographed with 6-(isopent-2-enyl)-aminopurine nucleotide (iP-NT) and a lesser zone with Z and DZ nucleotides. Hence cytokinin activity in the extracts appeared to be due mainly to bases, ribosides and nucleotides of the common cytokinins Z and/or DZ and possibly iP-NT. Detailed studies of cytokinin levels based on radioimmunoassay (RIA) after extensive purification of extracts are in accord with this result and are presented below (Table 1). Cytokinin *O*-glucosides were quantified by immunoassay, but these cytokinins have very low activity in the betacyanin bioassay (Letham, Palni, Tao, Gollnow, & Bates, 1983). Cytokinin bases, ribosides and nucleotides were also quantified. When the base and riboside fractions were further purified by HPLC (C_8 column), very similar values for cytokinin levels were obtained.

Six days after corolla opening, senescence of the corolla was first evident as slight wilting of the upper edge. At this time, the total level of cytokinin bases, ribosides and nucleotides, and the total level of unsaturated (Z-type) cytokinins were 1.2 times those on the day of corolla opening (Table 1). However, the total levels of saturated (DZ-type) cytokinins and of glucosylated cytokinins increased 2.7 and 3.9 times respectively, while the level of the principal glucoside, (OG)DZ, increased 6.3 times. Hence onset of corolla senescence was associated with increases in levels of saturated and glucosylated cytokinins. To assess if this was induced by ethylene production, the metabolism studies outlined below were conducted.

Table 1
The levels of cytokinins in *Petunia* corollas determined by RIA of purified extracts. Each value is the mean for two samples of corollas harvested on different occasions. Abbreviations for cytokinins not already defined in the text are listed as a footnote

	Cytokinin level (pmol g^{-1})	
	day of opening	6 d after opening[a]
Bases and ribosides[b]		
Z	0.92	1.28
[9*R*]Z	3.13	2.22
DZ	0.66	1.74[a]
[9*R*]DZ[c]	0.99	1.76
[9*R*]iP[d]	0.63	0.53
Nucleotides		
iP-NT[d]	4.27	4.09
Z-NT	0.29	1.24[a]
DZ-NT	0.66	0.88
Glucosides		
(OG)Z	0.03	0.32[a]
(OG)DZ	0.76	4.75[a]
(OG)[9*R*]Z	0.07	0.13
(OG)[9*R*]DZ	0.77	1.22
Overall total	13.18	20.16

[a] Denotes that the mean differs significantly ($P < 0.05$) from the mean determined for the day of corolla opening.
[b] After further purification by HPLC, similar values were obtained.
[c] Abbreviations: dihydrozeatin riboside, [9*R*]DZ; nucleotide, NT; *O*-glucosylzeatin, (OG)Z; *O*-glucosylzeatin riboside, (OG)[9*R*]Z; *O*-glucosyldihydrozeatin, (OG)DZ; 6-(isopent-2-enyl)aminopurine riboside, [9*R*]iP.
[d] See comment on identity in Section 3.

2.2. Preliminary studies of cytokinin metabolism

To simulate the natural supply of cytokinin via the xylem, [^{3}H]–[9*R*]Z solution was introduced through a wick inserted into the peduncle. Radioactivity recovered by extraction after 18 h was largely derived from the corolla and the calyx plus receptacle (Table 2); however radioactivity g^{-1} fresh weight was greatest in the calyx plus receptacle and in the ovary, indicating preferential accumulation in these tissues on a unit weight basis. One-dimensional (1D) TLC indicated that unmetabolized [9*R*]Z accounted for about 10% of

Table 2
Distribution of radioactivity within *Petunia* flowers after supply of [^{3}H]–[9*R*]Z (0.5 μM) via wicks inserted into the peduncles

