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Title: The 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.

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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 the ribonucleoside.

The [^{14}C]i^{6}Ado metabolism was also examined in cv. Jackson Wonder tissues grown in the simultaneous presence of Thidiazuron and zeatin. Regardless of the zeatin concentration present, at suboptimal Thidiazuron concentration, the [^{14}C]i^{6}Ado metabolism resembled that of tissues grown on zeatin alone; while at optimal concentration of Thidiazuron, nucleotide formation was inhibited. Cytokinin-autonomous growth was observed in tissues transferred from medium containing optimal Thidiazuron concentrations (and any concentration of zeatin), as well as suboptimal concentrations of the two compounds. Moreover, uptake of Thidiazuron and zeatin was found to be independent. Thus, although the cytokinin-active phenylureas and adenine derivatives differ in their effects on cytokinin autonomy as well as nucleotide formation, the two effects do not seem to be related.

The Effects of Thidiazuron on Cytokinin Autonomy and the Metabolism of $N^6(\Delta^2$-isopentenyl) [8-^{14}C]adenosine in Callus Tissues of *Phaseolus lunatus* L.

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Typed by Donna Lee Norvell-Race for Susan C. Capelle

Dedicated to the memory of

my friend . . .

Tante Lore

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LIST OF ABBREVIATIONS

Ado: adenosine

DPU: N,N'-diphenylurea

HPLC: High-performance Liquid Chromatography

i^6Ade: N^6-(Δ^2-isopentenyl)adenine

i^6Ado: N^6-(Δ^2-isopentenyl)adenosine

[^{14}C]i^6Ado: N^6-(Δ^2-isopentenyl)[8-^{14}C]adenosine

i^6AMP: N^6-(Δ^2-isopentenyl)adenosine 5'-monophosphate

i^6ADP: N^6-(Δ^2-isopentenyl)adenosine 5'-diphosphate

i^6ATP: N^6-(Δ^2-isopentenyl)adenosine 5'-triphosphate

TEA: triethylamine

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

The Effects of Thidiazuron on Cytokinin Autonomy and the Metabolism of $N^6(\Delta^2$-isopentenyl)[8-^{14}C]adenosine in Callus Tissues of *Phaseolus lunatus* L.

I. INTRODUCTION

The generic term "cytokinin" was proposed by Skoog *et al.* (1965) to describe those natural and synthetic plant growth substances which display kinetin-like biological activities. These activities include the promotion of cell division and bud formation, the release of lateral buds from apical dominance, and the retardation of senescence. Kinetin was the first of such compounds isolated, from an autoclaved herring sperm DNA preparation, and was identified as 6-furfurylaminopurine (Miller *et al.*, 1955, 1956). The first naturally occurring cytokinin was isolated from immature maize endosperm (Letham, 1963; Miller, 1961a), and hence called zeatin. It was characterized as 6-(4-hydroxy-3-methyl-*trans*-2-butenylamino)purine (Letham *et al.*, 1964), and is thus structurally similar to kinetin. Numerous compounds exhibiting cytokinin activity have subsequently been discovered and synthesized.

To detect cytokinin activity various bioassays based upon the growth regulatory activities of cytokinins are utilized. Cell division activity is commonly measured in the tobacco callus (Linsmaier and Skoog, 1965), soybean callus (Miller, 1960), *Phaseolus* callus (Mok *et al.*, 1978), and carrot phloem (Letham, 1967) bioassays. For kinetic studies, a modified tobacco assay

which measures growth rate rather than final yield of tissues has been devised (Hegelson et al., 1969). Cytokinin activities may also be assayed by the chlorophyll retention of radish leaf disks (Bruce et al., 1965), formation of lateral buds in Pisum sativum (Thimann and Sachs, 1966), and pigment formation in Amaranthus (Bamberger and Mayer, 1960). In the tobacco callus assay, kinetin concentrations as low as 10^{-9} M can be detected (Miller et al., 1955, 1956). Zeatin is approximately 100 times more active than kinetin in this system.

Systematic tests of cytokinin analogs have revealed the important structural features which confer cytokinin activity. These have been reviewed by Skoog and Armstrong (1970), Strong (1958), and Matsubara (1980). Basically, the aminopurine moiety and a N^6-substituent are essential for high activity. Furthermore, side chains which enhance the planarity of the molecule promote biological activity (Hecht et al., 1970).

Certain substituted urea compounds such as N,N'-diphenylurea (DPU) display cytokinin activity in callus culture as well as other bioassays (Bruce and Zwar, 1966; Bruce et al., 1965; Isogai, 1981; Mok et al., 1979; Shantz and Steward, 1955; Takahashi et al., 1978). Although DPU is a rather weakly active cytokinin, other phenylurea compounds such as the N-phenyl-N'-(4-pyridyl)urea derivatives and N-phenyl-N'-1,2,3-thiadiazol-5-ylurea (Thidiazuron) have been shown to possess high cytokinin activity (Isogai et al., 1976; Mok et al., 1982a; Takahashi

et al., 1978). Thus, cytokinin activity is a property of two distinct classes of compounds: N^6-substituted adenine derivatives and substituted phenylureas.

Previous studies on the activity of DPU in Phaseolus lunatus callus bioassays (Armstrong et al., 1981; Mok et al., 1979) 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.

The extremely high cytokinin activity of Thidiazuron in the P. lunatus callus system (Mok et al., 1982a) 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. 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, are examined in this study.

II. LITERATURE REVIEW

A. Cytokinin Activity of Phenylurea Derivatives

The cytokinin activity of diphenylurea (DPU) was first discovered by Shantz and Steward (1952, 1955) using the carrot phloem assay, and later confirmed in tobacco callus (Strong, 1958; Miller, 1961b; Bottomley et al., 1963), and soybean callus bioassays (Miller, 1960). DPU was also shown to be active in numerous other cytokinin bioassays: senescence retardation of radish leaf disks (Bruce et al., 1965; Kefford et al., 1973), pea lateral bud growth (Bruce et al., 1965; Kefford et al., 1966), promotion of lettuce seed germination (Bruce et al., 1965; Kefford et al., 1965), and the alteration of sex expression in Luffa acutangula (Bose and Nitsch, 1970). However, the activity of DPU was weak and variable in some of these tests.

Bruce and Zwar (1966) studied the structure-activity relationships of mono- and di-substituted urea and thiourea derivatives using the tobacco pith assay and later also the radish leaf disk chlorophyll retention bioassay (Kefford et al., 1973). Phenylurea was found to be the simplest active compound. Its characteristic structural properties, the planar ring and urea side chain, were found to be essential for the activity of all urea derivatives. Substitutions in the phenyl ring generally increased activity particularly with electronegative substituents

such as nitro or halogen groups in the following order: ortho< para<meta for the tobacco callus cell division assay, and ortho< meta<para in the chlorophyll retention assay. Addition of a phenyl group to the other side of the urea bridge (but not a thiourea bridge) enhanced activity.

Thiourea derivatives were found to be only slightly active in the tobacco pith assay (Bruce et al., 1965). However, Erez (1977) reported that thiourea promoted growth of cytokinin-requiring soybean, tobacco, and apple callus cultures. A synergistic effect between this compound and zeatin was observed in the stimulation of soybean callus growth. This result was taken as evidence in favor of the involvement of thiourea in the promotion of cell division by a different mechanism than either the urea or the purine-type cytokinins. The structure-activity relationships of the urea and thiourea derivatives were found to differ between tests measuring the promotion of germination and the stimulation of cell division (Kefford et al., 1965). In addition, thiourea and several dimethylphenylurea derivatives were almost completely inactive in stimulating cell division, but nevertheless lowered the light requirement of germinating seeds. From these results it was concluded that cytokinin activity of urea derivatives may not be a prerequisite for the ability of these compounds to promote lettuce seed germination.

Derivatives of pyridylurea which were moderately active or inactive in the tobacco pith assay were highly active in the

chlorophyll retention bioassay (Kefford et al., 1973), in the lettuce seed germination test (Kefford et al., 1965), and in the promotion of lateral pea bud development (Kefford et al., 1966). The N-phenyl-N'-(4-pyridyl)urea position-isomer was found to be particularly active in all of these assays. Okamoto et al. (1974) retested this compound and found that it was 10 times less active than kinetin in the tobacco callus bioassay but gave 10-20% greater callus yield at optimal concentration than the purine cytokinins. It was also shown to be highly active in the promotion of organ formation in tobacco tissue cultures (Isogai et al., 1976; Okamoto et al., 1978).

Derivatives of N-phenyl-N'-(4-pyridyl)urea varied in activity in the tobacco callus bioassay, depending upon the substituent and its position (Takahashi et al., 1978). Generally, substitution into the phenyl ring decreased activity, in the order meta> ortho>para, in notable contrast to the increase in activity by substitution in the phenyl ring of DPU (Bruce and Zwar, 1966). An exception to this was the marked increase in activity by the introduction of an electronegative Cl molecule into the 2-position of the pyridyl ring. N-phenyl-N'-(2-chloro-4-pyridyl)urea was found to be 10^4 times more active than DPU and 10^2 times more active than N-phenyl-N'-(4-pyridyl)urea in the tobacco callus bioassay and in the induction of shoot formation in tobacco cultures (Okamoto et al., 1978). In search of more potent cytokinin active urea derivatives, Okamoto et al. (1981) synthesized

several di-substituted pyridylurea derivatives. Of these, N-(2,6-dichloro-4-pyridyl)-N'-phenylurea, N-(2,6,dibromo-4-pyridyl)-N'-phenylurea , and N-(2,6-dichloro-4-pyridyl)-N'-m-fluorophenylurea were extremely active in promoting tobacco callus growth, exceeding that of all known adenine-type cytokinins.

