INTERNATIONAL JOURNAL OF PLANT, ANIMAL AND ENVIRONMENTAL SCIENCES
IJPAES
Volume-3, Issue-1, Jan-Mar-2013 ISSN 2231-4490
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Received: 17th Jan-2013 *Revised: 21st Jan-2013* *Accepted: 27th Jan -2013*

DIRECT SOMATIC EMBRYOGENESIS AND PLANT REGENERATION FROM LEAF AND STEM EXPLANTS OF *NOTHAPODYTES FOETIDA*: A CRITICALLY ENDANGERED PLANT SPECIES

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ABSTRACT: An efficient protocol for the regeneration of 'critically endangered' *Nothapodytes foetida* plants via direct somatic embryogenesis was developed. Somatic embryos were induced from leaf and stem explants cultured on Murashige and Skoog medium supplemented with 0.5-3.0 mg l^{-1} thidiazuron and 20% coconut water. Histological examination revealed that somatic embryos were induced directly from the explants. Maturation of somatic embryos was accomplished with same induction medium. Germination and regeneration efficiency of somatic embryos was found to be 90.3±3.20. The regenerated plants were morphologically similar to seed-derived plants. This system may prove to be beneficial for ex-situ conservation as well as for amenability of this endangered plant for genetic manipulation. This is the first report of direct somatic embryogenesis in *Nothapodytes foetida* with significantly high plantlet regeneration frequency.

Key words: Coconut water, direct somatic embryogenesis, *Nothapodytes foetida*, plant regeneration; thidiazuron

INTRODUCTION

Plant regeneration through somatic embryogenesis is one of the main prerequisites for the potential applications of clonal propagation, genetic transformation and in vitro conservation of germplasm of woody plants [1, 2, 3]. *Nothapodytes foetida* Sleumer belongs to family *Icacinaceae*. It is a small tree, naturally distributed in many parts of the Western Ghats, South India, Assam, the Himalayan foothills, Sri Lanka, Myanmar and Thailand. *N. foetida* is a potential source of campothecine (CPT) (about 0.3% on a dry weight basis) and its analogs [4]. Camptothecin (CPT), an isoquinoline alkaloid, is one of the most promising anti-cancer drugs of the twenty-first century [5, 6]. CPT is currently being used for treating colorectal and ovarian cancer [7, 8]. The projected global demand for CPT in 2002 was valued at US$ 4045 million [9]. Apart from the absence of synthetic sources and lack of commercially viable method of synthesis, the low yield of intact plant, poor seed germination and its high market price have prompted interest to look for alternative method(s) for production of CPT and its analogs from *N. foetida.* To serve global demand, this alkaloid is extracted from naturally existing populations of *N. foetida* from the Western Ghats, India. Consequently, in the last decade alone, over 20% of the population of the species has been lost from the Western Ghats [10, 11]. In fact due to the extremely high pressure, the species has been declared as endangered [12]. In recent years, several independent groups have addressed the need to conserve the species and to explore the possibilities of identifying high-yielding individuals or populations for the development of *in vitro* production systems [13, 14]. Although tissue culture methods for *N. foetida* propagation were not established, Fulzele et al. [15] developed a regeneration method through indirect somatic embryogenesis. Propagation through direct somatic embryogenesis, however, may have advantages over indirect somatic embryogenesis, since it is amenable to scaling-up in bioreactors and to production of synthetic seeds [16]. Additionally, direct somatic embryogenesis has a lower probability of genetic variation than other propagation methods [17]. Somatic embryogenesis has several other advantages, including the efficiency of process (i.e. the formation of plantlets in fewer steps, with a concomitant reduction in labour, time and cost) and the morphological and cytological uniformity of the plantlets [18]. To date, regeneration of *N. foetida* through direct somatic embryogenesis has not been reported.

N. foetida can be propagated from seeds but the seeds remain dormant for a long time after sowing, germinate poorly, and early seedling growth is also slower as compared to plants derived from tissue culture [18]. Therefore, efficient vegetative propagation is essential for conservation of this plant and has many advantages over seedling production. The plant regeneration with indirect somatic embryogenesis, adventitious shoots from immature zygotic embryos and seedling explant sources of *N. foetida* have been reported previously [15, 20]. The objective of this study was to develop a protocol for induction of direct somatic embryogenesis for conservation and for deriving plant material for *in vitro* production systems. To the best of our knowledge, this is the first report of direct somatic embryogenesis with significantly high plantlet regeneration frequency.