Tissue	Radioactivity extracted	
	Bq g^{-1} fr. wt.	% of total
Corolla	250	60
Calyx + receptacle	1158	37
Ovary	1117	2
Stigma + style	107	0.4
Stamen	125	0.6

the ^{3}H recovered from corolla tissue and that the principal metabolite in this tissue was adenosine; a similar pattern of metabolites was found in the calyx plus receptacle and in the ovary. In excised corollas which had taken up [^{3}H]–[9*R*]Z (0.12 μM) directly via the transpiration stream, the same metabolite pattern was noted. The metabolites in extracts of these corollas were studied in greater detail by elution of 1D TLC zones, treatment of eluted nucleotide and glucoside fractions with phosphatase and glucosidase respectively, and rechromatography. Adenosine (Ados) and adenine (Ade) nucleotides were the principal metabolites identified and accounted for 28 and 10%, respectively, of the extracted ^{3}H, while 9% was due to Z in base, riboside and nucleotide forms. *O*-Glucosides were minor metabolites (< 2% of ^{3}H). In contrast, Ados and Ade nucleotides were not detected as metabolites of [^{3}H]-*S*-DZ (0.12 μM) supplied to the excised corollas; glucosides appeared to be the dominant metabolites.

To determine if cytokinins normally present in the corollas are translocated to other parts of the flower, solutions of [^{3}H]–[9*R*]DZ and [^{3}H]–(OG)DZ, cytokinins with the metabolically stable dihydro sidechain which occur as endogenous cytokinins in corolla tissue, were applied directly (painted) on to the surface of corollas of flowers attached to the plant. After 5 d when early senescence was evident, radioactivity levels in the various parts of the flowers and adjacent shoot were assessed. About 90% of the total radioactivity recovered by extraction was located in the treated corollas and 1D TLC indicated it was largely (about 70%) in glucoside form in both (OG)DZ- and [9*R*]DZ-treated corollas. However, low levels of ^{3}H were present in the other floral parts extracted (calyx, receptacle plus peduncle, and ovary) and glucosides were the dominant metabolites in each tissue. Hence active translocation of cytokinin out of the corolla did not appear to precede onset of senescence.

Because of the apparent importance of glucosides as cytokinin metabolites in the floral tissues, these and other metabolites formed in the corolla from DZ, [9*R*]DZ and (OG)DZ were identified critically (Table 3). The principal metabolites of both DZ and [9*R*]DZ were the *O*-glucosides, (OG)DZ and (OG)[9*R*]DZ, and the *N*-glucoside, the 7-glucoside of DZ ([7G]DZ) while [9G]DZ was a minor metabolite. (OG)DZ was converted to the 9-riboside and a small proportion to [7G]DZ.

2.3. The effect of ethylene on metabolism of DZ and [9R]Z

Excised corollas with associated stamen were supplied with water (controls) or with ethrel for one day (no senescence evident) or two days (wilting at the corolla edges), via the transpiration stream. All corollas were then transferred to cytokinin solutions ([^{3}H]–[9*R*]Z or [^{3}H]-*S*-DZ) for 24 h prior to extraction of the corolla tissue. 1D TLC of extracts indicated that the two ethrel pretreatments promoted conversion of DZ to glucosides to the same extent and the metabolites formed after uptake of ethrel for two days were identified (Table 4). The formation of *O*-glucosides, but not of the 7-glucoside, from DZ was increased considerably by ethrel pretreatment. Uptake of ethrel for 24 h promoted formation of Ados and AMP from [9*R*]Z (Table 4) and this response was not increased by uptake of ethrel for two days, a pretreatment which induced early senescence.

2.4. Cellulose TLC of cytokinins

In the present studies, cellulose thin layers were used for separation of cytokinins and confirmation of metabolite identity. These methods complemented separations on silica gel layers. All commercial prespread cellulose layers tested were unsatisfactory due to the diffuse nature of the spots. Several types of cellulose and solvents were assessed for cytokinin separation, and Sigmacell microcrystalline cellulose and solvents B and C were selected as the most suitable for general use based on compactness of spots and separations achieved. The R_f values for natural cytokinins and related adenine derivatives with solvent C were as follows (compounds arranged in order of increasing R_f): AMP, 0.12; Z-NT, 0.34; DZ-NT, 0.37; Ade, 0.41; Ados, 0.46; (OG)Z, 0.50; (OG)[9*R*]Z, 0.50; lupinic

Table 3
The radioactivity due to metabolites extracted from corollas to which ^{3}H-labelled *S*-DZ, *R*,*S*-[9*R*]DZ and *R*,*S*-(OG)DZ had been applied. Cytokinin solutions were painted on the surface of corollas which were extracted 4 d later. Each corolla received 1.7 kBq

