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Localization of Cytokinin Biosynthetic Sites in Pea Plants and Carrot Roots[1]

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ABSTRACT

The biosynthesis of cytokinins was examined in pea (*Pisum sativum* L.) plant organs and carrot (*Daucus carota* L.) root tissues. When pea roots, stems, and leaves were grown separately for three weeks on a culture medium containing [8-^{14}C]adenine without an exogenous supply of cytokinin and auxin, radioactive cytokinins were synthesized by each of these organs. Incubation of carrot root cambium and noncambium tissues for three days in a liquid culture medium containing [8-^{14}C]adenine without cytokinin demonstrates that radioactive cytokinins were synthesized in the cambium but not in the noncambium tissue preparation. The radioactive cytokinins extracted from each of these tissues were analyzed by Sephadex LH-20 columns, reverse phase high pressure liquid chromatography, paper chromatography in various solvent systems, and paper electrophoresis. The main species of cytokinins detectable by these methods are N^6-(Δ^2-isopentyl)adenine-5′-monophosphate, 6-(4-hydroxy-3-methyl-2-butenyl-amino)-9-β-ribofuranosylpurine-5′-monophosphate, N^6-(Δ^2-isopentenyl)adenosine, 6-(4-hydroxy-3-methyl-2-butenylamino)-9-β-ribofuranosylpurine, N^6-(Δ^2-isopentenyl)adenine, and 6-(4-hydroxy-3-methyl-2-butenylamino)purine. On the basis of the amounts of cytokinin synthesized per gram fresh tissues, these results indicate that the root is the major site, but not the only site, of cytokinin biosynthesis. Furthermore, cambium and possibly all actively dividing tissues are responsible for the synthesis of this group of plant hormones.

There is evidence that cytokinins exist in the roots (15, 16) and that cytokinins from xylem exudate may come from the roots (9, 17). This evidence has been reviewed by Torrey (18). However, there is little experimental data supporting the widely accepted notion that cytokinin biosynthesis is limited to the root site. We have previously demonstrated that cultured rootless tobacco plants supplied with [8-^{14}C]Ade[2] are capable of synthesizing radioactive cytokinins (5), but the question of whether stem and/or leaf, cambium and/or noncambium serve as sites for cytokinin biosynthesis remains to be answered.

This paper reports the biosynthesis of radioactive cytokinins from [8-^{14}C]Ade in the pea roots, stems, and leaves as well as in the cambium of carrot roots. Noncambium carrot root tissues do not serve as a site for cytokinin biosynthesis.

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[2] Abbreviations: Ade, adenine; Ado, adenosine; i^{6}Ade, N^6-(Δ^2-isopentenyl)adenine; i^{6}Ado, N^6-(Δ^2-isopentenyl)adenosine; io^{6}Ade, 6-(4-hydroxy-3-methyl-2-butenylamino) purine; io^{6}Ado, 6-(4-hydroxy-3-methyl-2-butenylamino)-9-β-ribofuranosylpurine.

MATERIALS AND METHODS

Plant Materials and Chemicals. Pea (*Pisum sativum* L.) seeds were purchased from Northrup King Seeds Co. (Minneapolis). Fresh carrots (*Daucus carota* L.) were obtained from a local market. [8-^{14}C]Ade (50 mCi/mmol) and [8-^{14}C]Ado (53 mCi/mmol) were from Schwarz/Mann. Ribonucleosides, i^{6}Ade, i^{6}Ado, and 5′-nucleotidase (*Crotalus adamanteus* venom) were from Sigma Chemical Co.; i^{6}AMP was purchased from P-L Biochemical Co.; *trans*-io^{6}Ade and *trans*-io^{6}Ado were from Calbiochem. A cytokinin nucleotide, *trans*-io^{6}AMP, was a generous gift from R. Horgan (University College of Wales, UK).

Cultures of Pea Plants and Organs. Pea seeds (30 seeds) were imbibed for 16 h with running tap water, and surface-sterilized for 10 min with 0.5% NaOCl. The seeds were thoroughly rinsed with sterile distilled H_2O, and three seeds were grown on 50 ml of a basic culture medium (8) in a flask. After 21 d of growth, the pea plants were divided into leaves, stems (including apical meristem), and roots under aseptic conditions, and each organ was cultured separately on 100 ml of the basic medium containing filter-sterilized [8-^{14}C]Ade (2.2 μCi/flask). Intact pea plants were also transferred to the [8-^{14}C]Ade containing 100 ml of basic medium.

