Plant Physiol. (1983) 73, 796–802
0032-0889/83/73/0796/07/$00.50/0

Effects of Thidiazuron on Cytokinin Autonomy and the Metabolism of N^6-(Δ^2-Isopentenyl)[8-^{14}C]Adenosine in Callus Tissues of *Phaseolus lunatus* L.[1]

Received for publication May 16, 1983 and in revised form July 22, 1983

SUSAN C. CAPELLE, DAVID W. S. MOK, SANDRA C. KIRCHNER[2], AND MACHTELD C. MOK
Department of Horticulture, Oregon State University, Corvallis, Oregon 97331

ABSTRACT

The effects of a highly cytokinin-active urea derivative, *N*-phenyl-*N'*-1,2,3-thiadiazol-5-ylurea (Thidiazuron), and zeatin on cytokinin-autonomous growth and the metabolism of N^6-(Δ^2-isopentenyl)[8-^{14}C]adenosine ([^{14}C]i^{6}Ado) were examined in callus tissues of two *Phaseolus lunatus* genotypes, cv Jackson Wonder and P.I. 260415. Tissues of cv Jackson Wonder maintained on any concentration of Thidiazuron became cytokinin autonomous, whereas only tissues exposed to suboptimal concentrations of zeatin displayed cytokinin-autonomous growth. Tissues of P.I. 260415 remained cytokinin dependent under all these conditions. The metabolism of [^{14}C]i^{6}Ado was similar for the two genotypes, but differed with the medium used. [^{14}C]i^{6}Ado was rapidly converted to N^6-(Δ^2-isopentenyl)[8-^{14}C]adenosine 5'-P ([^{14}C]i^{6}AMP) by tissues grown on zeatin-containing medium, whereas only traces of the nucleotide were formed in tissues grown on medium with Thidiazuron. Incubation with [^{14}C]i^{6}AMP of tissues grown in the presence of Thidiazuron resulted in rapid conversion to [^{14}C]i^{6}Ado, while [^{14}C]i^{6}AMP persisted in tissues maintained on zeatin. Thus, Thidiazuron appears to stimulate enzyme activity converting the ribonucleotide to ribonucleoside. Although the cytokinin-active phenylureas and adenine derivatives differ in their effects on cytokinin autonomy as well as nucleotide formation, the two types of effects do not seem to be related.

The ability of certain substituted urea compounds such as DPU[3] to replace cytokinin-active adenine derivatives has been demonstrated in callus culture as well as other bioassays (3, 4, 6, 17, 21, 22). Although DPU is a rather weakly active cytokinin, other phenylurea compounds such as the *N*-phenyl-*N'*-(4-pyridyl)urea derivatives and Thidiazuron have been shown to possess high cytokinin activity (6, 17, 22). Thus, cytokinin activity is a property of two distinct classes of compounds: N^6-substituted adenine derivatives and substituted phenylureas.

Our previous studies on the activity of DPU in *Phaseolus lunatus* callus bioassays (1, 14) indicated pronounced genotypic differences in the ability of tissues to grow on DPU-containing medium. Furthermore, tissues of genotypes responsive to DPU displayed the tendency to become cytokinin autonomous after exposure to DPU, whereas comparable tissues grown on optimal concentrations of kinetin remained cytokinin dependent. These results seem to suggest a difference in the biological action of cytokinin-active phenylurea compounds and adenine derivatives. Our recent discovery that Thidiazuron exhibits extremely high cytokinin activity in the *P. lunatus* callus system (17) provides an opportunity to further compare the biological activities of the two types of cytokinin-active compounds in tissues derived from different genotypes (*i.e.*, responsive and unresponsive to DPU). Moreover, the possibility that phenylurea derivatives affect the biosynthesis or metabolism of adenine-type cytokinins can now be tested. We report here the effects of Thidiazuron and zeatin on cytokinin-autonomous growth and the metabolism of [^{14}C]i^{6}Ado in callus tissues of two *P. lunatus* genotypes, cv Jackson Wonder and P.I. 260415.

MATERIALS AND METHODS

Plant Materials. Seeds of *Phaseolus lunatus* L. cv Jackson Wonder and P.I. 260415 were obtained from Asgrow Seed Co. and the Regional Plant Introduction Station (Washington State University, Pullman, WA), respectively.

Chemicals. Kinetin, *trans*-zeatin, *trans*-ribosylzeatin, Ado, i^{6}Ade, i^{6}Ado, 3'-nucleotidase (ryegrass), 5'-nucleotidase (*Crotalus adamanteus* venom), and Sephadex LH-20 were purchased from Sigma. i^{6}AMP, i^{6}ADP, and i^{6}ATP were obtained from P-L Biochemicals. Thidiazuron and [^{14}C]Thidiazuron were gifts from Nor-Am Agricultural Chemicals and Schering AG. [8-^{14}C]Ado and 6-Cl[8-^{14}C]purine (used, respectively, for the synthesis of [^{14}C]i^{6}Ado and [^{14}C]zeatin) were obtained from Amersham Co. Picloram was a gift from Dow Chemical. Ready-Solv MP is a Beckman product.

Growth and Harvest of *Phaseolus* Callus Cultures. Callus cultures were established from hypocotyls of 5-d-old seedlings as previously described (15). Four replicate callus lines, each derived from a different seedling, were established for each genotype in each experiment. Callus tissues formed on the explants were transferred once (first passage) to medium containing 5 μM kinetin. Experiments were performed using second passage callus tissues derived from 4-week-old first passage stock cultures. Three pieces of callus (each weighing approximately 15 mg) were planted per flask. Tissues were grown in the dark at 27°C, and were harvested, weighed, and transferred to cytokinin-free medium at appropriate time intervals (as specified in "Results").

[1] Supported by the Oregon Agricultural Experiment Station and by a grant from the United States Department of Agriculture Competitive Grants Office (82-CRCR-1-1066). This is Technical Paper No. 6809 of the Oregon Agricultural Experiment Station.

[2] Present address: Department of Biology, Syracuse University, Syracuse, NY 13210.

