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Effect of Thidiazuron, a Cytokinin-Active Urea Derivative, in Cytokinin-Dependent Ethylene Production Systems[1]

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ABSTRACT

Cytokinins are known to stimulate ethylene production in mungbean hypocotyls synergistically with indoleacetic acid (IAA), in mungbean hypocotyls synergistically with Ca^{2+}, and in wilted wheat leaves. Thidiazuron, a substituted urea compound, mimicked the effect of benzyladenine (BA) in all three systems. In the Ca^{2+} + cytokinin system and the IAA + cytokinin systems of mungbean hypocotyls, thiadiazuron was slightly more active than BA at equimolar concentration. In mungbean hypocotyls exogenously applied IAA was rapidly conjugated into IAA asparate, and this conjugation process was effectively inhibited by thidiazuron, as by cytokinins. In the wilted wheat leaves system, 10 micromolar thidiazuron exerted stress ethylene production equal to that exerted by 1 millimolar BA, indicating that thidiazuron is more active than BA by two orders. The structure-activity relationship of thidiazuron and its thiadiazolylurea analogs in stimulating Ca^{2+}-dependent ethylene production in mungbean hypocotyls was found to agree well with the structure-activity relationship of these derivatives in promoting the growth of callus tissues. These results indicate that thidiazuron and its derivatives are highly active to mimic the adenine-type cytokinin responses in promoting ethylene production and that the structure-activity relationship in promoting the growth of callus and in promoting ethylene production is similar.

Two distinct groups of chemicals are known to have cytokinin effects. The N^6-substituted adenine derivatives are the classical cytokinins that can induce callus growth in tissues cultures. However, certain substituted urea compounds, such as diphenylurea, can replace adenine derivatives in callus growth (2, 19), and in other bioassay systems (4). Thidiazuron (for structure see Table II), a substituted urea compound, is known to be a growth regulator inducing cotton defoliation (3) and promoting the growth of cytokinin-dependent callus cultures of *Phaseolus lunatus* cv Kington (17). The cytokinin activity of thidiazuron was reported to be similar to the most active cytokinins of the adenine-type (17). Recently, Suttle (18) showed that thidiazuron promotes ethylene synthesis in mungbean hypocotyl segments.

Ethylene production by plant tissues is known to be promoted by many compounds, including auxins and cytokinins. In vegetative tissues, the promotion of ethylene production by auxins, in general, is much greater than that by cytokinins. The magnitude of cytokinin-promoted ethylene production is only several-folds above the controls. There are, however, three reported ethylene production systems in which ethylene production is specifically promoted by cytokinins. The first two are stimulating of ethylene production synergistically with IAA (12) or synergistically with Ca^{2+}, in mungbean hypocotyls (13, 14, 24). Based on the observations that the structure-activity relationship of different adenine-type cytokinins for the callus growth is similar to that for the synergistic stimulation of ethylene production, it has been proposed that this synergistic promotion of ethylene production may be advantageously adopted as a convenient, supplemental bioassay for cytokinins (15, 24). The third system is based on the ability of cytokinins to promote ethylene production in wilted wheat leaves (7). When detached wheat leaves are stressed with a water deficit, there is a massive synthesis of ethylene (16, 20). However, when the stressed leaves are rehydrated and restressed again, there is little synthesis of stress ethylene unless a cytokinin is supplied during the rehydration process (7).

The high ability of thidiazuron to replace cytokinin-active adenine derivatives in callus culture bioassay has been demonstrated (5, 17). In this study we compare the structure-activity relationship of thidiazuron and six of its analogs on three cytokinin-dependent ethylene production systems with that of BA.

MATERIALS AND METHODS

Plant Materials and Incubation Conditions. Seeds of mungbean (*Vigna radiata* L.) were thoroughly washed, imbibed in aerated water overnight, and then grown in moist paper towers for 3 d in darkness at 25°C before used. In the Ca^{2+} + cytokinin system, sample of 10 mungbean hypocotyls (2 cm, 1–3 cm below the hook), excised from the seedlings in dim green light (12), were incubated in a 50-ml Erlenmeyer flask which contained 5 ml medium consisting of 50 mM K-phosphate (pH 5.8), 2% sucrose, 10 mM $CaCl_2$, and various concentrations of thidiazuron or BA. In the IAA + cytokinin system, the incubation mixtures were as described above except that $CaCl_2$ was replaced by 10 μM of IAA. The flasks were then sealed with rubber serum caps and incubated in a shaker at 25°C in darkness. Ethylene accumulated during the 12-h incubation for the Ca^{2+} + cytokinin system or during the 9-h incubation for IAA + cytokinin system was determined.

