Indian Journal of Biotechnology
Vol 11, April 2012, pp 205-209

Organogenesis from the cultures of *Nothapodytes foetida* (Wight) Sleumer raised on TDZ supplemented media

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Received 18 November 2010; revised 9 September 2011; accepted 21 October 2011

Mature embryos of *Nothapodytes foetida* (Wight) Sleumer were inoculated on Murashige and Skoog (MS), modified MS (MMS) and Phillips and Collins (L2) media supplemented with TDZ either alone or with BAP and L-glutamine. Both shoot proliferation from the apical region of shoots and differentiation of shoots from the callus were observed from the cultures depending on the type of medium and the hormone combination. Proliferation of shoots from the apical region of shoots and also organogenesis from the callus, formed at the transition zone of primary root and hypocotyl of the young seedling, was obtained on L2+TDZ (0.44 μ*M*)+BAP (2.22 μ*M*)+L-glutamine (0.03 m*M*). Regeneration from the callus cultures was reported for the first time. The dual role of TDZ in promoting proliferation of shoots and shoot differentiation from the callus, along with beneficial effects of cytokinin and L-glutamine on the morphogenesis, was discussed.

Keywords: Auxin-cytokinin like activity, callus cultures, camptothecin, *Nothapodytes foetida*, thidiazuron

Introduction

Nothapodytes foetida (Wight) Sleumer belongs to the family *Icacinaceae* and grows wildly in the forests of North-east India and Western Ghats of Deccan Peninsula. The plant is an Indian source of camptothecin (CPT), an expensive anticancer drug costing about US $ 170 to 250 per mg. Accumulation of CPT was reported in young leaves, roots, stem, bark and also seeds[1]. Chemical profiling of the taxon revealed the natural variability in CPT content among the individuals across a wide geographical range[2]. Because of the deforestation and unscientific collection of the material, the taxon has been categorized under threatened status[3]. Hower, micropropagation has been proved to be an efficient *in vitro* method for multiplication and conservation of elite germplasms.

Thidiazuron (TDZ) is a urea derivative, which contains no purine ring but found more effective than purine type cytokinins[4]. Since 1980's TDZ is being used in *in vitro* culture and has been reported to induce adventitious shoot formation in a number of species, especially woody plants. Both cytokinin and auxin-cytokinin activity has been attributed to TDZ[4,5].

Perusal of literature shows that regeneration from the callus cultures has not been reported in *N. foetida*, though an adventitious shoot regeneration from the culture of hypocotyl, leaf and cotyledon has been achieved[6,7]. Regeneration from the callus is a prerequisite for the selection of variants (somaclones) and to employ biotechnological approaches for the genetic improvement. Hence, the present study is an attempt to induce callus from embryo explants of four populations collected from different locations of western Ghats and differentiation of shoot buds from the callus cultures.

Material and Methods

Seeds were collected from four different geographical areas, such as, Mahabaleshwar (North Western Ghats), Bisle ghat (Central Western Ghats), B R Hills and Ooty (South Western Ghats), keeping in view of earlier reports on variations in CPT contents among different populations[1,8]. The seeds were soaked for overnight in GA_3 (100 mg/L), since GA_3 is known to be effective in overcoming dormancy[9]. The seeds were decorticated before thorough washing in running water for 30 min. They were then surface sterilized in saturated chlorine water for 10 min and treated with mercuric chloride (0.1%) for 1 min. Mature-embryos were dissected in a laminar flow hood and used as explants. They were inoculated onto culture media supplemented with TDZ either alone or with various growth regulators. Three different media, such as, Marashige and

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Skoog (MS)[10], Phillips and Collins leguminous medium (L2)[11] and modified MS medium (MMS), containing salts of MS and vitamins of L2 medium, were used to raise the cultures. The media were autoclaved at 108 kpa and *p*H was maintained at 5.6. The cultures were maintained at 16:8 h light and dark regime under the illumination of about 50 µmol m^{-2} s^{-1} provided by white fluorescent tubes. For histological studies, the organogenic callus segments were fixed in FAA (formalin:acetic acid:ethyl alcohol) for 48 h. Customary paraffin technique was followed. Sections were cut at 15 µm thick and stained with Haidenhains haematoxylin and counter stained with Orange-G/Eosin. Photomicrographs were taken with Canon camera using a Nikon binocular microscope.

Data Analysis

All the experiments were repeated at least thrice. The data thus obtained were subjected to statistical analysis—one way ANOVA. Significant 'F' ratios between group means were further subjected to least significance difference (LSD). Probability (P) values <0.05 were considered significant[12].

