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***Communication***

# Morphoregulatory Role of Thidiazuron[1]

## Substitution of Auxin and Cytokinin Requirement for the Induction of Somatic Embryogenesis in Geranium Hypocotyl Cultures

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### ABSTRACT

**Somatic embryogenesis was induced in hypocotyl explants of geranium (*Pelargonium* × *hortorum*) cultured on media supplemented with various concentrations of *N*-phenyl-*N'*-1,2,3-thiadiazol-5-ylurea (thidiazuron). In less than 2 weeks, somatic embryos were observed in treatments containing levels of thidiazuron (TDZ) ranging from 0.2 to 1.0 micromolar. The use of $N^6$-benzylaminopurine in combination with indole-3-acetic acid also evoked embryogenesis, but the efficiency of somatic embryo production was significantly lower than that obtained with TDZ. Hypocotyl culture for only 2 days on TDZ-supplemented medium before transfer to a basal medium was sufficient for inducing somatic embryogenesis. This distinction between the induction and expression of embryogenesis may provide an experimental system for studying the developmental biology of somatic embryogenesis. Substitution of the auxin-cytokinin requirement for the induction of somatic embryogenesis by TDZ suggests the possibility of a novel mode of its action by modulation of endogenous growth regulators.**

TDZ[3], a substituted phenylurea (*N*-phenyl-*N'*-1,2,3-thiadiazol-5-ylurea; Dropp), is primarily used as a cotton defoliant (1) and has been shown in various cytokinin bioassays to exhibit strong cytokinin-like activity similar to that of $N^6$-substituted adenine derivatives (12). In many cytokinin-dependent callus cultures, the application of TDZ evoked a comparable or higher degree of growth response than adenine-based cytokinins (5, 20). The morphogenetic responses in which TDZ has been found to mimic cytokinin-like activity include release of lateral buds from dormancy (21) and shoot formation in cultures of a wide variety of plant species (7, 9).

The precise mechanism of action of TDZ is as yet unknown; however, there are two hypotheses in this regard. It is possible that TDZ directly promotes growth due to its own biological activity in a fashion similar to that of $N^6$-substituted cytokinins, or it may induce the synthesis and (or) accumulation of endogenous cytokinins (5, 11). The latter notion is based on the effects of the high ability of TDZ in inducing cytokinin-dependent shoot regeneration and modulation of endogenous levels of cytokinins. In this communication, we demonstrate the high efficiency of TDZ in stimulating somatic embryogenesis in geranium hypocotyl cultures, a response usually mediated by an appropriate combination of an auxin and a cytokinin. Our results suggest for the first time that auxin(s) may also be involved in the induction and/or expression of TDZ-induced morphogenic differentiation.

### MATERIALS AND METHODS

Seeds of the diploid zonal geranium (*Pelargonium* × *hortorum* Bailey) cv Scarlet Orbit Improved were surface sterilized by dipping in 95% ethanol for 30 s and then immersing in a 1.2% solution of sodium hypochlorite containing two drops of Tween 20 per 200 mL. Seeds were agitated in this solution for 20 min and then rinsed three times in sterile distilled water. Ten seeds were aseptically cultured per 100 × 15 mm Petri dish on 30 mL of MS (13) medium supplemented with 1 $mg \cdot L^{-1}$ nicotinic acid, 10 $mg \cdot L^{-1}$ thiamine HCl, 1 $mg \cdot L^{-1}$ pyridoxine HCl, 100 $mg \cdot L^{-1}$ myoinositol, and 30 $g \cdot L^{-1}$ sucrose. This medium was adjusted to pH 5.5 and solidified with 3 $g \cdot L^{-1}$ Gelrite (Schweizerhall, South Plainfield NJ). The plates were sealed with Parafilm and the seeds germinated at 24°C in the dark.

After 7 d, the extended hypocotyls were aseptically cut into three, approximately 1-cm long, sections each. These segments were placed in a Petri plate on MSO or MS medium amended with various growth regulators. The additions to the MS medium were 8 μM BAP plus 1 μM IAA, 8 μM BAP alone, or TDZ at a concentration of 0.2, 0.4, 0.8, 1.0, 5.0, 8.0, or 10.0 μM. In one experiment, the hypocotyl sections were also cultured on concentrations of TDZ (0.2–10.0 μM as above) together with 1.0 μM IAA. The Petri dishes were sealed with Parafilm and incubated at 24°C in the light at 30 to 50 $\mu E \cdot m^{-2} \cdot s^{-1}$ with a 16-h photoperiod.

