Environment and Ecology 41 (3B) : 1844—1851, July—September 2023
Article DOI: https://doi.org/10.60151/envec/XDPE8980
ISSN 0970-0420

In vitro Somatic Embryogenis and Plant Regeneration from Cell Suspension Culture of Endangered Plant 'Virali' *Dodonaea viscosa*

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Received 9 March 2023, Accepted 19 June 2023, Published on 21 August 2023

ABSTRACT

An effective protocol was developed for *in vitro* propagation of 'Virali' via somatic embryogenesis in cell suspension culture. Embryogenic callus was obtained on Murashige and Skoog (MS) medium by a relatively moderate concentration of 2,4-dichlorophenoxyacetic acid (2,4-D) (4 mg/L), high concentrations of proline and glutamine (both 300 mg/L) and coconut water to develop induction of calli from explant. A suspension culture of the callus-derived embryogenic cells in Murashige and Skoog (MS) basal medium (pH 5.3) with 2 mg/L 2,4-D, 1 mg/L indole-3-acetic acid (IAA), 1 mg/L NAA, 45 g/L sucrose, and 20 g/L maltose resulted in synchronously proliferating cells with the capacity to be induced into somatic embryos on an 8g/L agarose, 20 g/L maltose, 10 g/L dextrose, 40 g/L sucrose, 1 mg/L naphthaleneacetic acid (NAA), and 0.2 mg/L. On an 8 g/L agarose-solidified MS basal medium (pH 5.7) with 1 mg/L NAA, 100 ml/L coconut water, 30 g/L sucrose, 30 g/L maltose, and 100 mg/L glutamine, the young somatic embryos differentiated into regenerable mature somatic embryos. In a 2.5 g/L gelrite solidified MS-based medium with 2.5 mg/L 6-benzylaminopurine (BAP), 1 mg/L gibberellic acid (GA3), 100 mg/L L-glutamine, 30 g/L sucrose, and 2.5 mg thidiazuron, the mature somatic embryos regenerated into plantlets (TDZ). For the formation of plantlets with a height of 10 to 12 cm that could endure the hardening process, it took a total of 6 months from the explant stage. The plantlets' regular morphology suggests that there was no somaclonal variation in their development. The current regeneration approach for "Virali" has a significant deal of potential for micro propagating endangered germplasm and improving it through transgenic technology.

Keywords Somatic embryogenesis, Hop Bush, Virali, *Dodonaea viscosa*, Plant growth regulator.

INTRODUCTION

Hopbush (*Dodonaea viscosa* Linn.) belonging to the family sapindacease known indigenous medicinal plant (Rani and Mohan 2009). It originated from Australia having cosmopolitan distribution in tropical, subtropical and warm temperature regions of Africa, America and Southern Asia (Muqaddas *et al*. 2018). In India commonly known the name stated as puli-vailu, gollpulledu, bandedu (Telugu), wilayatimehandi, Jaglianar (Hindi), Bandare (Kannada), Virali (Tamil), Latchmi, Paorki (Marathi) in the different

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geographical states. Within a single plant, several portions may have various active chemicals. It has numerous medicinal qualities derived from various plant components, such as leaves, roots, bark, fruit, seeds, and flowers (Hossain 2019). The stems and leaves are used to treat the fever and seed (in mixing with other plants and coated in honey) used to treat malaria (Orpin *et al*. 2018). Roots and ingested by women in east Africa to stimulate milk production and to treat drysmenorrhea and irregular menstruation (Jiao *et al*. 2022). In worldwide, it is used in traditional medicine and reported for the treatment of sore throat, cold fever, rheumatism, indigestion, ulcers, diarrhea, constipation to expel round worms and also used for anti- inflammatory, anti-viral, spasmolytic, laxative, anti-microbial, hypotensive potentials, anti-bacterial, anti-fungal and anti-ulcerogenic activities (Malik *et al*. 2022).

Followed by several medicinal value of *Dodonaea viscosa* Linn is rapidly disappearing the plant those facing extinction to address the increasing commercial demands. According to an IUCA report around 50-80 thousand flowering plant species are used for medicinal purposes and among these around 15 thousand are on the verge of extinction (Chen *et al*. 2016). The reason as the increasing population increasing market demands over exploitation, over harvesting and habitat destruction (Anderson *et al*. 2011).

