

**Research Article**

## **REGENERATION OF SHOOTS FROM LEAF CALLUS CULTURES OF DRYMARIA CORDATA (L) WILLD EX ROEM AND SCHULT**

**\*D. H. Tejavathi<sup>1</sup> and M. N. Indira<sup>2</sup>**

<sup>1</sup>Department of Botany, Bangalore University, Bangalore-560056, India

<sup>2</sup>Department of Botany, CMR Institute of Management Studies, Bangalore -560043, India

\*Author for correspondence

### **ABSTRACT**

An efficient protocol for rapid regeneration of shoots from leaf callus cultures of *Drymaria cordata* was developed. Creamish white profuse callus proliferation was obtained on MS supplemented with NAA (2.69 $\mu$ M) + BAP (4.44 $\mu$ M) after three weeks of culture. The callus became green, compact and nodular on transfer to TDZ (0.91 $\mu$ M) supplemented medium. Shoot buds were initiated from the callus after 8 weeks of culture on the solid medium. The frequency of initiation of shoot buds could be enhanced if the green nodulated callus was transferred to filter paper placed on liquid MS medium supplemented with TDZ (2.27 – 4.54 $\mu$ M). The shoots have developed roots on IBA (0.49 $\mu$ M) supplemented MS solid medium. Thus obtained plantlets were successfully hardened and transferred to soil. Nearly 90% survival rate was recorded.

**Key Words:** Callus cultures, Indirect Organogenesis, Liquid medium, *Drymaria Cordata*

### **INTRODUCTION**

*Drymaria cordata* (L.) Willd. Ex. Roem and Schult, a member of Caryophyllaceae, has been traditionally used as one of the ingredient in many indigenous poly herbal formulations. The plant is being used in the treatment of a various ailments (Rao, 1981; Focho *et al.*, 2009 and Borah *et al.*, 2006). Its antitussive activity (Mukherjee *et al.*, 1997a), antibacterial efficacy (Mukherjee *et al.*, 1997b) and anti-inflammatory effects (Mukherjee *et al.*, 1998) make it a potential medicinal herb. A number of biologically active compounds have been isolated from the leaves of this taxon including drymaritin which exhibits anti HIV properties (Ding *et al.*, 1999; Ding *et al.*, 2000 and Hsieh *et al.*, 2004).

The plant is vegetatively propagated mainly by cuttings since it is a runner by its habit. Hence, the occurrence of genetic variability in the progenies is negligible. Further seed germination potential and percent viability are also very low (Ghimire *et al.*, 2010). Significance of somaclonal variations in the genetic improvement of the economically important plants is now well established (Larkin and Scowcroft, 1982). Micropropagation technique offers an alternative method for cloning plants (Unander, 1991 and Santos *et al.*, 1994). Somaclonal variant selection in *in vitro* culture is often achieved through indirect regenerations through callus cultures (Sen and Sharma, 1991). Since *Drymaria cordata* is a potential herb having anti-HIV properties, a clone with high content of the active principle has tremendous prospects in the future. An efficient regeneration protocol from the callus cultures is a prerequisite to employ any biotechnological approaches for genetic improvement of the taxa. Perusal of literature revealed that direct regeneration from leaf (Ghimire *et al.*, 2010) and nodal explants (Tejavathi and Indira, 2011) has been achieved in this taxon. Hence the present study is an attempt to develop an effective and reproducible protocol for mass multiplication through leaf callus cultures and successful establishment of plantlets in natural conditions.

### **MATERIALS AND METHODS**

#### **Explant Source**

Young, fully expanded leaves (0.77-0.96 cm<sup>2</sup>) from *in vitro* shoots raised on MS basal media were used as explant source. The leaves were wounded by few cuts and cultured abaxial side on the medium.

## Research Article

### Culture Media and Conditions

Explants were inoculated onto Murashige and Skoog's medium (Murashige and Skoog, 1962) supplemented with various auxins and cytokinins at different concentrations and combinations. Sucrose (3%) and Bacteriological Agar (0.8%) were used as carbon source and gelling agent respectively. The pH of the medium was adjusted to 5.8 prior to the addition of agar and autoclaved for 15 min at 108 kpa. The cultures were incubated at  $25 \pm 2^\circ\text{C}$  under 16:8h light and dark photoperiod. The cultures were illuminated by white fluorescent tubes at light intensity of  $25\mu\text{mol m}^{-2}\text{s}^{-1}$ . The cultures were periodically evaluated for the record of data.