Isolation and Cultivation of Cambium and Noncambium Carrot Root Tissues. Fresh carrot roots were cleaned in a 1% Alconox detergent solution, washed with water and 50% ethanol. After peeling the surface, the roots were soaked in a 0.5% NaOCl solution for 10 min, rinsed with sterilized H_2O five times, and the cambium tissue was separated from the noncambium tissue under aseptic conditions. The tissues were incubated in 100 ml of a liquid culture medium (8) containing 10 μCi of [8-^{14}C]Ade (0.9 μCi/g tissue) at room temperature for 3 d in a gyrotary shaker.

Extraction of Cytokinins. Two extraction methods were compared for their efficiency in recovering various exogenous cytokinins from plant tissues. In method A, cultured tobacco pith tissue (20 g) was frozen with liquid N_2 and ground to a powder. A cytokinin (10 O.D. units), [8-^{14}C]Ade, or [8-^{14}C]Ado was then added to the powder, and the mixture was extracted with 95% ethanol, followed by 50% ethylacetate and 60% ethanol (10 ml of each solvent/g fresh tissue). In method B, each cytokinin was mixed with tissue powder as in method A, and the mixture was extracted once or twice with a solvent system consisting of methanol:chloroform:formic acid:water (60:25:5:10) (1). In control experiments, identical extraction procedures were employed except that no cytokinin or radioactive compound was added to the tissue powders.

Analytical Methods. Radioactivity was measured in a Tracor Analytic Mark III liquid scintillation system. For liquid samples, an aliquot of no more than 0.1 ml was added to 10 ml of Bray's solution (2). For paper chromatograms, 1 × 2 cm sections were placed in vials containing scintillation fluid (6).

Biosynthesized cytokinins were partially characterized by

Sephadex LH-20 columns (2.5 × 32 cm) which were equilibrated and eluted with 35% ethanol HPLC (4), and paper chromatography in various solvent systems. Paper chromatography (Whatman No. 1MM) was carried out in a descending fashion in the following solvent systems (v/v): (a) 1-propanol:concentrated NH_4OH:H_2O (60:20:20); (B) ethylacetate:1-propanol:H_2O (4:1:2); (c) 2-propanol:H_2O:concentrated NH_4OH (7:2:1). The techniques used for paper electrophoresis were described previously (5).

RESULTS

Recovery of Cytokinins from Tissues. Cytokinins exist in plant tissues as free bases, ribonucleosides, ribonucleotides, and glucosides (11). The quality and quantity of these cytokinins isolated from tissues depend upon the extraction solvent system used. Two extraction solvent systems (methods A and B) were compared for the recovery of cytokinins added to plant tissues. Cytokinins extracted either by method A or by method B were separated on a Sephadex LH-20 column (2.5 × 32 cm), and the relative mobilities of fractionated A_{254} absorption samples were compared with the relative mobilities of authentic cytokinins. Each presumed cytokinin sample was collected and analyzed by UV absorption spectra at pH 2, 7, and 12. Backgrounds in the peak areas, measured from the control sample, were subtracted.

Recovery of various cytokinins, radioactive Ade and Ado from the tissues is shown in Table I. These results show that extraction method A recovered a greater amount of cytokinin than method B. Repeated extraction of the tissues with method A or extraction of tissue with method A followed by method B, did not significantly increase the recovery of cytokinins. (Only about 0.3–0.6% of additional cytokinins were recovered). On the other hand, method B requires two extractions to recover cytokinin nucleotides, and only about 2% of i^6Ade and 15% of i^6Ado added to the tissues were recovered by this method. These extraction methods estimate only the recovery of cytokinins from tissues; they do not truly represent the effectiveness of extracting internal cytokinins from tissues.

Table I. *Amounts of Cytokinin and Other Compounds Recovered by Two Extraction Methods*

In method A, a cytokinin or related compound was mixed with frozen tissue powder, and the powder was extracted with 95% ethanol, followed by 50% ethylacetate and 60% ethanol. In method B, cytokinin in tissue powder was extracted once or twice with a solvent system consisting of methanol:chloroform:formic acid:water (60:25:5:10). The recovered cytokinins were separated and characterized by Sephadex LH-20 columns, HPLC, and UV absorption spectra. Per cents are means ± SE of three experiments.

