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Induction of Somatic Embryogeneis in *Janakia arayalpathra* Joseph & Chandras. - A RET Medicinal Plant

K. Thangavel[1*], P. Ravichandran[2], R. Mahesh[3] and R. Uma[3]

[1] Department of Botany and Biotechnology, Thiagarajar College (Autonomous), Madurai - 625 009.
[2] Department of Plant Sciences, Manonmaniam Sundaranar University, Tirunelveli - 627 012.
[3]Department of Botany, South Travancore Hindu College, Nagercoil – 629 002, Tamil Nadu.
*for correspondence email: thangamspkces@gmail.com

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Abstract

An efficient and reproducible protocol for the induction of somatic embryos from leaf derived calli of *Janakia arayalpathra* has been devised. Efficient callus induction was obtained from leaf disc explants in MS medium supplemented with NAA and further proliferation of callus was rapid when BAP and PVP were co-supplemented in the medium. 2 % and 1.2 % of sucrose and agar respectively was found to be ideal for callus induction and establishment. Medium supplemented with NAA + CM + YE influenced differentiation of calli into cell aggregates followed by embryogenic cell mass.

Key Words: Suspension culture, Somatic embryogenesis, *Janakia arayalpathra.*

Abbreviations:

BAP	-	6-benzylaminopurine
BM	-	basal medium
CM	-	coconut milk
cm	-	centimetre
IBA	-	indole – 3 – butyric acid
KIN	-	kinetin
MS	-	Murashige & Skoog's medium
NAA	-	naphthalene acetic acid
PGRs	-	plant growth regulators
PVP	-	polyvinylpyrollidone
2,4-D	-	2,4-dichloro phenoxy acetic acid

Introduction

Micropropagation through somatic embryogenesis is an option for the rapid production of uniform plants which offers a powerful alternative to other vegetative propagation methods. Somatic embryos obtained by direct embryogenesis are less numerous than embryos derived from indirect embryogenesis. Thus, the indirect embryogenesis is generally preferred for large-scale propagation. Plant regeneration via somatic embryogenesis from single cells has been demonstrated in many medicinal plant species. Arumugam and Bhojwani (1990) reported the development of somatic embryos from zygotic embryos of *Podophyllum hexandrum* on MS medium supplemented with NAA and Kinetin. According to Zimmerman (1993), removal of auxin from the culture medium is a prerequisite to inactivate genes or synthesis of its products necessary for embryo development. *In vitro* studies and somatic embryogenesis have been carried out on many members of Asclepiadaceae such as *Hemidesmus indicus* (Sarasan *et al.,* 1994) and *Ceropegia spp*. (Patil, 1998). Somatic embryogenesis among medicinally important and red listed plants have been widely known in *Crataeva nurvala* (Inamdar *et al.,*1990*), Tylophora indica* (Jayanthi and Mandal, 2001), *Gymnema sylvestre* (Kumar *et al.,* 2002) and *Arachis hypogia* (Venkatachalam *et al.,* 2007), *Cicera rietinum* L (Ghanti *et al*., 2009.), *Cleome rosea* Vahl. (Simoes *et al*., 2010), *Arabidopsis thaliana* (2011), *Leucojum aestivum* (Ptak *et al*, 2013), *Cuminumcyminum* (Bahmankar *et al*., 2017). The pivotal role of plant growth regulators in controlling somatic embryogenesis has been well established in different plant species (Wang *et al.,* 2008; Simoes *et al*., 2010; Bahmankar *et al*., 2017).

Janakia arayalpathra (Periplocaceae) as a new type genus and species was described by Joseph and Chandrasekharan (1978). *Janakia arayalpathra* is a medium sized shrub growing in a peculiar habitat of rock crevices and on sedimentary rocky slopes at an altitude of above 800 meters. It is a monotypic endemic genus with the species having narrow and restricted distribution with a threat status of CR-Critically Endangered (B1 & 2c, d) - Globally (Ravikumar and Ved, 2000). This species is an endemic plant to Southern Western Ghats - Kerala and Tamil Nadu states. The tubers are being ruthlessly collected from its natural habitat by the