### Filter Paper Culture Method

Whatman (No.1) filter paper segments measuring about  $20\text{cm}^2$  were placed on MS liquid medium supplemented with TDZ at various concentrations. The culture bottles were then autoclaved for 15 min at 108 kpa. All other conditions remained same as explained under culture media and conditions.

### Rooting and Acclimatization of Plantlets

The shoots from the various cultures were transferred to MS solid medium supplemented with IBA at different concentrations. The rooted plantlets were washed in sterile distilled water carefully and transferred to plastic cups containing sterilized mixture of cocopeat, soilrite and perlite in the ratio of 1:1:1 for hardening. The plantlets were nourished with half strength MS liquid medium. The hardened plants were transferred to pots containing garden soil and maintained in the green house.

### Histological Studies

The organogenic callus segments were fixed in FAA (Formalin:Acetic acid:Ethyl Alcohol). Paraffin embedded plant materials were sectioned on a microtome at  $20\text{-}23\mu\text{m}$  thickness and stained with Haidenhain's haematoxylin and counter stained with eosin. Photomicrographs were taken with Canon camera using a Nikon binocular microscope.

## RESULTS AND DISCUSSION

Callus induction was observed within 15 to 20 days of incubation of leaf explants on MS medium containing different concentrations and combinations of 2,4-D/ NAA and BAP. The callus thus obtained was soft and creamish white in colour (Fig.1a). BAP ( $4.44\mu\text{M}$ ) in combination with 2, 4-D ( $4.52\mu\text{M}$ ) / NAA ( $2.69\mu\text{M}$ ) promoted callus from the explants. Auxin and cytokinin combinations act synergistically to promote cell division and expansion depending upon other factors in the cell system (Setterfield, 1963). Though profuse callus was obtained on 2, 4-D and BAP combinations in the initial phase of the culture, it gradually turned brown and necrotic as reported by Banerjee *et al.*, (2011) in *Arachis hypogea*. However callus remained healthy on BAP ( $4.44\mu\text{M}$ ) in combination with NAA ( $2.69\mu\text{M}$ ) supplemented medium. Similar combinations were found to be suitable for induction of callus from the culture of *Acmella calva* (Senthilkumar *et al.*, 2007) and *Indigofera enneaphylla* (Sindhu *et al.*, 2011). No organogenesis was recorded on this combination even after maintaining the callus for six months. Whereas Ghimire *et al.*, (2010) reported adventitious shoot bud regeneration from the leaf segments of *Drymaria cordata* on MS supplemented with NAA in combination with BAP or TDZ.

In the present studies, However BAP in combination with NAA promoted only callus formation. When this creamish callus was transferred to TDZ supplemented medium, compact green and nodular callus proliferated from the source callus. TDZ at the combination of  $0.91\mu\text{M}$  was sufficient to induce rapid proliferation of green nodular callus after four weeks of culture. Shoot buds ( $30.18 \pm 1.05$ ) have taken 3-4 months to emerge from this green nodular callus on this medium (Table 1, Figure 1b).