^{3}H-Cytokinin supplied	Radioactivity due to metabolites (% of total extracted)					
	DZ	[9*R*]DZ	(OG)DZ	(OG)[9*R*]DZ	[7G]DZ	[9G]DZ
R,*S*-(OG)DZ	0.3	0.6	42.5	31.0	6.8	0.2
R,*S*-[9*R*]DZ	0.3	1.2	14.6	40.7	5.4	1.5
S-DZ	0.8	0.9	20.2	34.8	10.3	0.2

Table 4
Radioactivity due to metabolites of [^{3}H]–[9*R*]Z and [^{3}H]-*S*-DZ formed in *Petunia* corollas. The corollas were allowed to take up water or ethrel solution (1.4 mM) and were then transferred to solutions (0.8 μM) of the radioactive cytokinins for 24 h

Cytokinin supplied and pre-treatment	Radioactivity (% of total ^{3}H extracted)												
	Z	DZ	[9*R*]Z	[9*R*]DZ	Ade	Ados	AMP	Z-NT	DZ-NT	total glucosides	(OG)DZ	(OG)[9*R*]DZ	[7G]DZ
[^{3}H]-*S*-DZ													
Water (48 h)	0.0	43.3	0.0	1.5	< 1.0	< 1.0	< 1.0	0.0	< 3.0	34.5	2.9	11.5	20.1
Ethrel (48 h)	0.0	25.4	0.0	2.7	< 1.0	< 1.0	< 1.0	0.0	< 3.0	50.4	8.8	20.7	20.9
[^{3}H]–[9*R*]Z													
Water (24 h)	4.1	0.0	66.2	3.3	1.2	10.8	2.6	0.8	0.0	3.5	ND[a]	ND[a]	ND[a]
Ethrel (24 h)	1.0	0.0	53.8	2.4	0.7	19.9	7.7	0.4	0.0	4.8	ND[a]	ND[a]	ND[a]

[a] Not determined.

acid, 0.51; iP-NT, 0.52; dihydrolupinic acid, 0.53; (OG)DZ, 0.56; (OG)[9*R*]DZ, 0.56; [7G]Z, 0.57; [9G]Z, 0.61; [9G]DZ, 0.65; Z, 0.72; [9*R*]Z, 0.74; *cis*Z, 0.75; *cis*[9*R*]Z, 0.76; DZ, 0.76; [9*R*]DZ, 0.78; [9*R*]iP, 0.87; iP, 0.87.

One useful feature of the solvent is the separation of saturated cytokinins (DZ sidechain) from unsaturated cytokinins (Z sidechain), and this applied to bases, ribosides, nucleotides and *O*- and *N*-glucosides. However, the solvent does not separate cytokinin bases from ribosides and only one solvent (D) was found to achieve this separation on cellulose without use of borate. Solvent D also separated the *cis* isomers of Z and [9*R*]Z from Z and [9*R*]Z respectively, the R_f values being: Z, 0.40; DZ, 0.45; *cis*Z, 0.46; [9*R*]Z, 0.50; *cis*[9*R*]Z, 0.55; [9*R*]DZ, 0.56.

3. Discussion

NP and RP TLC with silica layers have been used extensively for identification of metabolites of radiolabelled cytokinins and complement HPLC procedures. All known natural cytokinins can be separated by a combination of NP and RP TLC on silica gel. However, these TLC methods have one major unsatisfactory feature: eluates of silica TLC zones prepared with the polar solvents necessary for recovery of most cytokinins contain colloidal silica which can interfere in mass spectrometry, some bioassay methods and in subsequent HPLC. Removal of silica with a C_{18} cartridge is often necessary for effective use of these procedures. To avoid the above problem, and to provide a system of separation different from those obtained with silica gel, separation of cytokinins on cellulose was studied systematically and the methods reported herein were developed. The cellulose and silica gel TLC procedures are particularly useful in dealing with crude extracts which readily foul HPLC columns.