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local people for trade. This has led to the acute scarcity of this plant. Consequently, it has been enlisted as critically endangered (B1 & 2c, d) - Globally (Ravikumar and Ved, 2000). The moniliform tuberous roots of this plant are highly aromatic and the native Kani tribes use it as an effective remedy for peptic ulcer, cancer-like afflictions and as a rejuvenating tonic. The active principle (2-hydroxy -4-methoxy benzaldehyde) of the fleshy tuberous roots of this plant was extracted and bioactive potential have been characterized (Sudha and Seeni 2001; Thangavel, 2008; Srivastava, 2011). *In vitro* establishment of normal root cultures of *Janakia arayalpathra* and the hormonal supplementations were analyzed towards extraction of the aromatic compounds (Sudha and Seeni, 2001). Regeneration of whole plants through micropropagation has been reported with single shoot development from shoot tip cultures (Thangvel *et al*., 2011). However, a lack of comprehensive understanding of the morphogenetic potential for large scale multiplication and the restoration of the species is necessary. Hence, the present study focused on development of economically viable and rapid tissue culture protocol through somatic embryogenesis.

Materials and Methods

Fresh plant materials of *Janakia arayalpathra* was collected from its natural habitat Naraikadu forest - Kottangathatti Hills - at Thirukurangudi range, Kalakad Mundanthurai Tiger Reserve (Southern Western Ghats. Murashige and Skoog (1962) medium (MS) was used for initition and establishment of calli and somatic embryogenesis from leaf disc explants. Basal Medium was supplemented with different concentrations (0.5µM to 20µM) of Plant growth regulators such as naphthalene acetic acid (NAA), 6-benzyl-aminopurine (BAP). Liquid coconut endosperm was also used as natural plant growth regulator was at a concentration of 5-15 % (added before adjusting the pH). Polyvinylpyrollidone was used at a concentration of 0.05% to overcome the problems associated with the phenolic exudation. Leaf disc were surface sterilized stepwise as follows: The explants were initially washed with tap water and then soaked in liquid commercial bleach (0.5-1 % v/v NaOCl) containing a few drops of "Teepol" as surfactant for 5 minutes. This was followed by a thorough wash in running tap water. Explants were transferred to sterile laminar air flow cabinet, under such sterile conditions they were immersed in 0.05 % mercuric chloride ($HgCl_2$) solution for 5 min and rinsed thoroughly in sterile distilled water. Surface sterilized explants were trimmed into optimum size (0.5-1.0 cm) and aseptically placed in culture vial. Ten replicates were maintained for each treatment. The temperature of culture room was maintained at 25 ± 2ºC. Sub-culture was done in 15 days interval.

Results

Leaf explants cultured on MS basal medium (BM) remained green up to 30 days without forming any callus. In case of the explants inoculated in PGR supplemented media callus induction was bowered within 7 days in dark. The callus was friable in nature and appeared white. BAP did not induce callus yet, when supplemented along with NAA callus induction and proliferation was rapid. 6 µM NAA along with PVP promoted quick induction and rapid proliferation of callus compared to the rest of treatments. Conversely, NAA along with BAP induced more callus growth than any other medium composition. The leaf explants often leached phenolic substances that controlled the callus growth considerably, but this effect was suppressed by the addition of 0.05% PVP maximally. Out of various levels of sugar and agar tested 2 % and 1.2 % respectively was found to be ideal for required callus induction and mass multiplication. Suspension culture was initiated with friable calli continued to proliferate and increased cell biomass and formation of cell aggregates. Though a wide variation was provided in hormonal balance and combination (Table-1), only the medium supplemented with NAA + CM +YE influenced differentiation of cell aggregates and pro-embryogenic cell mass within 75 days in suspension culture with periodic subculture at every 15 days. There was a clear distinction between normal cell mass and pro-embryogenic mass (Fig. 3). Such embryogenic cell mass was brownish and somewhat spherical in shape and often formed clusters. The differentiation of such masses seems to be originated form single cells which were uniform in size and shape unlike the callus cells with regular outlines (Fig. 4). Such cells initially had large vacuoles and had dense cytoplasm. Once the cells differentiated they often detached from the cell mass and showed typical spherical zygote like appearance compactly filled with cytoplasmic contents. The cytoplasmic contents were abundant in starch grains. Pro-embryogenic cells had prominent nucleus which was confirmed by the nuclear stain haematoxylin. The cells in the embryonic mass were very tiny, tightly linked to each other and appeared like a globular somatic embryo (Fig.5). Every cell in the unit was rich in starch and that could be observed by starch specific I_2KI reagent where all the cell contents turned brown. The differentiation of such somatic embryos in suspension culture was favored by NAA + CM +YE at varying concentrations (Table 2). 6 µM NAA along with the supplements showed more differentiation of somatic embryos and the maximum average number of globules were 14. Hormone free medium, 2,4-D and NAA individually supplemented medium did not evoke somatic embryo development.