Overcoming this problem, micropropagation (or) *in vitro* regeneration is used to achieve large scale development and conservation of medicinal plans with unique traits in a short period of time (Adhikary *et al*. 2021). As a result, the current study was developed for *Dodonaea viscosa* Linn's rapid multiplication and conservation in order to investigate the effects of various concentrations of plant growth regulators (PGRs), explant source (*in vitro* grown seedlings, pot-grown plants, and wild plants), and explant type (shoot tips and nodal segments associated to their position along the shoot axis).

MATERIALS AND METHODS

Young nodal explants Hop bush (*Dodonaea viscosa* L.) plants were obtained from a Kannankudi village, Tevakotai Taluk, Sivangai district, Tamil Nadu, India.

Explants were carefully cleaned for 15 minutes with running water (Fig. 1A). They were then pre-treated by soaking in a fungicide solution of 1% (w/v) Bavistin for 30 minutes, followed by 15 minutes of rinsing in a liquid detergent of 5% (v/v) labolene, before being repeatedly washed in sterile double distil water (DDW). Nutrient medium comprising of MS (Murashige and Skoog 1962) on MS-based embryogenic callus induction (ECI) medium-1, 2, 3, 4 and 5 (Table 1) in 100×120 mm Petri dishes (Fig. 1B). At 18°C, the plates were incubated in the dark. Up until the development of embryogenic calli, sub-culturing was carried out once every 10 days.

A 'Virali' embryogenic cell suspension culture was initiated using six-month-old embryogenic calli (Fig. 1C). In 10 ml of liquid medium that contained Murashige and Skoog (1962) (MS)-based embryogenic cell suspension (ECS) medium (pH 5.3), 30 g/L sucrose, 30 g/L maltose, along with 4 mg/L 2,4-dichlorophenoxyacetic acid (2, 4-D), and 1 mg/L indole-3, 100 mg of embryogenic calli were added (Fig. 1D). The ECS culture was incubated at 18 to 25 ±1°C with continual shaking (100 rpm), kept under a 16 and 8-hr light/dark cycle, and had the used medium replaced once a week with new medium. At each sub-culturing stage, an aliquot of the suspension culture was examined under a light microscope to check for the presence of embryogenic cells. One milliliter of regenerable embryogenic cell suspension was added to each of the three semisolid Somatic embryo induction (SEIM) media, MS-based SEI medium-5, Somatic embryo maturation medium-3, and SEGM Somatic embryo germination medium -3 (Schenk and Hildebrandt 1972) (Fig. 1E) (Table 1). Until somatic embryos were apparent the cultures were incubated at 26°C in a growth chamber with a 16/8 h light/dark cycle, respectively.On 1x MS-based somatic embryo maturation media (SEMM)-1, 2 and 3, the somatic embryos were cultured for two months (Table 1). Mature somatic embryos were moved to 0.5x MS-based regeneration medium (RM) 1, 2, and 3 after two months (Fig. 1F) (Table 2).

The regenerated plantlets were taken out of the culture medium, washed to remove the gelrite, and

Table 1. Medium composition used for regeneration protocol for Virali.

Medium composition	SEIM1	SEIM2	SEIM3	SEIM4	SEIM5	SEMM1	SEMM2	SEMM3	SEGM1	SEGM2	SEGM3
MS components	+	+	+	+	+	+	+	+	+	+	+
SH components	-	-	-	-	-	-	-	-	-	-	-
2,4-D (mg)	6	2	4	4	-	-	-	-	-	-	-
Dicamba (mg)	-	-	-	-	4	-	-	-	-	-	-
IAA (mg)	1	1	1	1	1	-	-	-	-	-	-
NAA (mg)	1	1	1	1	1	-	-	1	-	-	-
2Ip (mg)	-	-	-	-	-	-	-	-	-	-	-
BAP (mg)	-	-	-	-	-	-	-	-	0.25	0.5	1.0
GA3 (mg)	-	-	-	-	-	-	-	-	-	1	1
TDZ (mg)	-	-	-	-	-	-	-	-	-	1.5	-
MS vitamins	+	+	+	+	+	+	+	+	+	+	+
MAs vitamins	-	-	-	-	-	-	-	-	-	-	-
CW (ml)	-	100	100	-	-	-	-	100	-	-	-
L-Proline (mg)	150	150	300	150	150	-	-	-	-	-	-
L-Glutamine (mg)	-	-	150	-	-	-	-	100	100	100	100
Biotin (mg)	1	1	1	1	1	-	-	-	-	-	-
Sucrose (g)	30	30	30	30	30	-	30	30	30	30	30
Maltose (g)	-	-	-	-	-	30	-	30	-	-	-
Dextrose (g)	-	-	-	-	-	-	-	-	-	-	-
Phytagel (g)	-	3	1.5	3	3	-	-	-	-	-	-
Agarose (g)	8	-	-	-	-	4	2.5	8	-	-	-
Gelrite (g)	-	-	-	-	-	-	-	-	2.5	2.5	2.5
pH	5.3	5.3	5.3	5.3	5.3	5.7	5.7	5.7	5.7	5.7	5.7