Karanov et al. (1968) investigated the structure-activity relationships of N-allyl-N'-phenylthiourea derivatives in the senescence retardation and growth promotion assays using radish leaf disks. The results indicate that the effects of the introduction of substituents into the phenyl ring depend on the particular substituent and its position in the ring. However, even the most active compounds tested, N-phenyl-N'-p-carboxy-phenylthiourea and N-allyl-N'-3-hydroxy-4-carboxy-phenylthiourea, were less active than kinetin.

Of other phenylureas tested, p-phenylthioureidosalicylic acid was found to be active in the Amaranthus bioassay (Vassilev et al., 1978), retardation of senescence in radish leaf disks (Vassilev and Karanov, 1972), and in the tobacco callus bioassay (Vassilev and Kamínek, 1981). N-2-(oxo-1-imidazolidinyl)ethyl-N'-phenylurea, an experimentally used antiozonant was effective in retarding senescence in red clover (Lee et al., 1981). However, it has not been tested for activity in any of the standard cytokinin bioassays.

Recently, a cotton defoliant, N-phenyl-N'-1,2,3-thiadiazol-

5-ylurea (Thidiazuron), was shown to possess cytokinin activity (Mok et al., 1982a). It was as active as some of the N-phenyl-N'-(4-pyridyl)urea derivatives and slightly more active than zeatin in the P. lunatus bioassay. In citrus tissue cultures Thidiazuron stimulated callus formation and abscission of buds (Taha and Stino, 1981). Thidiazuron was also found to be an uncoupler of photosynthetic and mitochondrial phosphorylation (Hauska et al., 1975).

Several hypotheses have been presented to explain the cytokinin activity of phenylurea derivatives. Kefford et al. (1968) and Isogai (1981) reported that certain benzylurea derivatives antagonize the activity of adenine derivatives as well as substituted phenylureas, and suggested that the two types of compounds may have a common site of action. Iwamura et al. (1980), using physico-chemical substituent parameters, have argued that cytokinin-active adenine derivatives and phenylureas have some features in common. Miller (1961b) proposed that DPU may be converted to active adenine-type compounds by serving as a precursor for the N^6-side chain. In support of this hypothesis, the phenylureidopurines were found to possess cytokinin activity (McDonald et al., 1971). In addition, the cytokinin activity of DPU derivatives has been shown to be associated with the ability of tissues to metabolize DPU (Dyson et al., 1972). However, the only metabolite to be isolated and identified has been an inactive glucosyl derivative (Burrows and Leworthy, 1976). The metabolism

of the more active phenylurea derivatives in plant systems has not been reported. The metabolism of Thidiazuron has been determined in rats (Crecelius and Knowles, 1978), goats and chickens (Benezet et al., 1978). In all cases the primary metabolites recovered in free or conjugated form were 4-hydroxyphenylthidiazuron and phenylurea. The metabolic pathway was found to involve the hydroxylation of the aniline moiety at the 4-position with subsequent conjugations to form the glucuronic acid or the ethereal sulfate.

B. The Metabolism of N^6-(Δ^2-isopentenyl) adenosine(i^6Ado) and its Derivatives

Paces et al. (1971) identified enzyme activity in crude extracts of tobacco tissue that could cleave the side chain of i^6Ado and other adenine derivatives with unsaturated N^6-side chains. An enzyme capable of such activity was partially purified from corn kernels by Whitty and Hall (1974). The enzyme, N^6-(Δ^2-isopentenyl) adenosine oxidase, or cytokinin oxidase, catalyzed the conversion of i^6Ado to adenosine (Ado) through the formation of an unstable intermediate later identified as an isopentenyl aldehyde (Brownlee et al., 1975). Terrine and Laloue (1980) have demonstrated that degradative activity of this kind varies with endogenous cytokinin levels. Side chain removal activity was shown to be blocked in N^6-(Δ^2-isopentenyl)adenine(i^6Ade) by 7-glucosylation (Laloue et al., 1977), possibly accounting for the 10-fold greater biological activity of i^6Ade over i^6Ado (Schmitz et al., 1972). Laloue (1977)

has further shown that 7-glucosylation is not necessary for the expression of biological activity, which supports the earlier contention that 7-glucosides are storage forms of cytokinins (Parker et al., 1973).

Converson of i^6Ado to the corresponding 5' mono-, di-, and trinucleotides has been shown in tobacco and Acer pseudoplatanus cell cultures (Dorée and Guern, 1973; Laloue et al., 1974, 1975). Although Dorée and Terrine (1973) were able to synthesize 5'-mononucleotides from various N^6-substituted adenosine derivatives in vitro using commercial adenosine kinase preparations, they were unsuccessful in isolating any plant enzyme systems capable of catalyzing the same reaction in vivo. However, Chen and Eckert (1977) reported the formation of N^6-(Δ^2-isopentenyl)adenosine-5'-monophosphate (i^6AMP) from i^6Ado and AMP by an adenosine kinase enzyme obtained from wheat germ. In addition, nucleotides could be formed directly from the free base by the enzyme adenine phosphoribosyltransferase (Chen et al., 1982). The reverse reaction, dephosphorylation of nucleotides to corresponding nucleosides, has been shown to occur in partially purified wheat germ preparations (Chen and Kristopeit, 1981a). i^6Ado has also been shown to be deribosylated to i^6Ade by the enzyme adenosine nucleosidase (Chen and Kristopeit, 1981b), and the ribosylation of i^6Ade to i^6Ado was catalyzed by adenosine phosphorylase (Chen and Petschow, 1978). The significance of these reactions and their relevance to the control of cytokinin activity in the cell remains to be

determined. However, Laloue and Pethe (1982) postulated that the free base may be the "active" form of cytokinins while nucleotide formation may be involved in the regulation of endogenous levels of cytokinin-active bases and ribonucleosides.

The natural occurrence of i^6Ade and i^6Ado has been reported in cytokinin-autonomous tobacco cultures (Dyson and Hall, 1972; Einset and Skoog, 1973), and in the female plants of Mercurialis ambiqua (Dauphin et al., 1979). Chen and Melitz (1979) have isolated and partially purified, from cytokinin-autonomous tobacco cultures, an enzyme system which catalyzes the formation of i^6AMP from isopentenylpyrophosphate and 5'-AMP. Modified nucleotides with the isopentenyl side chain have also been detected adjacent to the 3'-end of tRNA anticodons that recognize codons beginning with U in corn, peas, and spinach (Hall et al., 1967), tobacco (Chen and Hall, 1969), and wheat germ (Burrows et al., 1970). The formation of these modified nucleotides has been shown to occur by attachment of the Δ^2-isopentenyl group to preformed tRNA molecules, and not by the incorporation of intact i^6AMP onto tRNA (Chen and Hall, 1969). Thus, free cytokinins can be synthesized de novo from adenine monomers or formed by hydrolysis of polynucleotides. However, there is no agreement on the relative contribution of the two pathways (Maas and Klämbt, 1981).

C. Cytokinin Autonomy in Plant Tissue Cultures

Cytokinin-dependent callus tissues occasionally lose their requirement for exogenously supplied cytokinins and subsequently continue to proliferate in the absence of this hormone. This phenomenon originally termed "habituation" by Gautheret (1942) has also been called "autonomy" by Braun (1958) to describe hormone-independent crown gall cultures. It has been most extensively described for tobacco (Binns and Meins, 1973; Einset and Skoog, 1973; Fox, 1963) and soybean tissue cultures (Miura and Miller, 1969). Some of the autonomous tissues were found to have sufficient levels of endogenous cytokinins to support growth (Einset and Skoog, 1973; Mirua and Miller, 1969).

Gautheret (1955) hypothesized that habituation was a gradual process, involving epigenetic changes rather than classical mutations. Epigenetic changes must be directed, regularly reversible, occur at high rates, and leave the heritably altered cell totipotent (Meins, 1972; Meins and Lutz, 1980). Meins and Binns (1977, 1978), studying autonomous tobacco cultures, have proposed that the habituation process fulfills these criteria. They have shown that the habituation rates were 100 to 1000 times higher than normal mutation rates. Furthermore, single cell clones originating from the same habituated tissue inherited the habituated phenotype. Although differences in degree of habituation indicated a broad range of habituated states, this

variation was not found to be due to altered chromosome number (Binns and Meins, 1980). Habituated cultures were also capable of returning to the normal cytokinin-requiring phenotype, and of regenerating normal plants. Interestingly, cultures initiated from regenerated plants were all found to be cytokinin-dependent, regardless of the cytokinin requirements of the original cultures from which the plants were regenerated (Meins and Binns, 1977).

Evidence that cytokinin autonomy is also a genetic trait has been demonstrated in the Phaseolus callus system (Mok et al., 1980). P. vulgaris genotypes generally displayed higher levels of autonomy than P. lunatus lines. Reciprocal crosses between strictly cytokinin-requiring and autonomous varieties of P. vulgaris resulted in progeny with intermediate cytokinin-requiring phenotypes, indicating the nuclear control of this trait. Furthermore, genetic ratios recovered in the F_2 generation and in backcrosses demonstrate that the cytokinin-dependent phenotype in these Phaseolus species is controlled by one set of alleles.

