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Light Mediates Endogenous Plant Growth Substances in Thidiazuron-induced Somatic Embryogenesis in Geranium Hypocotyl Cultures

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Thidiazuron-induced somatic embryogenesis in geranium (*Pelargonium x hortorum Bailey*) was readily achieved under both light and darkness. The hypocotyl explants treated with TDZ formed well differentiated embryos when incubated under conditions of continuous light, complete darkness or 16-h photoperiod. However, embryogenesis was significantly impaired by light at both 16-h and continuous light exposure. Maintaining the cultures in the dark increased the frequency of the somatic embryos formed with a corresponding moderate elevation of the endogenous plant growth substances measured. In the complete absence of TDZ, all of the hypocotyl explants cultured in the dark remained thin but formed etiolated roots. The number of explants that formed roots decreased with increasing light exposure time; in addition the roots formed were shorter in length. The levels of endogenous adenine, adenosine, DHZ, zeatin, isopentenyladenine (2iP), tryptamine, IAA and ABA remained elevated for the first 2 days of culture in explants maintained on MSO and under continuous light, while they decreased on day 3 of culture. We provide evidence that the interaction between TDZ and light treatments modulated the endogenous plant growth substances, which in turn affected the embryogenic process in geranium hypocotyl explants.

Key words: *Pelargonium x hortorum* Bailey, physical and chemical stimuli, phytohormones, photoperiod, somatic embryogenesis.

Growth and morphogenic effects of light (physical stimulus) and phytohormones (chemical/hormonal stimulus) are well documented but their modes of action and mutual interactions are far from clear. The physical stimulus (light) has been shown to have both promotive and inhibiting effects on somatic embryogenesis depending on the species under study, as well as the nature and interaction, with the inductive hormonal stimulus given. Among the hormonal stimuli studied in somatic embryogenesis systems, 2,4-D, a synthetic auxin, has been used most frequently to induce the embryogenic response. Previously, we have demonstrated the usefulness of thidiazuron (TDZ), a phenyl urea, for enhanced embryogenic response in geranium under a 16-h photoperiod, adequately substituting the auxin-cytokinin requirements for somatic embryogenesis (1,2). On the other hand, TDZ has been shown to cause significant changes in endogenous levels of auxins and cytokinins in *Phaseolus* (3), peanut (4) and geranium (5) under 16-h photoperiod. One of the important questions is whether at least some of the morphogenic effects of TDZ, light and/or their interaction are mediated by changes in phytohormone levels.

The major objective of the present investigation was to evaluate the embryogenic response of geranium hypocotyl tissues to TDZ under different photoperiodic conditions and to assess if the observed differences were mediated, in part, by changes in endogenous levels of phytohormones.

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Materials and Methods

Seed sterilization and explant culture — Seeds of the diploid Zonal geranium, *Pelargonium x hortorum* Bailey cv. Scarlet Orbit Improved (Stokes Seed Co, St Catherines, Ontario), were surface sterilized by dipping in 95% ethanol for 45 s and then immersing in 1.5% sodium hypochlorite solution containing 2 drops/100 ml solution of 'Tween 20'. Seeds were agitated continuously in the sterilizing solution for 20 min and rinsed 3 times in sterile-deionized water. Ten seeds were germinated aseptically in each 100 x 15 mm disposable Petri dish containing 25 ml water-agar (0.85% purified agar, Sigma, in distilled water). The Petri dishes were sealed with Parafilm and incubated in the dark at 25°C for 6 d.