Results and Discussion

Among the media used, L2 medium was found to be the best in terms of per cent response for callus formation, followed by MMS and MS (data not shown). L2 medium was first formulated by Phillips and Collins[11] to initiate callus and suspension cultures of red clovers. Subsequently, it is being used for most legume cultures. The morphogenetic response of the explants from different geographical locations was similar but varied in percentage. The explants from Ooty accessions showed highest percentage of response to culture conditions compared to others (data not shown). Creamish coloured embryos turned green within 4-5 d of culture on both basal media and hormone(s) supplemented media. However, further growth was arrested on basal and IAA/IBA/Kin/2-ip supplemented media. Whereas on 2,4-D/zeatin supplemented media, the green embryos callused but no morphogenesis occurred even after many subcultures. When the medium was supplemented with TDZ at various concentrations, the response of the explants varied with the media and the other supplemented growth hormones. On all the TDZ supplemented media, the embryo developed into a seedling. When the seedling grew about 4-5 cm length with well developed primary root, proliferation of shoots from the shoot apical region was observed on L2 medium supplemented with TDZ (0.44 µ*M*) (Fig. 1a; Table 1). However, on MS+TDZ (0.44 µ*M*), callus formation was observed at the transition zone of the primary root and the hypocotyl without the proliferation of shoot apex. When these cultures were left on the same media for 4 to 5 wk, numerous shoot buds started differentiating from the callus (Figs 1b & 2a). Though the regenerated shoots from both direct and indirect organogenesis on TDZ supplemented media appear healthy, the leaves were narrow and light green in colour. However, presence

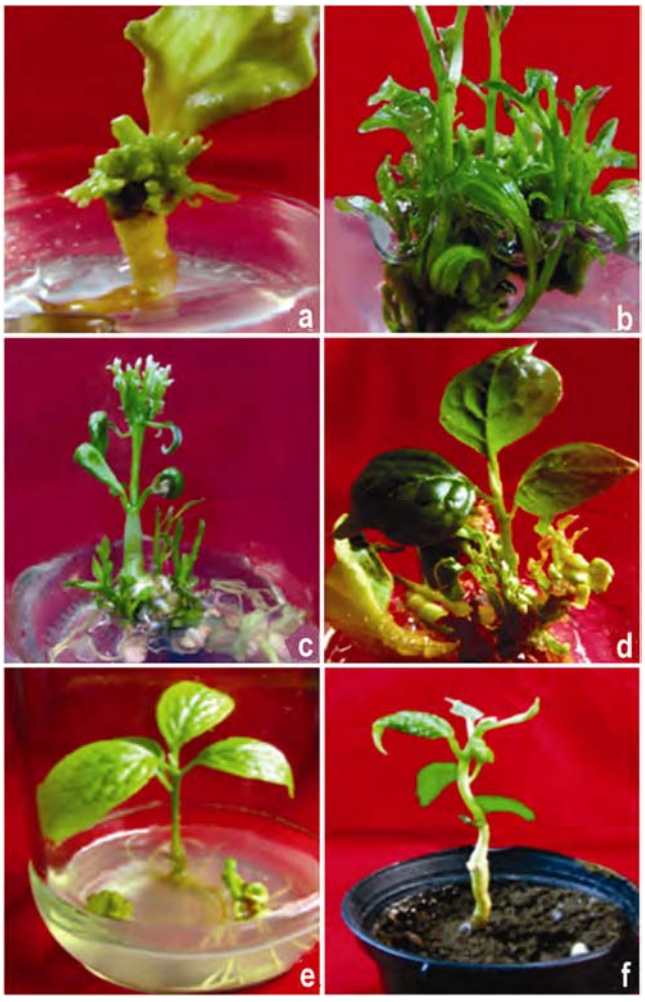

Fig. 1 (a-f)—*In* vitro propagation of *N. foetida*: a. Shoot proliferation from the apical region on L2+TDZ (0.44 µ*M*); b. Multiple shoots induction from the callus derived from the transition zone of primary root and hypocotyls on MS+TDZ (0.44 µ*M*); c. Both shoot proliferation from shoot apical region and shoot differentiation from the callus on MMS+TDZ (0.44 µ*M*); d. Well developed shoots on L2+BAP (2.22 µ*M*)+TDZ (0.44 µ*M*); e. Initiation of roots from the basal region of the regenerated shoots on MMS+IBA (2.46 µ*M*); f. Regenerated plant in pot containing soil, sand and manure in 1:1:1 ratio.