[3] Abbreviations: DPU, *N,N'*-diphenylurea; Thidiazuron, *N*-phenyl-*N'*-1,2,3,-thiadiazol-5-ylurea; [^{14}C]Thidiazuron, *N*-[U-^{14}C]phenyl-*N'*-1,2,3-thiadiazol-5-ylurea; i^{6}Ado, N^6-(Δ^2-isopentenyl)adenosine; [^{14}C]i^{6}Ado, N^6-(Δ^2-isopentenyl) [8-^{14}C]adenosine; Ado, adenosine; i^{6}Ade, N^6-(Δ^2-isopentenyl)adenine; i^{6}AMP, N^6-(Δ^2-isopentenyl)adenosine 5'-monophosphate; i^{6}ADP, N^6-(Δ^2-isopentenyl)adenosine 5'-diphosphate; i^{6}ATP, N^6-(Δ^2-isopentenyl)adenosine 5'-triphosphate; TEA, triethylamine.

Each experiment consisted of four replicate flasks per treatment. All tests were repeated at least once.

Tissue Culture Medium. The tissue culture medium consisted of the mineral nutrients described by Murashige and Skoog (19) with the following organic substances added: sucrose (30 g/l), *myo*-inositol (100 mg/l), thiamine·HCl (1 mg/l), nicotinic acid (5 mg/l), pyridoxine·HCl (0.5 mg/l), and picloram (2.5 μM) which satisfies the auxin requirement of *Phaseolus* tissue cultures (15). Kinetin (5 μM) was included in the medium used for callus initiation (explant) and stock cultures. The pH of the medium was adjusted to 5.7 and Difco Bacto-agar (10 g/l) was added. The medium was dispensed into 125 ml Erlenmeyer flasks (50 ml/flask) and autoclaved at 120°C for 15 min. Cytokinin-active compounds (zeatin and Thidiazuron) used for experiments in the second passage were dissolved in dimethyl sulfoxide (20) and added to the autoclaved flasks before the medium solidified.

Metabolism of [^{14}C]i^{6}Ado and [^{14}C]i^{6}AMP. The metabolism of [^{14}C]i^{6}Ado was examined in second passage callus tissues of cv Jackson Wonder and P.I. 260415 grown on medium containing Thidiazuron (0.01 μM) or zeatin (0.1 and 0.3 μM, respectively, for the two genotypes). The synthesis of [^{14}C]i^{6}Ado (25 mCi/mmol) has been reported previously (18). Callus tissues were selected for the labeling studies when the average fresh weight per flask was 10 ± 1 g (21 d).[^{14}C]i^{6}Ado, 0.05 μCi (0.002 μmol) in 1 ml distilled H_20, was aseptically applied to the surface of the callus pieces in each flask. Additional growth points (5 ± 0.5 g, 17 d and 20 ± 1 g, 27 d) of cv Jackson Wonder tissue were used to determine the effects of tissue age on [^{14}C]i^{6}Ado metabolism. The same concentrations of [^{14}C]i^{6}Ado were applied to these tissues, *i.e.* 0.025 μCi (0.001 μmol) [^{14}C]i^{6}Ado in 0.5 ml distilled H_20 was applied to 5 g callus tissues, and 0.1 μCi (0.004 μmol) in 2 ml distilled H_2O to 20 g tissues. The [^{14}C]i^{6}Ado metabolism was also examined in cv Jackson Wonder tissues grown on media containing both Thidiazuron (0.001 or 0.01 μM) and zeatin (0.01 or 0.1 μM). The tissues were incubated at 27°C in the dark for 0.25, 0.5, 1, 2, and 4 h.

To extract metabolites, the tissues were homogenized in 2.5 parts (v/w) of cold 95% ethanol. Cell debris was removed by successive filtrations through Whatman paper (No. 1 filter) and Millipore filters (0.45 μm). The ethanol extract was taken to dryness *in vacuo* at 35°C, redissolved in 4 ml of 33% (v/v) ethanol, and centrifuged at 23,500*g* for 20 min. The supernatant was fractionated on a Sephadex LH-20 column (2 × 60 cm) in 33% (v/v) ethanol. Fractions of 9 ml (0.05 bed volume) were collected. Aliquots (4 ml) were taken from each fraction, evaporated to dryness in scintillation vials, dissolved in Bray's scintillation fluid (2), and counted with a Beckman LS 7000 scintillation counter. Authentic cytokinin standards were chromatographed on the same column immediately after the experimental samples. Each treatment was repeated at least once. In the case of 0.5-h time points, experiments were also repeated using a second extraction procedure involving homogenization of tissues in cold 1 N $HClO_4$ (1:1, w/v) and isolation of the cytokinin metabolites as described by Laloue *et al.* (11).

The extraction procedure with 95% cold ethanol was used to examine the metabolism of [^{14}C]i^{6}AMP in callus tissues of cv Jackson Wonder (10 g) grown on media containing 0.01 μM Thidiazuron or 0.1 μM zeatin. (The labeled compound was purified from ethanol extracts of tissues incubated with [^{14}C] i^{6}Ado by chromatography on Sephadex LH-20 and repurified by HPLC on reversed-phase C_{18} [see below].) The amount of [^{14}C] i^{6}AMP applied to each tissue was 0.02 μCi (0.008 μmol) in 1 ml H_20, and the incubation periods were 0.5, 1, and 2h. All incubation experiments were repeated at least once.

Identification of Metabolites of [^{14}C]i^{6}Ado. Two of the three major peaks of radioactivity eluting off Sephadex LH-20 correspond with the elution position of Ado and i^{6}Ado. The identity of these two peaks of radioactivity was confirmed by HPLC on reversed-phase C_{18} (see below). The peak of radioactivity eluting off Sephadex LH-20 in fractions 16 and 17 was identified in previous studies as a nucleotide of i^{6}Ado (18). To further characterize this compound, the dried fractions were redissolved in 0.05 M Tris-HCl buffer (pH 8.6) containing 5 mM $MgCl_2$, and aliquots of 300 μl were incubated with one unit of 3′-nucleotidase or 5′-nucleotidase for 0.5 h at 37°C. Ethanol (1.5 ml) was added and the solution was centrifuged at 23,500*g* for 20 min. The supernatant was taken to dryness *in vacuo* at room temperature. The residue was dissolved in 100 μl of 15% methanol and fractionated by HPLC. A Beckman model 110 dual pump HPLC system with a prepacked column of reversed-phase C_{18} (Ultrasphere ODS 5 μm, 4.6 × 250 mm; Altex) was used. The aqueous buffer consisted of 0.2 M acetic acid, adjusted to pH 3.5 with TEA. Samples were eluted with a linear gradient of methanol (15–70% over 55 min) in TEA buffer at a flow rate of 1 ml/min. Fractions of 1 ml were collected and counted in Ready-Solv MP scintillation fluid with a Beckman LS 7000 scintillation counter.