Six-day-old etiolated hypocotyl sections were cut into four or five 8 mm long segments. Ten explants were cultured in one Petri dish containing 25 ml of the induction medium (IM) consisting of MS (7) salts, B5 (6) vitamins, 30g l^{-1} sucrose, and 10μM TDZ. After 3 d on the IM, the explants were transferred onto a basal expression medium

(EM) for development of the somatic embryos. The pH of all media was adjusted to 5.5 prior to autoclaving (1.19kg cm^{-2}) for 20 min. The cultures were placed in growth chambers set at 25°C and illuminated (16-h and 24-h photoperiod; 70-78 μmol m^{-2} s^{-1}) by Cool white fluorescent tubes (F40/CW/RS/EW-II; Phillips Canada, Scarborough, ON, Canada). Experiments for complete dark treatment were carried out under green light (24 W Decolour solid light, GE lighting Canada, Mississauga, ON, Canada) and Petri dishes were wrapped with aluminium foil for the entire period of culture in the 24-h photoperiod growth chamber. The mean number of somatic embryos per hypocotyl section, an indicator of the efficacy of the inductive stimulus, was determined after 30 d.

Analysis of endogenous plant growth substances — Analytical methods and validation were as described previously (4). Hypocotyls cultured on basal or media supplemented with 10 μM TDZ and incubated either in complete darkness or under continuous light, were sampled over a period of three days and stored at -80°C. Samples were then freeze dried. To extract the plant growth substances, samples were first shaken in 4 ml of 80% methanol for 24 h at 4°C in the dark on an orbital shaker (Lab-Line Instruments Inc, Melrose Park, IL). Subsequently, the samples were centrifuged using an IEC HN-SII centrifuge (International Equipment Co, Needham Heights, MA) for 10 min at 2.000 xg to remove particulate matter. The supernatant was then dried under vacuum using SpeedVac (Model DD-20, Precision Scientific Inc, Chicago, IL). The extracts were resuspended in a sample diluent (0.5 mM phosphate buffer, pH 7.4 with 5% acetonitrile) and an alilquot of 20 μl of each sample was injected into the HPLC system for analysis of auxins, cytokinins and ABA. The HPLC equipment and elution gradient were the same as previously published (4). Growth substances were quantified by comparison of peak area to regression equations calculated from repeated injections of standards of adenine, adenosine, absicisic acid (ABA), dihydrozeatin (DHZ), indole-3-acetic acid (IAA), isopentenyladenine (2iP), tryptamine (TRPM), zeatin, (Sigma Chemical Co, St Louis, MO) and TDZ (NOR-AM. Chemical Co Inc, Wilmington, DE). Recovery of all plant growth regulators was greater than 90% and peak identification was verified by GC-MS as previously described (4).

In all experiments, each treatment consisted of three to four replications and all experiments were repeated at least twice. Analysis of variance was carried out using the General Linear Model Procedure of SAS (8) and means compared by Least Significant Differences (LSD) test at P = 0.05.

Results

Somatic embryogenesis in geranium hypocotyl cultures was achieved under both light (continuous light and 16-h photoperiod) and dark conditions by first maintaining etiolated hypocotyl explants for 3 days on an induction medium containing 10 μM TDZ and then transferring them to a basal expression medium lacking TDZ. All the hypocotyl sections cultured on MSO and maintained in complete darkness remained thin, pale white in colour but formed long roots averaging 4.73 cm while those maintained under continuous light formed no roots. Only 50% of the explants maintaind under 16-h photoperiod formed roots averaging 1.29 cm in length. All the hypocotyls maintained under 16-h photoperiod were dark green in colour while those under continuous light displayed an array of colours of green and pink.

More somatic embryos were formed when explants, cultured on TDZ- supplemented media, were incubated in complete darkness than under continuous lilght or under a 16-h photoperiod (Table 1). Dark-grown cultures, in addition, produced embryos that were highly synchronized in their development than those grown under continuous light. Explants that were maintained continuously on 10μM TDZ and not transferred to a basal expression medium after the initial 3 days of induction did not form any somatic embryos under both light incubation conditions studied (Table 1). However, those incubated in complete dark formed an average of 2-3 embryos per explant. The

Table 1. Effect of dark and light regimes on mean number of somatic embryos formed on geranium (*Pelargonium x hortorum* Bailey *cv.* Scarlet Orbit Improved) hypocotyl cultures.