Table 1—Effect of TDZ and other growth regulators on induction of multiple shoots from embryo cultures of *N. foetida* (Wight) Sleumer

No.	Medium+growth regulators (μ*M*)	% response	No. of shoots/culture (mean±SE)
1	L2+TDZ (0.44)***	48.6	15 ± 0.58 [c]
2	L2+TDZ (0.44)+BAP (2.22)*	63.3	19 ± 0.51 [c]
3	L2+TDZ (0.44)+BAP (2.22)+L-glutamine (0.03 m*M*)*	85.0	26 ± 0.39 [a]
4	L2+IAA (2.85)+BAP (2.22)+L-glutamine (0.03 m*M*)**	66.0	20 ± 0.29 [b]
5	L2+IBA (2.46)+BAP (2.22)+L-glutamine (0.03 m*M*)**	72.3	22 ± 0.46 [a]
6	MS+TDZ (0.44)**	35.3	11 ± 0.31 [c]
7	MS+TDZ (0.44)+BAP (2.22)**	64.0	19 ± 0.52 [a]
8	MS+TDZ (0.44)+BAP (2.22)+L-glutamine (0.03 m*M*) **	76.0	23 ± 0.42 [b]
9	MS+IAA (2.85)+BAP (4.44)+L-glutamine(0.03 m*M*)*	51.3	15 ± 0.47 [c]
10	MS+IBA (2.46)+BAP (4.44)+L-glutamine (0.03m*M*)*	68.6	21 ± 0.31 [b]
11	MMS+TDZ (0.44)*	36.6	11 ± 0.51 [c]
12	MMS+TDZ (0.44)+ BAP (2.22)*	75.0	15 ± 0.44 [c]
13	MMS+TDZ (0.44)+BAP (2.22)+L-glutamine (0.03 m*M*)*	76.0	23 ± 0.42 [b]
14	MMS+IAA (2.85)+BAP (2.22)+L-glutamine (0.03 m*M*)*	74.6	22 ± 0.44 [a]
15	MMS+IBA (2.46)+BAP (2.22)+L-glutamine (0.03 m*M*)*	70.0	21 ± 0.39 [c]

In each treatment total no. of cultures were 30.
*Both direct and indirect regeneration, **only indirect regeneration, ***only direct regeneration. The mean values within the column having different letters are significantly different ($p \leq 0.05$) by Duncan multiple range test.

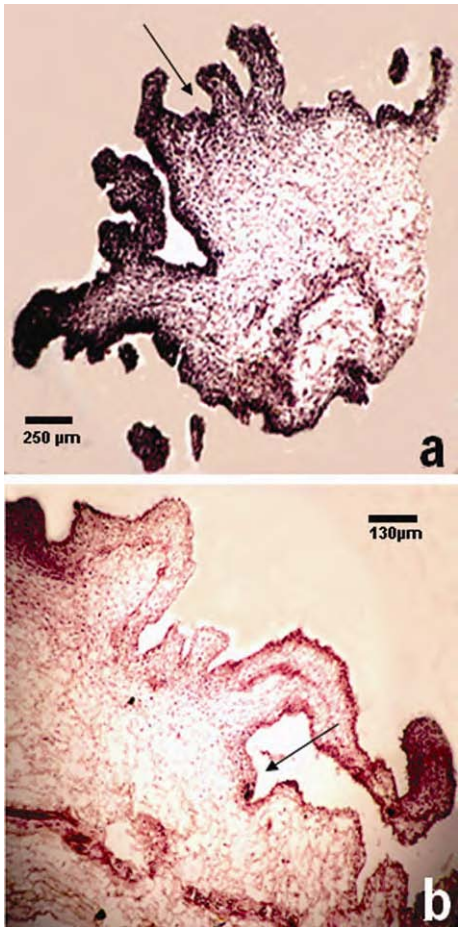

Fig. 2 (a & b)—Sections of the organogenic callus raised on MS+TDZ (0.44 μ*M*) (a) and MMS+TDZ(0.44 μ*M*) (b) showing differentiation of shoot buds from the callus

of TDZ (0.44 μ*M*) in MMS medium, which contains salts of MS and vitamins of L2 medium, promoted both the proliferation of shoot apex in the beginning and differentiation of shoot buds from the callus derived from the transition zone of the hypocotyl and primary root in later period of the culture on the same medium (Figs 1c & 2a). Thengane *et al*[6] reported high percentage of adventitious shoot regeneration from leaf explants of *N. foetida* at lower concentrations of TDZ in the MS medium, while higher concentrations favoured direct shoot induction from cotylendon and hypocotyl explants. This was later confirmed by Rai[7] in hypocotyl explants where high concentrations of TDZ supplemented to MS favoured large number of adventitious shoots from the cut surfaces of hypocotyl segments.