SEIM: Somatic embryo induction medium-5 Media, SEMM: Somatic embryo maturation medium-3 media , SEGM: Somatic embryo germination medium-3 media.

then transplanted into 90-well trays with sterilized soil mixture (sand: clay soil: coir pith = 1:1:1) and watered with Hoagland's medium. The plantlets were about 10 to 15 cm tall and had two to three leaves (Hoagland 1950). To keep the trays from drying out, they were wrapped in transparent plastic bags. Each plant was transferred into a container measuring 9 x 9 x 36 cm after three to four weeks of primary hardening and kept in a greenhouse until it reached a height of 20 to 25 cm. The plants were moved to the field after being hardened.

Data analysis

The statistical program AGRES was used to analyze the data. Duncan's multiple range test (DMRT) was used to differentiate mean values at a 5% probability level (Duncan 1955). In case of percentage, arcs in (p%/100) ½ transformation of the variable was per-

Table 2. Effect of NAA and IBA on rooting medium and effect of TDZ in shoot development (RM).

Variables medium -MS salt with	NAA/ mg/L	IBA / mg/L	TDZ/ mg/L	Average no. of roots/ propagule (cm)	Average root length (cm)	Average shoot length (cm)	Rooting percentage (%)
RM1	1	-	0.2	1.1-1.3	0.5-1	10.0-12.0	45
RM2	0.5	0.5	1.5	2.4-2.8	3.0-3.5	15.5-16.0	85
RM3	-	0.3	0.5	1.1-1.2	0.3	6.0-8.0	10

