BIOLOGIA PLANTARUM 44 (4): 611-613, 2001

BRIEF COMMUNICATION

Stimulation of shoot regeneration on *Linum* hypocotyl segments by thidiazuron and its response to light and calcium

P. JAIN and A. RASHID*

Department of Botany, University of Delhi, Delhi-110007, India

Abstract

The basal segment from hypocotyl of *Linum usitatissimum* L. seedling readily regenerates, to produce a large number of shoots, in a short period of 5 - 7 d. This response was stimulated by a low concentration (0.1 μM) of thidiazuron (TDZ). TDZ was also effective in inducing regeneration in dark. A drastic reduction in regeneration response on hormone-free as well as TDZ-supplemented medium was found after inclusion of an inhibitor of calcium-uptake, lanthanum (La^{3+}). An essentiality of calcium in the regeneration was also evidenced by an increased response with increasing concentration of calcium. At Ca^{2+} concentration insufficient for regeneration, inclusion of TDZ resulted in shoot formation.

Additional key words: calcium-inhibitor, calmodulin-inhibitor, chlorpromazine, flax, lanthanum, trifluoperazine.

Flax seedlings are characterized by an unusual developmental response: after decapitation the hypocotyl readily regenerates shoot-buds (Link and Eggers 1946). This process of regeneration was also described under *in vitro* conditions (Gamborg and Shyluk 1976, Lane 1979, Bretagne *et al.* 1994). In this investigation the rapid regeneration response of hypocotyl segments is utilized to study the role of thidiazuron (TDZ), a phenylurea derivative that simulates cytokinin activity and is known for a diverse array of subcellular and cellular responses ranging from tissue proliferation to induction of shoots and somatic embryos (Mok *et al.* 1982, Thomas and Katterman 1986, Malik and Saxena 1992, Iantcheva *et al.* 1999). Some new aspects of structure-activity relationship of TDZ are unravelled and an attempt is made to decipher its mode of action.

The seeds of common flax (*Linum usitatissimum* L. cv. Neelam) were washed with *Teepol* for 10 min and then dipped in 70 % ethanol for 30 s. After rinsing with sterile distilled water these seeds were gently agitated in chlorine water for 5 min. Finally the seeds were rinsed thrice in distilled water and placed for germination on a nutrient medium, that was based on mineral formulation of Chu *et al.* (1975) known as N_6 medium. This medium was gelled with 0.8 % agar and 2 % sucrose served as carbon source. The pH of the medium was adjusted to 5.8, before autoclaving.

The basal nutrient medium comprised [mg dm^{-3}]: KNO_3 2830, $(NH_4)_2SO_4$ 463, KH_2PO_4 400, $CaCl_2 . 2\ H_2O$ 166, $MgSO_4 . 7\ H_2O$ 185, Fe-EDDHA 5.00, $MnSO_4 . 4\ H_2O$ 4.4, $ZnSO_4 . 7H_2O$ 1.5, H_3BO_3 1.6, KI 0.8, inositol 100, glycine 2, thiamine HCl 1, pyridoxine HCl 0.5, and nicotinic acid 0.5.

Cultures of hypocotyl segments isolated from 7 to 8-d-old seedlings were raised in screw cap vials (15 cm^3) with 1.0 cm^3 of liquid medium. These were maintained at 25 ± 2 °C and continuous light (15 μmol m^{-2} s^{-1}). For every treatment at least 20 replicate cultures were raised. Each experiment was repeated more than once, with identical results.

The regenerative capacity of hypocotyl segments, in concurrence with the findings of Bretagne *et al.* (1994), increased with an increasing distance from apex. Although the basal segment of 5 mm length was most regenerative, still better regeneration response was possible from 2.5 mm hypocotyl region and 2.5 mm root region, without any contribution from root segment. However, it was not desirable to divide the basal segment

Received 20 November 2000, *accepted* 27 February 2001.

Abbreviations: BAP - benzylaminopurine; CPZ - chlorpromazine; NAA - naphthaleneacetic acid; TDZ - thidiazuron; TFP - trifluoperazine.

* Corresponding author

of hypocotyl into still smaller segments due to ensuing necrosis and poor regeneration response. Hence, 5 mm long basal segment of hypocotyl was selected as an explant for further studies.

Linum cultivar employed in this investigation is highly regenerative, it formed 8 - 10 regenerants per basal segment on hormone-free medium (Table 1) and this frequency could be further increased to 13 - 15 regenerants on medium with 0.1 μM of TDZ. An advantage of this system is reproducibility of results and rapid response. In other cultivars of *Linum* shoot regeneration frequency on benzylaminopurine (BAP) medium ranged between 2.5 to 3.2 (Lane 1979) and 2 to 5 shoots per segment (Bretagne *et al.* 1994). This frequency could be increased to 8.6 regenerants on medium with 0.1 μM TDZ and 0.01 μM NAA. TDZ (0.1 μM) alone gave about 4.6 regenerants (Bretagne *et al.* 1994).