Treatment	Incubation Condition	Number of Somatic Embryos
MSO	Complete Dark	0
	16-photoperiod	0
	Continuous light	0
Continuous TDZ Exposure	Complete Dark	2.4
	16-h photoperiod	0
	Continuous Light	0
3-day Incubation with TDZ	Complete Dark	49.3
	16-h photoperiod	33.9
	Continuous Light	35.7

Note : Explants were maintained on an induction medium supplemented wih 10 μM TDZ either for 3 days with transfer to basal expression medium or continuously for the 30 days. Means followed by the same letter are not significantly different at P = 0.05.

explants exposed to continuous light or 16-h photoperiod turned brown and died while those maintained in the dark formed small amounts of callus at the cut ends. Few embryos were formed on these calli when the explants were transferred from darkness to a 16-h photoperiod. The analysis of variance indicated the presence of significant effects for TDZ treatment, light incubation conditions, as well as the interaction between TDZ and light treatments with respect to their ability to induce somatic embryos (Table 2).

The levels of endogenous plant growth substances, analyzed over the 3-day induction period (9) were found to be dependent on the incubation conditions, the TDZ treatment and the length of the incubation period. The endogenous levels of IAA, ABA, 2iP and tryptamine were significantly influenced by the incubation conditions and the length of time in culture (Tables 3 & 4). During the first day of culture, explants cultured on basal medium (MSO) and incubated under continuous light and significantly greater amounts of adenine, adenosine, DHZ and zeatin but similar amounts of 2iP, IAA, tryptamine and ABA compared to those incubated in the dark (Table 3). The tissues treated with TDZ had higher levels of endogenous adenine, adenosine, DHZ, zeatin, 2iP, IAA, tryptamine and ABA over the 'MSO' control tissues irrespective of light conditions during incubation (Table 3). On the second day, tissues treated with 10 μM TDZ and cultured under continuous light had accumulated significantly greater amounts of adenine, adenosine, DHZ, zeatin, 2iP, tryptamine, IAA and ABA than similarly treated tissues cultured in the dark (Table 3). However, on the third day, there was dramatic increase in the accumulation of endogenous adenine, adenosine, DHZ, zeatin, and moderate increases in the levels of 2iP and IAA levels present in the tissues treated with 10μM TDZ and incubated in the dark (Table 3).

Discussion

Thidiazuron, a phenylurea, is a potent plant growth regulator that evokes a series of responses *in vitro* (10). The role of TDZ in hormonal regulation of somatic embryogenesis is interesting in view of the numerous reports indicating that auxins are the primary inducers of the embryogenic process in a wide variety of species (11). In a previous study, we reported that geranium hypocotyl tissues developed somatic embryos when treated with TDZ for 3 days and subsequently transferred to basal medium (9). The 3 day period corresponds to the induction period of somatic embryogenesis and the plant growth substances were analyzed during this period. Earlier, TDZ has been shown to modulate the endogenous levels of phytohormones *in vitro* (4,12). There is also substantial evidence indicating that light modifies endogenous levels of plant growth substances and/or alters the sensitivity of cells to hormones (13,14). The objective of the current study was to evaluate thidiazuron-induced somatic embryogenesis under different light conditions in geranium hypocotyl cultures and whether some of the TDZ responses are mediated in part by light. To date, little information is available to compare the effects of light on endogenous plant growth substances in both dark and light-grown cultures in relation to specific morphogenic activity such as somatic embryogenesis. In this communication, we show that there is a significant interaction between TDZ and light in modulating the endogenous levels of plant growth substances during the induction period of somatic embryogenesis in geranium. Some of the morphogenic responses of TDZ may therefore be mediated, in part, by light-induced changes in the levels of endogenous plant growth substances.

All components of light, quality, quantity and photoperiodicity, can modulate plant growth and development *in vitro* mainly through photomorphogenic role since photosynthesis is limited under tissue culture

Table 2. Analysis* of variance of TDZ and incubation conditions on mean number of somatic embryos formed on geranium (*Pelargonium x hortorum* Bailey) hypocotyl cultures.