Compound	Method A	Method B	
		One extraction	Two extractions
		% recovery	
[8-^{14}C]Ade	67 ± 5	60 ± 4	60 ± 5
[8-^{14}C]Ado	66 ± 7	53 ± 5	57 ± 5
i^6Ade	63 ± 6	2 ± 1	2 ± 1
i^6Ado	84 ± 8	15 ± 3	15 ± 3
AMP	82 ± 7	65 ± 6	77 ± 6
i^6AMP	68 ± 5	25 ± 3	39 ± 4
i^6ADP	77 ± 6	19 ± 3	55 ± 4
ATP	65 ± 6	24 ± 4	83 ± 7

Isolation of Biosynthesized Cytokinins. Intact pea plants or individual pea organs were cultured on a medium containing [8-^{14}C]Ade for 21 d. Uptake of radioactive Ade by the organs from the medium ranged from 4 to 8.6% depending upon the cultured organs (Table II). Roots took up 16 to 45% and 51 to 115% more radioactive material than stems and leaves, respectively. After 21 d of growth, about 2.2% of [8-^{14}C]Ade was taken up from the medium by the intact plants. The amounts of radioactive Ade taken up by the cultured tissue are rough estimates, since radioactive Ade may be incorporated into fractions that were not extracted by the extraction method. These intact plants (57 plants, average fresh weight 1.19 g/plant) were divided into roots (average 0.39 g/plant), stems (average 0.52 g/plant), and leaves (average 0.28 g/plant).

Radioactive materials in the pea organs were extracted with 95% ethanol followed by 50% ethylacetate and 60% ethanol. The ethanol-ethylacetate soluble fractions were combined, reduced to less than 2 ml in a flash evaporator (40°C), and the final volume was adjusted to 2 ml with 35% ethanol. Aliquots of the extracts were removed and radioactivity was determined using Bray's solution (2).

Biosynthesized radioactive cytokinins were initially fractionated by a Sephadex LH-20 column which was equilibrated and eluted with 35% ethanol. Figure 1 shows a separation pattern of the radioactive materials extracted from leaves, stems, and roots. The results were compared with the relative mobilities of authentic samples. The seven pooled fractions (Fig. 1, pool I–VII) from Sephadex LH-20 column were further separated by HPLC. Peak fractions of HPLC were collected and radioactivity counted.

Identification of Cytokinins. Isolated radioactive cytokinins were partially characterized by Sephadex LH-20 columns, HPLC (Table III), and paper chromatography in three solvent systems. Cytokinin nucleotides, i^6AMP and io^6AMP, were further analyzed by paper electrophoresis with authentic compounds as markers. Both of these nucleotides had relative mobility of +1.5 to +1.7, with relative mobility of inosine as −1.0. The suspected i^6AMP and io^6AMP collected from HPLC fractionations were also digested with 5′-nucleotidase of *C. adamanteus* venom in 0.05M Tris-HCl buffer (pH 7.5) at 37°C for 30 min, and the reaction products were separated by paper electrophoresis. After the treatment, the presumed radioactive i^6Ado sample migrated with unlabeled i^6Ado, and the radioactive io^6Ado sample moved with unlabeled io^6Ado. These results also indicate that the bio-

Table II. *Uptake of [8-^{14}C]Ade by Pea Organs*

Pea organs were cultured on a medium containing [8-^{14}C]Ade (4.88×10^6 dpm) for 21 d and fresh weight upon harvesting was measured. Radioactive materials in the tissues were extracted with 95% ethanol, followed by 50% ethylacetate and 60% ethanol.

Organ	Fresh Wt		Extractable Radioactive Materials in the Organs		Extractable Radioactivity Taken up from the Media	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2	Experiment 1	Experiment 2
	g		*dpm × 10^{-5}*		*%*	
Roots	27.8	22.8	3.84	2.88	8.6	6.5
Stems	26.6	20.9	2.63	2.48	5.9	5.6
Leaves	19.8	18.7	1.79	1.91	4.0	4.3

