Biological and Biochemical Effects of Cytokinin-active Phenylurea Derivatives in Tissue Culture Systems

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Plant growth regulator studies and plant tissue culture research have been closely related and mutually supportive. The manipulation of plant cells, tissues, and organs in culture, with important applications in propagation and genetic modification of plants, is highly dependent on the use of appropriate growth regulator regimes. Conversely, tissue culture systems are useful as bioassays to define the growth-regulating activity of many compounds. The discovery of the cytokinin N-(2-furanylmethyl)-1H-purin-6-amine (kinetin) by Miller et al. (17) was particularly relevant in this respect. Whereas the testing of this cytokinin and its structural analogues for biological activity was dependent on callus culture bioassays, the subsequent availability of synthetic cytokinins created many new opportunities in the field of plant tissue culture.

The naturally occurring cytokinins, as well as many synthetic ones, contain adenine and a side chain at the N6 position. However, as early as 1955, an unrelated compound, N,N'-diphenylurea (DPU), was found to possess growth-promoting properties (24). Although the cytokinin activity of DPU was generally low compared to the adenine derivatives, highly active phenylurea compounds were subsequently discovered. Some examples are N-phenyl-N'-(2-chloro-4-pyridyl)urea (4PU-30) (28) and N-phenyl-N'-1,2,3-thiadiazol-5-ylurea (thidiazuron, Dropp; see Fig. 1) (18, 21), which exhibit activities higher than (E)-2-methyl-4-(1H-purin-6-ylamino)-2-buten-1-ol (zeatin) in several bioassays.

Compounds like the phenylureas, with chemical structures widely different from those of the adenine-type cytokinins and yet possessing apparently similar biological activities, may be important for studies of the mode of action of cytokinins. In addition, due to their high activities, some of these compounds could be useful and economical for tissue culture applications. This article deals with the biological effects and biochemical properties of the cytokinin-active phenylureas, focusing mainly on thidiazuron as a representative of this group.

Biological effects of thidiazuron

Thidiazuron was developed by Schering AG (Berlin, F.R.G) as a cotton (Gossypium hirsutum L.) defoliant (1). Interestingly, the compound induces defoliation without apparent breakdown of chlorophyll. The defoliating effects appear to be limited to plants of the cotton family, since concentrations of up to 100 μM failed to cause...
Fig. 2. The effects of thidiazuron (A) and zeatin (B) on callus growth of the cotton cultivar Acala.

leaf drop in several of the dicotyledonary species (Schering AG, personal communication). Cytokinin activity of thidiazuron was detected in a Phaseolus lunatus L. cv. Kingston callus bioassay, where it was more active than zeatin and about as active as 4PU-30 (18, 21). All available analogues of thidiazuron were less active than the parent compound in this bioassay (21). In addition, thidiazuron could substitute for adenine-type cytokinins in promoting the growth of tobacco (Nicotiana tabacum L.) cell cultures (2) and cotton callus cultures. The high activity of thidiazuron as compared to zeatin in the latter system is shown in Fig. 2.

The effects of thidiazuron on shoot formation also have been determined in several test systems. The compound could substitute for N^6-(Δ^2-isopentenyl)adenine (i6 Ade) or N^6-benzyladenine in micropropagation of woody species (6, 11, 22, 23). The biological activity of thidiazuron was considerably higher than i6 Ade in the multiplication of broccoli [Brassica oleracea L. (Italica Group)] shoots (Fig. 3). Although the optimal concentration of i6 Ade was 10 μM, thidiazuron was very effective at 0.3 μM, and at 10 μM induced abnormal growth and vitrification. Other cytokinin-like effects of thidiazuron include stimulation of budbreak of dormant apple (Malus domestica Borkh.) trees (31), promotion of lettuce (Lactuca sativa L.) seed germination (2), retardation of leaf senescence (2), enhancement of ovary abscission in Citrus (25), and induction of nitrate reductase activity (12). In all instances, the biological activity of thidiazuron was higher than or comparable to that of the most active adenine-type cytokinins.