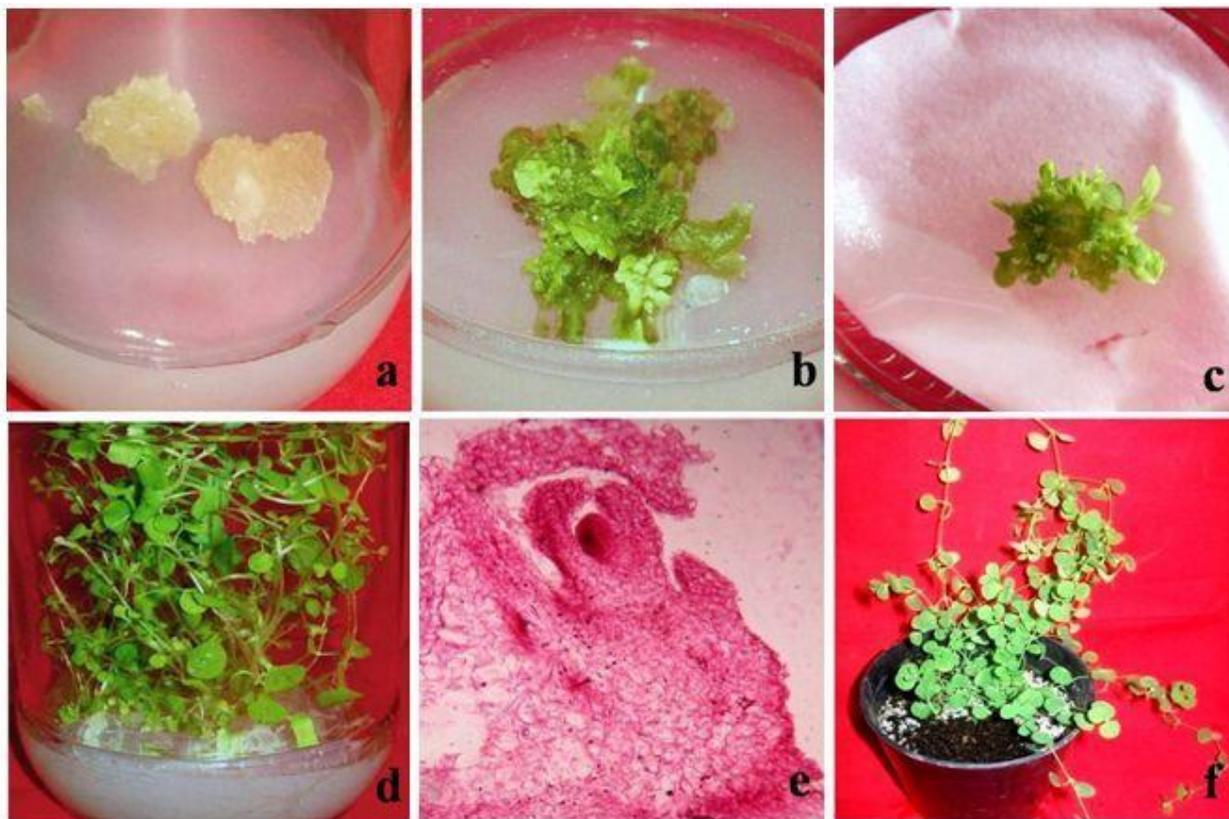
The frequency of initiation of shoot buds could be enhanced if this compact nodulated callus was transferred to filter paper placed on the liquid MS medium supplemented with TDZ at higher concentrations ( $2.27\text{-}4.54\mu\text{M}$ ). Filter paper method proved to be faster method to induce regeneration of shoot buds within 25 days of culture (Table 1, Figure 1c). Other cytokinins like Kin or 2-ip either alone or in combinations failed to induce shoot buds from the callus on either solid or liquid medium. Adventitious shoot regeneration from the leaves on TDZ supplemented medium has been reported in *Prunus avium*

**Research Article**

(Bhagwat and David, 2004) and *Prunus serotina* (Xiaomei *et al.*, 2008). TDZ is the most potent of the diphenyl ureas that have been evaluated for use in plant tissue culture (Mok *et al.*, 1987). Lower concentrations can induce greater axillary proliferation than many other purine based cytokinins. However higher concentrations of TDZ can stimulate callus induction as reported in many woody species (Huetteman and Preece, 1993). Dual effect of TDZ involving proliferation of shoots from the apical meristem and differentiation of shoots from the callus has been reported in *Nothapodytes foetida* (Tejavathi *et al.*, 2012) and Pigeon Pea (Singh *et al.*, 2003). Further studies are needed to understand the role of TDZ on the organogenesis.

**Table 1: Effect of TDZ on shoot proliferation from leaf derieved callus**

Medium	MS + TDZ (µM)	% Response	No of shoots/ culture (mean± SD)
Solid medium	0.45	63.33	23.36 ± 0.78
	0.91	76.67	30.18 ± 1.05
	1.82	73.33	17.56 ± 0.97
	4.54	43.33	7.77 ± 0.29
Liquid medium	2.27	50.00	18.26 ± 0.82
	4.54	83.33	<b>30.06 ± 0.50</b>
	6.81	63.33	17.35 ± 0.46



**Figure 1: A-** Creamish callus on 4.44 µM BAP + 2.69 µM NAA. **B-** Regeneration on 0.91 µM TDZ. **C-** Regeneration on filter paper 4.54 µM TDZ. **D-** Rooting on 0.49µM IBA. **E-** Section of the organogenic callus showing indirect organogenesis. **F-**Hardened plant