In *Petunia* corollas, [^{3}H]–[9*R*]Z was metabolized principally to adenosine and AMP, indicating active degradation by cytokinin oxidase, whereas *O*-glucosides and [7G]DZ were the principal metabolites of DZ and [9*R*]DZ. Formation of both *O*- and *N*-glucosides as major metabolites in a plant tissue is unusual. Ethylene has been reported to promote catabolism and conjugation of the auxin IAA (Sagee, Riov, & Goren, 1990) and the present studies with *Petunia* corollas show that ethylene can also promote these aspects of cytokinin metabolism previously known to be affected by exogenous auxin (Zhang, Letham, Wong, Nooden, & Parker, 1987; Zhang et al., 1995). When *S*-DZ was supplied to mature *Petunia* corolla through the transpiration stream, ethylene promoted its conversion to the *O*-glucosides, (OG)DZ and (OG)[9*R*]DZ. Ethylene also promoted the degradation of [9*R*]Z to adenosine and AMP. This effect of ethylene on glucoside formation and [9*R*]Z metabolism appears to be reflected in the changes in endogenous [9*R*]Z and *O*-glucoside levels associated with onset of senescence. The present studies reveal a diversity of cytokinins in *Petunia* corollas and a similar situation appears to exist in carnation petals (Van Staden, Featonby-Smith, Mayak, Spiegelstein, & Halevy, 1987). Unexpectedly, in both studies, iP-type cytokinins appeared to be present. However, this identification should be regarded as tentative because benzyladenine-type cytokinins, now known to occur in plants (Nandi, Letham, Palni, Wong, & Summons, 1989; Jones, Martinkova, Strnad, & Hanke, 1996), have chromatographic properties similar to those of iP-type cytokinins and cross-react with antibodies raised against [9*R*]iP (Badenoch-Jones, Parker, & Letham, 1987).

The limited studies of changes in cytokinin levels in flowers associated with their senescence do not provide a consistent picture. Thus, in carnation petals, the levels of Z and/or DZ and their ribosides declined, then rose and finally fell again (Van Staden, & Dimalla, 1980), while activity resembling glucosides declined throughout the period of senescence (Van Staden, & Dimalla, 1980). In contrast, in rose petals, onset of senescence was associated with a rise in cyto-

kinin levels (Mayak, Halevy, & Katz, 1972). In the present study, the total cytokinin level of petunia corollas exhibited a small increase prior to onset of senescence (Table 1). Hence the proposal that decline in endogenous cytokinin levels serves as a trigger for flower senescence initiated by ethylene production (Eisinger, 1977) is not supported by the present studies.

Based on the present studies, it is suggested that the ethylene production that triggers *Petunia* corolla senescence also promotes metabolism of senescence-retarding cytokinin bases and ribosides which reach the mature corolla by transpiration. The observed promotion of formation of *O*-glucosides such as (OG)[9*R*]DZ and (OG)DZ and of degradation of [9*R*]Z to adenosine and AMP would reduce cytokinin activity and facilitate the senescence process.

4. Experimental

4.1. Corolla senescence assays

Complete corollas with stamen attached were plucked from *Petunia* flowers (cv. Blue Spinnaker; nearly fully open but pre-pollination, anthers closed) leaving the ovary and calyx intact on the plant. The stigma plus style, although unattached, were included with each corolla. The base of each corolla explant was immediately placed in either water or cytokinin soln (15 ml) in a 20 ml glass scintillation vial. After 5–6 d at 23°C under laboratory light (5 μE m^{-2} s^{-1}), senescence was recorded. In further experiments, after uptake of water or cytokinin solution by the explants for one day, a senescence-inducing concentration of the ethylene-releasing compound ethrel was injected into each vial (final concentration 0.7 mM). After a further 4–5 d, senescence was assessed.

4.2. Radioactive cytokinins

Radioactive cytokinins used in metabolism studies were all labelled with ^{3}H at C-8 of the purine ring and were as follows: *S*(−)-dihydrozeatin, *S*-DZ, 407 TBq mol^{-1}; *R*,*S*(±)-dihydrozeatin riboside, [9*R*]DZ, 136 TBq mol^{-1}; *R*,*S*-*O*-glucosyldihydrozeatin, (OG)DZ, 400 TBq mol^{-1}; zeatin riboside, [9*R*]Z, 107 TBq mol^{-1}. All compounds were synthesized by methods described previously (Zhang, & Letham, 1990); radiochemical purity was >98%.