Wheat seeds (*Triticum aestivum* L. cv Anza), were soaked in aerated distilled H_2O for 3 h before planting in vermiculite. Plants were grown at 20°C with a 14 h light-10 h dark cycle under a light source which was composed of four Norelco Grolume lamps as described by McKeon *et al.* (16). In the water-stress system, 8-cm leaf segments cut from the top of 8-d-old wheat seedlings were wilted until they lost 10% of their fresh weight as described by Apelbaum and Yang (1). Each lot of 20 leaves was put into a 15-ml test tube and sealed with a serum-cap to prevent further water loss. To prevent excessive CO_2 and ethylene accumulation, tubes were flushed with water-saturated air every 4 h. At the end of 20 h incubation, the leaves were rehydrated by adding 0.5 ml of water containing different concentrations of thidiazuron or BA to the incubation tubes. After 14 h of recovery, leaves were subjected to a second wilting

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treatment until 10% of fresh weight was lost. Leaves were then enclosed in tubes and their ethylene production rates during the subsequent 2-h period were determined.

Determination of Ethylene. A 1-ml gas sample withdrawn with a hypodermic syringe from the enclosed tubes or flasks, was determined for its ethylene content with a gas chromatograph equipped with an alumina column at 90°C and a flame-ionization detector.

Determination of the Relatively Free IAA and IAAsp Levels in Mungbean Hypocotyls. Twenty segments were incubated in 5 ml of medium consisting of 2% sucrose, 50 mM of potassium phosphate buffer (pH 4.6), 0.22 μmol of [carboxyl-^{14}C]IAA (1 μCi), and with or without 0.5 μmol of thidiazuron. After 2.5 and 4.5 h of incubation, segments were washed with 10 μM of nonradioactive IAA for 5 min, and then with distilled H_2O. The segments were then extracted twice with 5 ml of 80% ethanol. After evaporation, the extract was brought to a final volume of 0.5 ml with water. Aliquots of 30 μl extracts were chromatographed on paper with unlabeled authentic IAA and IAAsp[2] using 1-butanol:3% NH_3 (1:1, v/v) as the developing solvent. IAA and IAAsp spots were located under UV light and their radioactivities were scanned with a radioactivity scanner; relative radioactivities in each spot were determined by their peak areas.

RESULTS

Comparison of Thidiazuron and BA on Ca^{2+}-Dependent Ethylene Production in Mungean Hypocotyls. Table I shows that 10 μM thidiazuron, 10 μM BA, or 10 mM Ca^{2+} alone promoted ethylene production slightly (3–5-fold) over the control. Such a slight promotion of ethylene production by thidiazuron in mungbean hypocotyls was recently reported by Suttle (18). However, when thidiazuron or BA was supplied in combination with Ca^{2+}, a marked synergistic effect on ethylene production was observed. The synergistic ratio, which is defined as the quotient of (ethylene produced due to the presence of both cytokinin and Ca^{2+}) ÷ ([ethylene produced due to the presence of cytokinin alone] + [ethylene produced due to the presence of Ca^{2+} alone]), was 3.2 for 10 μM BA and 4.2 for 10 μM of thidiazuron. The dependence of Ca^{2+}-dependent ethylene production on the concentrations of thidiazuron and BA was compared and is shown in Figure 1. As the concentration of thidiazuron or BA was increased from 0.01 to 100 μM, ethylene production increased progressively. Because of the solubility, higher concentrations were not examined. Figure 1 also shows that thidiazuron was slightly more effectively than BA at equimolar concentration.