	DF	SS	MSS	F	Pr<F
Model	8	9671.2	1208.9	226.32	0.0001
Treatment	2	9233.2	4616.6	864.29	0.0001
Condition	2	192.5	96.2	18.02	0.0001
Treatment x Condition	4	45.56	61.4	11.49	0.0001
Error	18	96.1	5.3		

* Statistical analysis of the data presented in Table 1.

Table 3. Effect of TDZ and light regimes on endogenous profiles of plant growth substances (μmolg^{-1}dry mass) in geranium (*Pelargonium x hortorum* Bailey *cv.* Scarlet Orbit Improved) hypocotyl cultures.

Day	Condition	Treatment	Adenine	Adenosine	DHZ	Zeatin	2iP	IAA	TRPM	ABA
1	Dark	MSO	2.1^{c}	1.7^{c}	1.5^{c}	24.5^{c}	25.4^{c}	45.2^{c}	15.6^{c}	44.8^{c}
		TDZ	3.3^{b}	2.7^{b}	2.4^{b}	38.5^{b}	26.6^{bc}	47.4^{bc}	16.3^{bc}	46.9^{bc}
	Light	MSO	4.2^{a}	3.3^{a}	2.9^{a}	48.2^{a}	29.1^{ab}	51.8^{ab}	17.8^{ab}	51.3^{ab}
		TDZ	3.9^{a}	3.1^{a}	2.8^{a}	45.2^{a}	3.13^{a}	55.7^{a}	19.2^{a}	55.2^{a}
2	Dark	MSO	2.4^{c}	1.9^{c}	1.7^{c}	27.3^{c}	18.8^{c}	33.6^{c}	11.6^{c}	33.3^{c}
		TDZ	3.3^{b}	2.7^{ab}	2.4^{ab}	38.6^{ab}	26.7^{ab}	47.6^{ab}	16.4^{ab}	47.1^{ab}
	Light	MSO	2.9^{b}	2.4^{b}	2.1^{b}	33.9^{b}	23.4^{b}	41.8^{b}	14.4^{b}	41.4^{b}
		TDZ	3.9^{a}	3.1^{a}	2.8^{a}	45.3^{a}	31.3^{a}	55.8^{a}	19.2^{a}	55.3^{a}
3	Dark	MSO	2.2^{c}	1.7^{c}	1.5^{b}	25.1^{b}	17.3^{b}	30.9^{b}	10.6^{c}	30.6^{b}
		TDZ	2.4^{bc}	5.4^{a}	4.8^{a}	27.5^{b}	19^{b}	33.8^{b}	11.7^{c}	33.5^{b}
	Light	MSO	6.7^{a}	1.9^{bc}	1.7^{b}	77.3^{b}	30.4^{a}	54.2^{a}	18.7^{a}	53.7^{a}
		TDZ	2.3^{bc}	1.9^{bc}	1.7^{b}	26.9^{b}	18.6^{b}	33.2^{b}	11.4^{c}	32.8^{b}

Note : Explants were maintained on TDZ supplemented media for 3 days under either continuous light or complete darkness. The incubation conditions 'dark' and 'light' refer to complete darkness and light, respectively. Means followed by the same letter on each day of analysis are not significantly different at P = 0.05.