The biosynthesis of ethylene is promoted by the synergistic action of auxin and cytokinin (14). Recently, it was shown that thidiazuron and its derivatives could substitute effectively for adenine-type cytokinins in enhancing ethylene production (32). Moreover, the structure–activity relationships of the thidiazuron analogues were similar to those observed in the Phaseolus callus bioassays (19, 32). This activity is in agreement with the earlier findings that application of thidiazuron resulted in an increase in ethylene production in beans and cotton (7, 26, 27).

From the observations cited above, the biological effects of thidiazuron conform, in general, to the properties established previously for the adenine-type cytokinins. There are, however, a few exceptions. One of the intriguing phenomena is the induction of defoliation in cotton only. Although increased ethylene production following the application of thidiazuron has been suggested as the basis of cotton defoliation (26, 27), other studies did not support this contention (7). The defoliation response of cotton plants to thidiazuron may be related to unusual metabolic or translocation patterns of the compound or, alternatively, to high sensitivity to thidiazuron, distinguishing cotton from other dicots. A second interesting phenomenon is the change in cytokinin requirement of callus tissues after exposure to thidiazuron. Cytokinin-dependent callus of some P. lunatus genotypes maintained on thidiazuron-

Fig. 3. The effects of thidiazuron (A) and N^6-(Δ^2-isopentenyl)adenine (B) on shoot multiplication of broccoli.
containing medium displayed cytokinin-independence (the capability to grow on cytokinin-free medium, also referred to as habituation) in subsequent passages; whereas comparable tissues maintained on optimal concentrations of kinetin or zeatin remained cytokinin-dependent (4). Transformation from a cytokinin-requiring state to cytokinin autonomy in these tissues appears to be associated with exposure to thidiazuron or a number of other phenylurea cytokinins, including DPU (19), suggesting some interesting possibilities regarding the mechanism of action, which are discussed below.

Biochemical effects of thidiazuron and implications regarding the site of action

Several hypotheses have been put forward to explain the cytokinin activity of the phenylurea derivatives. Miller (16) suggested that these compounds might serve as precursors for N6 side chains of cytokinin-active adenine derivatives. In support of this hypothesis, certain synthetic uridopurines are able to sustain the growth of tobacco callus (15). However, the activities of the uridopurines are quite low compared to those of thidiazuron and 4PU-30. Moreover, the main metabolites of thidiazuron in bean callus were found to be glucosyl derivatives (20). These and minor, unidentified metabolites were all inactive in the Phaseolus bioassay when supplied at levels optimal for thidiazuron activity; and, although activity of DPU was only observed in tissues that metabolized DPU (5), the only DPU metabolite to be isolated and identified from plant tissues has been a simple glucosyl derivative (3). Thus, cytokinin activity seems to be associated with thidiazuron and DPU rather than their metabolites.

The growth-promoting activities of the phenylureas may be due to their capability either to exert direct effect at the site of cytokinin action or, alternatively, to influence endogenous cytokinin biosynthesis or metabolism. The latter possibility seems to be supported by the capacity of thidiazuron to transform tissues from cytokinin dependence to cytokinin autonomy. One of the metabolic effects of thidiazuron that distinguishes it from zeatin became apparent in studies of interconversions between cytokinin ribonucleosides and ribonucleotides (supplied in radioactively labeled form to the tissue). The presence of thidiazuron in the medium preserved the ribonucleoside form of cytokins in the tissue, whereas zeatin in the medium supported conversion of ribonucleoside to the corresponding ribonucleotide (4). The inhibition of cytokinin nucleotide formation by thidiazuron was accompanied by high acid phosphatase levels (Fig. 4). Although these studies clearly demonstrate that the presence of thidiazuron may modify the metabolism of naturally occurring cytokinins, it is not certain whether the metabolic and enzymatic changes reflect direct effects of thidiazuron or are consequences of changes in callus growth. Also, the metabolic change may or may not result in a net increase of the active cytokinin. Interpretation is furthermore complicated since the question with regard to the active form of cytokinin has not yet been resolved, although indirect evidence has been presented in favor of the free base (13).