4.3. Application of ^{3}H-labelled cytokinins to flowers

Petunia flowers (cv. Blue Spinnaker or Old Glory Blue) were used on the day when the corolla had fully opened and ^{3}H-labelled cytokinin was applied in one of three ways. (a) *Via a wick*. A cotton thread was inserted through the peduncle 5 mm below the flower and each end was inserted into a small vial containing 0.5 ml of [^{3}H]–[9*R*]Z soln (0.5 μM). After 3–4 h, all the soln was taken up and 18 h later the flowers were dissected and extracted. (b) *Through the bases of the excised corollas by transpiration*. Corollas with attached stamen were plucked from the receptacles and their bases were placed in [^{3}H]–[9*R*]Z soln (0.12 μM) or in [^{3}H]-*S*-DZ soln (0.12 μM); the solns (pH 6.0) also contained the following nutrients (mM): KH_2PO_4 3.0; $MgCl_2{\cdot}6H_2O$ 1.0; $CaSO_4{\cdot}2H_2O$ 2.0; glutamine 3.0; and asparagine 2.9. After 18 h, the corolla bases which contacted the cytokinin soln and the stamen were discarded and the remaining corolla tissue was extracted. However, it was later found that the nutrients in the uptake soln had a negligible influence on the short term studies of cytokinin metabolism. Hence, to assess the effect of ethylene on cytokinin metabolism, the corolla bases were placed in ethrel soln (1.4 mM) or in water (control) for 24 or 48 h, and then transferred to ^{3}H-labelled [9*R*]Z or *S*-DZ solns (0.8 μM). After uptake of the cytokinins for 24 h, the corolla tissue was extracted (corolla bases in contact with the cytokinin soln and stamen discarded). During uptake of solns, all excised corollas were kept at 20°C under weak light (5 μE m^{-2} s^{-1}). (c) *By painting*. A soln of each ^{3}H-labelled cytokinin (*S*-DZ, *R*,*S*-[9*R*]DZ, *R*,*S*-(OG)DZ) in Tween 80 (0.04% w/v) was distributed with a very small paint brush over the upper surfaces of corollas of 10 flowers attached to the plants; each corolla received 1.7 kBq of ^{3}H. After 4 or 5 d, the flower bearing shoots were excised and dissected for extraction. Corollas were immersed in 0.04% (w/v) Tween 80 and then water (20 s for each) prior to extraction.

4.4. TLC

For all normal phase (NP) TLC, layers were spread with PF_{254} silica gel 60 (E. Merck, Darmstadt, Germany) or microcrystalline cellulose (Sigmacell type 20, 20 μm particle size, Sigma Chemical Co.). The latter was mixed with a fluorescent indicator F_{254} (0.6%, Merck) and the slurry was blended, sonicated briefly and then placed under vacuum (to remove air bubbles) prior to spreading of the layers (thickness 0.5 mm). For some purposes (e.g. direct probe mass spectrometry or bioassay of eluates), the dried layers should be washed by allowing eluting solvent to run to the top of the layers in a chromatography tank. Silica gel 60 GF_{254} (15 μm particle size, Merck) was used to prepare layers (0.3 mm) for impregnation with silicone fluid (Letham, Singh, & Willcocks, 1992) for reversed phase (RP) TLC. Solvents for NP TLC were (proportions are by vol): A, *n*-BuOH–H_2O–HOAc (12:5:3);

B, *n*-BuOH–H_2O–14 M NH_4OH (6:2:1, upper phase); C, *n*-BuOH–H_2O–14 M NH_4OH–PrOH–EtOH (6:3:1:1:1); D, MeCOEt–H_2O–14 M NH_4OH–PrOH (250:25:25:28). Solvents for RP TLC were: E, MeOH–H_2O (1:4); F, MeOH–H_2O (1:9) containing ammonia (0.7 M); G, MeOH–H_2O (1:9).