A final experiment was performed to test the effect of the dose and the duration of exposure to TDZ. Seven-day-old hypocotyls were excised as above and cultured on MS medium amended with 0.4, 0.8, or 5.0 μM TDZ. Petri dishes

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[3] Abbreviations: TDZ, thidiazuron; MS, Murashige and Skoog (1962) medium; BAP, $N^6$-benzylaminopurine; MSO, MS basal medium.

were sealed and incubated as described above. After culturing hypocotyl sections for periods of 2, 4, and 8 d on TDZ-enriched medium, the explants were removed from each treatment, subcultured on MSO medium, and returned to original culture conditions. In the control treatment, one set of hypocotyl sections was subjected to continuous exposure to the TDZ treatments and was not transferred to MSO medium. After their initial appearance, somatic embryos were counted once a week for 4 successive weeks. About 30 to 45 explants were cultured per treatment and the experiments were repeated twice.

## RESULTS AND DISCUSSION

Somatic embryogenesis was observed from the hypocotyl sections after 12 d of culture in treatments containing TDZ at concentrations ranging from 0.2 to 1.0 μM, as well as with BAP plus IAA (Table I). The appearance of somatic embryos was most rapid at 0.4 μM TDZ (Fig. 1C). However, after the fourth week, all TDZ treatments 1.0 μM and below showed a similar number of embryos, giving evidence that TDZ has a broad spectrum of activity. The use of 0.4 to 1.0 μM TDZ resulted in an approximately twofold increase in somatic embryo production over that achieved with 8 μM BAP plus 1 μM IAA used in a previously published protocol (22). The use of BAP alone caused swelling of the explants (Fig. 1A), but supported only an occasional appearance of somatic embryos. No somatic embryos were formed on MSO medium or MS supplemented with IAA; only roots appeared at the cut basal ends of the explants (Fig. 1B). TDZ concentrations above 1 μM were toxic to the explants, which caused browning. No somatic embryos were produced from the hypocotyls by culturing and maintaining them continuously on the medium containing TDZ concentrations of 5, 8, or 10 μM (Table I).

With IAA at 1 μM included in the TDZ treatments of the explants, the trend of somatic embryogenesis was similar to that observed in the presence of TDZ alone (data not shown). Addition of IAA neither increased the frequency of TDZ-induced somatic embryo formation nor subjugated the inhibitory effect of TDZ concentrations greater than 1 μM. TDZ-induced somatic embryos matured and germinated on a basal medium developing into plantlets (Fig. 1D).

The *in vitro* induction of somatic embryos under controlled and easily manipulated conditions allows a unique opportunity to study the developmental pathway leading to somatic embryogenesis. In a wide variety of species that produce

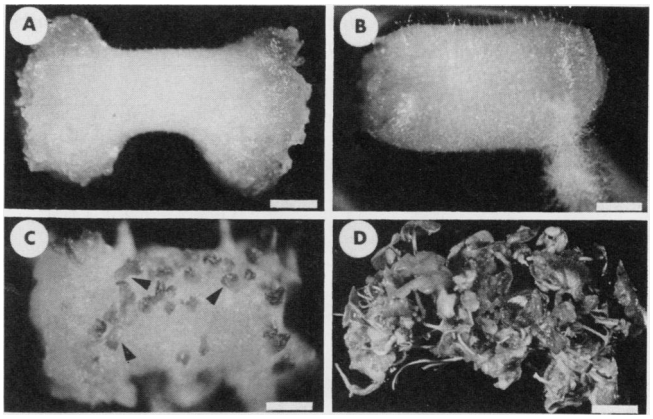


**Figure 1.** A-D, Somatic embryogenesis in hypocotyl cultures of geranium (*Pelargonium* × *hortorum* Bailey). Hypocotyl explants were cultured on MS medium supplemented with various growth regulators for a period of 3 weeks. A, Explants cultured in the presence of 8 μM BAP. Note the swelling of explant at cut ends; B, explants cultured in the presence of 1 μM IAA. Note the appearance of root. C, Differentiation of somatic embryos (arrow) from hypocotyl explants on a 0.4 μM TDZ-supplemented medium; D, multiple plantlets formed from somatic embryos after 7 weeks of culture. Bar in A = 1.5 mm; B = 2.0 mm; C = 1.8 mm; D = 1.5 mm.

