

Short Communication

Cytokinin Activity Induced by Thidiazuron¹

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ABSTRACT

The diphenylurea derivative thidiazuron induces a variety of cytokinin responses. Levels above 5×10^{-9} molar and 4×10^{-7} molar stimulate maximum soybean callus growth and radish cotyledon expansion, respectively. A wider range of dose response related effects follows thidiazuron induced tobacco plant regeneration. Analysis of soybean callus extracts strongly suggests that thidiazuron treatment creates an accumulation and/or synthesis of purine cytokinins, able to induce the growth, expansion and regeneration, mentioned above.

Naturally occurring cytokinins are most often described as N⁶-isopentenyl adenine derivatives (3, 12). While structurally unlike purine derivatives, some synthetic diphenylureas are even more active than their purine counterparts (7, 10, 13). A clear explanation of how DPUs³ and derivatives stimulate cytokinin-like effects is needed. One recent proposal is that urea type cytokinins promote the conversion of cytokinin ribonucleotides to the biologically more active ribonucleosides (1).

Purine type cytokinins can be judged by a variety of criteria. Radish cotyledon expansion without cell division occurs after cytokinin treatment (2). Cytokinin induced soybean callus growth and tobacco regeneration rely on both cell division and elongation (5, 11). DPU type cytokinins should also elicit these responses.

The purpose of this communication is to compare the relative activities of purine cytokinins and a DPU cytokinin. The well known N⁶-BA and zeatin were tested alongside the DPU cytokinin *N*-phenyl-*N'*-1,2,3-thiadiazol-5-ylurea (thidiazuron), by means of the classical bioassay methods mentioned above. Thidiazuron demonstrates powerful cytokinin activity in *Phaseolus lunatus* cell culture (8). Secondly, the content of adenine type cytokinins in soybean callus grown on a thidiazuron supplemented medium is also examined.

MATERIALS AND METHODS

Thidiazuron (Dropp, Nor-Am Agriculture Products) was obtained from Dr. R. Briggs (University of Arizona). Purine cytokinins were purchased from Sigma.

The radish (*Raphanus sativus* L.) cotyledon cytokinin bioassay was employed to test thidiazuron cytokinin activities (4). Seeds

(Early Scarlet Globe, Northrup King Seed Co.) were soaked for 5 min in 10% Clorox with 1 drop of Tween 20 in 250 ml, rinsed with sterile water, and germinated on Whatman No. 1 filter paper with sterile water in darkness at 22°C overnight. Ten to 12 cotyledons were aseptically excised and placed in a 25 × 100 mm glass Petri dish with 1 disc of Whatman No. 1 filter paper and 7 ml of 2 mM K-phosphate (pH 6.0) containing various concentrations of either BA or thidiazuron. Dishes were sealed with Parafilm and incubated in 1000 lux fluorescent light for 16 h at 25°C for 2.5 d. Afterward the cotyledons were removed, quickly blotted dry, and weighed.

Cotyledon callus was derived from *Glycine max* L. Merrill cv Acme according to Miller (5). Seeds were germinated aseptically and the cotyledons excised and placed in Miller's medium with 3% sucrose, 1% Difco Bacto agar, 5 mg/L IAA and 0.2 mg/L kinetin. Cultures were grown in 2000 lux fluorescent light for 16 h at 25°C. Callus was subcultured every 4 weeks on a similar medium (6), except 2 mg/L NAA and 0.2 mg/L BA were used as the plant phytohormones.

Thidiazuron was dissolved and serial dilutions made in DMSO. After autoclaving, the appropriate thidiazuron concentration was added when the medium had cooled to 55°C. Medium was then poured into sterile 125 ml flasks. Soybean callus (0.5 g/flask) was inoculated and grown as before. After 5 weeks, cultures were harvested and the fresh weights determined.