### Research Article

4 – 6 weeks old shoots were excised from the cultures and transferred to MS supplemented with IBA or IAA at various concentrations. IBA at 0.49 $\mu$ M was proved to be efficient in producing healthy and long roots from the nodal regions of the shoots (Figure 1d). Similar observations were made by Tejavathi and Indira (2011) in the same taxon when the proliferated shoots from the nodal cultures were transferred to rooting medium. The efficiency of IBA in inducing roots from the *in vitro* cultured shoots has been reported in several plants (Yeh-Jin *et al.*, 2007; Arockiasamy *et al.*, 2002; Muthu and Narayanaswamy Pillai, 2003; Sharma *et al.*, 2010 and Logesh *et al.*, 2011).

The well rooted plantlets were washed thoroughly in sterile distilled water and transferred to plastic cups containing sterilized cocopeat, soilrite and perlite (1:1:1 ratio) (Figure 1f). The plantlets were fed with liquid ½ MS medium for three weeks followed by distilled water. Six weeks old plantlets were transferred to pots containing garden soil and maintained in the green house. Nearly 90% of survival was recorded.

In the present study, a protocol of high frequency indirect shoot regeneration is achieved through leaf callus cultures. The technique can be effectively used for rapid propagation as well as for conservation of the taxon. Callus raised plantlets can be further exploited for somaclonal variations.

### REFERENCES

- Arockiasamy DI, Muthukumar B, Natarajan E and Britto SJ (2002).** Plant regeneration from node and internode explants of *Solanum trilobatum* L. *Plant Tissue Culture* **12**(2) 93-97.
- Banerjee P, Maity S and Banerjee N (2011).** *In vitro* propagation of *Arachis hypogaea* L. Var. AK 1224: Direct and Indirect plant regeneration. *Plant Cell Biotechnology and Molecular Biology* **12**(1-4) 57-62.
- Bhagwat B and David LW (2004).** *In vitro* shoot regeneration from leaves of sweet cherry (*Prunus avium*) ‘Lapins’ and ‘Sweetheart’. *Plant Cell, Tissue and Organ Culture* **78** 173-181.
- Borah PK, Gogoi P, Phukan AC and Mahanta J (2006).** Traditional medicine in the treatment of gastrointestinal diseases in Upper Assam. *Indian Journal of Traditional Knowledge* **5**(4) 510-512.
- Ding Z, Zhou J and Tan (1999).** A novel flavonoid glycoside from *Drymaria diandra*. *Planta Medica* **65** 578-579.
- Ding Z, Zhou J, Tan N and Teng R (2000).** Two new cyclic peptides from *Drymaria diandra*. *Planta Medica* **66** 368-388.
- Focho DA, Ndam WT and Fonge BA (2009).** Medicinal plants of Aguambu- Bamumbu in the Lebiale highlands, Southwest province of Cameroon. *African Journal of Pharmacy and Pharmacology* **3**(1) 001-013.
- Ghimire BK, Seong ES, Goh EJ, Kim NY, Kang, WH, Kim EH, Yu CY and Chung IM (2010).** High frequency direct shoot regeneration from *Drymaria cordata* Willd. leaves. *Plant Cell Tissue Organ Culture*. **100**(2) 209-217.
- Hsieh PW, Chang FR, Lee KH, Hwang TL, Chang SM and Wu YC (2004).** A new anti-HIV alkaloid, Drymaritin, and a new C-glycoside flavonoid, diandraflavone, from *Drymaria diandra*. *Journal of Natural Products* **67** 1175-1177.
- Huetteman CA and Preece JE (1993).** Thidiazuron: a potent cytokinin for woody plant tissue culture. *Plant cell Tissue Organ Culture* **33** 115-119.
- Larkin PJ and Scowcroft WR (1982).** Somaclonal variation- a novel source of variability from cell cultures for plant improvement. *Theoretical and Applied Genetics* **60**(4) 197-214.
- Logesh P, Settu A, Thangavel and Ganapathi A (2011).** *In vitro* callus induction and regeneration studies in *Withania somnifera* L. (Dunal). *Plant Cell Biotechnology and Molecular Biology* **12**(1-4) 51-56.
- Mok MC, Mok DWS, Turner JE and Muser CV (1987).** Biological and biochemical effects of cytokinin- active phenylurea derivatives in tissue culture systems. *HortScience* **22** 1194-1197.