MATERIALS AND METHODS

Collection of plant material and establishment of aseptic cultures

Nursery grown plants of *N. foetida* were collected in the month of June 2010, from Jejuri, Pune, Maharashtra, India and identified with the help of standard flora of the area. Collected plants were maintained in shade-net and young leaves and stem were used as explants. Leaf and stem segments from *N. foetida* were cleaned thoroughly by gently brushing under running tap water for 10 min and washing in liquid detergent (1–2 drops of Tween 10 in distilled water) followed by three rinses with sterile distilled water. Surface disinfection was carried out by submerging and shaking the explants in 70% (v/v) ethanol for 30sec and washing twice with sterile distilled water. The cleaned explants were then surface sterilized in 0.1% mercuric chloride with constant shaking for 3 min and rinsed three times with sterile distilled water under aseptic conditions. After surface sterilization, leaves and stem were cut into small pieces (approximately 1-2 cm) and the explants were inoculated in glass culture bottles (22mm x 150mm) containing 30 ml liquid MS (Murashige and Skoog 1962) medium [21]. MS medium was fortified with 3% sucrose, 15% CW and various concentrations of TDZ (Table 1). The pH of the culture medium was adjusted to 5.8 ± 0.2 before autoclaving at 120ºC and at 15 lb pressure. Filter paper Bridge was used for supporting, instead of agar. Culture bottles were maintained at 25 ± 2 ºC, 16/8 hours photo-period using cool white florescent light.

Induction and development of somatic embryos

Leaf and stem segments were excised from the shade net grown plants, surface sterilized and inoculated on MS medium fortified with different concentrations of TDZ with 20% coconut water (Table 1). MS basal medium was used as a control. Culture bottles were kept at 25 ± 2ºC, 16/8 hours photoperiod using cool white florescent light. Subculturing was done after every 6 wk. Observations were recorded regularly after 1 wk interval. Each experiment was replicated at least three times (n = 24).

Maturation and germination of somatic embryos

Leaf and stem induced direct somatic embryos were multiplied and maintained on same medium in sealed glass vessel and were subcultured monthly. Somatic embryos were transferred to MS basal medium without PGR for germination and cultures were incubated at 16 h photoperiod. Plant regeneration rate was calculated from number of plantlets obtained from three replicates of each treatment.

Histological analysis

Somatic embryos were collected and fixed by immersion in formalin:acetic acid:ethanol:water solution (FAA, 1:1:9:9 v/v) at room temperature for 48 h and stored in 70% ethanol at 4°C. After dehydration through an ethanol series (30, 50, 70, 85, 95, and 100% ethanol for 2 h each), the samples were embedded in paraffin wax (58-60°C). Eight µm thick sections were made from median longitudinal or cross-sectional axes of hypocotyls using a microtome (HM 340 E; Microm International GmbH, Walldorf, Germany) and fixed on glass slides. The sections were de-waxed in xylene for 5–10 min and observed under light microscope (Docuval, Carl Zeiss, Germany).

Plant regeneration and transplantation

Germinated embryos were subcultured on the same medium containing 3.0% sucrose, 20% coconut water supplemented with (0.5-3.0) mg l^{-1} TDZ with 20% CW. Germinated somatic embryos were cultured in glass bottle containing 30 ml liquid medium with filer paper bridge. Each experimental unit had twenty four explants with three replicates. Percentage of plantlet regeneration was calculated after 6 weeks of culture. Cultures were maintained at 24°C, with 16 h photoperiod.

Rooting of regenerated plantlets

Germinated embryos with shoots were transferred to full strength MS agar medium containing IBA, NAA, IAA, 3.0% sucrose and 20% CW for further growth. The survival rate was recorded for 6 weeks after transplantation.

Statistical analysis

The percentage of embryo induction and germination from stem and leaf explants was calculated and the data was subjected to Tukey-Kramer multiple comparisons test to analyze the level of significance in variation between the response of explants with reference to various concentrations of TDZ.