embryogenic cultures, auxins, namely 2,4-D and naphthaleneacetic acid, alone or in combination with cytokinins have been used in the induction and proliferation of somatic embryos (2, 3, 18). In geraniums, various combinations of auxins and cytokinins have been shown to induce somatic embryogenesis (10, 17). Our study marks the first time TDZ has been used for this purpose and indicates that the efficiency of this compound is much higher than that of the auxin-cytokinin complement used in previous procedures (10, 17, 22).

The induction of somatic embryogenesis by TDZ as the sole growth regulator appears to be unique in view of existing literature on its mechanism of action. Thus far, TDZ has been primarily shown to replace cytokinins. The ability of TDZ and other diphenylureas to substitute for cytokinin-active adenine derivatives has been demonstrated in various callus culture bioassays as well as in cytokinin bioassays based on Chl retention, bud development, and seed germination (4, 11, 12). Similarly, TDZ has been shown to promote shoot

**Table I.** *Mean Number of Somatic Embryos (± SE) per Hypocotyl Section after Culturing on IAA plus BAP, BAP, or TDZ at a Range of Concentrations*

Values are the means of five replications.

| Time after Culture | MSO | IAA (1 μM) + BAP (8 μM) | BAP (8 μM) | TDZ (μM) | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | 0.2 | 0.4 | 0.8 | 1.0 | 5, 8, 10 |
| *d* | | | | | | | | |
| 13 | 0.0 ± 0.0 | 1.5 ± 0.1 | 0.0 ± 0.0 | 1.5 ± 0.0 | 2.4 ± 0.3 | 0.3 ± 0.1 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 20 | 0.0 ± 0.0 | 8.0 ± 0.9 | 0.2 ± 0.0 | 12.1 ± 1.0 | 18.6 ± 1.0 | 5.5 ± 0.9 | 1.6 ± 0.3 | 0.0 ± 0.0 |
| 27 | 0.0 ± 0.0 | 18.6 ± 2.0 | 1.7 ± 0.6 | 23.4 ± 1.0 | 31.8 ± 3.8 | 17.8 ± 3.2 | 21.0 ± 3.1 | 0.0 ± 0.0 |
| 34 | 0.0 ± 0.0 | 18.5 ± 1.6 | 2.1 ± 0.7 | 28.1 ± 1.4 | 38.1 ± 2.9 | 25.3 ± 3.2 | 35.4 ± 3.3 | 0.0 ± 0.0 |

**Table II.** *Mean Number (± SE) of Somatic Embryos per Hypocotyl Section after Exposure for Various Durations to TDZ*

| Time after Culture | TDZ | Time of Exposure | | | |
|---|---|---|---|---|---|
| | | 2 | 4 | 8 | Continuous |
| *d* | μM | | *d* | | |
| 16 | 0.4 | 0.1 ± 0.0 | 1.5 ± 0.1 | 4.0 ± 0.4 | 3.0 ± 0.1 |
| | 0.8 | 3.4 ± 0.4 | 9.9 ± 0.1 | 8.6 ± 0.6 | 15.0 ± 1.3 |
| | 5.0 | 9.5 ± 0.5 | 14.2 ± 0.5 | 4.4 ± 0.1 | 0.3 ± 0.1 |
| 22 | 0.4 | 0.3 ± 0.1 | 2.8 ± 0.5 | 10.9 ± 2.4 | 9.9 ± 2.3 |
| | 0.8 | 9.1 ± 2.3 | 18.7 ± 1.7 | 29.0 ± 3.1 | 37.7 ± 4.7 |
| | 5.0 | 18.6 ± 1.6 | 43.6 ± 2.3 | 22.4 ± 3.3 | 0.3 ± 0.1 |
| 29 | 0.4 | 0.2 ± 0.1 | 2.6 ± 0.5 | 11.0 ± 1.3 | 11.8 ± 3.2 |
| | 0.8 | 10.7 ± 1.4 | 20.8 ± 1.8 | 33.6 ± 3.1 | 36.1 ± 5.4 |
| | 5.0 | 24.6 ± 0.5 | 45.8 ± 1.8 | 25.9 ± 4.4 | 0.2 ± 0.1 |
| 36 | 0.4 | 0.3 ± 0.3 | 1.0 ± 0.6 | 10.4 ± 2.3 | 19.0 ± 7.9 |
| | 0.8 | 15.0 ± 6.5 | 52.3 ± 9.2 | 36.3 ± 3.2 | 45.0 ± 6.6 |
| | 5.0 | 36.3 ± 4.7 | 64.7 ± 8.8 | 33.4 ± 3.4 | 0.0 ± 0.0 |