### Research Article

- Mukherjee PK, Saha K, Bhattacharya S, Giri, SN, Pal M and Saha BP (1997a).** Studies on Antitussive Activity of *Drymaria cordata* Willd. (Caryophyllaceae). *Journal of Ethnopharmacology* **56** 77-80.
- Mukherjee PK, Bhattacharya S, Saha K, Giri SN, Pal M and Saha BP (1997b).** Antibacterial evaluation of *Drymaria cordata* Willd. (Family - Caryophyllaceae) extract. *Phytotherapy Research John Wiley and Sons Ltd. U.K.* **11** 249-250.
- Mukherjee PK, Saha K, Bhattacharya S, Saha BP and Pal M (1998).** Studies on the anti-inflammatory effects of *Drymaria cordata* Willd. *Natural Product Sciences* **4**(2) 91-94.
- Murashige T and Skoog F (1962).** A revised medium for rapid growth and bioassays with tobacco cultures. *Physiological Plantarum* **15** 473-497.
- Muthu T and Narayanaswamy Pillai J (2003).** A protocol for efficient plantlet regeneration from leaf derived callus of Lablab Bean (*Lablab purpureus* var. *Lignosus* (L.) prain). *Pertanika Journal of Tropical and Agricultural Science* **26**(1) 35-40.
- Rao RR (1981).** Ethnobotany of Meghalaya: medicinal plants used by Khasi and Garo tribes. *Economic Botany.* **35** 4-9.
- Santos ARS, Filho VC, Niero R, Viana AM, Moreno FN, Campos MM, Yunes AR and Calixto JB (1994).** Analgesic effects of callus culture extracts from selected species of *Phyllanthus* in mice. *Journal of Pharmacy and Pharmacology* **46** 755-759.
- Sen J and Sharma AK (1991).** Micropropagation of *Withania somnifera* (L.) Dunal from germinating seeds and shoot tip. *Plant Cell Tissue organ Culture* **26** 71 -73.
- Senthilkumar P, Paulsamy S, Vijayakumar KK, and Kalimuthu K (2007).** *In vitro* regeneration of the medicinal herb of Nilgiri shola *Acmella calva* L. from leaf derived callus. *Plant Tissue Culture & Biotech* **17**(2) 109-114.
- Setterfield G (1963).** Growth regulation in excised slices of Jerusalem artichoke tuber tissues. *Symposia of the Society for Experimental Biology* **17** 98-126
- Sharma S, Kamal B, Rathi N, Chauhan S, Jadon V, Vats N, Gehlot A and Arya S (2010).** *In vitro* rapid and mass multiplication of highly valuable medicinal plant *Bacopa monnieri* (L.) Wettst. *African Journal of Biotechnology* **9**(49) 8318-8322.
- Sindhu KK and Mathew MM (2011).** *In vitro* culture studies on *Indigofera tinctoria* Linn. and *Indigofera enneaphylla* Linn. *Plant Cell Biotechnology and Molecular Biology* **12**(1-4) 31-40.
- Singh, Dolendro N, Sahoo Lingaraj, Sarin Bhalla N and Jaiwal Pawan K (2003).** The effect of TDZ on organogenesis and somatic embryogenesis in Pigeonpea (*Cajanus cajan* L. Millsp). *Plant Science* **164**(3) 341-347.
- Tejavathi DH and Indira MN (2011).** *In vitro* regeneration of multiple shoots from the Nodal explants of *Drymaria cordata* (L) Willd. Ex. Roem. and schult. *The Bioscan* **6**(4) 657-660.
- Tejavathi DH, Raveesha HR and Shobha K (2012).** Organogenesis from the cultures of *Nothapodytes foetida* (Wight) Sleumer raised on TDZ supplemented media. *Indian Journal of Biotechnology* **11** 205-209.
- Unander DW (1991).** Callus induction in *Phyllanthus* spp. and inhibition of viral enzymes by callus extracts. *Plant Cell Reports* **10** 461-466.
- Xiaomei L and Paula MP (2008).** Plant Regeneration from *in vitro* leaves of mature black cherry (*Prunus serotina*). *Plant Cell Tissue Organ Culture* **94** 113-123.
- Yeh-Jin A, Louisa V, Thomas A and Mc. Keon and Grace QC (2007).** High frequency plant regeneration through adventitious shoot formation in castor (*Ricinus communis* L.) *In vitro Cell Development Biology* **43** 9-15.