The differentiation assay was based on the regeneration of individual plantlets from *Nicotiana tabacum* W-38 leaf discs. Plants were aseptically germinated and grown on ½ strength M and S salts (9) with 0.4 mg/L thiamine·HCl, 0.5 mg/L pyridoxine·HCl, 0.5 mg/L nicotinic acid, 100 mg/L inositol, 1% sucrose, and 1% Difco Bacto agar. Sterile leaves were carefully removed and placed upon 3 or 4 sheets of sterile Whatman No. 1 filter paper. Leaf discs were punched with a sterile 10 mm cork borer, floated on water for 5 min, and placed upper epidermal side up on differentiation medium.

Differentiation medium contained M and S salts, 0.4 mg/L thiamine·HCl, 0.5 mg/L pyridoxine·HCl, 0.5 mg/L nicotinic acid, 2 mg/L glycine, 100 mg/L inositol, with 3% sucrose, 1% Difco Bacto agar, 2 mg/L BA and 10 μg/L NAA. Regeneration was tested in identical media without BA and with different concentrations of thidiazuron added after medium was autoclaved.

The fractionation of adenine type cytokinins followed the methods of Van Staden (14, 15). Soybean callus growing in either 0.1 or 1 mg/L thidiazuron was extracted (10 g fresh weight) in 90 ml of cold methanol then allowed to stand at 4°C overnight. The extract was filtered through 2 layers of Miracloth and the pH was adjusted to 2.5 with 1 N HCl. The resultant precipitate was removed by centrifugation at 10,000g for 10 min and the clear supernatant applied to a 1 × 30 cm Dowex column (50W-X8, 100-200 mesh, H⁺). The column was washed three times with 70% methanol and 2 volumes of deionized H₂O. The

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³ Abbreviations: DPU, diphenylurea; thidiazuron, *N*-phenyl-*N'*-1,2,3-thiadiazol-5-ylurea; NAA, naphthalene acetic acid.

adenine type cytokinins were eluted with 5 volumes of 5 M NH_4OH . The eluate was evaporated to dryness at 40°C and taken up in 2 ml of 35% methanol.

Fractionation of cytokinins was done on a Sephadex LH-20 column (1.5×50 cm). The column was standardized by eluting (0.5 ml/min) zeatin (Z), zeatin riboside (ZR), zeatin glucoside (ZG), AMP and 2-isopentenyladenine (2iP) in 35% methanol. Fractions (90) of 7 ml each were collected and monitored at 260 nm.

For soybean bioassay, 1 of the 2 ml of the extract was applied to the Sephadex column and eluted with 35% methanol. After elution, every two fractions were poured into French squares and dried at 40°C . Each fraction represents the equivalent of 0.11 g fresh weight of soybean callus. For bioassay, 20 ml of Miller's medium without cytokinin was added, and the bottles were capped and autoclaved for 20 min. Each bottle was inoculated with 0.25 g fresh weight of soybean cotyledon callus. Soybean callus was also inoculated on Miller's medium with increasing concentrations of zeatin to provide a cytokinin responsive control. Cultures were grown as stated earlier and the fresh weight of each culture was determined after 5 to 6 weeks. These experiments were repeated three times.

RESULTS

In order for cytokinin bioassays to estimate the cytokinin concentration in a given sample, the response of the bioassay must be dose dependent. Using BA, the radish bioassay response was linear for concentrations from 2×10^{-8} to 5×10^{-5} M (Fig. 1). Zeatin has been shown to stimulate radish cotyledon expansion in a similar dose dependent manner (4). This was not the case for thidiazuron (Fig. 1). Only at concentrations from 0 to 4×10^{-7} M was a linear response observed. At higher concentrations, greater expansion did not occur.

Thidiazuron also had a similar effect on the growth of cytokinin dependent soybean cotyledon callus (Fig. 2). The linear dose-response range was narrow, between 0 and 5×10^{-9} M. In comparison, a dose response range for kinetin was between 0 and 10^{-5} M (5). Zeatin (1×10^{-7} M) plus thidiazuron (5×10^{-7} M) had no cumulative effect on fresh weight increase of soybean callus as compared to thidiazuron (5×10^{-7} M) alone.