To determine whether the nucleotide was a mono-, di-, or triphosphate, paired-ion reversed-phase HPLC was used (8). The HPLC system was the same as described above. The buffer consisted of 0.3% (w/v) tetrabutylammonium phosphate, 0.65% (w/v) KH_2PO_4, and was adjusted to pH 5.8 with NH_4OH. Acetonitrile was used as the organic phase. The sample was applied in 100 μl buffer with 20% acetonitrile and eluted with a linear gradient of acetonitrile (20–60% over 20 min) in buffer at a flow rate of 1.5 ml/min. Fractions of 1 ml were collected and counted as described above.

Uptake of [^{14}C]Thidiazuron and [^{14}C]Zeatin. Second passage callus tissues of cv Jackson Wonder were grown on medium containing 0.1 μM [^{14}C]zeatin (±0.01 μM Thidiazuron) or 0.01 μM [^{14}C]Thidiazuron (±0.1 μM zeatin). The specific radioactivity of [^{14}C]zeatin was 24 mCi/mmol. (The methodology used to synthesize [^{14}C]zeatin from 6-Cl[8-^{14}C]purine will be published elsewhere.) The specific radioactivity of [^{14}C]Thidiazuron was 70 mCi/mmol. After 21 d of growth at 27°C in the dark, tissues were harvested and homogenized with 2.5 parts (v/w) of cold 95% (v/v) ethanol. The cell debris was removed by successive filtrations through Whatman No. 1 filter paper and Millipore filters. The media were heated for 5 min at 100°C. The radioactivity in tissue extracts and media was determined by counting aliquots dissolved in Ready-Solv MP.

RESULTS

Effects of Thidiazuron and Zeatin on Callus Growth and Cytokinin Autonomy. The effects of Thidiazuron and zeatin on callus growth and cytokinin autonomy were tested in the second passage using tissues of two genotypes, cv Jackson Wonder and P.I. 260415. (The choice of these two genotypes was based on their differential responses to DPU [1].) Thidiazuron was highly active in stimulating callus growth of both genotypes (Figs. 1A and 2A). Its activity was slightly higher than that of zeatin in callus tissues of cv Jackson Wonder, and about 30 times higher in tissues of P.I. 260415.

To determine whether Thidiazuron promotes cytokinin-autonomous growth, similarly to DPU (14), the second passage callus tissues were transferred to cytokinin-free medium (third passage). Tissues of cv Jackson Wonder exposed to Thidiazuron in the second passage continued to proliferate in the absence of cytokinin (Fig. 1B). The autonomous growth also persisted in subsequent passages. In contrast, only tissues transferred from suboptimal concentrations of zeatin were able to grow in the absence of cytokinin in the third passage. Tissues transferred from optimal or near-optimal levels of zeatin remained cytokinin dependent. Tissues of P.I. 260415 did not become cytokinin autonomous after transfer from either Thidiazuron- or zeatin-containing medium (Fig. 2B). The growth of tissues transferred from medium containing 1 or 3 μM Thidiazuron was probably due to residual effects, since growth ceased upon additional transfer to medium without cytokinin (fourth passage).

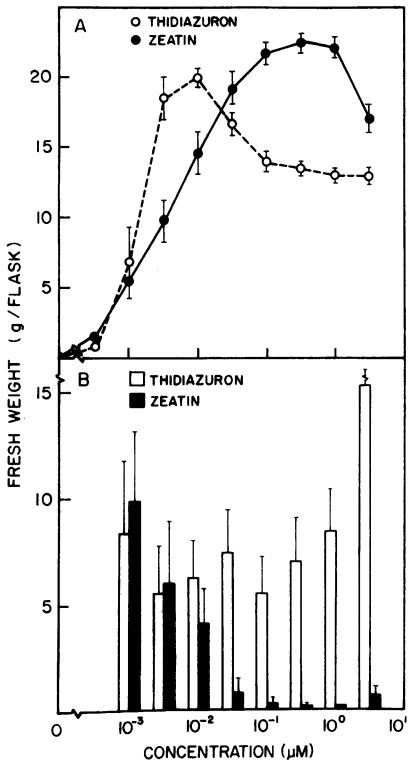

FIG. 1. Callus growth of cv Jackson Wonder in the second passage on media containing Thidiazuron and zeatin (A), and in the third passage on cytokinin-free medium (B). The growth periods were 28 and 35 d, respectively, for the two passages. Vertical lines indicate standard errors.

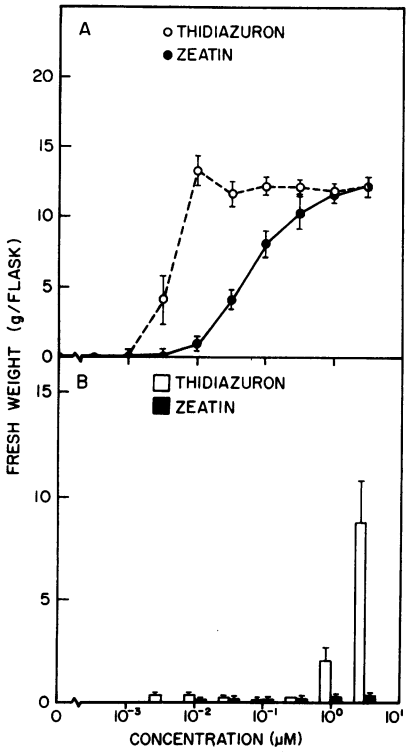

FIG. 2. Callus growth of P.I. 260415 in the second passage on media containing Thidiazuron and zeatin (A), and in the third passage on cytokinin-free medium (B). The growth periods were 28 and 35 d, respectively, for the two passages. Vertical lines indicate standard errors.

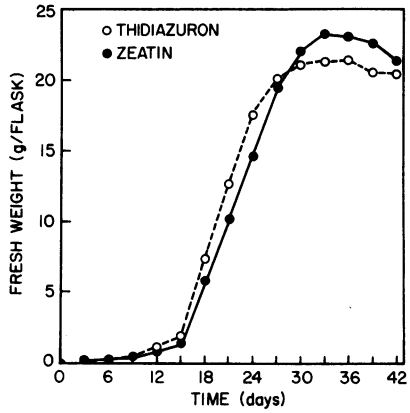

FIG. 3. Growth curves of cv Jackson Wonder callus tissues on media containing 10^{-2} μM Thidiazuron (O) and 10^{-1} μM zeatin (●).