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Table -1: Initiation and establishment of callus culture from leaf disc explants of *Janakia arayalpathra*

S. No.	Medium Composition	Callus induction efficiency	Initial wt. of callus (g)	Wt. after 20 days[a] (10 replicates)	ANOVA[b] P - Value	F - Value
1	MS – BM	-	0.5	0.5	-	-
2	MS + 2 µM NAA + 2.5 % S + 0.8 % A + 0.05 % PVP	+	0.5	0.68±0.18	0.1042 NS	2.927
3	MS + 4 µM NAA + 2.5 % S + 0.8 % A + 0.05 % PVP	+	0.5	0.72±0.16	0.0019 **	13.198
4	MS + 6 µM NAA + 2.5 % S + 1.0 % A + 0.05 % PVP	+++	0.5	0.72±0.21	0.0019 **	13.198
5	MS + 8 µM NAA + 2 % S + 1.0 % A + 0.05 % PVP	+	0.5	0.76±0.25	1.97E-06 **	47.287
6	MS + 10 µM NAA + 2 % S + 1.2 % A + 0.05 % PVP	+	0.5	0.78±0.19	3.85E-06 **	42.682
7	MS + 2 µM 2,4-D + 2 % S + 1.2 % A + 0.05 % PVP	+	0.5	0.80±0.21	5.38E-07 **	57.241
8	MS + 4 µM 2,4-D + 2 % S + 1.2 % A + 0.05 % PVP	+	0.5	0.80±0.24	5.38E-07 **	57.241
9	MS + 6 µM 2,4-D + 2 % S + 1.2 % A + 0.05 % PVP	+ +	0.5	1.60±0.50	1.11E-20 **	2443.606
10	MS + 8 µM 2,4-D + 2 % S + 1.2 % A + 0.05 % PVP	+ +	0.5	1.70±0.53	1.37E-20 **	2386.983
11	MS + 10 µM 2,4-D + 2 % S + 1.2 % A + 0.05 % PVP	+ +	0.5	1.70±0.62	1.37E-20 **	2386.893
12	MS + 2 µM NAA + 2 µM BAP + 10 % CM + 2 % S + 1.2 % A + 0.05 % PVP	+ +	0.5	1.78±0.59	3.34E-23 **	4674.064
13	MS + 4 µM NAA + 2 µM BAP + 10 % CM + 2 % S + 1.2 % A + 0.05 % PVP	+ +	0.5	1.8±0.61	1.37E-20 **	2387.344
14	MS + 6 µM NAA + 2 µM BAP + 10 % CM + 2 % S + 1.2 % A + 0.05 % PVP	+ + + +	0.5	2.9±0.64	8.98E-26 **	9039.249
15	MS + 2 µM 2,4-D + 2 µM BAP + 10 % CM + 2 % S + 1.2 % A + 0.05 % PVP	+ + +	0.5	2.0±0.58	2.9E-21 **	2839.667
16	MS + 4 µM 2,4-D + 2 µM BAP + 10 % CM + 2 % S + 1.0 % A + 0.05 % PVP	+ + +	0.5	2.1±0.62	1.49E-22 **	3956.045
17	MS + 6 µM 2,4-D + 2 µM BAP + 10 % CM + 2 % S + 1.2 % A + 0.05 % PVP	+ +	0.5	1.6±0.56	1.27E-19 **	1860.319
18	MS + 6 µM NAA + 2 µM BAP + 10 % CM + 2 % S + 1.2 % A + 0.05 % YE + 0.05 % PVP	+ +	0.5	1.55±0.58	7.07E-17 **	912.910
19	MS + 6 µM 2,4-D + 2 µM BAP + 10 % CM + 2 % S + 1.2 % A + 0.05 % YE + 0.05 % PVP	+ +	0.5	1.52±0.72	1.79E-19 **	1789.952