Since Briggs *et al*[13] had observed better quality shoots in the culture of azaleas on TDZ and purine cytokinin supplemented media, BAP was added along with TDZ to the media to know the synergistic effect of TDZ and BAP on the morphology of the regenerated shoots. On L2+TDZ (0.44 μ*M*)+BAP (2.22 μ*M*), along with the proliferation of shoots at the apical region of the seedling, as was recorded on L2 + TDZ (0. 44 μ*M*), the basal callus induction at the transition zone of the root and hypocotyls was obtained. Subsequently, differentiation of shoots was noticed after 4-5 wk of culture. However, addition of BAP (2.22 μ*M*) to MMS/MS+TDZ (0.44 μ*M*) could not make any difference in the response of the

explants from the earlier observations made on TDZ (0.44 μ*M*) alone supplemented media. Though, BAP enhanced the rate of callus formation and subsequent shoot bud differentiation with TDZ in MS/MMS media along with multiple shoot induction. In addition, presence of BAP in L2/MS/MMS media fortified with TDZ improved the quality of shoots in terms of leaf morphology and enhanced the growth of the seedlings (Fig. 1d).

L-glutamine (0.03 m*M*) was added to MMS/MS/L2+TDZ (0.44 μ*M*)+BAP (2.22 μ*M*), since L-glutamine is a potent amino acid used extensively in several systems to improve the morphogenetic potential of the cultures[14]. Presence of L-glutamine further improved the morphology of the shoots as well as the per cent induction of multiple shoots from the callus. The shoots, thus, developed were dark green in colour and were also elongated compared to the shoots raised on only TDZ supplemented medium.

In both the previous reports on adventitious shoot proliferation in *N. foetida*[6,7], MS medium was used to raise the cultures along with TDZ. In the present studies, three different media along with TDZ either alone or with BAP and L-glutamine were used. L2 medium contains less ammonium salts but increased levels of Ca and Mg as compared to MS medium. But the organic composition of the MS includes nicotinic acid, which is absent in L2 medium. Presence of nicotinic acid in the medium was reported to have a promotory effect on the callus formation[10]. Hence, TDZ alone in L2 medium could only promote the adventitious shoot formation but not the callus formation, whereas it stimulated the formation of callus and subsequent shoot differentiation in MS medium. Dual effect of TDZ involving proliferation of shoots and differentiation of shoot buds from the callus was observed when it was supplemented to MMS. However, along with BAP, which is known for its ability to stimulate cell division, TDZ favoured the callus formation even in L2 medium. Thus, MS/MMS/L2 media supplemented with TDZ+BAP+L-glutamine were found to be best for direct regeneration in the beginning and indirect regeneration through the callus formation in the later period of the cultures, indicating the dual nature of the TDZ depending on the media composition and other supplements (Table 1). Singh *et al*[5] has also observed dual nature of the TDZ in the differentiation of somatic embryos in the intact seedlings of pigeon pea. TDZ exhibited a cytokinin like activity that promoted cell division and differentiation and an auxin like activity that was crucial for induction of embryogenic competence. However, Mundhara and Rashid[15] attributed triple role to TDZ in the cultures of *Linum*, where it was involved in the inhibition of root elongation, shortening and swelling of hypocotyls and tightening of cotyledons towards apex. The morphogenetic effect of TDZ is highly debatable. Though it is not having a purine ring common to adenine, it stimulates the synthesis of endogenous cytokinins or inhibits their degradation leading to accumulation[16]. Further, Capelle *et al*[17] are of the opinion that TDZ promotes the conversion of cytokinin ribonucleotides to the biologically more active ribonucleosides. However, further studies are needed to understand the role of TDZ.