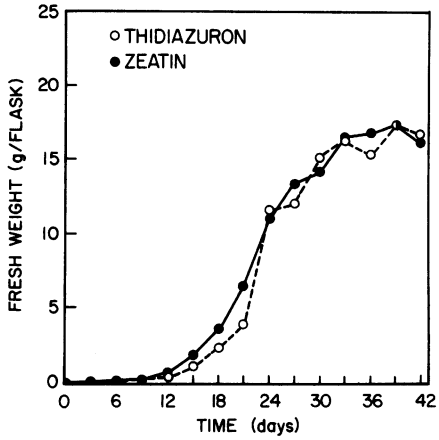

FIG. 4. Growth curves of P.I. 260415 callus tissues on media containing 10^{-2} μM Thidiazuron (O) and 3.10^{-1} μM zeatin (●).

Growth of Callus Tissues at Optimal Concentrations of Thidiazuron and Zeatin. Based on the results presented in Figures 1 and 2, 0.01 μM Thidiazuron and 0.1 and 0.3 μM zeatin (for cv Jackson Wonder and P.I. 260415, respectively) were chosen for subsequent biological and biochemical studies. The fresh weights of tissues determined at 3-d intervals are presented in Figures 3 and 4. Tissues of each genotype proliferated at about the same rate on both types of compounds at the concentrations used. Although the growth rate of cv Jackson Wonder tissues was faster than that of P.I. 260415, the exponential growth period was similar for both genotypes.

Characterization of Major Metabolites of [^{14}C]i^{6}Ado in Selected Callus Tissues. Ethanol extracts of callus tissues incubated with [^{14}C]i^{6}Ado were chromatographed on Sephadex LH-20 columns. Representative elution profiles of tissues grown on Thidiazuron and zeatin are shown in Figure 5. (The results presented were obtained from 10 g tissues of cv Jackson Wonder incubated with [^{14}C]i^{6}Ado for 0.5 h.) A prominent peak at fractions 16 and 17 was obtained from tissues grown on zeatin (Fig. 5B), while only small amounts of this metabolite were formed in tissues maintained on Thidiazuron (Fig. 5A). The elution position of this metabolite was identical to that of a nucleotide of i^{6}Ado previously identified (18). To further characterize the metabolite, aliquots of fractions 16 and 17 were incubated with 5′-nucleotidase and fractionated by HPLC on a reversed-phase C_{18} column (Fig. 6). The untreated labeled compound (Fig. 6B) coeluted with i^{6}AMP, i^{6}ADP, and i^{6}ATP (Fig. 6, A and B). Incubation with 5′-nucleotidase resulted in a shift of the radioactivity from the nucleotide position to that of i^{6}Ado

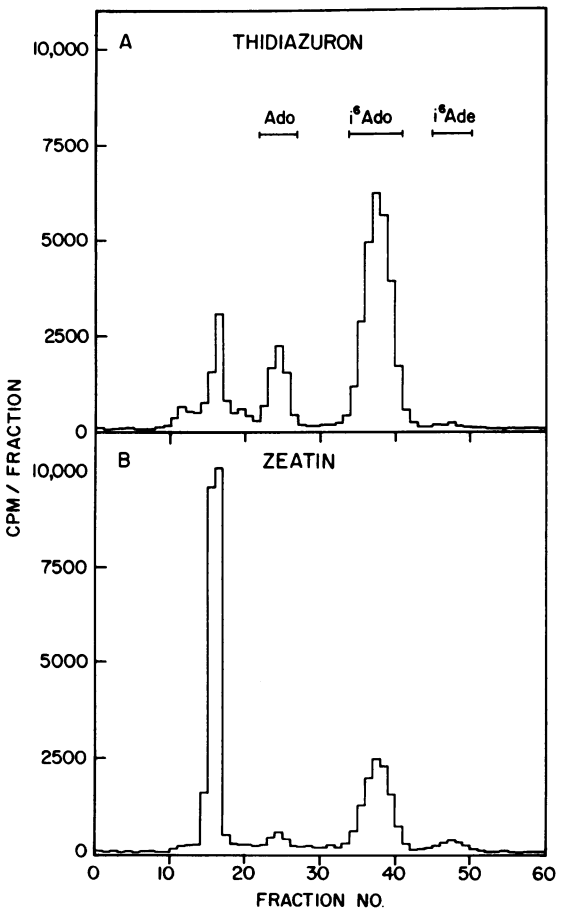

FIG. 5. Chromatographic separation on Sephadex LH-20 of radioactive metabolites extracted from cv Jackson Wonder tissues incubated with [^{14}C]i^{6}Ado for 0.5 h. A, Tissues grown in the presence of 10^{-2} μM Thidiazuron; B, tissues grown in the presence of 10^{-1} μM zeatin.

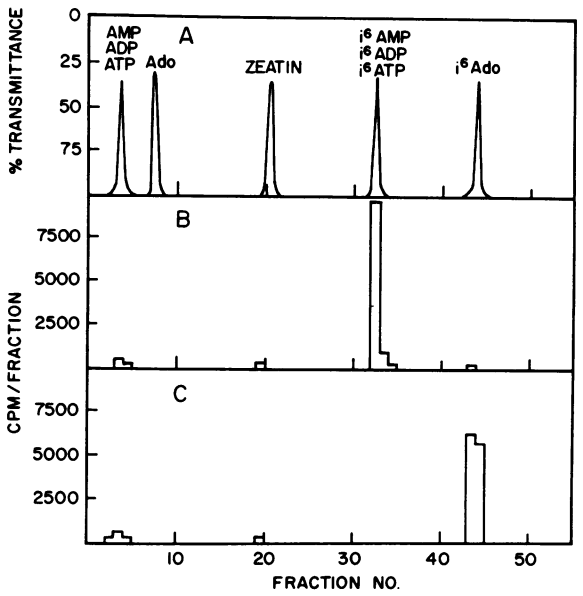

FIG. 6. Analysis by HPLC on reversed-phase C_{18} of fractions 16 and 17 (Fig. 5B) after incubation with 5′-nucleotidase. A, Cytokinin standards; B, control; C, sample incubated with 5′-nucleotidase.