Cytokinin autonomy is inducible by certain cultural conditions. Incubation at superoptimal temperatures increased the incidence of cytokinin autonomy (Meins, 1974). The size of explants and the seasonal effects of culture initiation (Meins and Lutz, 1980; Meins et al., 1980) were also shown to be important factors. Furthermore, treatment with certain chemical agents such as carcinogenic morphactins and aminofluorenes (Bednar and Linsmaier-Bednar, 1971a, 1971b, 1971c; Bednar et al., 1973), and

streptomycin (Kamínec and Lu̍stinec, 1974b) promoted cytokinin autonomy.

Disorders in chloroplast function and enzyme activity have been shown to be associated with cytokinin autonomy. Kamínek and Lu̍stinec (1974a) observed that cytokinin-autonomous strains of tobacco had reduced amounts of chlorophyll and numbers of plastids per cell compared to cytokinin-requiring strains. Furthermore, the isozyme patterns of several chloroplast and non-plastid enzymes have been found to be altered in cytokinin-autonomous strains of tobacco (Kamínek et al., 1981). It was suggested that reduced chlorophyll synthesis may result in decreased utilization of endogenous cytokinins, thus enhancing cytokinin autonomy. In support of this hypothesis, Vyskot and Novak (1977) found increased incidence of cytokinin autonomy in tobacco cultures carrying chlorophyll-deficiency mutations located either on the chromosomes or plastid genomes.

To explain the habituation phenomenon, Meins and Binns (1978) have proposed a "positive feedback loop" hypothesis in which cell division factors act as reaction intermediates and induce their own production or block their own degradation. As evidence in favor of this hypothesis they have shown that cultures in which habituation was suppressed by low temperatures could be returned to the habituated state by addition of kinetin. However, Terrine and Laloue (1980) have reported that cytokinin degradation could

be induced by the presence of cytokinins in tobacco cell cultures. Thus, it appears that both negative and positive control mechanisms may regulate cytokinin levels in plant tissues.

III. 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^6Ade, i^6Ado, 3'-nucleotidase (ryegrass), 5'-nucleotidase (Crotalus adamanteus venom) and Sephadex LH-20 were purchased from Sigma. i^6AMP, i^6ADP, and i^6ATP were obtained from P-L Biochemicals. Thidiazuron and [^{14}C]Thidiazuron are 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^6Ado and [^{14}C]zeatin) were obtained from Amersham Co. Picloram is 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 five-day-old seedlings as previously described (Mok and Mok, 1977). 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 four-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 the Results section). 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 (1962) with the following organic substances added: sucrose (30 g/l), myo-inositol (100 mg/l), thiamine. HCl (0.5 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 (Mok and Mok, 1977). 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 (Schmitz and Skoog, 1970) 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 µM and 0.3 µM, respectively, for the two genotypes). The synthesis of [^{14}C]i^{6}Ado (25 mCi/mmol) has been reported previously (Mok et al., 1982b). 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_2O, 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_2O 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 (.45μ). 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 x 60 cm) in 33% (v/v) ethanol. Fractions of 9 ml (0.5 bed volume) were collected. Aliquots (4 ml) were taken from each fraction, evaporated to dryness in scintillation vials, dissolved in Bray's scintillation fluid (Bray, 1960) 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 1N $HClO_4$ (1:1, w/v) and isolation of the cytokinin metabolites as described by Laloue *et al*. (1974).

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_2O, and the incubation periods were 0.5, 1, and 2 h. 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 (Mok et al., 1982b). To further characterize this compound, the dried fractions were redissolved in 0.05 M Tris-HCl buffer (pH 8.6) containing 0.005 M $MgCl_2$, 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 µ, 4.6 x 250 mm; Altex) was used. The aqueous buffer consisted of 0.2 M acetic acid, adjusted to pH 3.5 with triethylamine (TEA). Samples were eluted with a linear gradient of methanol (15 to 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 (Juengling and Kammermeir, 1980). 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 to 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 (+ or - 0.01 µM Thidiazuron) or 0.01 µM [^{14}C]Thidiazuron (+ or - 0.1 µM zeatin). The specific activity 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 activity of [^{14}C]Thidiazuron was 70 mCi/mmol. After 21 days 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.

IV. 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 (Armstrong et al., 1981.) 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.

In order to determine whether Thidiazuron promotes cytokinin-autonomous growth, similarly to DPU (Mok et al., 1979), 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

FIGURE 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.

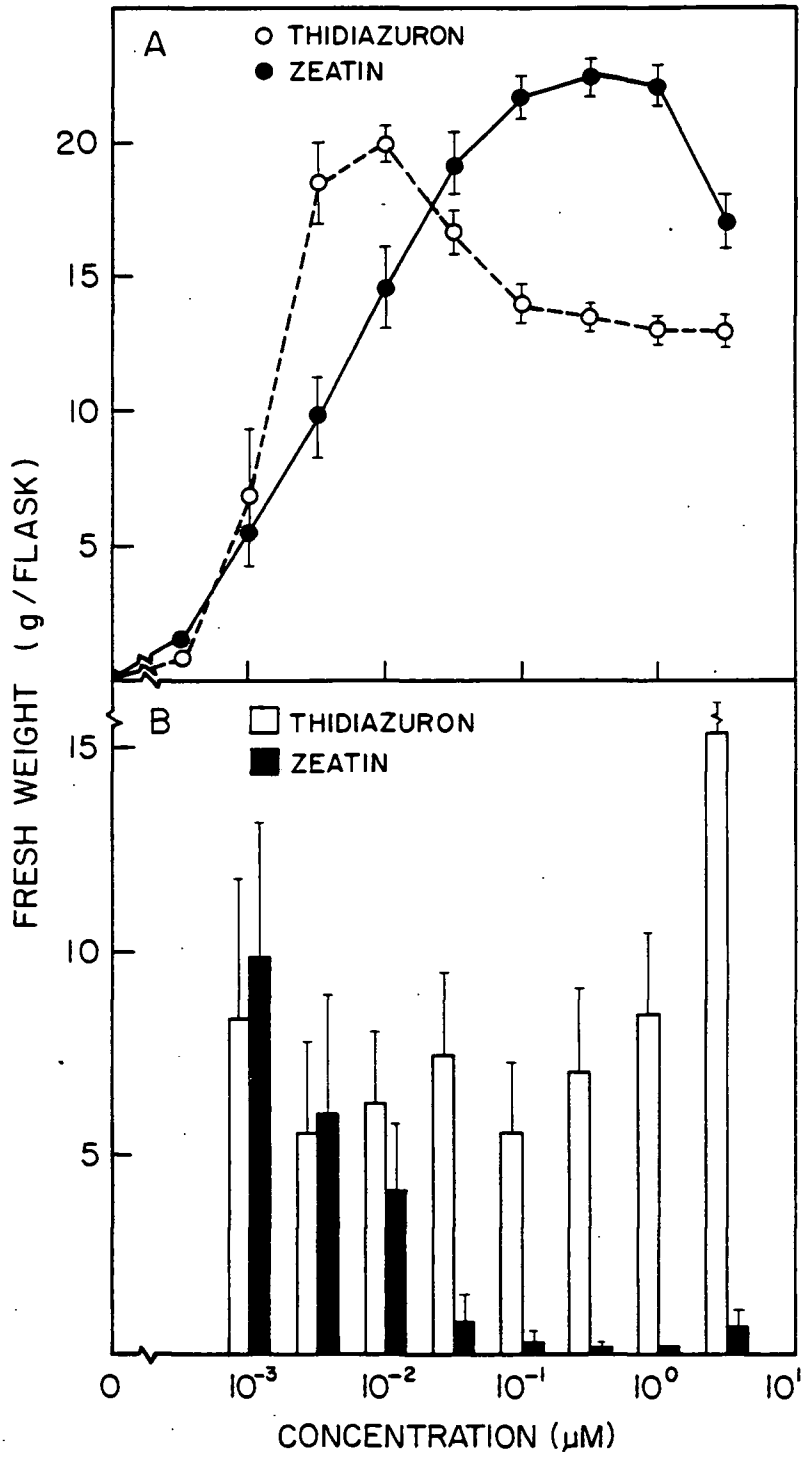
A
THIDIAZURON
ZEATIN
20
15
10
5
B
THIDIAZURON
ZEATIN
15
10
5
FRESH WEIGHT (g/FLASK)
0
10^{-3}
10^{-2}
10^{-1}
10^{0}
10^{1}
CONCENTRATION (μM)

Figure 1.

FIGURE 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.