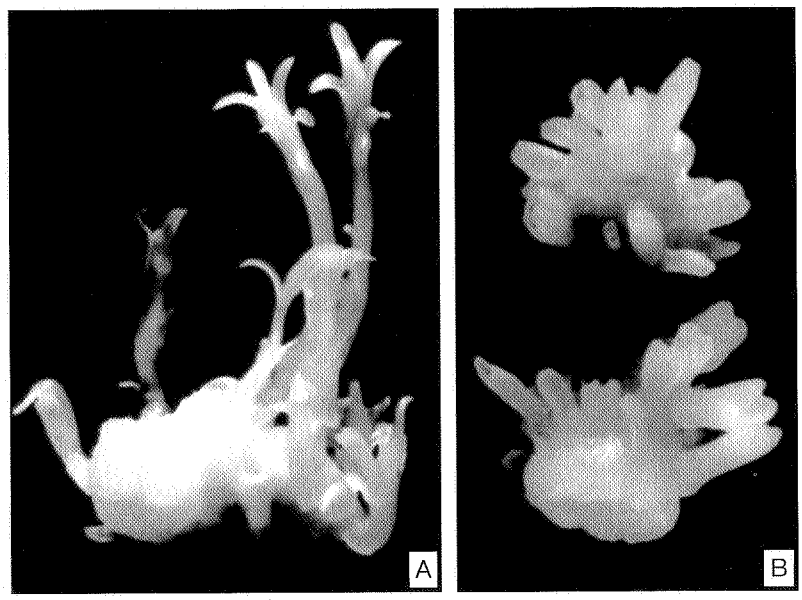

Fig. 1. Shoot-bud regeneration on basal segment of hypocotyl of *Linum* seedling, on medium supplemented with TDZ in dark (A) or in light (B). Note elongation of shoots in dark.

Besides an increase in frequency of regeneration TDZ induced regeneration in dark (Table 1). In dark on a hormone-free medium there was not more than one shoot-bud per responding explant as compared to 8 - 10 shoots in light. The shoots developed on TDZ medium in dark were, however, elongated (Fig. 1*A*). This corresponds to an etiolation symptom. In earlier studies on other systems the shoots formed in response to TDZ remained stunted (Chalupa 1987, Huetteman and Preece 1993, Sankhla *et al.* 1996).

An essentiality of calcium in regeneration was evidenced by an increased response with increasing concentration of calcium. At the lowest concentration 10 mg dm^{-3} there was practically no regeneration. However, at this concentration, a low frequency regeneration was possible on TDZ-medium (Table 1).

An inhibitor of calcium-uptake, lanthanum (La^{3+}), at different concentrations (0.01, 0.1 and 1.0 mM) reduced the frequency of regeneration in terms of number of regenerants per explant (Table 2). Since La^{3+} does not enter plant cells it is ascribed to inhibit an influx of calcium from the medium to the tissue (Wayne and Hepler 1984). When La^{3+} was employed along with TDZ, a low frequency regeneration (Table 2) was possible.

Table 1. Frequency of shoot-bud regeneration on basal segment of hypocotyl of *Linum* seedling, in light or dark on hormone-free basal medium (BM) and on medium supplemented with TDZ. Effect of different concentrations of calcium on hormone-free medium and on medium supplemented with 0.1 μM TDZ.

Medium	Number of shoots [segment^{-1}]	
	light	dark
BM	8.6 ± 1.0	1.5 ± 0.5
BM + 0.1 μM TDZ	13.5 ± 1.5	10.4 ± 0.6
BM + 10 mg dm^{-3} Ca	0.2 ± 0.0	
BM + 42 mg dm^{-3} Ca	5.6 ± 1.2	
BM + 83 mg dm^{-3} Ca	7.1 ± 0.7	
BM + 166 mg dm^{-3} Ca	8.6 ± 1.0	
BM + 332 mg dm^{-3} Ca	3.0 ± 1.0	
BM + 10 mg dm^{-3} Ca + 0.1 μM TDZ	2.9 ± 0.5	

In view of involvement of calmodulin in Ca^{2+}-mediated processes effects of trifluoperazine (TFP) and chlorpromazine (CPZ), which inhibit calmodulin-dependent processes in animals, was studied. Both these drugs inhibited regeneration (Table 3) at a low

concentration (0.1 μM). CPZ was not so effective when given either for first 5 d or last 5 d. First 5 d seem to be the stage of initiation and determination and the last 5 d are stage of development. If CPZ is not present during initiation and determination it is ineffective to prevent the development of initiated shoot-buds, when given during last 5 d. This was not true for TFP (Table 3), it was effective when given for first 5 d and was also effective when given for last 5 d.