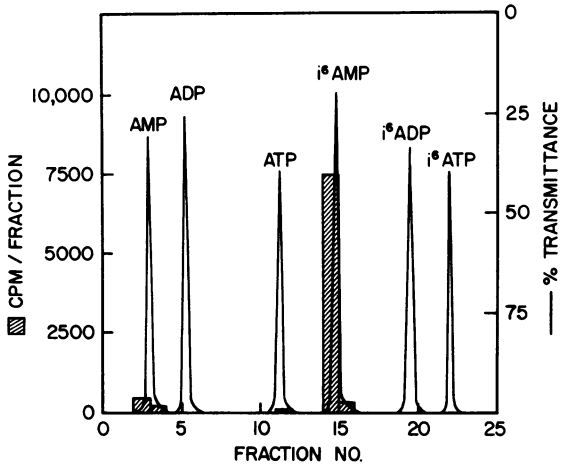

FIG. 7. Analysis by HPLC on paired-ion reversed-phase C_{18} of fractions 16 and 17 (from Fig. 5B).

(Fig. 6C). Treatment with 3′-nucleotidase did not change the elution position of the nucleotide. Thus, the metabolite appears to be a 5′-nucleotide of i^{6}Ado. The nucleotide was also rechromatographed using an HPLC system with a paired-ion reversed-phase C_{18} column. This chromatographic procedure allows clear separation of mono-, di-, and trinucleotides. The radioactivity coeluted with i^{6}AMP (Fig. 7).

The peak of radioactivity coeluting with Ado (Fig. 5A, fractions 23–27) from the Sephadex LH-20 columns was further identified by HPLC on a reversed-phase C_{18} column. This chromatographic system resolves Ado and ribosylzeatin which cochromatograph on Sephadex LH-20. The label coeluted with the Ado standard from the C_{18} column.

Although the data presented are for the tissues incubated with [^{14}C]i^{6}Ado for 0.5 h, similar results were obtained with the corresponding fractions recovered from other samples. In addition, essentially identical elution profiles were obtained with the $HClO_4$ extraction procedure. There was no indication that any significant amounts of di- and trinucleotides were formed.

Metabolism of [^{14}C]i^{6}Ado in Callus Tissues grown on Media Containing Thidiazuron and Zeatin. The metabolism of [^{14}C] i^{6}Ado was initially examined in callus tissues incubated at about the midpoint of growth (10 g) with the labeled compound for various time periods (0.25 to 4 h). The total radioactivity recovered and the distribution of label in the i^{6}Ado, i^{6}AMP, and Ado peaks, calculated as percentages of the total radioactivity recovered at time 0, are presented in Figures 8 and 9, respectively, for tissues of cv Jackson Wonder and P.I. 260415. In tissues of both genotypes, [^{14}C]i^{6}Ado was rapidly converted to [^{14}C]i^{6}AMP when zeatin was present in the medium (although at a somewhat faster rate in tissues of cv Jackson Wonder), while very little [^{14}C] i^{6}AMP was formed in the presence of Thidiazuron. Slightly higher amounts of [^{14}C]Ado were recovered from tissues grown on Thidiazuron. The total extractable radioactivity decreased with incubation time.

The effects of callus age on the metabolism of [^{14}C]i^{6}Ado were determined using young (5 g) and older (20 g) callus tissues of cv Jackson Wonder (Fig. 10). The metabolism of [^{14}C]i^{6}Ado in both the 5 g and 20 g tissues was very similar to that in the 10 g tissues. However, relatively more [^{14}C]Ado was formed in the older tissues grown in the presence of Thidiazuron. Also, the total radioactivity recovered was higher for the older tissues maintained on Thidiazuron. The higher levels of label recovered may be due to either slower uptake of [^{14}C]i^{6}Ado, slower conversion to metabolites inextractable by ethanol, or both.

Incubation of cv Jackson Wonder Tissues with [^{14}C]i^{6}AMP. Tissues of cv Jackson Wonder grown on Thidiazuron- and zeatin-containing medium were incubated with [^{14}C]i^{6}AMP (re-

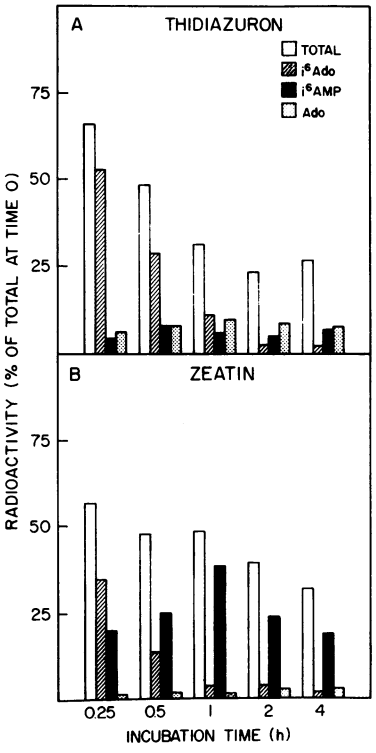

FIG. 8. Distribution of radioactive metabolites extracted from cv Jackson Wonder tissues incubated with [^{14}C]i^{6}Ado for various time periods. A, Tissues grown in the presence of 10^{-2} μM Thidiazuron; B, tissues grown in the presence of 10^{-1} μM zeatin. The radioactivity in each fraction is expressed as percentage of the total radioactivity recovered from the control (0 h).

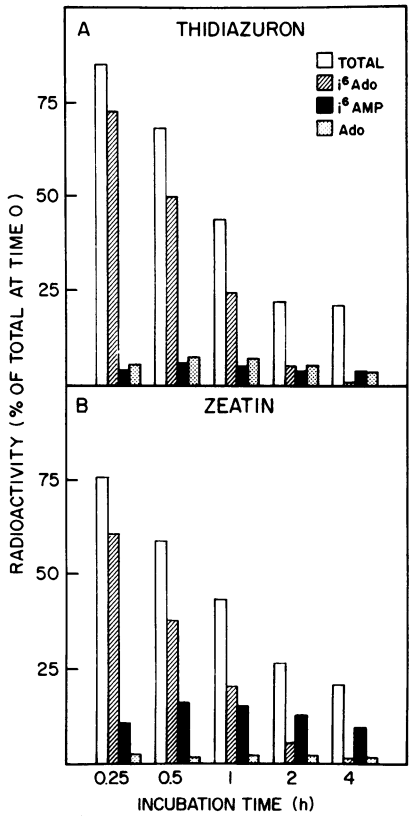

FIG. 9. Distribution of radioactive metabolites extracted from P.I. 260415 tissues incubated with [^{14}C]i^{6}Ado for various time periods. A, Tissues grown in the presence of 10^{-2} μM Thidiazuron; B, tissues grown in the presence of 3.10^{-1} μM zeatin. The radioactivity in each fraction is expressed as percentage of the total radioactivity recovered from the control (0 h).

covered from tissues grown on zeatin and purified by HPLC on reversed-phase C_{18} columns) for 0.5, 1, and 2 h (Fig. 11). In the presence of Thidiazuron, [^{14}C]i^{6}AMP was rapidly converted to i^{6}Ado, while large amounts of the nucleotide remained in zeatin-grown tissues. In addition, the total radioactivity recovered decreased much more rapidly in the former tissues. Since plant cells do not readily take up cytokinin nucleotides (10), it is likely that the nucleotide was first converted to i^{6}Ado outside the cells and that subsequently the i^{6}Ado was taken up and metabolized as in the previous tests.