regeneration with an efficiency comparable to or greater than that of cytokinins (7, 9). The effectiveness of TDZ to inhibit stomatal closure and to stimulate ethylene production in bean is also consistent with the properties of a cytokinin (6), although the pattern of the lag period in elevation of ethylene production by TDZ-treated mung bean tissue was similar to that induced by an auxin (19).

However, in our study, both exogenous BAP and IAA were replaced by TDZ. In tissue cultures of geranium, the presence of IAA or other auxin-like compounds such as phenylacetic acid (17) in combination with BAP is essential for the onset of somatic embryogenesis; if used alone, these compounds were found to be ineffective. The substitution of the IAA-BAP requirement of embryogenic induction demonstrates that in addition to displaying cytokinin-like activity, TDZ may also impinge upon endogenous auxins by their modified biosynthesis and (or) their protection *in vivo*.

The assumption of TDZ-mediated modulation of endogenous auxins gains further support from our recent observation of a similar activity of TDZ in inducing differentiation of somatic embryos from structurally intact seedlings of peanut (15). Somatic embryogenesis in peanut cultures has been previously shown to be dependent upon the presence of auxins in the culture medium (8, 14). According to Skoog and Miller's theory of regeneration (16), quantitative interactions between diverse growth factors, mainly phytohormones, rather than specific morphogenetic substances, provide a common mechanism for the regulation of all types of morphogenetic phenomena in plants. The high efficiency with which TDZ induced somatic embryogenesis in our study may be a reflection of its ability to create optimal endogenous auxin/cytokinin levels within the cultured tissue.

The experiment studying the effect of the duration of exposure to various concentrations of TDZ also provided interesting information. Exposure to TDZ for only 2 d, although at a higher concentration (5 μM) than when continuously subjected to TDZ (Table I), was sufficient to evoke an embryogenic response in the hypocotyl sections (Table II). The production of somatic embryos after being exposed to TDZ for such a short period allows for a very narrow temporal window for analyzing embryogenic developmental processes. A 4-d exposure to 5.0 μM TDZ resulted in the highest degree of somatic embryo formation (Table II), perhaps indicating that optimal auxin and cytokinin levels were reached at this point. Continuous hypocotyl culture on 5.0 μM TDZ was lethal to the explants, and no somatic embryos were produced. For an 8-d preculture period, 0.8 μM was the most effective of the three levels of TDZ used in the induction of somatic embryos. Prolonged exposure to TDZ required concentrations 1.0 μM or less (Table I) for efficient somatic embryogenesis. A level of 0.4 μM TDZ had almost no effect until after at least 8 d of exposure (Table II).

In conclusion, the present study reveals the potential of TDZ in regulation of somatic embryogenesis. Of particular interest is the probability of the modulation by TDZ of endogenous auxins or auxin-like bioregulators, which in synergism with cytokinins, may constitute the inductive signal for embryogenic expression. The ability of TDZ to induce somatic embryogenesis with only 2 d of exposure is expected to facilitate further characterization of the induction and expression phases of embryo development *in vitro*. Irrespective of the mode of action, the high-frequency production of somatic embryos obtained in our experiment represents a significant improvement in the techniques of micropropagation of geranium.

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