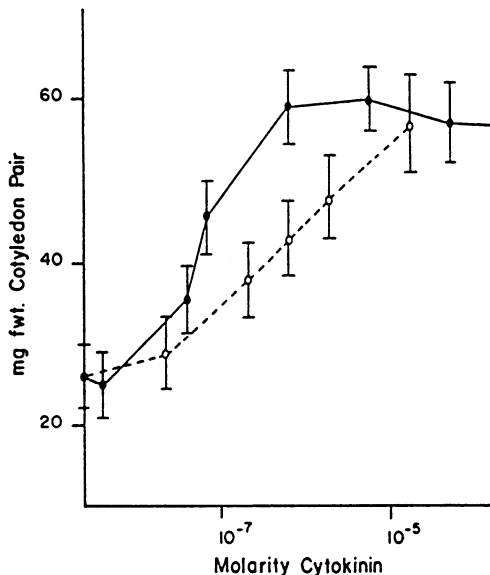


FIG. 1. Effect of increasing thidiazuron (●—●) and 6 BA (○—○) concentrations on radish cotyledon expansion. Each point represents the average and standard deviation of three separate experiments using 10 cotyledons/experiment.

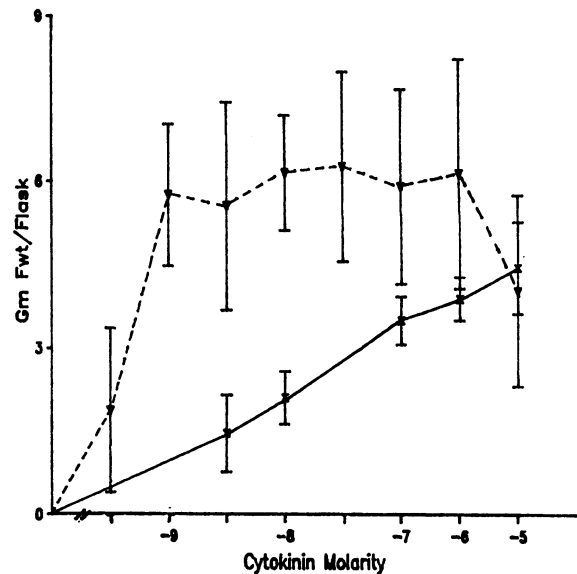


FIG. 2. Soybean callus growth on various zeatin (—) and thidiazuron (---) concentrations. Each point represents the average fresh weight/flask and standard deviation of three separate experiments each done in triplicate.

Table I. Regeneration of Tobacco Plantlets Induced by Thidiazuron

Experiments were done twice in triplicate and the average and standard deviations of all six explants represented below.

Cytokinin Concentration	Plantlets Regenerated/Explant
M	
0	0
Thidiazuron	
5×10^{-9}	0
5×10^{-8}	1.4 ± 2.1
5×10^{-7}	9.4 ± 5.3
5×10^{-6}	11.5 ± 5.9
5×10^{-5}	16.2 ± 5.9
5×10^{-4}	26.7 ± 10.8
BA	
1×10^{-5}	30.2 ± 11.9
Kinetin	
1×10^{-5}	10.3 ± 5.0

The differentiation of tobacco leaf discs to form multiple shoots was induced effectively by thidiazuron (Table I). The concentrations of thidiazuron used to induce plantlet regeneration are similar to both BA and kinetin. Generally, the greater concentrations of thidiazuron produced greater number of plantlets; however, they were quite small in height. Larger plants that rooted spontaneously were obtained at lower concentrations (5×10^{-8} M).