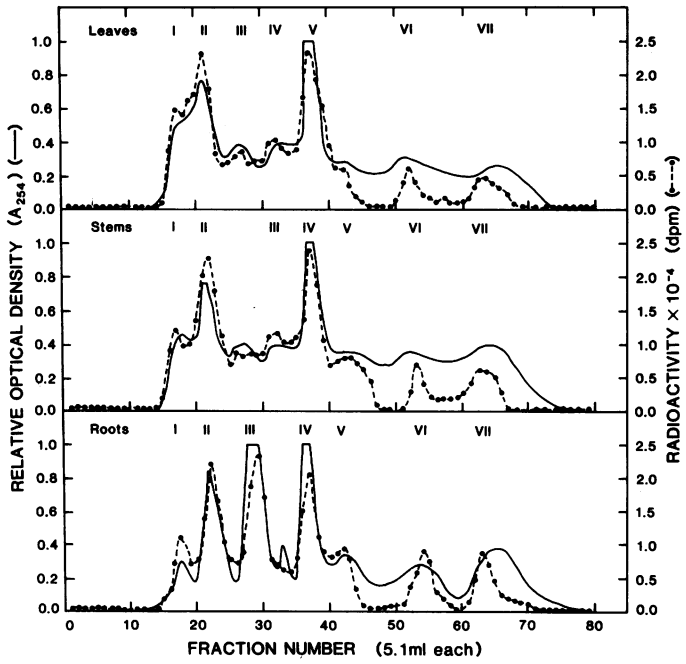

FIG. 1. Elution profile of radioactive materials extracted from cultured pea organs on Sephadex LH-20 column. Pea organs, grown on a medium containing [8-^{14}C]Ade for 21 d, were extracted with 95% ethanol, followed by 50% ethylacetate and 60% ethanol. The extracted material was applied onto a column (2.5 × 32 cm) which was equilibrated and eluted with 35% ethanol.

Table III. *HPLC Retention Times and Sephadex LH-20 Column Mobilities of Cytokinins and Related Compounds*

HPLC was performed on a Waters Associates ALC-200 liquid chromatograph using a Li Chrosorb (RP-18, 10 μm) column (25 cm × 4.6 mm). The mobile phase consisted of a gradient of (A) 4% aqueous acetic acid and (B) distilled 100% methanol. Cytokinins were eluted with a linear gradient of increasing methanol concentration (0–100%) at 2 ml/min over 20 min.

Compound	HPLC			Sephadex LH-20 Column
	Retention time	Retention time	Eluted by methanol	Relative mobility
	min	*relative*	%	*ratio*
Ade	7.4	1.00	37	1.36
Ado	5.0	0.68	25	1.20
AMP	2.8	0.39	14	0.61
i^{6}Ade	14.4	1.95	72	1.89
i^{6}Ado	12.4	1.68	62	1.70
i^{6}AMP	2.2	0.30	11	0.53
io^{6}Ade	9.2	1.24	46	1.30
io^{6}Ado	5.2	0.70	26	1.15
io^{6}AMP	1.8	0.24	9	0.42

synthesized cytokinin nucleotides are 5′-monophosphate.

Since there were not sufficient quantity of purified, biosynthesized radioactive cytokinins for mass spectrometric identification, the biosynthesized cytokinin nucleosides, i^{6}Ado and io^{6}Ado, were also analyzed by chemical reactions unique to these compounds. The presumed radioactive io^{6}Ado was mixed with three A_{269} units of unlabeled io^{6}Ado and the mixture was oxidized with 1 M $KMnO_4$ in a sealed tube at 100°C for 30 min. The reaction products were chromatographed on Whatman No. 1MM paper in solvent C. Three radioactive spots comigrating with three oxidation products (R_f values: 0.14, 0.18 and 0.55) of unlabeled io^{6}Ado were detected. Similarily, the putative radioactive i^{6}Ado sample was mixed with 3 A_{269} units of unlabeled i^{6}Ado and hydrolyzed with HCl (14), the hydrolysate was chromatographed on Whatman No. 1MM paper in solvent B. Two major radioactive spots migrated together with two UV-detectable hydrolytic products. These results demonstrate that these radioactive samples are indeed io^{6}Ado and i^{6}Ado.