Effects of Simultaneous Presence of Thidiazuron and Zeatin on the [^{14}C]i^{6}Ado Metabolism and Cytokinin Autonomy. The effects of the simultaneous presence of Thidiazuron and zeatin on the metabolism of [^{14}C]i^{6}Ado were examined using callus tissues of cv Jackson Wonder at the midpoint of growth (10 g). When Thidiazuron was supplied at suboptimal (0.001 μM) concentration and zeatin at either suboptimal (0.01 μM) or optimal (0.1 μM) concentrations, the patterns of [^{14}C]i^{6}Ado metabolism resembled that of tissues grown on zeatin as the only cytokinin (Fig. 12, A and B). However, the presence of Thidiazuron at optimal concentration (0.01 μM) suppressed nucleotide formation regardless of the concentration of zeatin supplied (Fig. 12, C and D). Thus, in the presence of zeatin, a threshold concentration of Thidiazuron needs to be exceeded before the effects of this compound on the [^{14}C]i^{6}Ado metabolism become apparent.

Comparable tissues grown in the presence of both Thidiazuron and zeatin were transferred to cytokinin-free medium after 21 d. The fresh weights after a growth period of 35 d are presented in Table I. Cytokinin-autonomous growth was expressed in three of the four types of tissues; only tissues previously maintained on medium containing optimal zeatin plus suboptimal Thidiazuron concentrations remained cytokinin dependent.

Uptake of [^{14}C]Thidiazuron and [^{14}C]Zeatin. The effects of the combined presence of Thidiazuron and zeatin described above could, at least in part, be the result of competitive uptake of these two compounds. To test this possibility, callus tissues of cv Jackson Wonder were grown on media containing [^{14}C]Thidiazuron (with or without unlabeled zeatin) or [^{14}C]zeatin (with or without unlabeled Thidiazuron). The levels of radioactivity in tissues and medium were examined after a 21-d growth period (Table II). A much larger proportion of [^{14}C]zeatin than [^{14}C] Thidiazuron was taken up by the tissues, even through the concentration of [^{14}C]zeatin in the medium (0.1 μM) was 10 times higher than that of Thidiazuron (0.01 μM). In addition, the same amounts of [^{14}C]Thidiazuron remained in the medium with or without zeatin added. Also, [^{14}C]zeatin uptake was not affected by the presence of Thidiazuron. Therefore, the uptake of the two compounds by the tissues is independent of each other, at least when supplied at optimal concentrations for growth.

DISCUSSION

Thidiazuron is extremely active in stimulating callus growth of *P. lunatus*. Its activity is greater than that of zeatin, which is the most active N^6-substituted adenine derivative tested to date in the *P. lunatus* system. The results of the experiment measuring uptake of [^{14}C]Thidiazuron and [^{14}C]zeatin from the medium indicate that comparatively little [^{14}C]Thidiazuron is taken up. Thus, Thidiazuron is probably even more active in the tissues than the dose-response curves indicate.

The high biological activity of Thidiazuron has permitted a

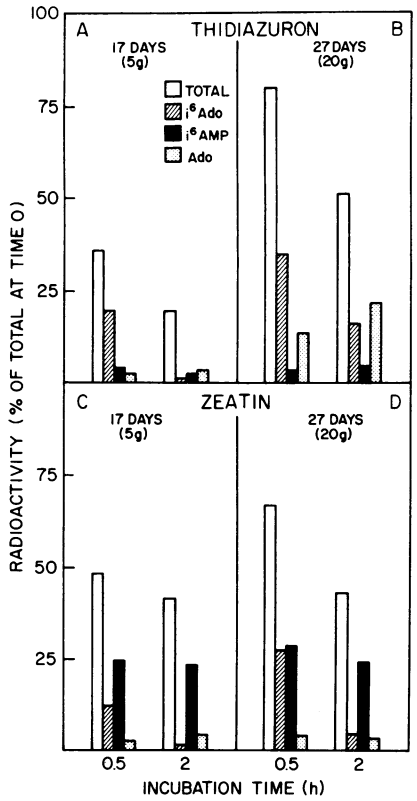

FIG. 10. The effects of tissue age on the metabolism of [^{14}C]i^{6}Ado in cv Jackson Wonder tissues. A, 17 d, 10^{-2} μM Thidiazuron; B, 27 d, 10^{-2} μM Thidiazuron; C, 17 d, 10^{-1} μM zeatin; D, 27 d, 10^{-1} μM zeatin. The radioactivity in each fraction is expressed as percentage of the total radioactivity recovered from the control (0 h).

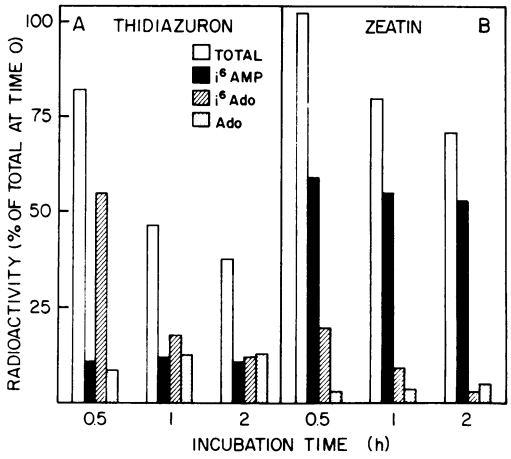

FIG. 11. Distribution of radioactive metabolites extracted from cv Jackson Wonder tissues incubated with [^{14}C]i^{6}AMP for various time periods. A, Tissues grown in the presence of 10^{-2} μM Thidiazuron; B, tissues grown in the presence of 10^{-1} μM zeatin. The radioactivity in each fraction is expressed as percentage of the total radioactivity recovered from the control (0 h).