The result of cytokinin extraction and fractionation from soybean callus grown on 0.1 and 1 mg/L thidiazuron is shown in Figure 3. Normally, this soybean variety requires purine cytokinins added in the culture medium in order to grow (5). Thidiazuron allows this callus to grow and to produce compounds that co-chromatograph with and demonstrate a biological activity similar to purine cytokinins.

CONCLUSIONS

Exogenous application of a DPU type (thidiazuron) and purine cytokinins zeatin or BA both elicit effects traditionally ascribed

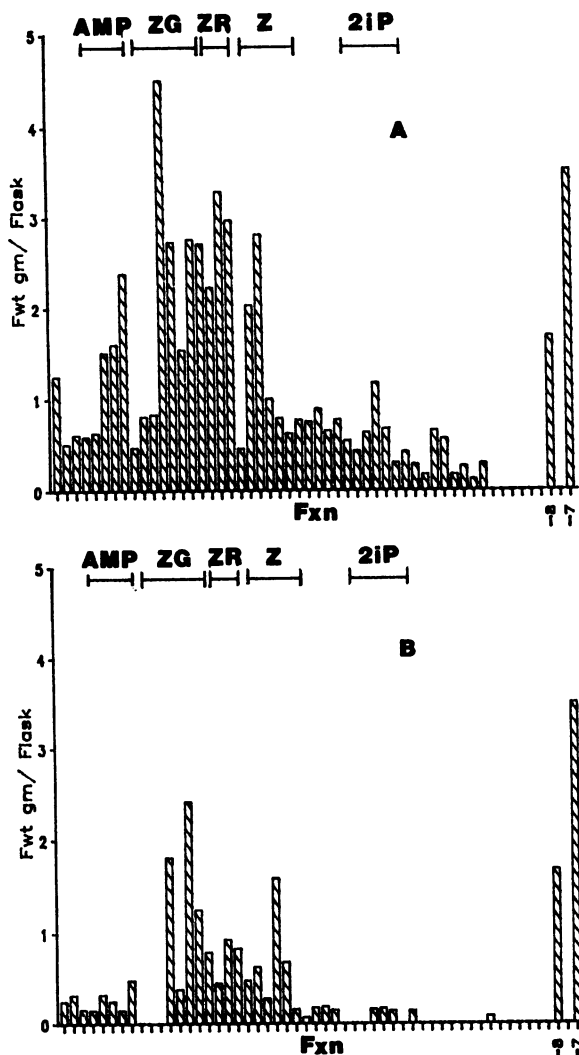


FIG. 3. Endogenous supply of purine cytokinins present in soybean callus grown in Miller's medium (6) with 2 mg/L NAA and either 1 (A) or 0.1 (B) mg/L thidiazuron. Every two fractions were pooled and bioassayed with soybean callus as described in "Materials and Methods." The elution position of standard purine cytokinins are indicated above the figure. AMP (adenosine 5'-monophosphate), ZG (zeatin glucoside), ZR (zeatin riboside) and 2iP (N^6 -[Δ^2 -isopentenyl]adenosine). Two zeatin concentrations are also shown ($-8: 10^{-8}$ M and $-7: 10^{-7}$ M).

to cytokinins in the nondifferentiating conditions of the radish and callus assays (Figs. 1 and 2). The dose response effect of thidiazuron in either bioassay is very narrow. These data indicate that the general growth and cell division stimulation becomes saturated at low thidiazuron levels. On a concentration basis, thidiazuron is 10^2 times more effective in the radish and 10^4 times more effective in the soybean callus cytokinin assays than the purine cytokinins tested.

The purpose of endogenous cytokinin extraction from soybean callus grown on thidiazuron was to address how thidiazuron influences endogenous purine cytokinin levels. Since thidiazuron ($100 \pm 2\%$) is washed from the Dowex column prior to NH_4OH elution, the direct effect of thidiazuron on endogenous purine cytokinin concentrations can be observed.