Shoot regeneration on hypocotyl segments of *Linum* is a rapid response. The inhibitors of calmodulin (CPZ and TFP) were effective in reducing the number of shoot-buds at very low level of 0.1 μM in this system. This is comparable to concentration effective in animal system, and was regardless whether inhibitors were included in basal medium or medium supplemented with TDZ.

Table 2. Frequency of shoot-bud regeneration on basal segment of hypocotyl of *Linum* seedling, cultured on different concentrations of lanthanum chloride and with or without TDZ.

Medium	Shoot number [segment^{-1}]
BM	8.6 ± 1.0
BM + 0.01 mM La^{3+}	7.1 ± 1.0
BM + 0.10 mM La^{3+}	6.0 ± 1.0
BM + 1.00 mM La^{3+}	2.1 ± 1.0
BM + 0.1 μM TDZ	13.5 ± 1.5
BM + 0.1 μM TDZ + 1 mM La^{3+}	2.8 ± 1.3

Table 3. Frequency of shoot-bud regeneration on basal segment of hypocotyl of *Linum* seedling on BM medium supplemented with chlorpromazine (CPZ) or trifluoperazine (TFP) during different periods.

0 - 5 d	5 - 10 d	Shoot number [segment^{-1}]
BM	BM	8.6 ± 1.0
BM + 0.1 μM CPZ	BM + 0.1 μM CPZ	2.0 ± 0.3
BM + 0.5 μM CPZ	BM + 0.5 μM CPZ	1.6 ± 0.5
BM + 0.1 μM TFP	BM + 0.1 μM TFP	3.0 ± 1.0
BM + 0.5 μM TFP	BM + 0.5 μM TFP	2.5 ± 0.5
BM	BM + 0.1 μM TFP	3.1 ± 0.8
BM + 0.1 μM CPZ	BM	4.3 ± 0.3
BM	BM + 0.1 μM CPZ	6.0 ± 0.8
BM + 0.1 μM TFP	BM	3.3 ± 0.3

References

Bretagne, B., Chupeau, M.C., Chupeau, V., Fouilloux, G.: Improved flax regeneration from hypocotyls using thidiazuron as a cytokinin source. - Plant Cell Rep. **14**: 120-124, 1994.

Chalupa, V.: Effect of benzylaminopurine and thidiazuron on *in vitro* shoot proliferation of *Tilia cordata* Mill., *Sorbus aucuparia* L. and *Robinia pseudoacacia* L. - Biol. Plant. **29**: 425-429, 1987.

Chu, C.C., Wang, C.C., Sun, C.S., Hsu, C., Yin, K.C., Chu, C.Y., Bi, F.Y.: Establishment of an efficient medium for anther culture of rice through comparative experiments on nitrogen sources. - Sci. sin. **18**: 659-668, 1975.

Gamborg, O.L., Shyluk, J.P.: Tissue culture, protoplasts and morphogenesis in flax. - Bot. Gaz. **137**: 301-306, 1976.

Huetteman, C.A., Preece, J.E.: Thidiazuron: a potent cytokinin for woody plant tissue culture. - Plant Cell Tissue Organ Cult. **33**: 105-119, 1993.

Iantcheva, A., Vlahova, M., Bakalova, E., Kondorsi, E., Elliott, M.C., Atanassov, A.: Regeneration of diploid annual medics via direct somatic embryogenesis promoted by thidiazuron and benzylaminopurine. - Plant Cell Rep. **18**: 904-910, 1999.

Lane, D.W.: Influence of growth regulators on root and shoot initiation from flax meristem tips and hypocotyls *in vitro*. - Physiol. Plant. **45**: 260-264, 1979.

Link, G.K.K., Eggers, V.: Mode, site, and time of initiation of hypocotyledonary bud primordia in *Linum usitatissimum* L. - Bot. Gaz. **107**: 441-454, 1946.

Malik, K.A., Saxena, P.K.: Regeneration in *Phaseolus vulgaris* L. High frequency induction of direct shoot formation in intact seedlings of N^6-benzylaminopurine and thidiazuron. - Planta **186**: 384-389, 1992.

Mok, M.C., Mok, D.W.S., Armstrong, D.J., Shudo, K., Isogai, Y., Okamoto, T.: Cytokinin activity of N-phenyl-N'-1,2,3-thidiazol-5-urea (thidiazuron). - Phytochemistry **21**: 1509-1511, 1982.

Sankhla, D., Davis, T.D., Sankhla, N.: *In vitro* regeneration of silktree (*Albizzia julibrissin*) from excised roots. - Plant Cell Tissue Organ Cult. **44**: 83-86, 1996.

Thomas, J.C., Katterman, F.R.: Cytokinin activity induced by thidiazuron. - Plant Physiol. **81**: 681-683, 1986.

Wayne, R., Hepler, P.K.: The role of calcium ions in phytochrome-mediated germination of spores of *Onoclea sensibilis* L. - Planta **160**: 12-20, 1984.