Comparison of Thidiazuron and other Thiadiazolylurea Derivatives on the Ca^{2+}-Dependent Ethylene Production in Mungbean Hypocotyls. The structure-activity responses of these analogs in promoting the growth of callus culture have been reported by Mok *et al.* (17). In the present study, thidiazuron and its analogs were tested for their activities to promote ethylene production by mungbean hypocotyls in the presence of 10 mM $CaCl_2$ (Table II). Thidiazuron was the most active thiadiazolylurea derivative to trigger the synergistic ethylene production based on its synergistic ratio. Replacement of *N*-phenyl group of thidiazuron with other ring stuctures resulted in a reduction in activity in the following order: phenyl-(**1**) > 2-pyridyl-(**2**) > furfuryl-(**3**) = methyl,phenyl-(**4**) = benzyl-(**5**) > thiadiazolyl-(**6**). Similar to the results of Table I and Figure 1, thidiazuron was more active than BA in this experiment. However, diphenylurea (**7**) at 10 μM showed little synergistic effect on ethylene production as in the callus growth system (17). These results agree well with the activity of these compounds in promotion of the growth of callus tissues as reported by Mok *et al.* (17).

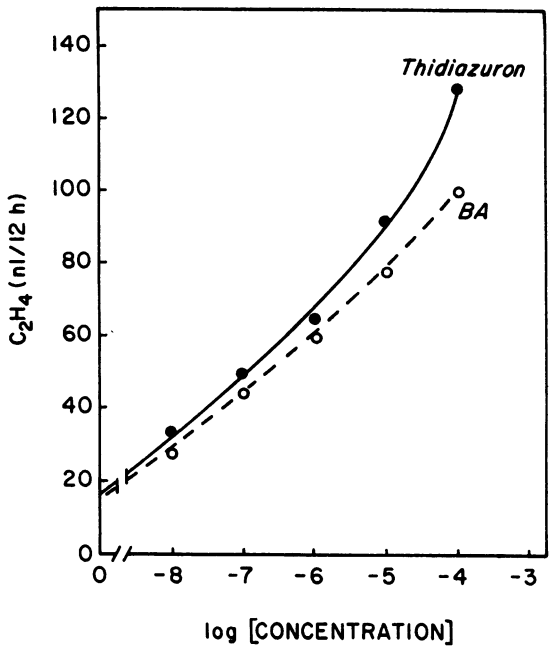


FIG. 1. Effect of various concentrations of thidiazuron or BA on Ca^{2+}-dependent ethylene production by mungbean hypocotyls. The assay conditions were the same as in Table I, except that 0.01 to 100 μM thidiazuron or BA was employed.

Table I. *Synergistic Effect of Thidiazuron or BA on Ca^{2+}-Dependent Ethylene Production in Mungbean Hypocotyls*

Ten segments of mungbean hypocotyls were incubated in a 50-ml Erlenmeyer flask which contained 5 ml medium consisting of 50 mM K-phosphate (pH 5.8), 2% sucrose, and various additions where indicated. Ethylene produced during the 12-h incubation periods at 25°C was determined.

Additions	Ethylene
	nl
None	4.6
BA (10 μM)	14.5
Thidiazuron (10 μM)	17.7
Ca^{2+} (10 mM)	19.8
BA (10 μM) + Ca^{2+} (10 mM)	84.7
Thidiazuron (10 μM) + Ca^{2+} (10 mM)	123.9

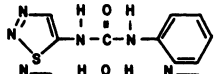
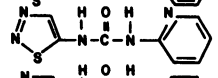
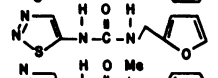
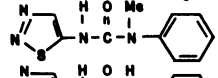
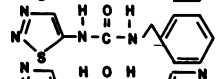
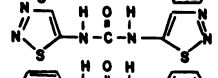
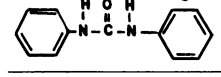
Comparison of Thidiazuron and BA on IAA-Dependent Ethylene Production in Mungbean Hypocotyls. The synergistic effect of thidiazuron or BA on the IAA-dependent ethylene production is shown in Table III. IAA at 10 μM markedly stimulated ethylene production nearly 10-fold above the control. In the presence of 10 μM of IAA, addition of 0.1, 1, or 10 μM of thidiazuron resulted in synergistic increase of ethylene production with the synergistic ratios of 2.3, 3.4, and 2.9, respectively. When BA was employed, the synergistic effect was not significant at 0.1 μM, but was significant at 1 and 10 μM with synergistic ratios of 2.5 and 1.9, respectively. These data also indicate that thidiazuron is more active than BA in the promotion of IAA-dependent ethylene production.

