

Direct somatic Embryogenesis and plant Regeneration from Leaf Explants of *Nymphoides cristatum*: A Medicinally Important Plant

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Abstract: *In vitro* multiplication of *Nymphoides cristatum* through direct regeneration was attempted using leaf segments. Direct somatic embryos were produced in clusters directly from epidermal and mesophyll cells of leaf tips and wound surfaces without an intervening callus within one month when cultured on MS medium supplemented with dosage (0.0-3.0 mg l⁻¹) of TDZ and 2, 4-D (0.0-3.0 mg l⁻¹) Sub culturing of these embryo clusters produced more embryos and subsequently plantlet formation on the same medium. The high-frequency embryos of these leaf cells in this *Nymphoides cristatum* is strong evidence of their totipotency. The regenerants were more vigorous in their growth and useful for mass propagation, which can be exploited further for their medicinal use.

Key words: Somatic embryo • Leaf explant • *Nymphoides cristatum* • Medicinal plant

INTRODUCTION

Nymphoides cristatum (Roxb) a member of Gentianaceae is an aquatic medicinal herb [1]. The plant is used to cure fever and hepatoprotective [2]. Stalks and leaves are pounded with oil and applied to ulcers and insect bites; and a decoction is used as a wash for parasitic skin infections. Seeds are considered anthelmintic [3]. The urgent need for mass propagation of *Nymphoides cristatum* hence the over exploitation of this plant and for its medicinal properties. The indirect method of plant regeneration through floral bud of *Nymphoides cristatum* has been reported previously by [4]. However there are no reports of regeneration of the *Nymphoides cristatum* from the leaves, stem and roots. In this communication we report a method for the direct regeneration of *Nymphoides cristatum* from leaves.

In this investigation reported here, young leaf explants were used for initiating somatic embryos. The Somatic embryos which were initially observed after days cultures arose directly from the epidermal and mesophyll cells without the formation of a callus intermediate. Histological observation of the development of somatic embryos and subsequent PLBs from mesophyll cells and epidermal cells were made.

The present investigation has been undertaken to develop a protocol for *in vitro* culture of this medicinally important herb.

MATERIALS AND METHODS

Nymphoides cristatum was collected from Habbalur Lake, Shimoga, Karnataka, India and maintained in our herbal garden pond. Leaf explants were excised and kept in tap water for 30 minutes. These were first surface sterilized with bavistin for 15 minutes and washed thoroughly using distilled water under aseptic condition; explants were treated with 0.1% HgCl₂ for 3 minutes and then rinsed 5-6 times with sterile distilled water. These explants were trimmed evenly about 2-3 cm in size and inoculated on MS medium [5] supplemented with TDZ (0.0-1.0 mg l⁻¹) and 2,4-D (0.0 -3.0 mg l⁻¹) the medium contained 3% (w/v) sucrose and 0.8% (w/v) agar and the pH of the medium was adjusted to 5.7 before autoclaving. The cultures were incubated under a 16:8h photoperiod at 28 -36 μ mole m⁻² s⁻¹ (daylight fluorescent tubes) and 25±2°C. Twenty five replicates were taken for each treatment and for each experiment. Observations were made after 2-4 weeks and growth. The percentage of embryo-produced explants and the average number of embryos per explant were determined for each trial with Duncan's multiple range test [6].

Tissues for histological observations were fixed in FAA (95% ethyl alcohol : glacial acetic acid : formaldehyde : water, 10:1:2:7), dehydrates in a tertiary-butyl alcohol series, embedded in paraffin wax, sectioned

at a 10 μm thickness and stained with 0.5% safranin and 0.1% fast green [7].

RESULTS

Direct embryo/PLB Proliferation: Initially, almost all the leaf explants remained green when cultured on MS medium with TDZ and 2, 4-D. After 20 days of cultural on medium containing TDZ. Clusters of nodular masses produced from the wound surfaces and epidermal cell layers (Fig. 1a). No such nodular masses formed on the explants grown on media devoid of TDZ or supplemented with 2, 4-D alone. The appearance of these nodular masses was followed by embryo production, which became visible with in 5-8 days. Initially, the embryos appeared as white small globular masses (Fig. b), which germinated and passed through successive developmental stages, ultimately giving rise to PLB's (Fig.1b-c).

The effect of TDZ and 2, 4-D on frequency of embryos and eventually PLBs formation per explant is described in Table 1. TDZ had a marked effect on embryo formation. Twenty to thirty percent of the explants from the leaves 2-4 cm in length and 10-35% of the explants from the leaves 5-7 cm in length cultured. On MS medium containing 0.5-1.0 mg l^{-1} TDZ produced embryos. The presence of 2, 4-D had no inductive effect on embryo/PLB formation and when added with TDZ, the former even retarded the inductive effect of TDZ. Embryos and PLBs also arose through lower in numbers in the medium containing 2, 4-D and TDZ, but these showed less vigour upon further development.