4.5. Identification of ^{3}H-labelled cytokinin metabolites

All tissue samples were extracted with MeOH–H_2O–HCO_2H (15:4:1 by vol.) as described previously (Singh et al., 1988). Blue pigment was removed from evaporated corolla extracts by passage of an aq soln (pH 3) through a column of polyvinylpyrrolidone (Wang et al., 1995). All extracts with added cytokinin markers were chromatographed initially by 1D NP TLC on silica gel layers using solvent B (for R_f data see Palni, Palmer, & Letham, 1984) yielding zones corresponding to (a) Z + DZ, (b) [9*R*]Z + [9*R*]DZ, (c) adenine, (d) adenosine, (e) glucosides of Z, DZ and their ribosides, and (f) nucleotides (zone at and just above the origin). For a more precise analysis, the zones were eluted (EtOH–H_2O–HOAc, 50:50:3) for further chromatography and evaporated eluates from (a), (b) and (e) were then dissolved in HOAc (0.05 M) and cytokinins were purified using SPE C_{18} columns (Wang et al., 1995). Further chromatography of fractions was as follows: (a), (b), (c) and (d) — NP TLC (silica, solvent A; cellulose, solvent C) and RP TLC (solvent E); (e) — RP TLC (solvents E and F). Eluate (e) was also treated with *β*-glucosidase (Sigma; 0.2 mg ml^{-1} in 0.05 M pyridine, pH to 5.4 with HOAc) which hydrolyzes *O*-, but not 7- and 9-, glucosides of cytokinins. The hydrolyzates were subjected to NP TLC (silica, solvent A) and RP TLC (solvents E and G) to determine ^{3}H in Z, DZ, [9*R*]Z, [9*R*]DZ, 7- and 9-glucosides. The identification of [7G]DZ as a metabolite of [^{3}H]DZ was confirmed by degradation (periodate oxidation followed by cleavage with cyclohexylamine (Letham, & Gollnow, 1985)) to a ^{3}H-compound which cochromatographed with DZ. The evaporated eluate of the nucleotide zone (f) was treated with alkaline phosphatase (Singh et al., 1988) and then subjected to NP TLC (silica gel, solvent B; cellulose, solvent C) and RP TLC (solvent E) to determine ^{3}H due to particular nucleotides.

4.6. Quantification of cytokinins in corolla extracts

Corollas were extracted and the blue pigment was removed from the extracts as detailed above. Each extract was then fractionated into a basic fraction (containing cytokinin bases, ribosides and glucosides) and an anionic fraction (containing nucleotides) (Badenoch-Jones et al., 1984). The former was purified using a paraffin-impregnated silica gel column (Hall, Badenoch-Jones, Parker, Letham, & Barlow, 1987) and then subjected to NP TLC (silica gel, solvent A) with marker dyes yielding two fractions (Palni et al., 1984; Singh et al., 1988): fraction (a) containing Z, DZ, [9*R*]Z and [9*R*]DZ; fraction (b) containing cytokinin glucosides. The cytokinins in these fractions were further purified with C_{18} SPE columns (Wang et al., 1995) and the eluted fraction from (a) was separated into a base (Z + DZ) and a riboside ([9*R*]Z + [9*R*]DZ) fraction with a column of boronate gel (Badenoch-Jones, Parker, Letham, & Singh, 1996). The eluted fraction from (b) was hydrolyzed with β-glucosidase (Duke, Letham, Parker, MacLeod, & Summons, 1979) and the products were extracted into *n*-BuOH and then separated into a base and riboside fraction with a boronate gel column. The nucleotide fraction (above) was treated with alkaline phosphatase (Singh et al., 1988), extracted with *n*-BuOH, and the released ribosides purified using C_{18} SPE and boronate columns. Cytokinins in the above purified fractions were quantified by RIA (Badenoch-Jones et al., 1987) and all values were corrected for losses during purification with ^{3}H-labelled cytokinins (Singh et al., 1988). After further purification of the base and riboside fractions derived from (a) above by HPLC (Badenoch-Jones et al., 1984), values for Z, DZ and their ribosides were redetermined by RIA. The *Amaranthus* betacyanin bioassay (Biddington, & Thomas, 1973) was used to determine cytokinin activity in chromatogram zones after cellulose TLC of corolla extract fractions. The zones were eluted with 50% EtOH for bioassay.

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