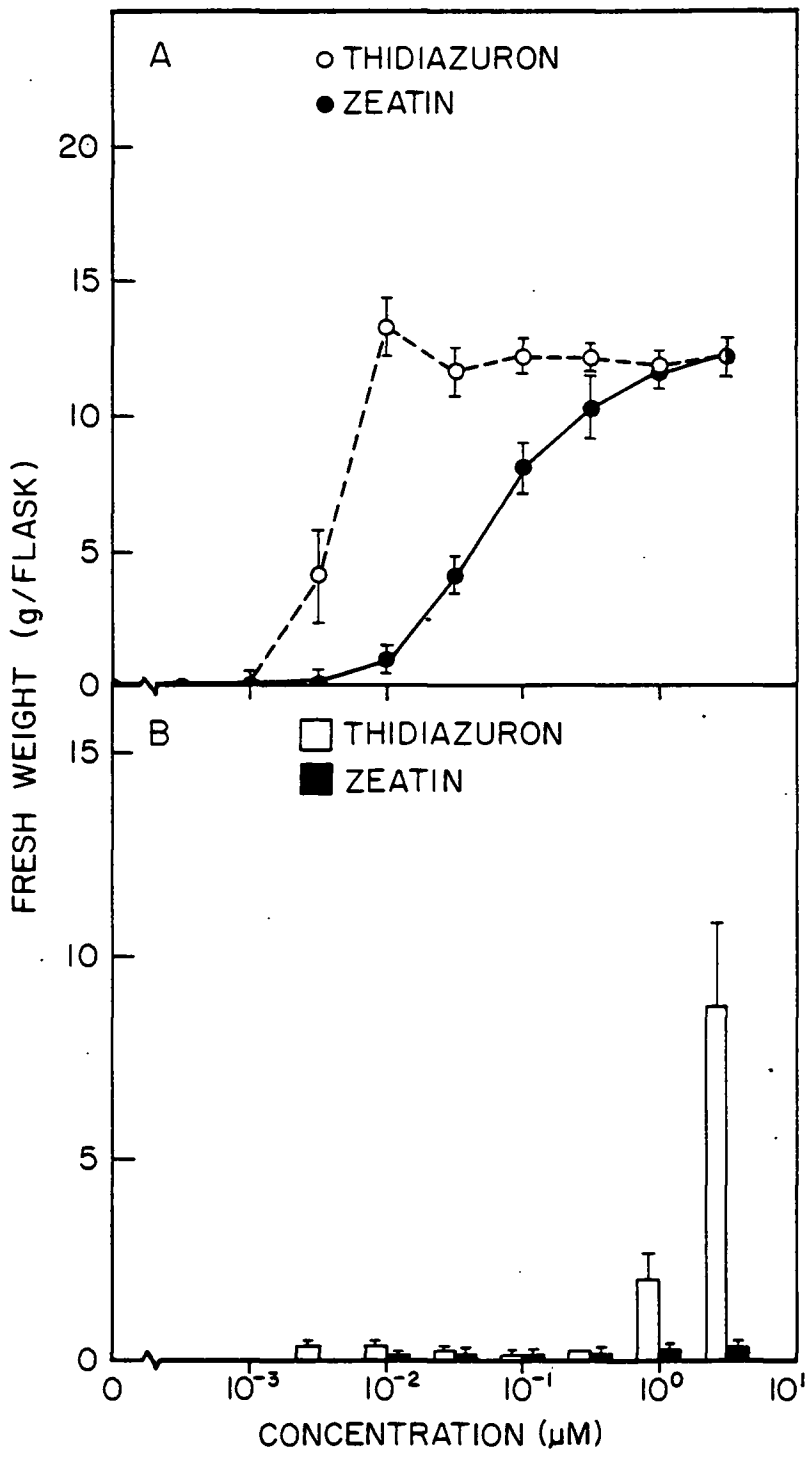
A
o THIDIAZURON
● ZEATIN
20
15
10
5
0
B
□ THIDIAZURON
■ ZEATIN
15
10
5
0
FRESH WEIGHT (g/FLASK)
0
10^{-3}
10^{-2}
10^{-1}
10^{0}
10^{1}
CONCENTRATION (μM)

Figure 2.

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).

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 three-day 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

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

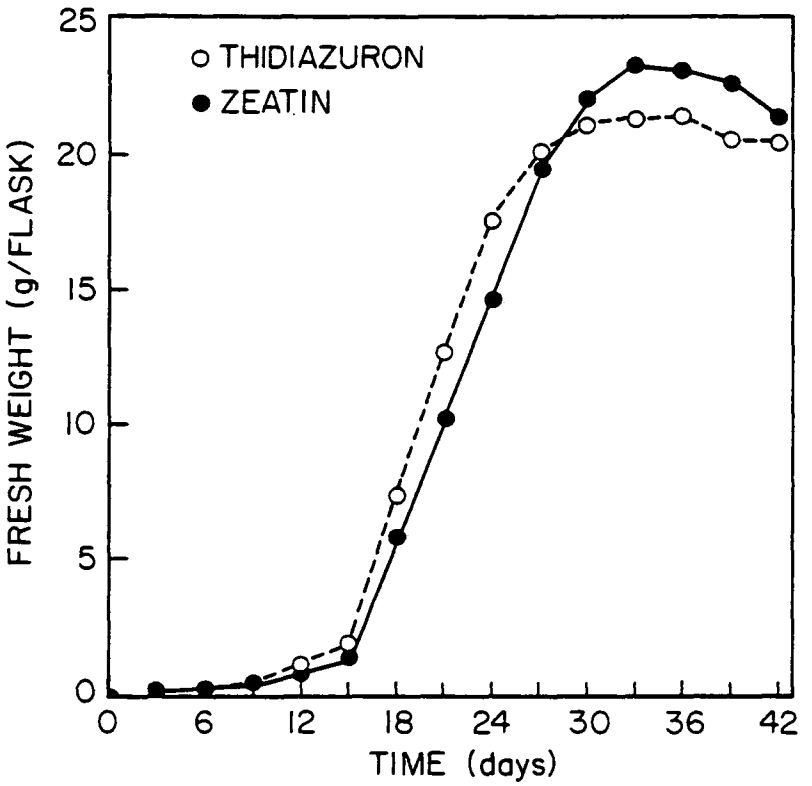
25
20
15
10
5
0
FRESH WEIGHT (g/FLASK)
THIDIAZURON
ZEATIN
0
6
12
18
24
30
36
42
TIME (days)

Figure 3.

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

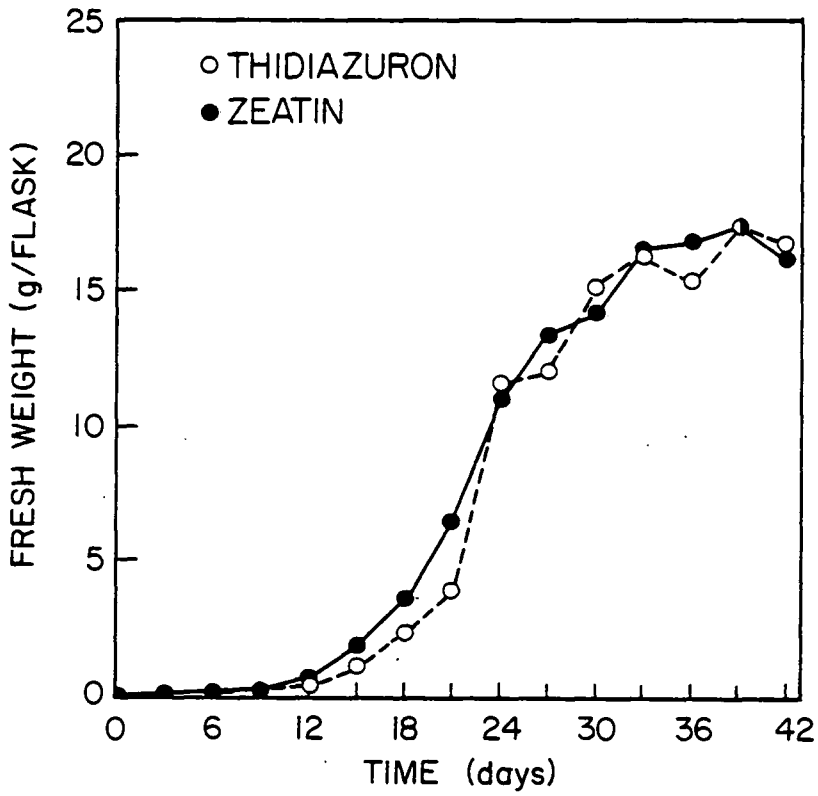
25
20
15
10
5
0
FRESH WEIGHT (g/FLASK)
○ THIDIAZURON
● ZEATIN
0
6
12
18
24
30
36
42
TIME (days)

Figure 4.

FIGURE 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.