RESULTS

Induction of direct somatic embryos

The cultures reflected morphological variations four weeks after culturing. The leaf explants showed swelling and expansion and a curvature indicating cellular growth while stem just showed swelling. The induction of embryos was visible within 9-10 weeks. Somatic embryos were induced directly at the midrib, at cut edges of explants, or on the leaf surface and embryos were green, small, and globular, arising individually or in group (Fig. 1). MS medium fortified with 0.5 mg 1^{-1}TDZ showed faster response to induce direct somatic embryos as compared to other concentrations. The induction frequency of embryos was significantly high at lower concentration of the TDZ The frequency of direct somatic embryo induction was highest in the leaf explants (23.3±1.52) as compared to stem explants (11.5±0.83) on 0.5 mg 1^{-1} TDZ with 20% coconut water (Table 1). These somatic embryos formed either individually or in groups, directly on the explants without callus formation. Most of the globular embryos gradually developed as bipolar structures resembling heart-, torpedo-, and cotyledon-stage embryos (fig.2).Histological examination confirmed the induction of somatic embryos directly from epidermal and subepidermal cells of the cultured leaf and stem, especially on media containing TDZ (fig.3).

Table 1: Effect of different concentration TDZ with coconut water on induction of embryos from leaf and stem explants of *Nothapodytes foetida*

Plant growth regulator (PGR) (mg 1^{-1})	Percentage of Embryo induction (%)		Percentage of germination (%)	
TDZ 20% cocnut water	Leaf	Stem	Leaf	Stem
0.5	23.3±1.52^{a}	11.5±0.83^{a}	90.3±3.20^{a}	81.9±2.32^{a}
1.0	13.0±0.99^{b}	8.8±0.72^{a,b}	84.7±2.32^{a,b}	79.1±1.52^{a}
1.5	11.0±0.76^{b,c}	8.4±0.61^{b,c}	83.3±1.53^{a,b}	76.4±0.86^{a,b}
2.0	9.1±0.65^{b,d}	6.3±0.52^{b,c,d}	91.6±3.05^{a}	77.7±0.86^{a}
2.5	7.8±0.74^{c,d,e}	6.2±0.51^{b,c,d,e}	84.7±0.89^{a,b}	73.6±3.82^{a,b}
3.0	6.3±0.52^{d,e,f}	4.9±0.41^{d,e,f}	75.0±3.05^{b}	68.0±2.33^{b}

Values are represented as mean ± standard error (SE) of three independent replications. Figures followed by different superscript letters in a column are statistically different (P≤0.01) from each other (Tukey Kramer Multiple Comparisons Test)

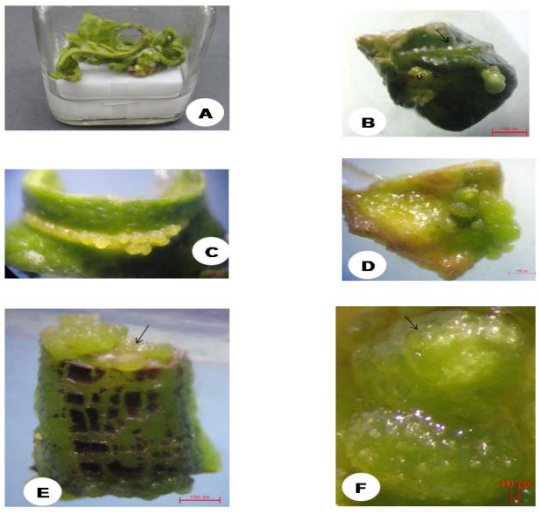

Figure 1

A- Expanded leaf explants on MS medium supplemented with 5.0 mg 1^{-1} TDZ
B- Stereo microscopic picture of embryo induction from leaf explants
C- D induction of direct somatic embryos from midrib and cut edges of leaf explants on MS medium supplemented with 5.0 mg 1^{-1} TDZ
E- Direct somatic embryos induction from stem explants on MS medium supplemented with 5.0 mg 1^{-1} TDZ
F- Matured embryo with induction of leaf primordia