Distribution of Biosynthesized Cytokinins in Pea Organs. Distribution of identifiable radioactive cytokinins in the cultured intact pea plants and individual organs is shown in Table IV. The identifiable cytokinins (pmol/g fresh tissue) isolated from the intact plants are: root, 8.8 to 11.2; stem, 6.4 to 8.4; and leaves, 5.0 to 7.1. In the cultured pea organs, the contents of identifiable cytokinins (pmol/g fresh tissue) are roots, 12.0 to 14.1; stem, 7.1 to 8.9; and leaves, 2.4 to 3.4. On the basis of the amounts of cytokinins synthesized per gram fresh tissues, these results indicate that the root is the major site, but not the only site for cytokinin biosynthesis. The values for the biosynthesized cytokinins are minimal since the dilution factor resulting from endogenously synthesized cytokinins is unknown and some cytokinins, such as cytokinin glucosides, are not identified.

Biosynthesis of Cytokinins in Cambium and Noncambium Tissues. Experiments were designed to study whether cambium and/or noncambium tissues serve as cytokinin biosynthetic sites. Carrot root cambium can be separated from noncambium cells by their morphological differences. In a cross-section, the cambium consists of small, thin-walled cells which form a ring around the xylem, and upon inoculation of *Agrobacterium rhizogens*, hairy roots are formed in the area of cambium ring. Carrot cambium cells (48.8–51.5 g each experiment) were separated from noncambium cells (49.2–51.3 g each experiment) under aseptic conditions, and to ensure noncambium cells containing no cambium, those cells in close contact with the cambium ring were discarded. Cambium and noncambium cells were incubated separately in a liquid culture medium (6) without cytokinin but containing [8-^{14}C]Ade for 3 d. Radioactive cytokinins were extracted sequentially with 95% ethanol, 50% ethylacetate, and 60% ethanol (10 ml of each solvent/g tissue). Extracted cytokinins were separated and characterized by Sephadex LH-20 columns, HPLC (Table III), paper chromatography in solvent systems A to C, and paper electrophoresis.

Results from three repeated experiments indicate that no radioactive cytokinin was detected in the noncambium tissues after 3 d of incubation. Under the identical experimental conditions, six species of identifiable radioactive cytokinins were isolated from the cultured cambium cells. These cytokinins are estimated to be (pmol/g tissue): i^{6}Ade (0.3–0.5), i^{6}Ado (0.3–0.6), io^{6}Ade (2.1–2.7), io^{6}Ado (1.8–2.2), i^{6}AMP (2.8–3.4), and io^{6}AMP (3.7–4.3). Cytokinin glucosides and dihydrozeatin may be also present, but were not identified. The amounts of cytokinins synthesized under *in vitro* conditions may not reflect the true ability of tissues to synthesize cytokinins under *in vivo* conditions. Nevertheless, these results indicate that cambium cells are capable of synthesizing cytokinins, and it is possible that all meristematic cells are capable of cytokinin biosynthesis.

DISCUSSION

The results reported here show that cytokinins are synthesized in the pea stems, leaves, and roots, and that the cambium tissues but not noncambium tissue is the site for cytokinin synthesis in carrot roots. Although no experiments were performed to prove that shoot cambium and meristem also synthesize cytokinins, it is reasonable to assume that all cambium and meristemetic tissues are likely the sites for cytokinin biosynthesis. Environmental factors and length of plant growth appear to alter cytokinin levels in plant tissues (10); furthermore, plant tissues con-

Table IV. *Distribution of Identifiable Radioactive Cytokinins in Cultured Intact Pea Plants and Individual Organs*

Compound	Intact Plants			Individual Organs		
	Roots	Stems	Leaves	Roots	Stems	Leaves
	pmol/g fresh wt			*pmol/g fresh wt*		
io^{6}AMP	2.4–2.8	1.2–1.5	0.2–0.4	3.8–4.2	1.9–2.3	0.2–0.3
io^{6}Ado	1.4–1.8	1.0–1.4	0.8–1.2	1.8–2.2	0.8–1.1	0.5–0.7
io^{6}Ade	1.6–2.0	1.1–1.5	1.2–1.6	1.5–1.8	1.4–1.7	0.7–1.0
i^{6}AMP	1.8–2.3	1.3–1.6	0.4–0.7	3.2–3.7	1.6–1.9	0.3–0.4
i^{6}Ado	0.8–1.1	0.5–0.8	1.1–1.5	0.6–0.8	0.3–0.5	0.3–0.4
i^{6}Ade	0.8–1.2	1.3–1.6	1.3–1.7	1.1–1.4	1.1–1.4	0.4–0.6
Total identifiable cytokinins	8.8–11.2	6.4–8.4	5.0–7.1	12.0–14.1	7.1–8.9	2.4–3.4

tain enzymes to interconvert cytokinin bases, nucleosides, and nucelotides (3), thus the amounts of cytokinins in the tissues reported here are not absolute. Cytokinin biosynthesis in explants is not necessarily the same as in whole plants; however, it is likely a valid measure of biosynthetic potential.