Recently, the endogenous cytokinin activity was determined in callus tissues of soybean [Glycine max (L.) Merr.] grown at two levels of thidiazuron (29). Higher endogenous cytokinin activity was measured at the high level (1 mg-liter\(^{-1}\)) of thidiazuron than at the low level (0.1 mg-liter\(^{-1}\)) of the compound. These data were interpreted as support for the hypothesis that thidiazuron induces endogenous cytokinin biosynthesis. However, the possibility that the activities of the exogenously supplied thidiazuron and its metabolites were also measured was not excluded. In studies using immobilized cells, the presence of N6-benzyladenine was shown to promote formation of zeatin and ribosylzeatin (30). This promotion raises the question whether all unnatural cytokinins, including the phenylureas and adenine derivatives (N6-benzyladenine, kinetin), may be able to induce the biosynthesis of zeatin, while only the latter (and/or its nucleoside or nucleotide) can exert direct effect at the site of cytokinin action.

Finally, direct cytokinin action by thidiazuron needs to be considered. The capacity of benzylurea derivatives to antagonize the effects of both types of cytokinin-active compounds may point to a common site of action (8, 10). In addition, calculations of physical-chemical parameters indicate that the two types of compounds may have some common properties (9). Nevertheless, these two lines of evidence also could be taken as indication that there may be a common induction site for cytokinin biosynthesis. However, if direct activity is indeed exerted by all cytokinin-like compounds, the possibility of cytokinin action through incorporation into macromolecules seems highly unlikely.

Application of thidiazuron in tissue culture

High concentrations of adenine-type cytokinins are often necessary for growth and differentiation of tissue cultures. A major reason is the occurrence of cytokinin-degrading enzymes (cytokinin oxides), which cleave unsaturated N6-isoprenoid side chains. Synthetic cytokinins, such as kinetin and BA, are less susceptible to degradative enzymes, but are generally less active than the naturally occurring cytokinins such as zeatin and \(\text{\textsuperscript{p}}\)Ade. Thidiazuron and 4PU-30 are resistant to oxides, stable, and biologically active at lower concentrations than the adenine-type cytokinins. These properties may enhance the future use of these compounds in tissue culture manipulations.

Conclusions

Cytokinins can be classified into at least two broad groups, the adenine and the phenylurea derivatives, according to their chemical structure. Cytokinin-active phenylureas, such as thidiazuron and 4PU-30, display biological properties qualitatively similar to those of adenine-type cytokinins: they promote callus growth, induce organogenesis, and stimulate the production of ethylene. An unusual effect of phenylurea cytokinins is the tendency to enhance cytokinin autonomy of tissues. This enhancement may be due to the capacity of thidiazuron to stimulate endogenous cytokinin biosynthesis or alter endogenous cytokinin metabolism, resulting in an increase of the natural cytokinins. However, other observations indicate that they may have a common site of action with the naturally occurring cytokinins. Interpretations of experimental results to date regarding the mode of action of phenylurea cytokinins in relation to adenine cytokinins remain speculative, due in large part to the lack of information regarding the site of action of plant growth regulators in general. In any event, the high activity and stability of some phenylurea derivatives may render them particularly valuable as sources of cytokin in tissue culture systems where degradation of growth regulators is a limiting factor.
Effects of Thidiazuron and CPPU on Meristem Formation and Shoot Proliferation

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N-phenyl-N’1,2,3-thiadiazol-5-ylurea (thidiazuron) and several substituted pyridyl phenylurea compounds have been demonstrated to stimulate in vitro meristem and shoot formation at unusually low concentrations. These compounds appear to have strong cytokinin-like effects on a wide range of species and on species that respond little to conventional cytokinins. Thidiazuron has been reported to stimulate shoot proliferation in several woody species (e.g., Acer and Malus). The addition of 0.5 μM N-(2-chloro-4-pyridyl)-N’-phenylurea (CPPU) to the culture medium caused dramatic shoot number increases in hardy deciduous azaleas (Rhododendron sp.) cultured in vitro. In potted Petunia × híbrida Hort. Vilm.-Andr.) leaf test systems both thidiazuron and CPPU caused greater proliferation when used as explant dips or in the medium than similar treatments with β-(phenylmethyl)-1’-purin-9(2H)-ylurea (CPPU), and pointed out that it was primarily active

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