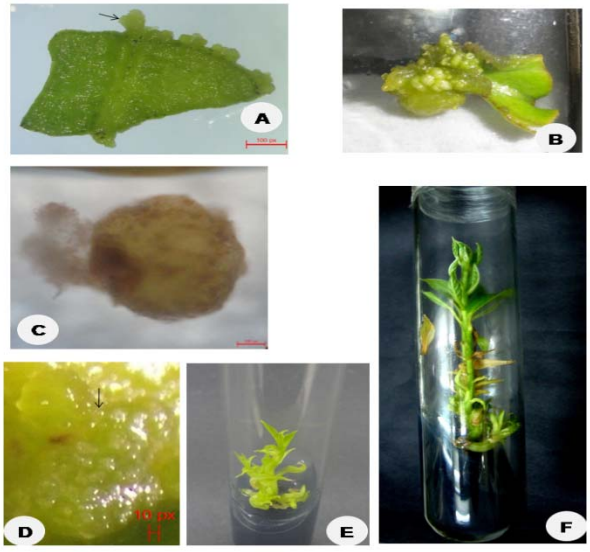

Figure 2

A- Cotyledonary stages of somatic embryos induced on leaf explants, scale bar 100 µm
B- Germinated embryos with prominent shoots
C- Germination embryo, scale bar 100 µm
D- Torpedo stage of embryo
E and F- Regenerated plantlet

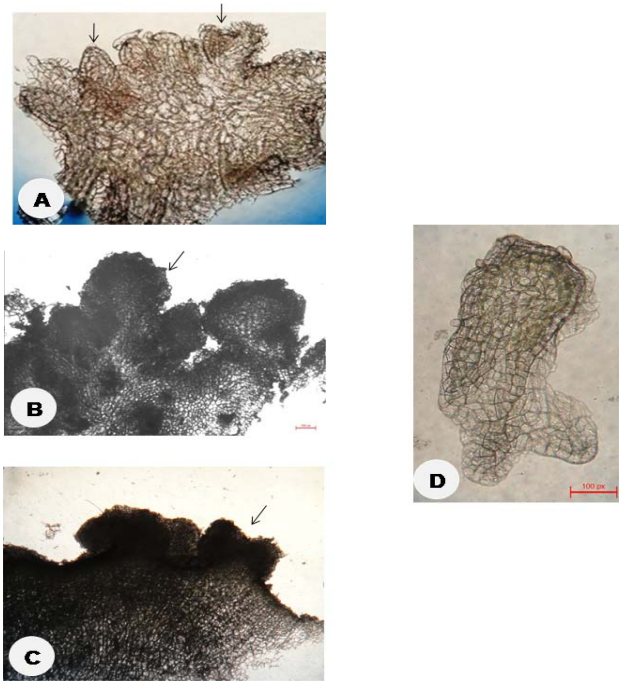

Figure 3

A- Section of leaf, somatic embryos at the early globular, early heart stage development, scale bar 100 µm
B and C- Section of a stem explant with globular, heart embryo-like structures, scale bar 100 µm
D- Torpedo stage of somatic embryos from leaf origin, scale bar 100 µm

Germination of embryos

After 9-10 wks, the cultures exhibited near-confluent growth, which turned green with patches of developing embryos (Fig.1). Embryo germination occurred within 3 wks of transfer of the embryos onto fresh medium with the same composition. Maximum regeneration frequency (90.3±3.20) of the somatic embryos was noticed in embryos generated from leaf explants on MS medium containing 0.5 mg 1^{-1} TDZ supplemented with 3% sucrose and 20% coconut water. Somatic embryos produced only shoots and the studies to induce rooting in these shoots are still in progress.