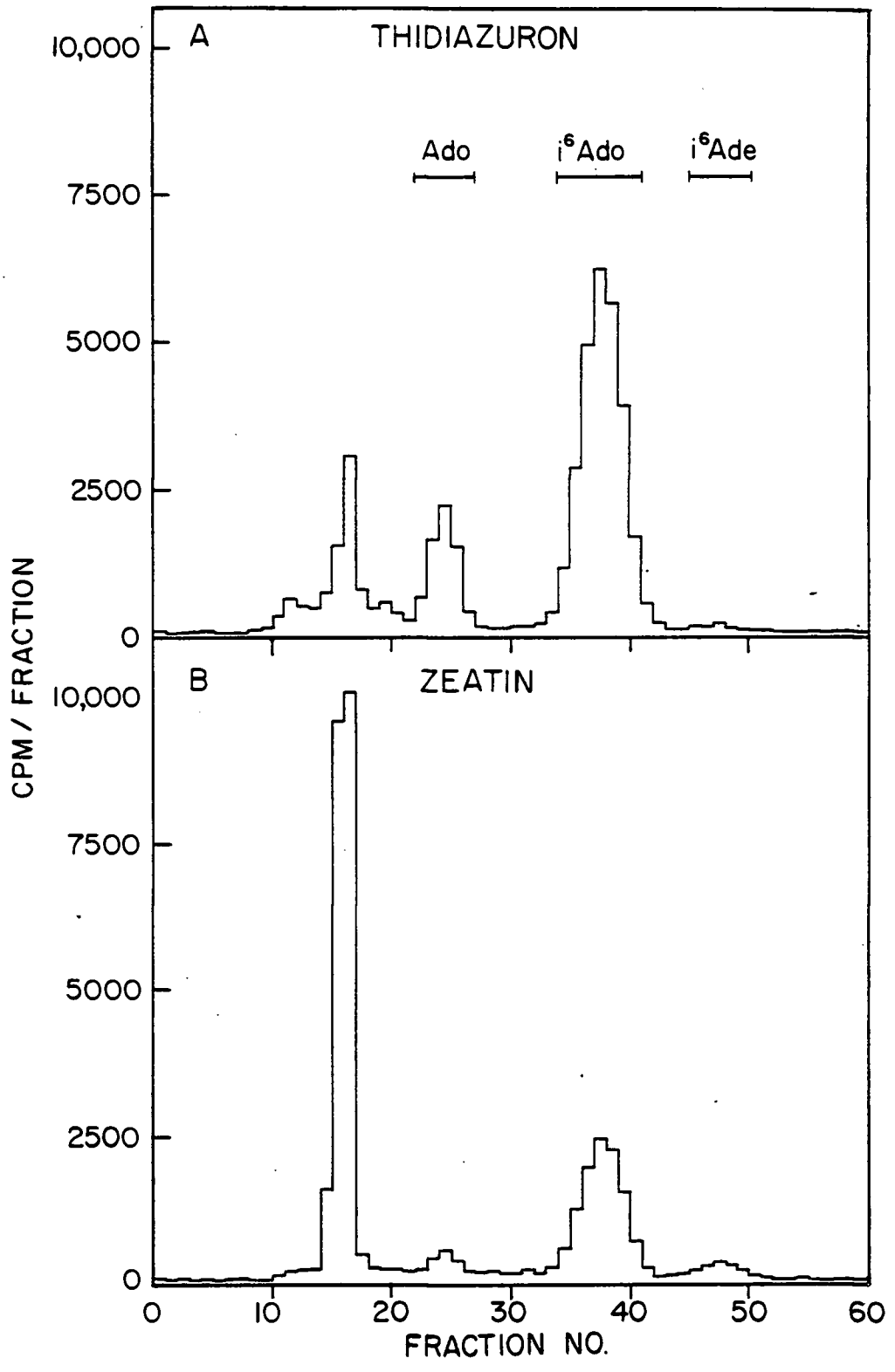
A
THIDIAZURON
Ado
i⁶Ado
i⁶Ade
10,000
7500
5000
2500
0
B
ZEATIN
10,000
7500
5000
2500
0
CPM / FRACTION
0
10
20
30
40
50
60
FRACTION NO.

Figure 5.

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^6Ado previously identified (Mok et al., 1982b). 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. The results are presented in Figure 6. The untreated labeled compound (Fig. 6B) coeluted with i^6AMP, i^6ADP, and i^6ATP (Fig. 6A, B). Incubation with 5'-nucleotidase resulted in a shift of the radioactivity from the nucleotide position to that of i^6Ado (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^6Ado. 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^6AMP (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.

FIGURE 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.

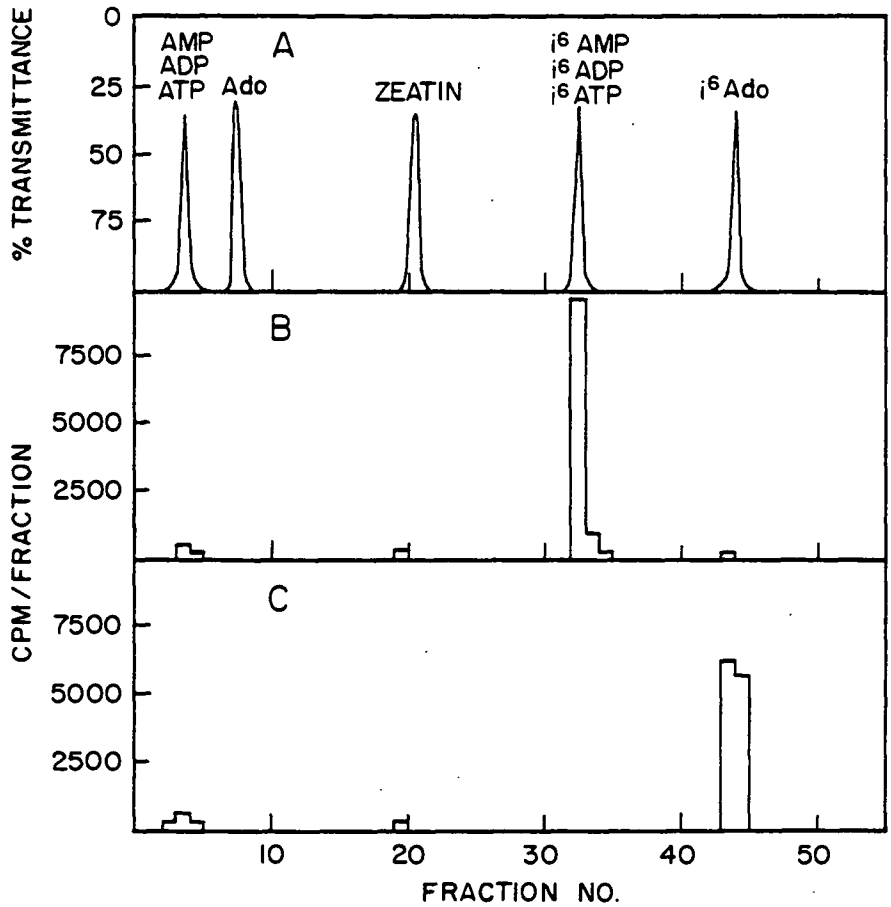
% TRANSMITTANCE
0
25
50
75
AMP
ADP
ATP
Ado
A
ZEATIN
i6 AMP
i6 ADP
i6 ATP
i6 Ado
B
7500
5000
2500
CPM/FRACTION
C
7500
5000
2500
10
20
30
40
50
FRACTION NO.

Figure 6.

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

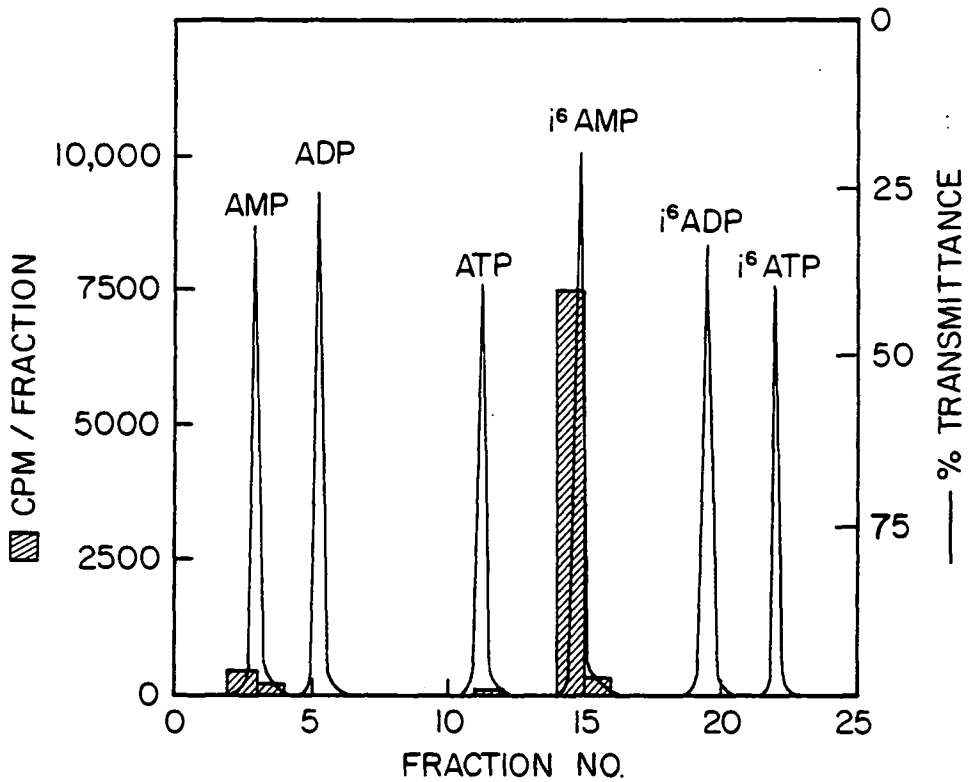
AMP
ADP
ATP
i⁶AMP
i⁶ADP
i⁶ATP
10,000
7500
5000
2500
0
CPM / FRACTION
0
5
10
15
20
25
FRACTION NO.
0
25
50
75
% TRANSMITTANCE

Figure 7.

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

FIGURE 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).