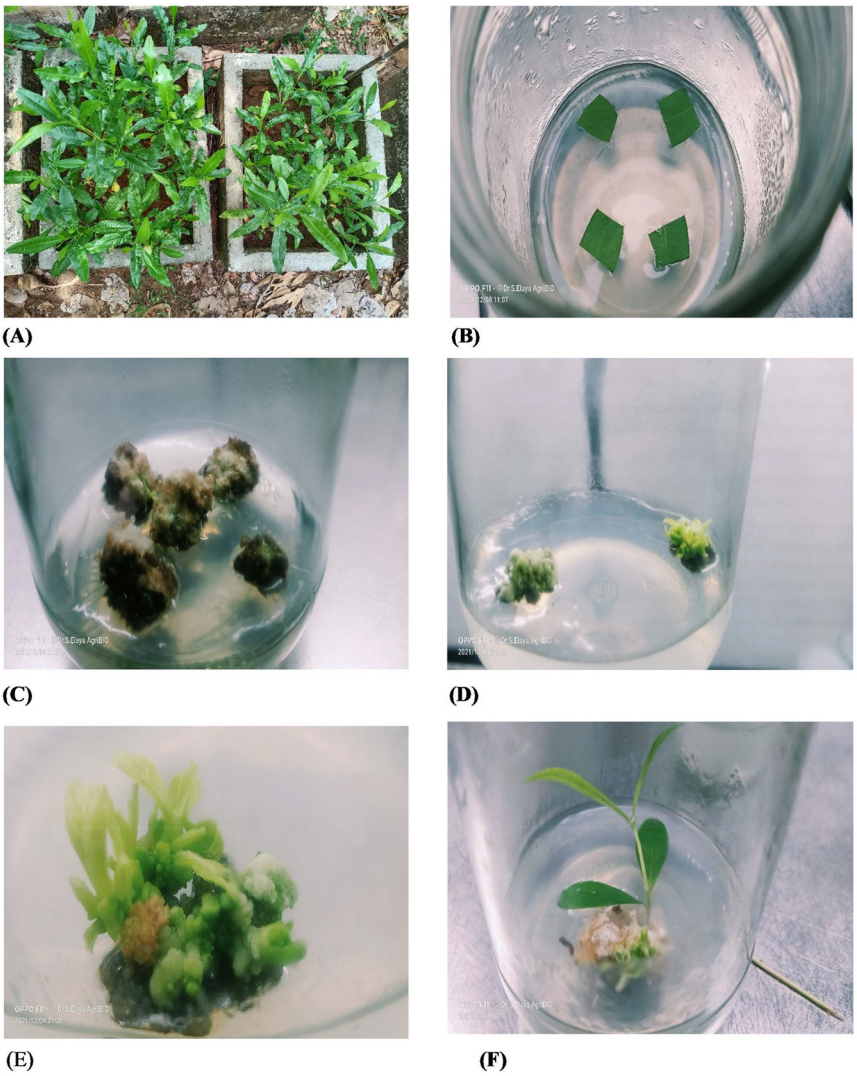

Fig. 1. (A) Mother plant (B) Inoculated explant leaf (C) Induction of embryogenic callus (D) Induction of somatic embryo (E) Leaf promodia on somatic embryo (F) Germination of somatic embryo.

formed before analysis, and were converted back to percentages for presentation.

RESULTS AND DISCUSSION

Among the different media tested for embryogenic callus induction of cultivar 'Virali' (Table 1), the highest frequency of callus formation (57.78) occurred in the MS based SEIM3 (Table 3). The composition of SEIM3 differed from those of the other media by having a moderate concentration of 2,4-D i.e., 4 mg/L, while the 2,4-D levels in other SEM media varied between 2 to 6 mg/L; a high concentration of proline i.e., 300 mg/L, while the others had only 150 mg/L; glutamine (150 mg/L), which was absent in other SEIM media, and 1.5 g/L phytagel. The other SEIM media either completely lacked phytagel or had concentrations of 3 g/L. Although, both SEIM2 and SEIM3 contained coconut water, they differed in the concentrations of growth factors and amino acids

Table 3. Somatic embryo induction, maturation and regeneration.

1.*Media	Number of SE (%±SE)	2.**Media	Number of SE matured ** (%±SE)	3.***Media	Number of SE germinated*** (%±SE)
SEIM1	46.67±1.1b	SEMM1	85.83±47b	SEGM1	56.66±12b
SEIM2	53.33±0.5ab	SEMM2	55.33±80c	SEGM2	92.53±12a
SEIM3	57.78±0.2a	SEMM3	90.33±26a	SEGM3	38.3±73c
SEIM4	46.67±1.1b				
SEIM5	40±1.5c				

*Three replications maintained with15 leaf bit explants per replicate. Data were taken 6 months after embryo genic callus (EC) Induction.
** Each treatment had 3 replications with 50 embryos per replication. Observations were taken after 3 months of somatic embryo maturation.
***Each treatment had 3 replications with 50 embryos per replication. Observations were taken after 3 months of somatic embryo germination.

(Table 1). A higher frequency of callus induction in media containing 2,4-D (4 mg/l) was also reported by Binte Mostafiz and Wagiran (2018), Malik *et al*. (2003), Upadhyaya *et al*. (2015), Lone *et al*. (2017).

A protrusion of callus tissue was visible at the explant's base. These embryogenic protrusions eventually transformed into masses of yellowish-white embryogenic calli after first emerging as larger translucent calli (Mai *et al*. 2017) (Fig. 1C). Browning of the calli and brown exudation in the surrounding medium were both signs that callus growth was frequently linked to the exudation of phenolic chemicals (Sabooni and Shekafandeh 2017). In plant tissue culture several issues that might arises in media i.e., Rapid browning, hyperhydricity, and eventual degradation of calli caused by the exudation of phenolics. Similar results noted in this study Abdalla *et al*. (2022) Sandhu *et al*. (2018). With the various media, varying levels of phenolic compound exudation were seen. In comparison with other media, the calli grown on coconut water and L-proline produced significantly less or lower quantities of phenolics. Moreover, the medium enriched with L-proline and coconut water promoted calli differentiation into somatic pre-embryos and improved calli growth.