The initial appearance of the foliar embryos appears to be affected by the stage of maturity of the leaves. More development of the foliar embryos occurred on the

older leaf explants (Table 1). The average number of embryos 17-34 were recorded on the TDZ (0.5-3.0 mg l^{-1}) effect on explants taken from the 5 to 7-cm long leaves, while explants taken from 2 to 4-cm, long leaves, produced an average of 5-13 embryos/PLBs on the same medium containing 0.5 to-3.0 mg l^{-1} TDZ.

Histological Observations on Direct Embryo Formation:

The embryos were generally formed from the epidermal layers or sub-epidermal layers of the explants and apparently initiated from a single cell (Fig. 2a-c). The first division seemed to be transverse, the basal resultant cell take part in farther divisions. As the differentiation progressed, embryos started to initiate from the region further from leaf surface. Developing embryos were composed of embryonic cells having a diameter of 8-10 μm ; the cells had densely stained cytoplasm and large nuclei. Subculture and proliferation of embryo/PLB clumps.

Clumps of the leaf-derived primary embryogenic nodule mass proliferated to produce more embryos/PLBs when sub cultured on the same medium containing 0.5 mg l^{-1} TDZ. Trials were conducted to determine the optimal TDZ dosage for embryo/PLB formation by inoculating nodular clumps (1 x 1mm) onto basal medium containing different dosages (0.01-1.0 mg l^{-1}) of TDZ (Table 2). The use of TDZ at 0.5 to 1.0 mg l^{-1} most favored embryo/PLB proliferation. However, spontaneous shoot development was found on basal medium without TDZ supplement.

Plant Regeneration and Conversion: The nodular most derived embryos or eventually PLBs produced shoots after 15 days, as shown in (Fig. 1d). Embryos or PLBs physically removed from the explants and placed on the

Table 1: Effect of Thidiazuron (TDZ) and 2, 4-Dichloro Phenoxy acetic acid (2, 4-D) on embryo proliferation from leaf explants of *Nymphoides cristatum*. Data were scored after one month of culture

Concentration (mg l^{-1})		Explant from 2 to 4 cm long donor leaf		Explants from 5 to 7 cm-long donor leaf	
2,4-D	TDZ	Percentage forming embryos	Embryos/explants	Percentage forming embryos	Embryos/Explants
0.0	0.0	0.0	0.0	0	0.0
0.0	0.5	15a,b,c	5.3	10b,c	19.0
0.0	1.0	20a,b	13.0	25a,b	24.4
0.0	3.0	25a	8.6	35a	17.0
3.0	0.0	0d	0.0	0c	0.0
3.0	0.3	5c,d	6.0	5c	8.0
3.0	1.0	0d	0.0	5c	12.0
3.0	3.0	5c,d	4.0	5c	9.0

Means of the 20 replicates with the same letters are not significantly different at $P < 0.05$

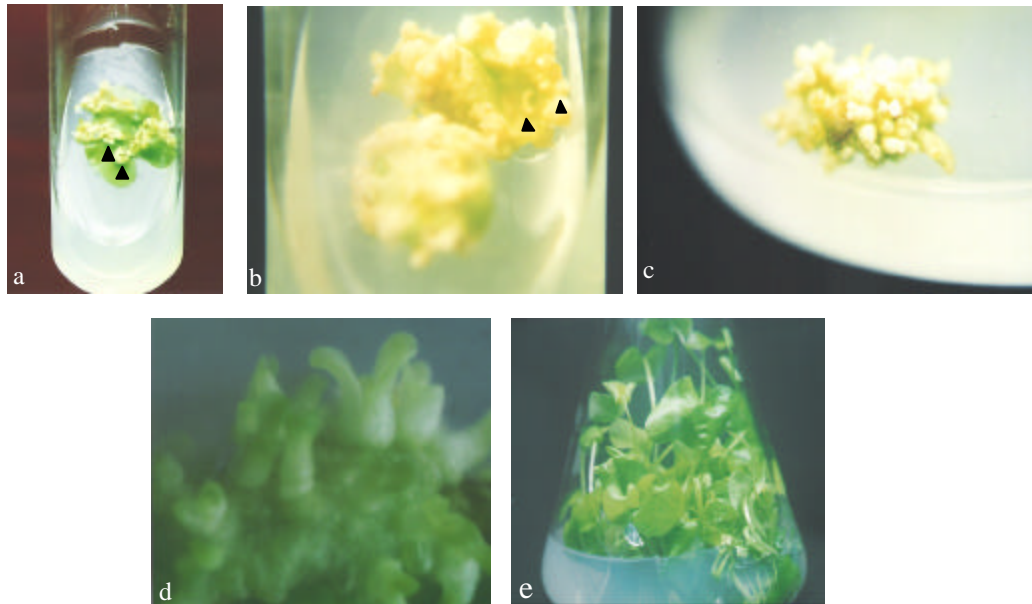


Fig.1a-e: *In vitro* morphogenesis of leaf explants and plant regeneration of *Nymphoides cristatum*