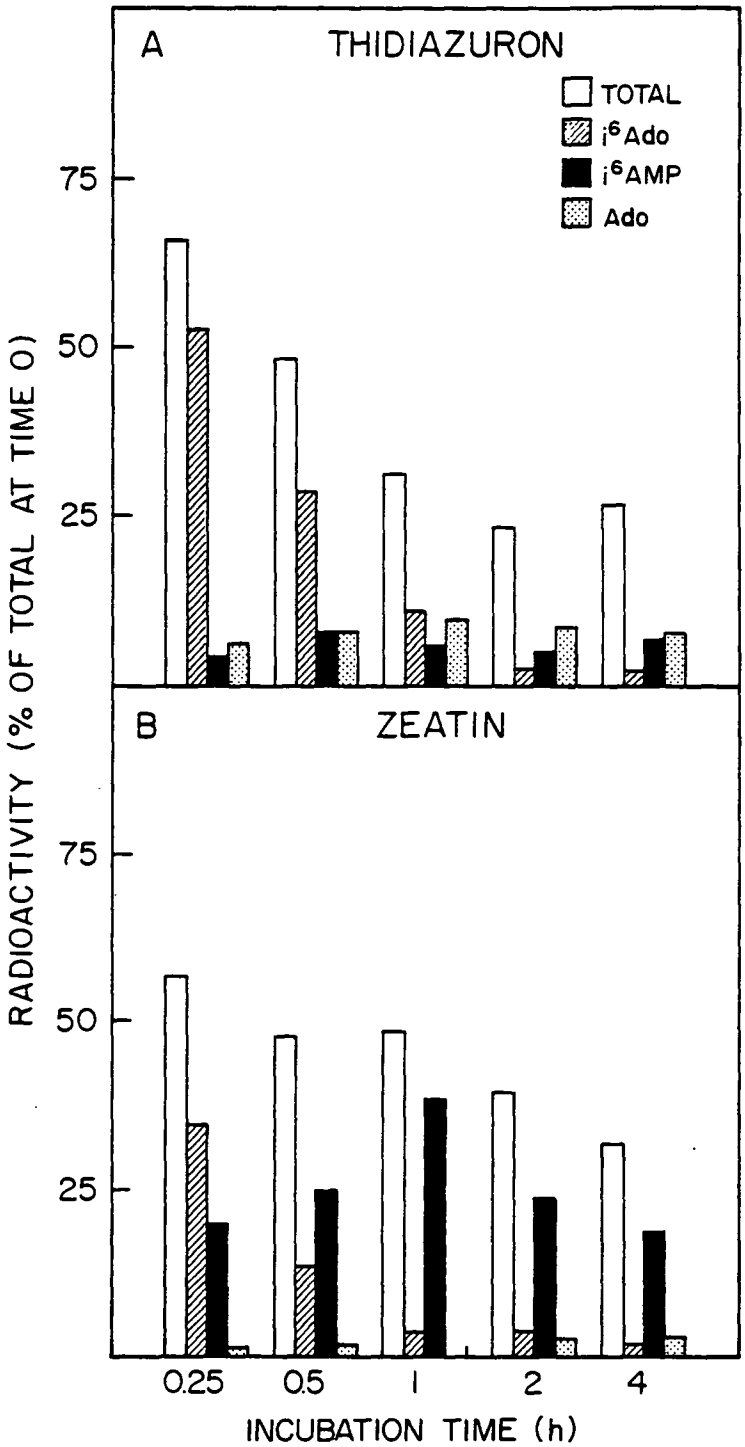
A
THIDIAZURON
TOTAL
i6Ado
i6AMP
Ado
75
50
25
B
ZEATIN
75
50
25
RADIOACTIVITY (% OF TOTAL AT TIME 0)
0.25
0.5
1
2
4
INCUBATION TIME (h)

Figure 8.

FIGURE 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).

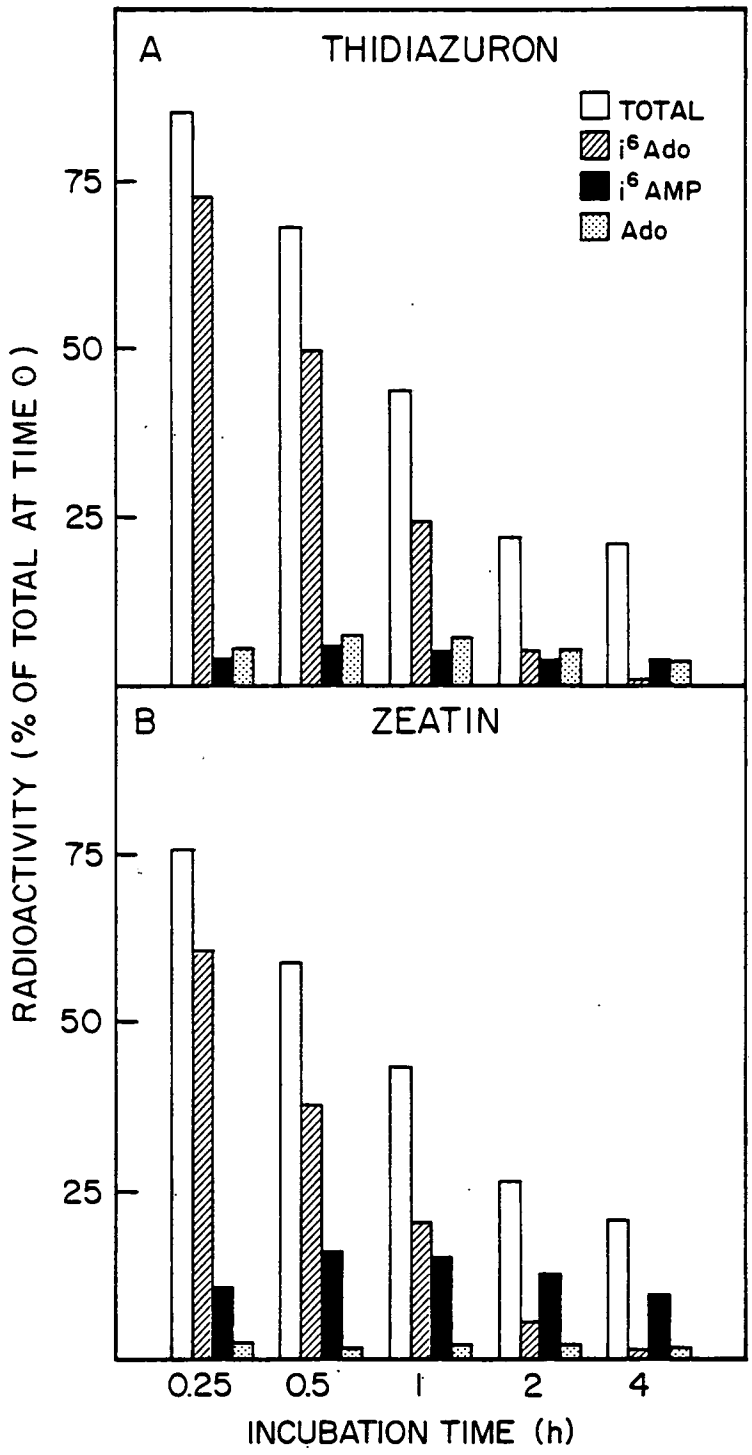
A
THIDIAZURON
TOTAL
i⁶Ado
i⁶AMP
Ado
75
50
25
B
ZEATIN
75
50
25
RADIOACTIVITY (% OF TOTAL AT TIME 0)
0.25
0.5
1
2
4
INCUBATION TIME (h)

Figure 9.

were determined using young (5 g) and older (20 g) callus tissues of cv. Jackson Wonder. The results of this test are presented in Figure 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 or zeatin-containing medium were incubated with [^{14}C]i^{6}AMP (recovered from tissues grown on zeatin and purified by HPLC on reversed-phase C_{18} columns) for 0.5, 1, and 2 h. The results of these tests are shown in Figure 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 (Laloue and Pethe, 1982), 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.

FIGURE 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).