The generated shoots from the above cultures were excised and inoculated onto MMS+IBA (2.46 μ*M*) for rooting. Initiation of roots was started at the basal part of the shoots after 4-5 wk of culture on rooting medium (Fig. 1e). However, Thengane *et al*[6] used ½ and ¼ strength MS medium for better induction of roots from the basal parts of the adventitious shoots. Both IAA and IBA in ¼ and full MS were both used to induce the roots from the adventitious shoots obtained through hypocotyl cultures of *N. foetida*[7]. The complete plantlets were then transferred to small plastic cups containing soilrite and fed with sterile distilled water. They were covered with perforated polythene bags for 4-5 wk before transferring them into pots containing soil:sand:manure in 1:1:1 ratio. Nearly 40% of survival was recorded (Fig. 1f). Thus, the regeneration from the callus cultures and subsequent successful transfer to the pots with significant survival percentage of regenerated plantlets was achieved and reported for the first time in this taxon. Accumulation of secondary metabolites is found to be more in specific differentiating tissues rather than undifferentiated callus tissues[18]. Hence, the present study not only helps in the conservation of the taxon by micropropagation but also paves the way for studies on extraction of CPT from the differentiating cultures of four populations growing at various geographical regions and to identify elite populations.

Acknowledgement

Authors sincerely acknowledge the Ministry of Environment and Forests, Government of India, New Delhi, for funding this project.

References

1 Padmanabha B V, Chandrashekar M, Ramesha B T, Hombe Gowda H C, Gunaga R P *et al*, Patterns of accumulation of camptothecin, an anti-cancer alkaloid in *Nothapodytes nimmoniana* Graham, in the Western Ghats, India: Implications for identifying high-yielding sources of alkaloid, *Curr Sci*, 90 (2006) 95-100.

2 Suhas S, Ramesha B T, Ravikanth G, Gunaga R P, Vasudeva R *et al*, Chemical profiling of *Nothapodytes nimmoniana* populations in the Western Ghats, India for anti cancer compound, camptothecin, *Curr Sci,* 92 (2007) 1142-1147.

3 Panneerselvam K, Bhavanisankar K, Jayapragasam M, Kumar A, Rathakrishnan P *et al*, Effect of growth regulators and planting media on rooting of cuttings in *Nothopododytes nimmoniana* Mabberly, *Indian J Plant Physiol*, 9 (2004) 308-312.

4 Lu C, The use of thidiazuron in tissue culture, *In Vitro Cell Dev Biol-Plant*, 29 (1993) 92-96.

5 Singh N D, Sahoo L, Sarin N B & Jaiwal P K, The effect of TDZ on organogenesis and somatic embryogenesis in pigeonpea [*Cajanus cajan* (L.) Millsp], *Plant Sci*, 164 (2003) 341-347.

6 Thengane S R, Kulkarni D K, Shrikhande V A & Krishnamurthy K V, Effect of thidiazuron on adventitious shoot regeneration from seedling explants of *Nothapodytes foetida, In Vitro Cell Dev Biol-Plant*, 37 (2001) 206-210.

7 Rai V R, Rapid clonal propagation of *Nothapodytes foetida* (Wight) Sleumer—A threatened medicinal tree, *In Vitro Cell Dev Biol-Plant,* 38 (2002) 347-351.

8 Anuradha M, Rajani R, Indu B K & Divakar K M, Production of anticancer compounds from cultures of *Nothapodytes foetida* of selected geographical origin, *Phytomorphology*, 54 (2004) 185-191.

9 Riley M R, Gibberellic acid for fruit set and seed germination, *CRFG J*, 19 (1987) 10-12.

10 Murashige T & Skoog F, A revised medium for rapid growth and bioassays with tobacco tissue cultures, *Physiol Plant*, 15 (1962) 476-497.

11 Phillips G C & Collins G B, *In vitro* tissue culture of selected legumes and plant regeneration from callus cultures of red clover, *Crop Sci*,19 (1979) 59-64.

12 Snedecor G W & Cochron W G, *Statistical methods,* 8th edn (Affiliated East-West Press, New Delhi, India & Iowa State University Press, Iowa, USA) 1994.

13 Briggs B A, McCulloch S M & Edick L A, Micropropagation of azaleas using thidiazuron, *Acta Hortic*, 226 (1988) 205-208.

14 George E F & Sherrington P D, *Plant propagation by tissue culture—Handbook and directory of commercial laboratories*, (Exegetics Ltd., Basingstoke, England) 1984, pp 709.

15 Mundhara R & Rashid A, TDZ-induced triple-response and shoot formation on intact seedlings of *Linum*, putative role of ethylene in regeneration, *Plant Sci*, 170 (2006) 185-190.

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

17 Capelle S C, Mok D W S, Kirchner S C & Mok M C, Effect 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., *Plant Physiol*, 73 (1983) 796-802.

18 Butcher D N, Secondary products in tissue cultures, in *Plant cell, tissue and organ culture*, edited by J Reinert & Y P S Bajaj (Narosa Publishing House, New Delhi) 1977, 668-693.