Since the main objective of this investigation is to show that stems and leaves also synthesize cytokinins, no effort was made to identify a group of cytokinin derivatives, the cytokinin glucosides, which have been reported to accumulate in various plant tissues (7, 11–13, 15). The quality and quantity of cytokinins extracted from plant tissues depend upon the solvent system used in recovering these cytokinins. Although reasons are unknown, extraction method A is shown to recover more cytokinins than method B (Table I), for both of these extraction methods contain a mixture of different solvents which have medium to high dielectric constants. All extraction procedures are performed at low temperature to minimize enzymic or chemical degradation; hence, isolated cytokinin bases and ribosides are not likely to be the degradation products of corresponding nucleotides during extraction. Although the biosynthesized cytokinins were not characterized by MS, analyses of the biosynthesized radioactive cytokinins by various analytical techniques clearly demonstrated that cytokinins are synthesized in pea stems and leaves in addition to the reported root site.

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LITERATURE CITED

1. Bieleski RL 1964 The problem of halting enzyme action when extracting plant tissues. Anal Biochem 9: 431–442
2. Bray G 1960 A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Anal Biochem 1: 279–285
3. Chen C-M 1981 Biosynthesis and enzymic regulation of the interconversion of cytokinin. *In* J Guern, C Peud-Lenoel, eds, Metabolism and Molecular Activities of Cytokinins. Springer-Verlag, New York, pp 34–43
4. Chen C-M, SM Leisner 1984 Modification of cytokinins by cauliflower microsomal enzymes. Plant Physiol 75: 442–446
5. Chen C-M, B Petschow 1978 Cytokinin biosynthesis in cultured rootless tobacco plants. Plant Physiol 62: 861–865
6. Chen C-M, OC Smith, JD McChesney 1975 Biosynthesis and cytokinin activity of 8-hydroxy and 2,8-dihydroxy derivatives of zeatin and N^6-(Δ^2-isopentenyl)-adenine. Biochemistry 14: 3088–3093
7. Fox JE, J Cornette, G Deleuze, W Dyson, C Ctiersak, P Niu, J Zapata, JD McChesney 1973 The formation, isolation and biological activity of a cytokinin 7-glucoside. Plant Physiol 52: 627–632
8. Gamborg OL, RA Miller, K Ojima 1968 Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50: 151–158
9. Kende H, D Sitton 1967 The physiological significance of kinetin and gibberellin-like root hormones. Ann NY Acad Sci 144: 235–243
10. Letham DS 1978 Cytokinins. *In* DS Letham, PB Goodwin, TJV Higgins, eds, Phytohormones and Related Compounds—A Comprehensive Treatise. Elsevier/North-Holland, Amsterdam, pp 205–263
11. Letham DS, LMS Palni 1983 The biosynthesis and metabolism of cytokinins. Annu Rev Plant Physiol 34: 163–197
12. Morris RO 1977 Mass spectroscopic identification of cytokinins. Plant Physiol 59: 1029–1033
13. Peterson JB, CO Miller 1977 Glucosylzeatin and glucosyl ribosyl-zeatin from *Vinca rosea* L. crown gall tumor tissue. Plant Physiol 59: 1026–1028
14. Robins MJ, RH Hall, R Thedford 1967 N^6-(Δ^2-isopentenyl)adenosine. A component of the transfer ribonucleic acid of yeast and of mammalian tissue, method of isolation, and characterization. Biochemistry 6: 1837–1848
15. Scott IM, R Horgan 1984 Root cytokinins of *Phaseolus vulgaris* L. Plant Sci Lett 34: 81–87
16. Short TC, JG Torrey 1972 Cytokinins in seedling roots of pea. Plant Physiol 49: 155–160
17. Skene KGM 1968 Increase in the level of cytokinins in the bleeding sap of *Vitis vinifera* L. after C C C treatment. Science 159: 1477–1478
18. Torrey JG 1976 Root hormones and plant growth. Annu Rev Plant Physiol 27: 435–459