Table 4. Statistical significance of the levels of the endogenous profiles of plant growth substances in geranium (*Pelargonium x hortorum* Bailey *cv.* Scarlet Orbit Improved) hypocotyl tissues treated with TDZ and incubated under complete darkness or complete light

	Adenine	Adenosine	DHZ	Zeatin	2iP	IAA	TRPM	ABA
Condition	NS	NS	NS	NS	+++	+++	+++	+++
Treatment	++	++	++	++	NS	NS	NS	NS
Day	++	++	++	++	+++	+++	+++	+++
Condition X Treatment	+	+	+	+	NS	NS	NS	NS
Condition X Day	+++	+++	+++	+++	+++	+++	+++	+++
Treatment X Day	NS	NS	NS	NS	+++	+++	+++	+++
Condition X Treatment X Day	+	+	+	+	+	+	+	+

NS = not significant; + = significant at P = 0.01; ++ = significant at P = 0.001; +++ = significant at P = 0.0001.

conditions. In the present study, explants treated with TDZ formed well differentiated embryos under incubation conditions of continuous light, complete darkness of 16-h photperiod. However, embryogenesis was significantly impaired under both light conditions (16-h photoperiod and continuous light). Maintaining the cultures in the dark increased the frequency of the somatic embryos formed with a corresponding increase in the amount of the endogenous plant growth substances in the tissues on the third day of culture. Continuous light significantly reduced the amount of endogenous plant growth substances on the third day of culture in the tissues treated with TDZ. These results indicate that in the presence of TDZ, light interferes with the accumulation of the endogenous plant growth substances. However, because there were no significant differences in the dark and light cultures during the first 2 days of culture, it may be suggested that light could be reducing tissue sensitivity to endogenous hormones during the induction of somatic embryos in geranium hypocotyl cultures. The reduced levels of auxins, cytokinins and ABA under continuous light, during the critical first 3 days of culture corresponding to the induction period, could have impaired the induction process leading to fewer embryos being formed. Reduced embryogenic response in the light-grown tissues could be explained in part by a red light mediated reduction in auxin production, auxin transport and/or a reduced sensitivity of cells to endogenous plant growth substances.

Light has been shown to either suppress or promote embryogenesis in different species: *Podophyllum hexandrum* (15) and *Solanum melongena* (16). Previously, we reported that TDZ promoted chlorophyll accumulation in the cucumber cotyledons (17). The formation of somatic embryos in the absence of light indicate that TDZ promotion of chlorophyll synthesis and embryo development are not closely linked. Cytokinins added to the culture medium have frequently been noted to promote chlorophyll development in callus or suspension cultures (18), or even to be essential for its formation in light (19). The increased pigmentation of tissues cultured under continuous light could be due to enhanced activity of the flavonoid biosynthetic pathway responsible among other factors for pigmentation.

Light also significantly influenced the production of roots on geranium explants mainteined continuously on basal medium. All the cultures maintained on basal medium and incubated in the dark formed single elongated roots while only 50% and none of the explants maintained on a 16-h or continuous light, respectively, formed roots. The complete inhibition of root formation in cultures maintained under continuous light and the corresponding decrease in extractable IAA on the 3rd day of culture may be an indication of light-mediated changes in endogenous IAA levels. It is possible that the reduced levels of IAA resulted from accelerated photo-degradation of IAA (20) leading to a possible reduction in the amount of DNA replication (21), which plays a key role in the regulation of nucleic acid biosynthesis. However, since dark and light grown cultures treated with TDZ showed no significant differences in the amounts of endogenous IAA during the first 2 days of culture, it may be postulated that an altered sensitivity of cells to endogenously present IAA may also be a significant factor during embryogenesis, in addition to the threshold values of IAA. The ABA content of tissues maintained under continuous ligh increased on the second day but significantly decreased on the third day of culture with a corresponding decrease in the embryogenic response of cultures maintained under continuous light. Previously the content of ABA in birch seedlings (22) and

spinach leaves (23) was shown to increase with increasing day-length and endogenous ABA has been correlated with induction of morphogenesis (9).

The study of TDZ and light regulation of somatic embryogenesis will add to our understanding of the modes of action and mutual interactions of TDZ and light in hormonal regulation of somatic embryogenesis *in vitro*. The results of this study suggest that light could be modulating the endogenous levels of plant growth substances and possibly tissue sensitivity to these plant growth substances during TDZ-regulated somatic embryogenesis in geranium hypocotyl cultures.

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