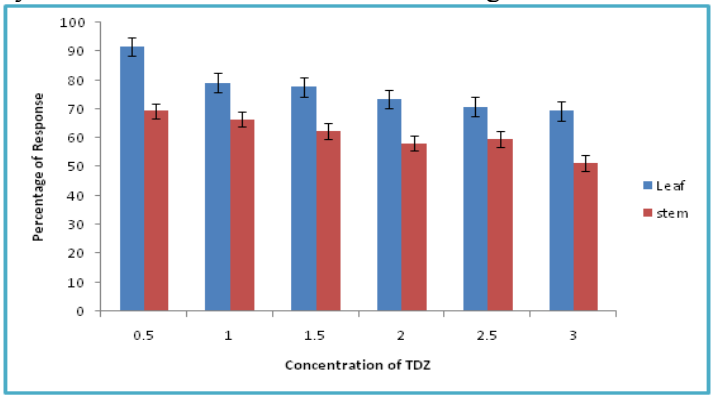

Figure 4: Percentage of response of explants to various concentrations of TDZ

DISCUSSION

The objectives of this study were to develop methods for induction of direct somatic embryos. To the best of our knowledge, this is the first report of direct somatic embryogenesis and subsequent plant regeneration of *N. foetida*. Somatic embryos formed directly on mid rib or at cut edges or on the surfaces of leaf sections, around cut ends of stem explants. Somatic embryos germinated and developed on the induction medium.

Embryogenesis was slower and asynchronous in leaf explants. Small, round, individual somatic embryos appeared 9-10 wks after culture. The formation of somatic embryos around stem segments was slower and more asynchronous; embryos matured and germinated after 3 wks, resulting in plantlets in 3–4 months. The induction of somatic embryos directly from mature tree tissues or at least from non-seed tissues such as leaf parts or shoot segments is an important objective in forestry practices given that the induction of somatic embryos appears to be associated with the cytological and genetic stability of the regenerated plantlets [22]. Propagation through somatic embryogenesis, however, may have advantages over organogenesis. Direct somatic embryogenesis can be potentially scaled-up using bioreactors and for production of synthetic seeds [16]. Additionally, direct somatic embryogenesis has a lower probability of genetic variation than other propagation methods [17]. To date, regeneration from direct somatic embryogenesis has not been reported in *N. foetida*. Somatic embryogenesis is a powerful tool for the improvement of forest trees and is considered to be the in vitro plant regeneration system of choice in woody plants [23]. Direct somatic embryogenesis has been reported for numerous species of forest trees [24, 25]. Most effective use of TDZ has been in regeneration of woody plant species [26, 27, 28] and TDZ alone is found to be a substitute for both auxin and cytokinin requirement of somatic embryogenesis in many species [29, 30, 31]. Low concentrations of TDZ have been reported to be more efficient in inducing organogenesis or somatic embryogenesis than other cytokinins, particularly in recalcitrant woody species [32]. Results in the present studies are in accordance with this observation and efficient somatic embryogenesis was achieved with low concentration of TDZ. In the present study, direct somatic embryos formed on MS medium with different concentrations of TDZ. Maximum induction of somatic embryo was observed at lowest concentration of TDZ. Lower concentrations of TDZ produced numerous somatic embryos but their germination was satisfactory following 12 wks of culture on the same medium. TDZ has been shown to promote differentiation of organized centers of growth in cultured tissues at much lower concentrations, with efficient somatic embryogenesis than that of other cytokinins (Data not shown here).

All stages of somatic embryos, from globular through cotyledonary embryos, developed on the surface of leaf and stem explants. In general, leaf explants were found to produce significantly more embryos than stem explants (Table 1, fig. 4). Medium containing BAP and kinetin were not suitable for embryo induction or were unable to stimulate an embryogenic response.

Although plant regeneration with direct and indirect organogenesis from various explant sources of *N. foetida* species have already been reported [20, 33], to date there is only one report on indirect somatic embryo formation from *N. foetida* [15].

In conclusion, it takes about 3–4 months of culture to obtain plantlets of *N. foetida* via direct somatic embryogenesis using TDZ. Direct somatic embryogenesis avoids the passage through callus and thus avoids the genetic instability often associated with somatic embryos obtained indirectly from callus. This standardized protocol can be used for regeneration of *N. foetida* through direct somatic embryogenesis for various purposed including ex-situ conservation, regeneration and for raising plant material for genetic manipulation.

ACKNOWLEDGEMENTS

Suresh P. Khadke is thankful to Prof. Ranjekar, Director, IRSHA and Prof. Kothari, Ex Principal, RGIT and BT for encouragement and support.

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