Effect of Thidiazuron on IAA Conjugation. It has been documented that exogenously supplied IAA is rapidly metabolized to IAAsp in mungbean hypocotyls and that cytokinin is capable of suppressing this conjugation of IAA to IAAsp (12). It is interesting to examine whether thidiazuron also displays such a cytokinin activity. Figure 2 illustrates that 0.1 mM of thidiazuron markedly suppressed the conjugation of exogenously supplied

[2] Abbreviations: IAAsp, *N*-(indole-3-acetyl)-asparate; PCIB, 4-chlorophenoxy-isobutyric acid; ACC, 1-aminocyclopropane-1-carboxylic acid.

Table II. *Comparison of Various Thidiazuron Derivatives on Ca^{2+}-Dependent Ethylene Production in Mungbean Hypocotyls*

The assay mixtures and conditions are as described in Table I, except where indicated, 10 μM BA, 10 μM of thidiazuron derivatives, and 10 mM $CaCl_2$ were added.

Additions	Ethylene		Synergistic Ratio
	$-Ca^{2+}$	$+Ca^{2+}$	
	nl		
None	5.2	18.8	1.0
BA	15.9	99.5	3.9
 (1)	18.6	117.1	4.2
 (2)	31.5	118.0	2.8
 (3)	29.7	81.6	2.0
 (4)	14.2	48.6	1.9
 (5)	25.6	68.5	1.9
 (6)	11.9	37.9	1.6
 (7)	2.7	22.5	1.6

(1) *N*-Phenyl-*N'*-1,2,3-thiadiazol-5-yl-urea (thidiazuron).
(2) *N*-2-Pyridyl-*N'*-1,2,3-thiadiazol-5-yl-urea.
(3) *N*-Furfuryl-*N'*-1,2,3-thiadiazol-5-yl-urea.
(4) *N*-Methyl-*N*-phenyl-*N'*-1,2,3-thiadiazol-5-yl-urea.
(5) *N*-Benzyl-*N'*-1,2,3-thiadiazol-5-yl-urea.
(6) *N,N'*-Di-1,2,3-thiadiazol-5-yl-urea.
(7) Diphenylurea.

Table III. *Comparison of BA and Thidiazuron on IAA-Dependent Ethylene Production in Mungbean Hypocotyls*

The assay conditions were those of Table I, except that various additions were employed where indicated and the incubation was for 9 h at 25°C.

Additions	Ethylene
	nl
None	3.0
IAA (10 μM)	28.7
Thidiazuron (0.1 μM)	5.6
Thidiazuron (0.1 μM) + IAA (10 μM)	68.9
Thidiazuron (1 μM)	11.8
Thidiazuron (1 μM) + IAA (10 μM)	122.4
Thidiazuron (10 μM)	18.7
Thidiazuron (10 μM) + IAA (10 μM)	130.6
BA (0.1 μM)	5.5
BA (0.1 μM) + IAA (10 μM)	38.8
BA (1 μM)	9.5
BA (1 μM) + IAA (10 μM)	83.5
BA (10 μM)	22.3
BA (10 μM) + IAA (10 μM)	91.4

[carboxyl-^{14}C]IAA during the course of incubation. The relative IAAsp and IAA contents were estimated from the peak areas of the paper radiochromatograms of the extracts. After 2.5 h of incubation, the IAAsp to IAA ratio was 0.9 in the control, but 0.3 in the presence of 0.1 mM of thidiazuron; after 4.5 h of incubation, the ratio was 14 in the control, but reduced to 1.3 in the presence of thidiazuron.