Calli phenolic compounds are known to inhibit callus tissue growth and differentiation (Palacio *et al*. 2012). Due to cellular competition for resources and space, phenolic chemicals in the calli and local catabolite accumulation surrounding the calli occurs stress. Depending on the different concentration of the media, the degree of calli browning appears to vary (Raza *et al*. 2022). Also, the concentrations of the growth promoters and other ingredients utilized may have an impact on how much phenolics exude and the resulting browning. Baker Mohammad (2018) reported browning of calli prior to pre-embryo stage in media containing high concentrations of 2,4-D, 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and picloram.

Frequent weekly sub-culturing prevented the exudation of phenolic chemicals that would otherwise have interfered with the formation of embryogenic calli. The least amount of phenolic compound exudation and browning occurred in SEIM3, which contained coconut water and just 1.5 g/L of phytagel. With a stereo microscope, embryogenic calli with pro-embryos were discovered after five months in the callus induction media.

To create somatic embryo cell suspension (SEM) medium, portions (0.1 g) of embryogenic calli with pro embryos were utilized. This MS basal media (pH 5.3) supplemented with 2 mg/L 2,4-D, 1 mg/L IAA, 1 mg/L NAA, 30 g/L sucrose, and 30 g/L maltose. The cells uniformly matured after three months (Fig. 1D). Due to the high quantity of carbon in the medium (30 g/l sucrose + 30 g/l maltose), the embryogenic cells multiplied quickly. Using a compound microscope (40 x)., dense starch granules were seen in the cytoplasm of the embryogenic cells. The elongated or irregularly shaped non-embryogenic cells missing starch granules may be distinguished from the spherical embryogenic cells with abundant starch granules. This study demonstrates that SEM3 (pH 5.7), a mixture of three carbon sources (30 g/L sucrose, 30 g/L maltose and agarose (8 g/L), produced the most embryos. Each milliliter of SEIM3 produced an estimated 57.78 ±0.2 a embryos (Fig. 1E).

According to Table 3, SEMM3 (i.e., 8 g/L agarose-solidified MS basal medium, pH 5. 7, with 1 mg/L NAA, MS vitamins, 100 mg/L glutamine,

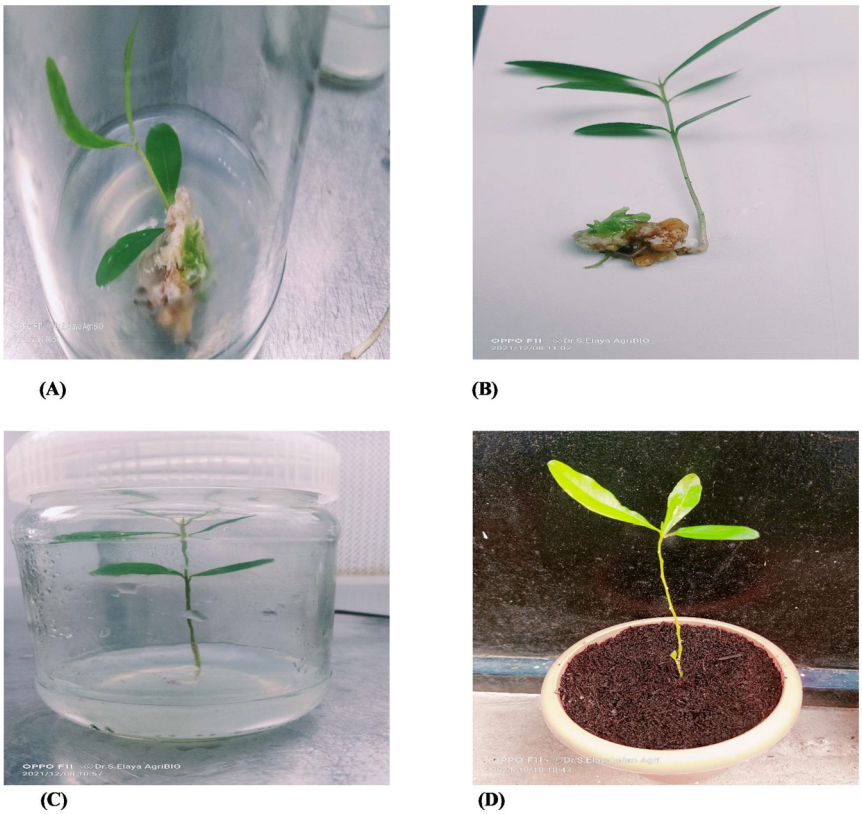

Fig. 2. (A) Germination of somatic embryo and shoot development (B) Shoot and root development. (C) Complete plantlet development (D) Hardening in plastic pot.