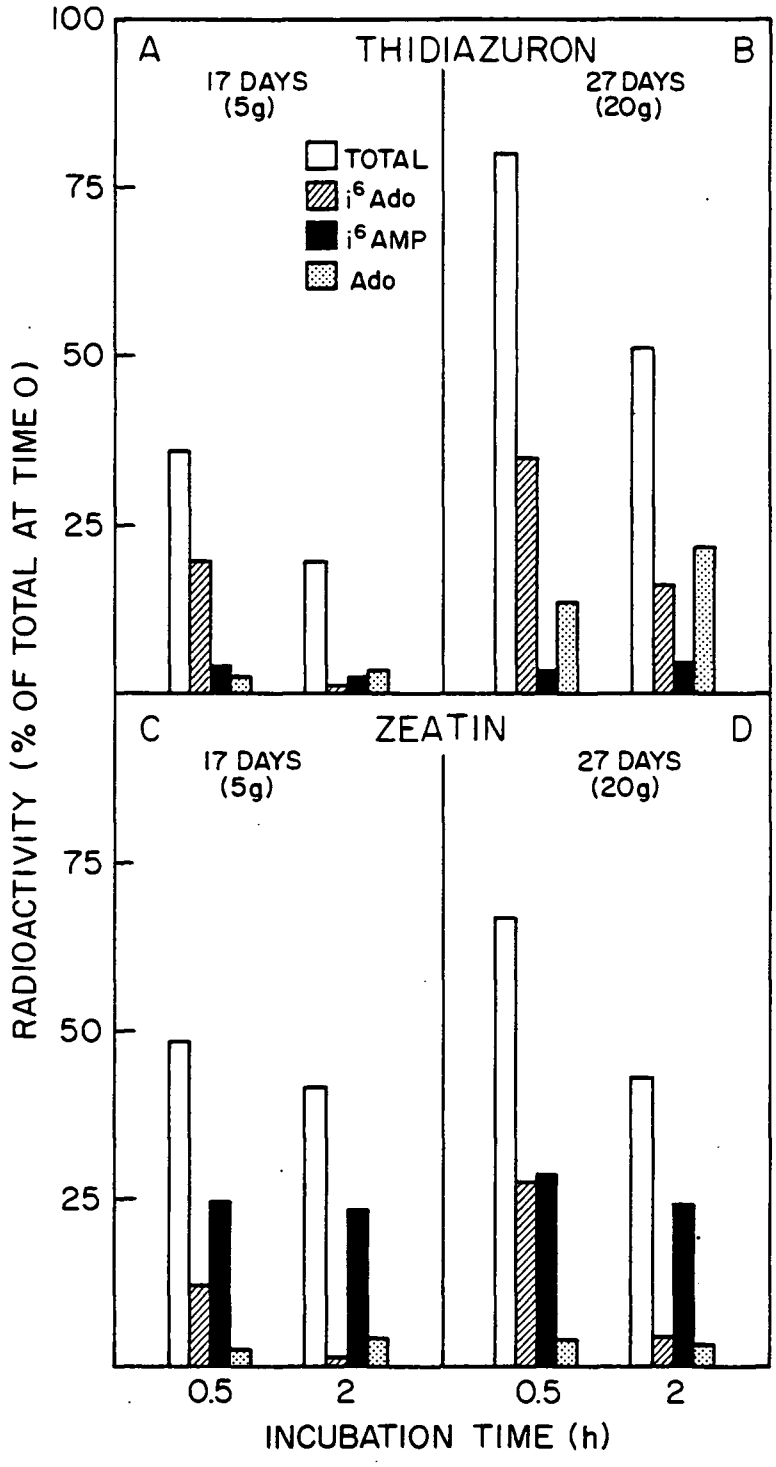
A
THIDIAZURON
B
17 DAYS
(5g)
27 DAYS
(20g)
TOTAL
i⁶Ado
i⁶AMP
Ado
C
ZEATIN
D
17 DAYS
(5g)
27 DAYS
(20g)
100
75
50
25
75
50
25
RADIOACTIVITY (% OF TOTAL AT TIME 0)
0.5
2
0.5
2
INCUBATION TIME (h)

Figure 10.

FIGURE 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).

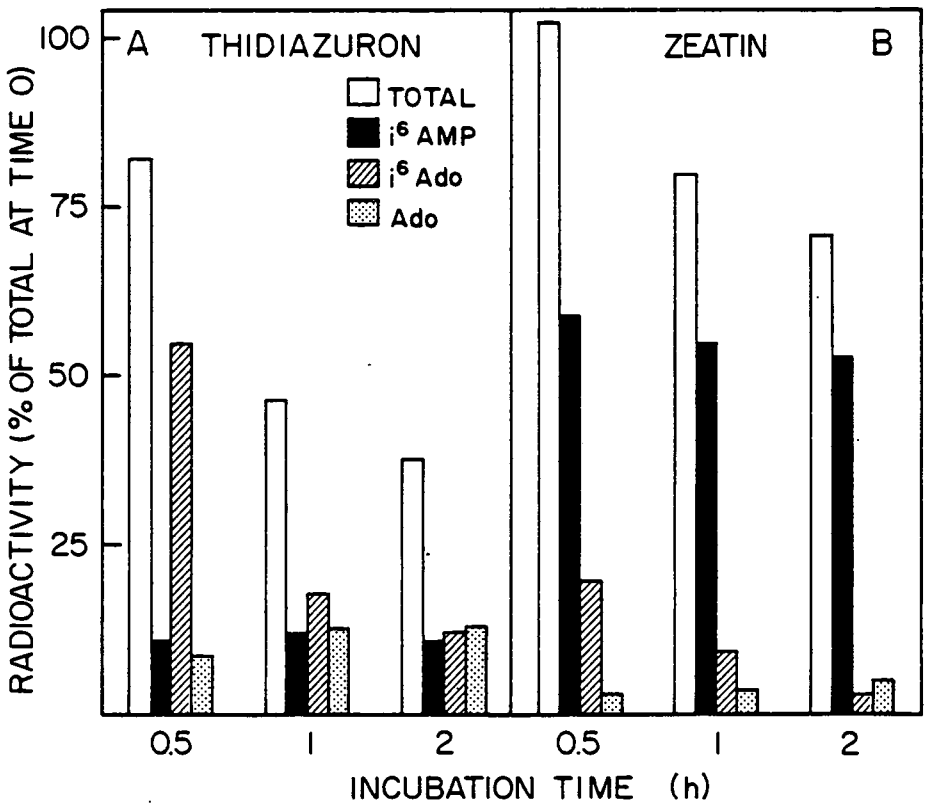
A
THIDIAZURON
ZEATIN
B
TOTAL
i⁶ AMP
i⁶ Ado
Ado
100
75
50
25
RADIOACTIVITY (% OF TOTAL AT TIME 0)
0.5
1
2
0.5
1
2
INCUBATION TIME (h)

Figure 11.

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 mid-point 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 (Figs. 12A and B). However, the presence of Thidiazuron at optimal concentration (0.01 μM) suppressed nucleotide formation regardless of the concentration of zeatin supplied (Figs. 12C and D). Thus 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 days. The fresh weights after a growth period of 35 days are presented in Table 1. 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

FIGURE 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).

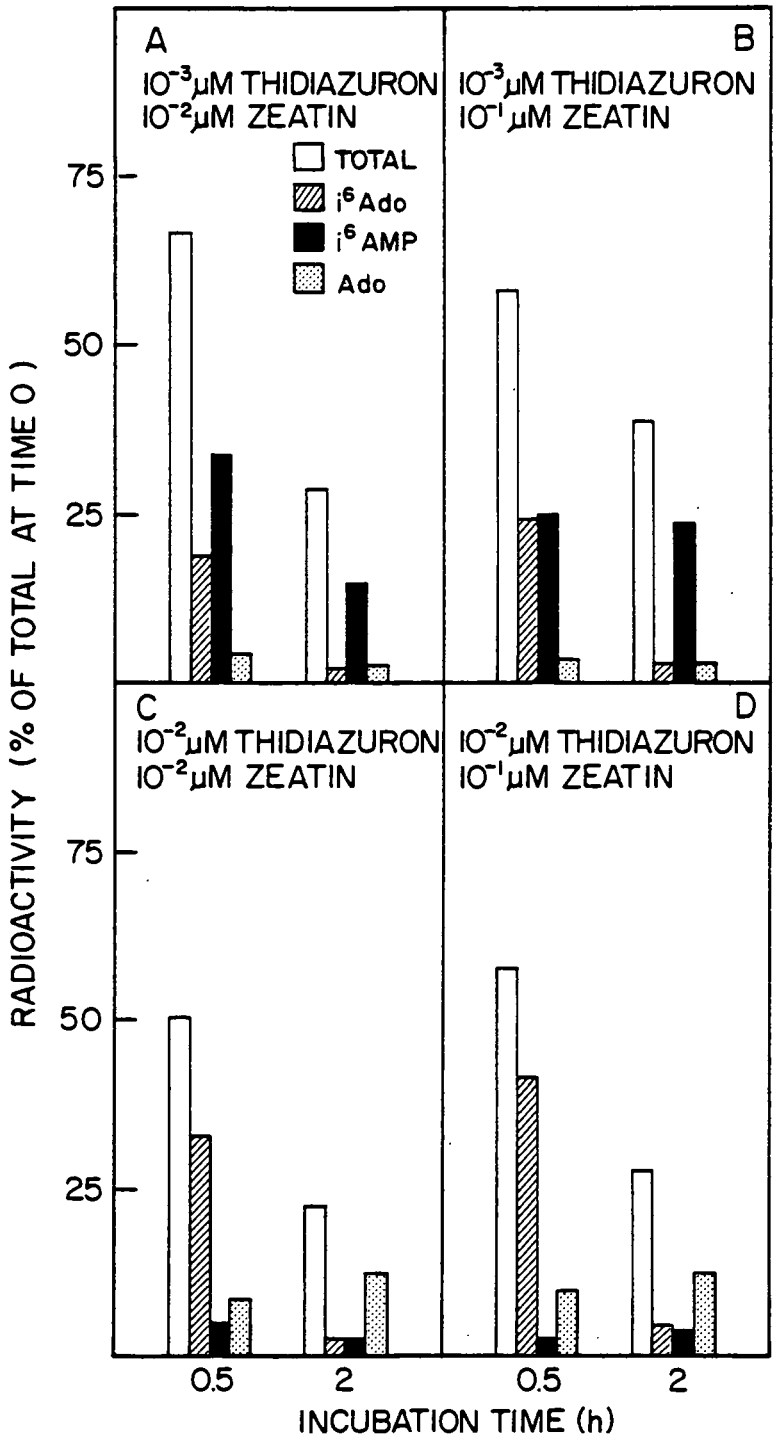
A
10^{-3}µM THIDIAZURON
10^{-2}µM ZEATIN
TOTAL
i⁶Ado
i⁶AMP
Ado
B
10^{-3}µM THIDIAZURON
10^{-1}µM ZEATIN
C
10^{-2}µM THIDIAZURON
10^{-2}µM ZEATIN
D
10^{-2}µM THIDIAZURON
10^{-1}µM ZEATIN
RADIOACTIVITY (% OF TOTAL AT TIME 0)
75
50
25
0.5
2
INCUBATION TIME (h)

Figure 12.