Based upon the chromatographic elution pattern (as compared to known purine cytokinin standards) and the biological activity

of each fraction in a cytokinin bioassay, thidiazuron treatment of soybean callus stimulates purine cytokinin accumulation and/or synthesis (Fig. 3). After growth on thidiazuron, purine cytokinins are found in quantities similar to those observed when soybean callus was cultured on zeatin and zeatin riboside supplemented media (14, 15).

Capelle *et al.* (1) have shown that thidiazuron stimulates the conversion of cytokinin nucleotides to nucleosides. Changes in the purine metabolism of soybean callus must also result from thidiazuron treatment, leading to an uncoupling of the normal inhibition of cytokinin biosynthesis. The effect of thidiazuron on cytokinin dependent soybean callus is to encourage the synthesis (or inhibit the breakdown) of purine cytokinins. As a result of an endogenous cytokinin supply, this callus will grow in the absence of medium supplemented purine cytokinins.

Thidiazuron may also have stimulated cytokinin metabolism leading to tobacco plantlet differentiation. At high concentrations, the suppression of cytokinin breakdown and/or the continued biosynthesis of purine cytokinins results in rooting inhibition and many smaller plantlets being produced. These concentration dependent responses are similar to those of purine cytokinins in tobacco (11) (Table I).

The urea type cytokinin thidiazuron is active in the traditional cytokinin dependent bioassays of soybean callus growth, radish cotyledon expansion and tobacco plantlet regeneration. The data in this report suggests that in soybean callus, thidiazuron promotes callus growth by encouraging the synthesis and/or accumulation of purine type cytokinins.

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LITERATURE CITED

1. CAPELLE SC, DWS MOK, SC KIRCHNER, MC MOK 1983 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. *Plant Physiol* 73: 796-802
2. GORDON ME, DS LETHAM 1975 Regulators of cell division in plant tissues. XXII. Physiological aspects of cytokinin-induced radish cotyledon growth. *Aust J Plant Physiol* 2: 129-154
3. LETHAM DS, LMS PALNI 1983 The biosynthesis and metabolism of cytokinins. *Annu Rev Plant Physiol* 34: 163-197
4. LETHAM DS 1971 Regulators of cell division in plant tissues. XII. A cytokinin bioassay using excised radish cotyledons. *Physiol Plant* 25: 391-396
5. MILLER CO 1963 Kinetin and kinetin-like compounds. In *Modern Methods of Plant Analysis*, HF Linskens, MV Tracey, eds, Vol 6. Springer-Verlag, Berlin, pp 194-202
6. MILLER CO 1967 Cytokinins in *Zea mays*. *Ann NY Acad Sci* 14: 251-257
7. 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
8. MOK MC, S-G 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
9. MURASHIGE T, F SKOOG 1962 A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiol Plant* 15: 473-497
10. OKAMOTO T, K SHUDO, S TAKASHI, T YATSUNAMI, Y ISOGAI, K YAMADA 1978 Shoot formation of tobacco callus by various cytokinin-active ureas and pyrimidines. *Chem Pharm Bull* 26: 3250-3252
11. SKOOG F, CO MILLER 1957 Chemical regulation of growth and organ formation in plant tissues cultured in vitro (Biological Action of Growth Substances). *Symp Soc Exp Biol* 11: 118-131
12. SKOOG F, DJ ARMSTRONG 1970 Cytokinins. *Annu Rev Plant Physiol* 21: 359-384
13. TAKAHASHI S, K SHUDO, T OKAMOTO, K YAMADA, I ISOGAI 1978 Cytokinin activity of *N*-phenyl-*N'*-(4-pyridyl)urea derivatives. *Phytochemistry* 17: 1201-1207
14. VAN STADEN J, JE DAVEY 1977 The metabolism of zeatin and zeatin riboside by soya bean callus. *Ann Bot* 41: 1041-1048
15. VAN STADEN J 1979 The effect of adenine and mevalonic acid on the endogenous cytokinins of a cytokinin-dependent strain of soya bean callus. *Ann Bot* 44: 671-675