Comparison of Thidiazuron and BA on Ethylene Production in Water-Stressed Wheat Leaves. The effect of thidiazuron or BA on ethylene production by wheat leaves, which was subjected to second wilting treatment, is shown in Table IV. Thidiazuron, at concentrations ranging from 1 to 500 μM, caused little ethylene production when applied to the rehydrated, turgid wheat leaves but caused marked increase in stress ethylene production following the wilting treatment. Similarly, BA at 1 mM also caused little ethylene production in nonstressed leaves but caused marked increase in stress ethylene production following wilting treatment. However, the potency of BA diminished rapidly as the concentration was decreased. Our results indicate that treatments with 1 mM BA and with 0.01 mM thidiazuron resulted in similar extent of stress ethylene production. These data indicate

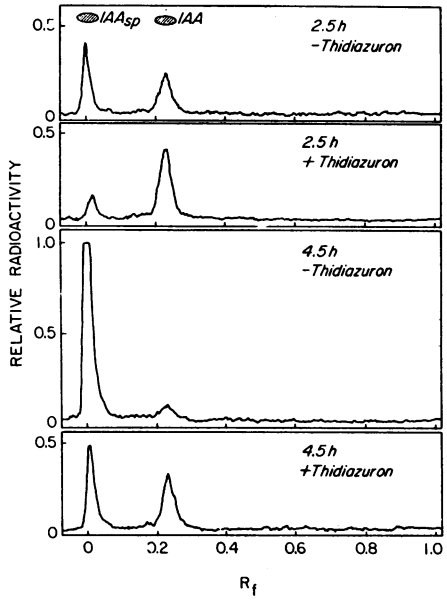


FIG. 2. Paper radiochromatograms of extracts of mungbean hypocotyls which were incubated with 0.22 μmol of [carboxy-^{14}C]IAA (1 μCi) with or without 0.5 μmol of thidiazuron. After incubation for 2.5 h, 0.043 μCi and 0.050 μCi of radioactivity were recovered from the minus and plus thidiazuron treated tissues, respectively, while after 4.5 h of incubation, the recovery of radioactivity was 0.136 μCi for the minus thidiazuron and 0.100 μCi for the plus thidiazuron treated tissues. Relative radioactivities of IAA and IAAsp after incubation for 2.5 h and 4.5 h are shown.

Table IV. *Comparison of BA and Thidiazuron on Wilting-Induced Ethylene Production in Detached Wheat Leaves*

Twenty excised leaves, which were wilted as described in "Materials and Methods," were placed in a 15-ml test tube and rehydrated with different concentrations of BA or thidiazuron. After 14 h of recovery, leaves were wilted to 90% of their original fresh weight and enclosed in test tubes for ethylene measurement.

Treatment	Ethylene		Total 0–7 h after stress
	Before stress	2.5 h after stress	
	nl/h		*nl*
Control	1.5	10.1	20.6
BA (1 μM)	3.8	9.8	18.2
BA (0.01 mM)	2.2	13.5	23.7
BA (0.1 mM)	3.0	19.2	38.0
BA (1 mM)	2.1	35.7	85.2
Thidiazuron (1 μM)	3.0	30.3	58.4
Thidiazuron (0.01 mM)	3.6	38.1	83.8
Thidiazuron (0.1 mM)	3.0	48.2	93.3
Thidiazuron (0.5 mM)	3.0	45.9	110.8

that thidiazuron is more active than BA by two orders of magnitude.

Effect of PCIB on IAA-, Thidiazuron-, and BA-Dependent Ethylene Production in Mungbean Hypocotyls. Based on the observation that PCIB, an anti-auxin compound, inhibited thidiazuron-mediated ethylene production, Suttle (18) has proposed that thidiazuron acts as an auxin-like compound. Suttle's conclusion is valid if the inhibition of ethylene production exerted by PCIB is specific to auxin-dependent systems. We have therefore examined the effects of PCIB on IAA-dependent, BA-dependent, and thidiazuron-dependent ethylene production and the results are presented in Table V. IAA at 100 μM promoted ethylene production 46 times, and this IAA-dependent ethylene production was inhibited 60% upon the addition of 1 mM PCIB in the medium. Thidiazuron or BA each at 100 μM promoted ethylene production 4 and 5 times, respectively. In the presence of 1 mM of PCIB, ethylene production was similarly (50%) inhibited in both cases. Since PCIB inhibited cytokinin-mediated and thidiazuron-mediated ethylene production to the same extent (8; Table V) as it inhibited IAA-mediated ethylene production (Table V; 8, 18), PCIB does not appear to be a specific inhibitor of auxin-mediated ethylene production. Thus, there is no basis to assume that thidiazuron acts as auxin-like compound. Moreover, the marked synergistic effect between thidiazuron and IAA in promoting ethylene in this system does not support the view that thidiazuron acts as an auxin.