TABLE 1. 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 (μM)		Fresh weight ± SE (g/flask)	
Thidiazuron +	zeatin	Second passage	Third passage (cytokinin-free medium)
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

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-day growth period (Table 2). A much larger proportion of [^{14}C]zeatin than [^{14}C]Thidiazuron was taken up by the tissues, even though 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.

TABLE 2. 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 (% of cpm added to the medium)	
	Medium	Tissues
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%

V. 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 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 previous studies (Mok et al., 1979) 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 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 (Mok et al., 1980). Results very similar to those described here for Thidiazuron and zeatin were obtained with N-phenyl-N'-(4-pyridyl)urea 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, it was possible to demonstrate that pronounced 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 (1982) 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 and were discussed in the Literature Review. The exact relationship between the two types of compounds, however, 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. 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.

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APPENDICES

APPENDIX I. Cytokinin activities of N-(2,6-dichloro-4-pyridyl)-N'-phenylurea and N-(2,6-dichloro-4-pyridyl)-N'-(3-fluorophenyl)urea in the P. lunatus callus bioassay.

Certain N-phenyl-N'-(4-pyridyl)urea derivatives exhibited high cytokinin activity (Takahashi et al., 1978). In particular, N-(2,6-dichloro-4-pyridyl)-N'-phenylurea and N-(2,6-dichloro-4-pyridyl)-N'-(3-fluorophenyl)urea were found to be extremely active in the tobacco callus bioassay (Okamoto et al., 1981). These two compounds were obtained from Dr. K. Shudo, University of Tokyo, Japan. The activities of Thidiazuron, N-(2,6-dichloro-4-pyridyl)-N'-phenylurea, and N-(2,6-dichloro-4-pyridyl)-N'-(3-fluorophenyl) urea in the promotion of P. lunatus cv. Kingston callus growth were compared. Tissues were harvested and weighed after a growth period of 35 days. The results of this test are presented in Figure I-1. Both N-phenyl-N'-(4-pyridyl)urea derivatives promoted vigorous callus growth, but Thidiazuron was slightly more active than either of these compounds.

FIGURE I-1. Callus growth of cv. Kingston in the second passage on media containing Thidiazuron (--●--), N-(2,6-dichloro-4-pyridyl)-N'-phenyl-urea (—■—), and N-(2,6-dichloro-4-pyridyl) N'-(3-fluorophenyl)urea (•••o•••).

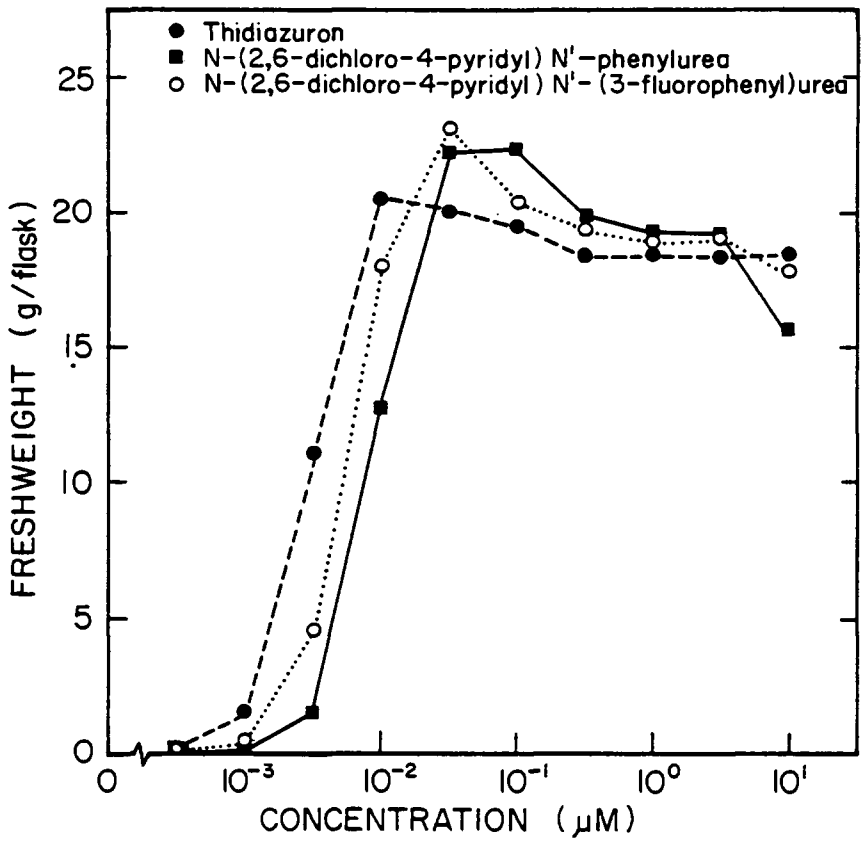
Thidiazuron
N-(2,6-dichloro-4-pyridyl) N'-phenylurea
N-(2,6-dichloro-4-pyridyl) N'-(3-fluorophenyl)urea
FRESHWEIGHT (g/flask)
25
20
15
10
5
0
0
10^{-3}
10^{-2}
10^{-1}
10^{0}
10^{1}
CONCENTRATION (μM)

Figure I-1.

APPENDIX II. The effects of N-benzyl-N'-3-nitrophenylurea on *P. lunatus* cv. Kingston callus growth.

Certain N-benzyl-N'phenylurea derivatives inhibit the activities of cytokinin-active phenylurea derivatives as well as N^6-substituted adenine derivatives in the tobacco callus (Isogai *et al.*, 1980) and radish leaf disc chlorophyll retention (Kefford *et al.*, 1968) bioassays. One such compound, N-benzyl-N'-3-nitrophenylurea was obtained from Dr. K. Shudo, University of Tokyo, Japan. The ability of this compound to antagonize the activities of Thidiazuron and zeatin was tested in *P. lunatus* callus cultures. Fresh weights of *P. lunatus* cv. Kingston callus tissues grown for 35 days on medium containing N-benzyl-N'-3-nitrophenylurea, and either Thidiazuron or zeatin, are presented in Tables II-1 and II-2. N-benzyl-N'-3-nitrophenylurea suppressed callus growth only when supplied at high concentrations (10 μM and 30 μM). The growth-inhibiting effects of this compound were apparent in the presence of Thidiazuron and zeatin. However, high concentrations of zeatin and Thidiazuron could overcome the growth-inhibiting effects of 10 μM N-benzyl-N'-3-nitrophenylurea. Thus, N-benzyl-N'-3-nitrophenylurea appears to be a cytokinin antagonist in *P. lunatus* callus tissues, as well as in other bioassay systems.

TABLE II-1. Fresh weight (±SE) of cv. Kingston callus tissues grown in the presence of Thidiazuron and N-benzyl-N'-3-nitrophenylurea.

Thidiazuron concentration	Antagonist Concentration (μM)						
	0	.1	.3	1	3	10	32
0	.04 ± .01	0.14 ± 0.1	2.15 ± 2.1	2.65 ± 1.3	1.9 ± 1.4	0.6	
.001	6.6 ± 4.0	12.38 ± 3.0	8.7 ± 3.1	4.8 ± 2.7	9.9 ± 4.4	0.05	
.003	20.7 ± 0.3	20.2 ± 0.1	19.7 ± 0.6	19.8 ± 0.6	19.4 ± 1.1	11.45	1.4
.01	20.1 ± 0.2	19.9 ± 0.3	20.4 ± 0.2	19.2 ± 0.2	19.2 ± 0.2	11.4	1.8
.03	18.7 ± 0.3	18.6 ± 0.4	18.9 ± 0.2	18.9 ± 0.3	18.5 ± 0.4	16.34	0.1
.1	18.5 ± 0.3	18.5 ± 0.2	18.7 ± 0.2	18.7 ± 0.2	17.8 ± 0.3	16.5	4.8
.3	18.6 ± 0.4	19.8 ± 0.2	19.1 ± 0.3	19.1 ± 0.4	18.8 ± 0.3	17.9	1.5

TABLE II-2. Fresh weight (±SE) of cv. Kingston callus tissues grown in the presence of zeatin and N-benzyl-N'-3-nitrophenylurea.

Zeatin concentration	Antagonist Concentration (μM)						
	0	.1	.3	1	3	10	32
0	0.04 ± .01	0.14 ± .1	2.15 ± 2.1	2.65 ± .13	1.9 ± 1.4	0.6	
.003	5.12 ± 2.8	1.9 ± 1.6	2.8 ± 1.6	0.71 ± 0.6	2.4 ± 1.7	0.25	
.01	13.4 ± 3.7	8.5 ± 3.6	11.0 ± 2.0	13.6 ± 4.4	9.0 ± 1.3	2.9	1.0
.03	22.0 ± 0.7	21.2 ± 0.3	18.2 ± 2.3	21.3 ± 0.3	20.1 ± 1.8	19.7	1.0
.1	23.6 ± 0.3	24.4 ± 0.2	24.1 ± 0.2	23.9 ± 0.2	23.8 ± 0.3	22.0	1.4
.3	23.5 ± 0.2	23.3 ± 0.3	24.0 ± 0.2	23.9 ± 0.3	23.7 ± 0.2	24.9	1.6
1.0	24.2 ± 0.3	23.7 ± 0.3	24.1 ± 0.3	23.3 ± 0.2	23.2 ± 0.3	24.0	2.0
3.0	21.4 ± 0.1	21.5 ± 0.2	21.7 ± 0.3	21.2 ± 0.3	20.9 ± 0.3	20.7	10.0