P^H 5.6

+ Delayed response with very minimum callus; + + Delayed response with normal callus; + + + earlier response with normal callus; + + + + earlier response with maximum callus

a - All values are mean of 10 replicates ± SD; NS – Not Significant; ** Significant at P< 0.01

b - All values have compared with MS BM

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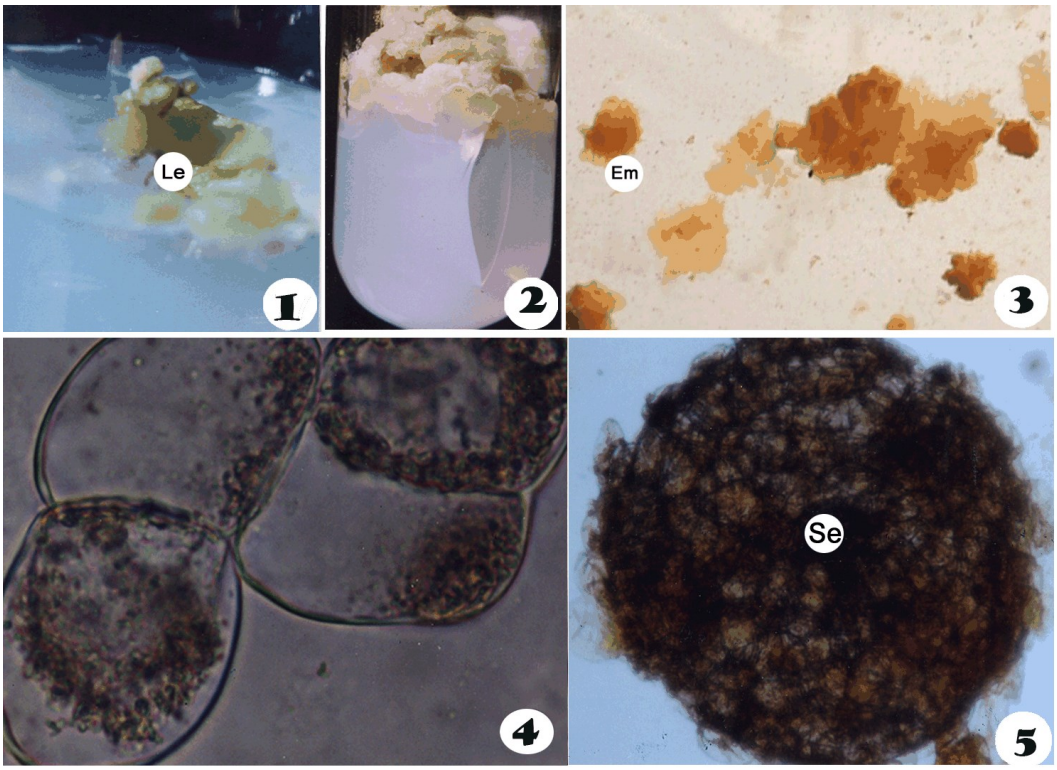
Table -2: Conversion of leaf disc derived calli into somatic embryo in suspension culture

S. No.	Medium Composition	Initial wt. of callus (g)	Avg. No. of Embryos[a] produced / 100ml
1	MS BM	0.5	-
2	MS + 2 µM NAA + 1.5 % S	0.5	-
3	MS + 4 µM NAA + 1.5 % S	0.5	-
4	MS + 6 µM NAA + 1.5 % S	0.5	-
5	MS + 8 µM NAA + 1.5 % S	0.5	-
6	MS + 10 µM NAA + 1.5 % S	0.5	-
7	MS + 2 µM 2,4-D + 1.5 % S	0.5	-
8	MS + 4 µM 2,4-D + 1.5 % S	0.5	-
9	MS + 6 µM 2,4-D + 1.5 % S	0.5	-
10	MS + 8 µM 2,4-D + 1.5 % S	0.5	-
11	MS + 10 µM 2,4-D + 1.5 % S	0.5	-
12	MS + 1 µM NAA + 1.5 % S + 0.05 % YE + 10 % CM	0.5	5±0.12
13	MS + 2 µM NAA + 1.5 % S + 0.05 % YE + 10 % CM	0.5	5±0.16
14	MS + 4 µM NAA + 1.5 % S + 0.05 % YE + 10 %CM	0.5	9±0.24
15	MS + 6 µM NAA + 1.5 % S + 0.05 % YE + 10 %CM	0.5	14±0.21
16	MS + 8 µM NAA + 1.5 % S + 0.05 % YE + 10 % CM	0.5	8±0.25
17	MS + 10 µM NAA + 1.5 % S + 0.05 % YE + 10 % CM	0.5	6±0.28