more detailed examination of the influence of cytokinin-active phenylurea derivatives on the development of cytokinin autonomy in *P. lunatus* callus tissues than was possible in our earlier work (14) with DPU. Callus tissues of cv Jackson Wonder displayed cytokinin-autonomous growth after exposure to either Thidiazuron or to suboptimal concentrations of zeatin. Callus tissues of P.I. 260415 remained cytokinin dependent under all

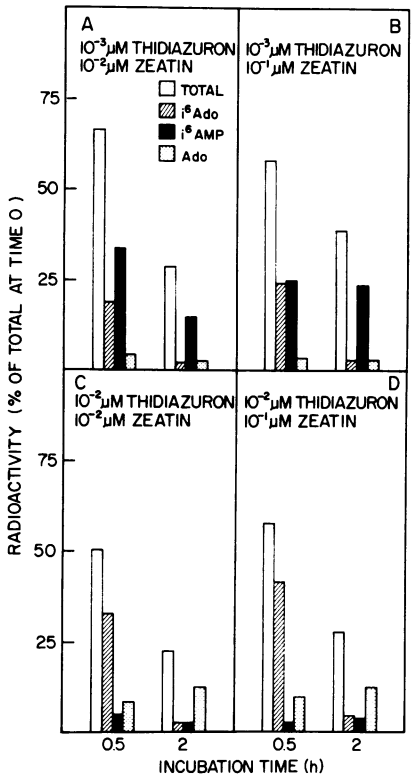

FIG. 12. The effects of simultaneous presence of Thidiazuron and zeatin on the metabolism of [^{14}C]i^{6}Ado in cv Jackson Wonder tissues. A, Suboptimal concentrations of Thidiazuron (10^{-3} μM) and zeatin (10^{-2} μM); B, suboptimal concentration of Thidiazuron (10^{-3} μM) and optimal concentration of zeatin (10^{-1} μM); C, optimal concentration of Thidiazuron (10^{-2} μM) and suboptimal concentration of zeatin (10^{-2} μM); D, optimal concentrations of Thidiazuron (10^{-2} μM) and zeatin (10^{-1} μM). The radioactivity in each fraction is expressed as percentage of the total radioactivity recovered from the control (0 h).

Table I. *Callus Growth of cv Jackson Wonder on Medium Containing Both Thidiazuron and Zeatin (Second Passage) and Medium without Cytokinin (Third Passage)*

The growth periods were 21 and 28 d, respectively, for the second and third passages.

Concentrations in Second Passage		Fresh Weight	
Thidiazuron	+ zeatin	Second passage	Third passage (cytokinin-free medium)
μM		*g/flask* ± *SE*	
10^{-3}	10^{-2}	8.6 ± 0.9	5.6 ± 1.6
10^{-3}	10^{-1}	13.6 ± 1.2	0.9 ± 0.6
10^{-2}	10^{-2}	13.4 ± 0.7	5.7 ± 1.6
10^{-2}	10^{-1}	14.1 ± 1.1	4.8 ± 1.7

conditions tested. Therefore, it appears that the ability of *P. lunatus* callus tissues to become cytokinin autonomous is determined by the genotype as previously observed in *P. vulgaris* (16). Results very similar to those described here for Thidiazuron and zeatin were obtained with *N*-phenyl-*N'*-pyridylurea and kinetin, respectively (unpublished). Thus, the ability to enhance cytokinin autonomy in callus tissues of certain *P. lunatus* genotypes seems to be a distinctive feature of the phenylurea derivatives.

By examining the metabolism of [^{14}C]i^{6}Ado in callus tissues of *P. lunatus*, we were able to demonstrate that pronounced

Table II. *Uptake of [^{14}C]Thidiazuron and [^{14}C]Zeatin by Tissues of cv Jackson Wonder after a Growth Period of 21 Days*

Cytokinin-active Compounds in Medium	Radioactivity Recovered	
	Medium	Tissues
	% cpm added to the medium	
10^{-2} μM [^{14}C]Thidiazuron	62	16
10^{-2} μM [^{14}C]Thidiazuron + 10^{-1} μM zeatin	60	16
10^{-1} μM [^{14}C]zeatin	21	13
10^{-1} μM [^{14}C]zeatin + 10^{-2} μM Thidiazuron	19	11

metabolic differences occur in the tissues depending on the type of cytokinin-active compound present. The small amounts of [^{14}C]i^{6}AMP formed in tissues grown on Thidiazuron imply either slow conversion of i^{6}Ado to i^{6}AMP or high conversion rates of i^{6}AMP to i^{6}Ado. The experiments using [^{14}C]i^{6}AMP indicate that the latter is the case. Preliminary experiments using [^{14}C] zeatin (which has recently been synthesized) indicate that little zeatin ribonucleotide is formed in tissues grown on Thidiazuron as compared to those maintained on zeatin- or kinetin-containing medium. Thus, Thidiazuron may in general promote the conversion of cytokinin ribonucleotides to ribonucleosides.

The different patterns of [^{14}C]i^{6}Ado metabolism by tissues grown on Thidiazuron and zeatin could not be the result of the presence of higher levels of adenine-type cytokinins in the tissues grown on zeatin. When both Thidiazuron and zeatin were present at optimal concentrations in the medium, [^{14}C]i^{6}AMP formation was suppressed; moreover, it was shown that zeatin was taken up to the same extent by these tissues as by those grown on zeatin alone. Therefore, Thidiazuron must exert a direct effect on the i^{6}Ado metabolism, independent of the level of N^6-substituted adenine derivatives present in the tissues.

It is not clear, at present, if the modification of the [^{14}C]i^{6}Ado metabolism by Thidiazuron has a direct bearing on its biological activity. Based on the biological activities observed in tobacco cell cultures under certain conditions, Laloue and Pethe (10) formed the hypothesis that the free base represents the active form of cytokinins. If this hypothesis is correct, the biological activity of Thidiazuron could, at least in part, be due to an increase of ribonucleoside levels and, therefore, possibly a higher level of free bases. However, several other explanations for the cytokinin activity of phenylurea derivatives have been put forward. Miller (13) speculated that substituted phenylureas may serve as precursors for the N^6-side chain of adenine derivatives. In support of this hypothesis, certain synthetic ureidopurines are known to possess cytokinin activity (12). However, adenine derivatives with cytokinin activity comparable to the extremely active phenylureas have not been found. Moreover, the only DPU metabolite identified from plant tissues is a simple glucosyl derivative (5). Alternatively, phenylurea compounds may have direct growth promoting activity. Based on results obtained in experiments using cytokinin antagonists, Kefford *et al.* (9) and Isogai (6) concluded that the two types of cytokinin-active compounds must have a common site of action. Iwamura *et al.* (7), using physicochemical substituent parameters, have argued that cytokinin-active adenine derivatives and phenylureas have some features in common. The exact relationship between the two types of compounds may not be elucidated until more is known about the site(s) of action of cytokinins.