DISCUSSION

Yu *et al.* (24) have shown that BA is as active as other highly active adenine-type cytokinins in inducing synergistic ethylene production with Ca^{2+} or IAA in mungbean hypocotyls, and that the structure-activity relationships among N^6-substituted adenine-type cytokinins for the callus tissue growth systems and for synergistic stimulation of ethylene production in mungbean hypocotyls were similar. In the present study we have shown that thidiazuron, a substituted urea, displays activity equivalent to or exceeding that of BA in three known cytokinin-dependent ethylene producing systems. Thus, as in callus culture bioassy (5, 17), the stimulation of ethylene production can be exerted by the adenine type as well as the substituted urea type cytokinins. The biological response to an exogenous compound depends not only on its function at action site, but also on its uptake, translocation, and metabolism. Nevertheless, the relationship between structure and activity of thidiazuron and other thiadiazolylurea derivatives (Table II), in inducing synergistic ethylene production with Ca^{2+} in mungbean hypocotyls, is similar to that in the growth of callus tissue (17). While thidiazuron and BA displayed comparable activity in inducing ethylene production in mungbean hypocotyls, thidiazuron displayed much higher activity than BA in inducing wilting-dependent ethylene production. This may be explained on the basis of different uptake and/or metabolism in different plant tissue rather than of different structural requirements.

Table V. *Effect of PCIB on IAA-, BA- or Thidiazuron-Dependent Ethylene Production in Mungbean Hypocotyls*

The assay conditions were as described in Table I, except where indicated, 0.1 mM IAA, 0.1 mM BA, 0.1 mM thidiazuron, and 1 mM PCIB were added.

Additions	Ethylene	
	−PCIB	+PCIB
	nl	
None	6.1	
IAA	277.5	110.6
BA	24.2	12.4
Thidiazuron	32.9	17.0

Since mungbean can replace the N^6-substituted adenine cytokinins in such a wide range of physiological systems, both N^6-substituted adenine derivatives and substituted urea derivatives probably have the same site(s) of action. Antagonistic studies of both types of compounds do support this point (9). Iwamura *et al.* (10) argued that both groups of compounds bear common molecular features. Recently, high affinity cytokinin binding sites have been found in wheat germ ribosomes (6), and mitochondrial fractions of mungbean seedlings (11). It is yet to be tested whether these binding sites also recognize thidiazuron and its allied compounds.

In the present Ca^{2+} + cytokinin synergistic ethylene production system phosphate ion is required. The synergistic effect can only occur when hypocotyls are incubated with phosphate at concentrations above 5 mM (W-K Yip and SF Yang, unpublished observation). This explains why no synergistic ethylene production was observed by Suttle (18), when mungbean hypocotyls were incubated in thidiazuron + Ca^{2+} + Mes. Although it is clear that Ca^{2+}, cytokinin, and phosphate induces the synergistic ethylene production by inducing the synthesis of ACC (21), it is not clear how Ca^{2+}, cytokinin and phosphate interact, and whether calmodulin is involved in the induced synthesis of ACC, by presumably inducing the synthesis of ACC synthase.

In the IAA + cytokinin synergistic ethylene producing system, the synthesis of ACC synthase is induced, resulting in increased ACC level and increased ethylene production rate (22, 23). Although the mode of cytokinin action in this system has not been fully understood (8), cytokinin is known to play a role by suppressing the conjugation of IAA to IAAsp, and thereby maintaining a higher IAA concentration within the tissue (12). Figure 2 shows that thidiazuron was also very effective in suppressing IAA conjugation, and thus maintained a high level of free IAA. BA promotes wilting-induced ethylene production by promoting the synthesis of ACC (16). However, the mode of BA action in this system is not clear. The results of Table IV indicate that thidiazuron is more active than BA in promoting this type of wilting-induced ethylene production. Thidiazuron has been used as an agent to induce the defoliation of cotton leaves. It is yet to be determined whether an increase in ethylene production is directly related to the action of thidiazuron as a cotton defoliant.

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