P^H 5.6 - No Differentiation a - All values are mean of 10 replicates ± SD



Figs. 1 - 2. Initiation and Establishment of Callus Culture from Leaf Disc Explants of J*anakia arayalpathra*

1. Leaf disc (Le) cultured on MS medium supplemented with 6 µM NAA + 2 µM BAP + 10 % CM + 0.05 % PVP. Callus induced after 7 days of inoculation.

2. Establishement of friable callus (Fc) after subculture on the same medium.

Figs. 3-5. Conversion of Leaf Disc Derived Calli into somatic Embryo in Suspension Culture.

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3.Differentiation of embryogenic (Em) calli in MS liquid medium supplemented with 8 µM NAA + 4 µM IBA + 10 % CM + 0.05 % YE + 0.05 % PVP - X 15

4. Differentiation of embryogenic cell mass in liquid medium supplemented with 6 µM NAA + 0.05 % YE + 10 % CM) stained with I_2 KI - X 200

5. Single globular somatic embryo (Se) stained with I_2 KI - X 800.

Discussion

Janakia arayalpathra require a reproducible *in vitro* regeneration and establishment in field conditions as it is categorized as critically endangered. Hence, this study was aimed at understanding the cytodifferntiaton potential of explants such as leaf disc for somatic embryogenesis. Calli obtained from leaf cultures could be mass multiplied on solid medium and maintained viable for a long time by regular subculture process in both the species (Fig 1-2) Further, the calli did not have any secondary compounds when they were subjected to staining for the localization of secondary compounds like polyphenols, 2H4MB and tannins. Cells and cell aggregates in suspension showed colour change due to a possible phenolic synthesis and leaching which controlled new cell divisions. Similar conditions were reported in many cases where the suspension cultures failed to proceed due to leaching of phenolic compounds.

Plant regeneration through somatic embryogenesis from single cells and various types of explants has been demonstrated in many medicinal and rare and endangered species such as *Podophyllum hexandrum* (Arumuagam and Bojwani, 1990), *Typhonium trilobatum* (Das, *et al.,* 2002), *Holostemma ada-kodien* (Martin, 2003) and *Hevea brasiliensis* (Venkatachalam *et al.,* 2007; *Cicera rietinum* L (Ghanti *et al*., 2009.), Cleome rosea Vahl (Simoes *et al*., 2010), Arabidopsis thaliana (2011), Leucojum aestivum (Ptak et al, 2013), *Cuminum cyminum* (Bahmankar *et al*., 2017).

Differentiation of somatic embryogenic mass of cells and cell aggregates was successful when the medium was constantly altered and subcultured at frequent intervals. Observations in the present study are in conformity with the earlier reports. However, addition of adjuvants like coconut milk and yeast extract was indispensable along with normal hormonal supplements tested. Except MS basal medium, all other media combinations were supplemented with 0.05% of yeast extract and 5-15% of coconut water. The actual role of such adjuvants could be their intervention with the indigenous concentration of auxins which supposed to be adjusted for somatic embryogenesis *in vitro* (Das, 2002; Simoes et al., 2010; Ptak et al, 2013). Somatic embryogenic mass differed in morphology and cell aggregation in suspension cultures. These aggregates were scooped out and examined under microscope for confirmation of their growth stage. The embryogenic cells had dense cytoplasm, were uniform in size and shape, and rich starch grains (Fig 3-5). Smearing a small aggregate indicated the presence of uniform embryogenic cells as that of normal embryo with dense cytoplasm by staining with TBO. Such embryogenic cell aggregates were transferred to solid medium to achieve maturation and germination of somatic embryos. With their relatively faster growth, ease of manipulation into organs and comparative homogeneity, suspension cultures are the most widely employed systems in the studies of secondary metabolism and somatic embryogenesis. Strong understanding of physiology and further attempts to standardize tissue culture conditions are required to achieve an efficient somatic embryogenesis in this important medicinal plant.

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