- a: Embryogenic nodular masses (arrowheads) protruding from the wound surfaces and epidermal layers of explant of 2 -3 cm long leaves
- b: Small globular embryos (arrowheads) formed from the mesophyll cells of the leaf surface
- c: Small protocorm - like bodies eventually formed from embryos on surface of leaf explant
- d: PLBs with young shoots
- e: Complete plantlets developed from isolated PLBs ready for transplanting to pots

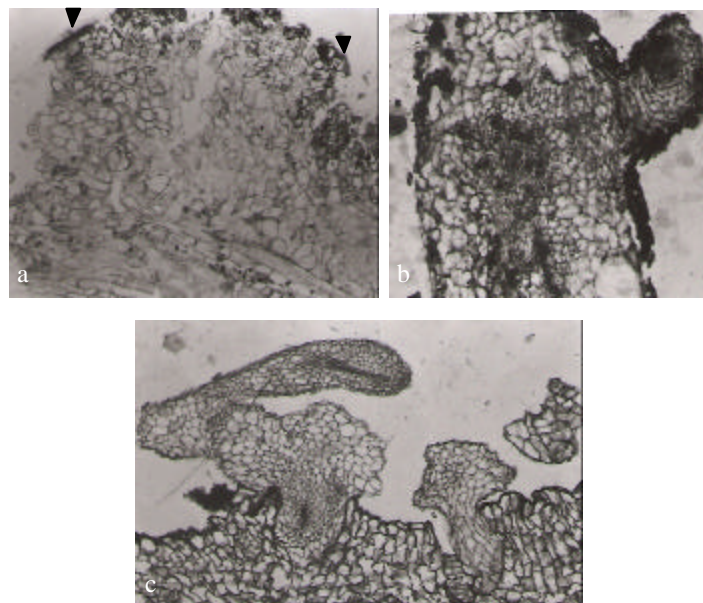


Fig. 2a-c: Morphogenesis and structure of embryos and PLBs

- a: Cross section of a leaf explants showing embryonic cells (arrowheads) of the epidermal layer with densely stained cytoplasm and nuclei.
- b: A developing embryo (arrowhead) with more embryonic cells.
- c: A well developed embryo or young PLB, protruding into adjacent surface

Table 2: Effect of TDZ on proliferation of leaf-derived embryo/PLB clumps and spontaneous plantlet formation of *Nymphoides cristatum*. Data were scored after one month of culture

TDZ (mg l ⁻¹)	Fresh weight of clump (mg)	Fresh weight of PLBs (mg)	Number of plantlets
00	450a	30b	16.8a
0.01	380a	60b	15.2a
0.10	390a	70b	14.3a
0.30	420a	360a	9.0b
1.0	360a	290a	13.0a,b

Means of 6 replicates with the same letters are not significantly different at P<0.05

same basal medium containing 0.5 mg l⁻¹ NAA also formed individual plantlets (Fig. 1e). About 80 regenerants about 3-4 cm in height with 3 to 5 leaves and 4 to 6 roots were then acclimatized in the green house. These plants grew well with an almost 90% survival rate. At present, all the regenerated plants appear to be morphologically normal.

DISCUSSION

Medium composition had a marked effect on direct somatic embryogenesis (Table 1). The medium with 2, 4-D (3.0 mg l⁻¹) and combination with TDZ (0.5 mg l⁻¹) produced somatic embryos more. Under these conditions cultured leaf explants produced the maximum number of somatic embryos and the percentage of response was also high. Similar results were observed in *Arachis Hypogaea* [8], in *Picea abies* [9], in *Camellia sinensis* [10] in *Anacardium occidentale* [11], in Grape vine [12].

In conclusion, the present experiment have shown that epidermal cells of *Nymphoides cristatum* are able to directly form somatic embryos in a defined medium supplemented with low dosage (0.01-1.0 mg l⁻¹) of TDZ. Clumps of these embryo masses proliferated to produce more embryos and eventually PLBs when sub cultured in the same medium. From these PLBs healthy plants developed with high survival rates when transplanted. This protocol is simple, easy to carry out and can provide large number of embryos and plants for mass propagation in a short period of time. Using this procedure not only plants can be regenerated on a large scale in a short span of time but the mother plant can also be conserved since using leaves as explant does not destroy the mother plant.

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ABBREVIATIONS

2, 4-D: 2, 4 = Dichlorophenoxyacetic acid,
MS = Murashige and Skoog's, basal medium,
NAA = Naphthalene acetic acid,
PLB = Protocorm-like body,
TDZ = Thidiazuron