The change in the metabolism of [^{14}C]i^{6}Ado induced by Thidiazuron did not necessarily signal a change in the cytokinin requirements of the tissues (*i.e.* transformation from cytokinin dependence to autonomy). Tissues of cv Jackson Wonder and P.I. 260415 exhibited similar patterns of [^{14}C]i^{6}Ado metabolism while only tissues of cv Jackson Wonder, displayed cytokinin autonomy. Conversely, transformation to cytokinin autonomy was not always accompanied by inhibition of nucleotide formation. Suboptimal concentrations of zeatin plus Thidiazuron, or zeatin alone, promoted cytokinin autonomy in cv Jackson Wonder, and as much nucleotide was formed in these tissues as those grown on medium with optimal zeatin concentration. (The metabolism data at suboptimal zeatin concentrations are not presented, but are similar to those obtained with tissues grown at optimal zeatin concentrations.) Therefore, the cytokinin-active phenylureas and adenine derivatives seem to differ in their effects on cytokinin autonomy as well as cytokinin nucleotide formation, but the two types of effects may not be related.

Acknowledgment—We thank Professor G. Shaw for providing the side chain precursor of [^{14}C]zeatin.

LITERATURE CITED

1. ARMSTRONG DJ, SG KIM, MC MOK, DWS MOK 1981 Genetic regulation of cytokinin metabolism in *Phaseolus* tissue cultures. *In* J Guern, C Péaud-Lenoël, eds, Metabolism and Molecular Activities of Cytokinins. Springer-Verlag, Berlin, pp 97–104
2. BRAY GA 1960 A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Anal Biochem 1:279–285
3. BRUCE MI, JA ZWAR 1966 Cytokinin activity of some substituted ureas and thioureas. Proc R Soc Lond Ser B 165: 245–265
4. BRUCE MI, JA ZWAR, NP KEFFORD 1965 Chemical structure and plant kinin activity—The activity of urea and thiourea derivatives. Life Sci 4: 461–466
5. BURROWS WJ, DP LEWORTHY 1976 Metabolism of *N,N'*-diphenylurea by cytokinin-dependent tobacco callus: Identification of the glucoside. Biochem Biophys Res Commun 70: 1109–1114
6. ISOGAI Y 1981 Cytokinin activities of *N*-phenyl-*N'*-(4-pyridyl) ureas. *In* J Guern, C Péaud-Lenoël, eds, Metabolism and Molecular Activities of Cytokinins. Springer-Verlag, Berlin, pp 115–128
7. IWAMURA H, T FUJITA, S KOYAMA, K KOSHIMIZU, Z KUMAZAWA 1980 Quantitative structure-activity relationship of cytokinin-active adenine and urea derivatives. Phytochemistry 19: 1309–1319
8. Juengling E, H Kammermeier 1980 Rapid assay of adenine nucleotides or creatin compounds in extracts of cardiac tissue by paired-ion reverse-phase high performance liquid chromatography. Anal Biochem 102: 358–361
9. KEFFORD NP, JA ZWAR, MI BRUCE 1968 Antagonism of purine and urea cytokinin activities by derivatives of benzylurea. *In* F Wightman, G Setterfields, eds, Biochemistry and Physiology of Plant Growth Substances. Runge, Ottawa, pp 61–69
10. LALOUE M, C PETHE 1982 Dynamics of cytokinin metabolism in tobacco cells. *In* PF Wareing, ed, Plant Growth Substances. Academic Press, New York, pp 185–195
11. LALOUE, M, C TERRINE, M GAWER 1974 Cytokinins: formation of the nucleoside-5'-triphosphate in tobacco and *Acer* cells. FEBS Lett 46: 45–50
12. MCDONALD JJ, NJ LEONARD, RY SCHMITZ, F SKOOG 1971 Cytokinins: synthesis and biological activity of ureidopurines. Phytochemistry 10: 1429–1439
13. MILLER CO 1961 Kinetin and related compounds in plant growth. Annu Rev Plant Physiol 12: 395–408
14. MOK MC, SG KIM, DJ ARMSTRONG, DWS MOK 1979 Induction of cytokinin autonomy by *N,N'*-diphenylurea in tissue cultures of *Phaseolus lunatus* L. Proc Natl Acad Sci USA 76: 3880–3884
15. MOK MC, DWS MOK 1977 Genotypic responses to auxins in tissue cultures of *Phaseolus*. Physiol Plant 40: 261–264
16. MOK MC, DWS MOK, DJ ARMSTRONG, A RABAKOARIHANTA, SG KIM 1980 Cytokinin autonomy in tissue cultures of *Phaseolus:* a genotype-specific and heritable trait. Genetics 94: 675–686
17. MOK MC, DWS MOK, DJ ARMSTRONG, K SHUDO, Y ISOGAI, T OKAMOTO 1982 Cytokinin activity of *N*-phenyl-*N'*-1,2,3-thiadiazol-5-ylurea (Thidiazuron). Phytochemistry 21: 1509–1511
18. MOK MC, DWS MOK, SC DIXON, DJ ARMSTRONG, G SHAW 1982 Cytokinin structure-activity relationships and the metabolism of N^6-(Δ^2-isopentenyl)adenosine-8-^{14}C in *Phaseolus* callus tissues. Plant Physiol 70: 173–178
19. MURASHIGE T, F SKOOG 1962 A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473–497
20. SCHMITZ RY, F SKOOG 1970 The use of dimethylsulfoxide as a solvent in the tobacco bioassay for cytokinins. Plant Physiol 45: 537–538
21. SHANTZ EM, FC STEWARD 1955 The identification of compound A from coconut milk as 1,3-diphenylurea. J Am Chem Soc 77: 6351–6353
22. TAKAHASHI S, K SHUDO, T OKAMOTO, K YAMADA, Y ISOGAI 1978 Cytokinin activity of *N*-phenyl-*N'*-(4-pyridyl)urea derivatives. Phytochemistry 17: 1201–1207