100 ml/L coconut water, 30 g/L sucrose, and 30 g/L maltose) was the best for somatic embryo maturation. Because SEMM3 contained NAA, coconut water, and glutamine, it was different from SEMM1 and SEMM2. In addition, SEMM3 had two carbon sources, whereas the other SEMMs only had one (30 g/L each of sucrose and maltose). Whereas the other SEMMs contained agarose at 2.5 or 4 g/L, SEMM3 employed agarose at a concentration of 8 g/L as the solidifying media.

The regeneration medium-2 [2.5 g/L gelrite-solidified MS basal medium (pH 5.7) supplemented with 2.5 mg/L 6-benzylaminopurine (BAP), 1 mg/L gibberellic acid (GA3), 2.5 mg/L thidiazuron (TDZ), MS vitamins, 100 mg/L glutamine, and 30 g/L sucrose] produced the highest regeneration efficiency (93%) of all the media tested. The diphenylurea derivative cytokinin TDZ was absent from the other mediums.

In the micropropagation hopbush, thiadiazuron has not been utilized frequently. The majority of hopbush micropropagation procedures have utilized adenine-derived cytokinins such BAP, 2-isopentenyladenine (2-2ip), and zeatin (Kahira *et al*. 2015). It has been demonstrated that thiidiazuron causes habituation and (relative) cytokinin independence in some species (Pischke 2004). Because of this advantageous biological characteristic, TDZ is an economical choice for micropropagation. The ability of *Dodonaea viscosa* shootlets to grow on hormone-free media in numerous Dodonaea cultivars demonstrated the habituation impact of TDZ (Abdalla *et al*. 2022). Although the other regeneration media, which solely contained adenine-derived cytokinins, were less effective, the high regeneration efficiency (93%) found with medium-2 in the current experiment is likely due to the action of TDZ.

To simulate various embryonic stages in SEM-2, the somatic embryos developed into translucent spherical to torpedo-shaped. These embryos developed adventitious buds that differentiated into green shootlets, some of which turned green due to the genesis of chloroplasts (Mazri *et al*. 2020). (Figs. 2A, B - C). This study demonstrates that the most efficient method for achieving uniform embryo maturation carried under MS-based maturation medium and coconut water together with a higher concentration of carbon sources and agarose instead of phytagel. Possibly the cytokinins in coconut water accelerated the maturation of somatic embryos (Eisa *et al*. 2022). It is understood that coconut water can cause plant cells to divide and develop quickly when added to a medium containing auxin. Coconut water was also shown to produce the most shoots and roots in Hop bush.

Within 45 days, in regeneration medium-2 the somatic embryos differentiated and produced green shoot primordia that grew into shootlets 1 to 2 cm in height (Figs. 2A - B). After 45 days of subculture on the same medium, the shootlets had grown to a height of 6 to 8 cm (Figs. 2C - D). The root primordia gave rise to roots during this time period. These plantlets were grown for a week in moisture-preserving transparent polyethylene bags, then for two weeks in direct green-house conditions when they reached a height of 10 to 12 cm. The plantlets produced juvenile lanceolate leaves, which are typical of 'Virali' that are vegetative developed plant.

The plantlets were then transplanted into individual (9 x 9 x 36 cm) pots filled with standard pot mixture and irrigated with 0.5x Hoagland's solution. Within three weeks, the plantlets had grown to a height of 15 to 16 cm and were transplanted into the field. The micro propagated plants were morphologically normal and looked similar. At this stage of plant development, no evidence of somaclonal variation was found.

CONCLUSION

This study produced optimized conditions for embryogenic callus induction, establishment of cell suspension culture, somatic embryo induction and development and regeneration for micropropagation of the endangered cultivar 'Virali'. The combined use of three different cytokinins, that is, the diphenyl urea-derived cytokinin TDZ (1.5 mg/l) and adenine-derived cytokinins IBA (0.5 mg/l) and NAA (1 mg/l) appeared to increase the regeneration efficiency of somatic embryos in 'Virali'. A correlation was found between cytokinin levels of the medium and shoot proliferation and subsequent development of plants.

ACKNOWLEDGMENT

The authors are grateful to the Department of Genetics and Plant Breeding, Faculty of Agriculture, Annamalai University, Tamil Nadu for supporting and providing all the necessary facilities to conduct this research.

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