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Callus induction, Indirect Organogenesis and Plantlet Regeneration from different explants of *Vernonia anthelmintica* (L.) Willd

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ABSTRACT

Present study was conducted to standardize callus development and indirect organogenesis from different explants of *Vernonia anthelmintica* (L.) Willd. Among, the various hormonal combinations tested: Indole Acetic Acid (IAA) and BA and IAA and Kinetin combinations were found to be optimum for inducing callusing within a time span of 10 days. Best callus response was observed in 1.5 mg l^{-1} IAA and 1.5 mg l^{-1} BA, which produced white friable callus. Best indirect shoot organogenesis was observed in 4 mg l^{-1} BA and 6 mg l^{-1} kinetin. Elongated shoots when transferred on to half strength Murashige and Skoog (MS) Medium supplemented with Indole-3-butyric acid (IBA), rooting was observed. MS medium fortified with 2 mg l^{-1} IBA showed better rooting than all other concentrations tested. This concentration produced maximum number of roots and maximum percentage of rooting. Plantlets developed by indirect organogenesis of *V. anthelmintica* were successfully acclimatized to field conditions.

1. INTRODUCTION

Vernonia anthelmintica (L.) Willd belongs to the family Asteraceae. Seeds of this plant are known as bitter cumin or "Kalijiri" because of its bitter taste. Other names of this plant include *Centratherum anthelminticum* and *Conyza anthelmintica*. It is an important constituent in the dietary practices of the local communities of India. Medicinal properties of *V. anthelmintica* include curative of ulcers, skin diseases, leucoderma, and fever. This plant is extensively used in Ayurveda for treating cough and diarrhea and also as anthelmintic, stomachic, and antiphlegmatic agent [1,2]. Several studies reported that this plant is pharmacologically active with properties, such as antihelminthic [3], antipyretic [4], larvicidal [5], antihyperglycemic [6], and anti-microbial activities [7]. Plant also contains secondary metabolites: mainly flavonoids, phenolic compounds, tannin, and saponins [8]. Major flavonoids found in *V. anthelmintica* are Butein and 5,6,7,4 tetra hydroxyl flavones [9]. Leaves contain centratherin; a sesquiterpene lactone possessing anti-inflammatory and anti-microbial property [10]. The plant is also reported to possess isorhamnetin [11].

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Callus is an undifferentiated mass of plant tissue produced on growth medium supplemented with appropriate hormones [12]. These undifferentiated tissue contains actively dividing cells, which are able to differentiate and regain their meristamatic properties [13]. As a result, callus is totipotent resulting in the development of root, shoot, flower, stem, etc. This process of formation of plantlets from callus is called indirect organogenesis. Plant hormones added to the culture media enhance the growth and development of plant cells and also enhance the metabolite synthesis [13,14]. Somaclonal variations are spontaneous genetic changes occurring in the callus. They can be promising routes for creating new cultivars and also for isolating cell lines with greater capacity of producing desired metabolites.

Production of secondary metabolites by *in vitro* cultures is usually accomplished by using undifferentiated calli, cell suspension cultures, or organized structures, such as shoots, roots, or somatic embryos [15]. Suspension cultures help to enhance the production of valuable metabolites present in plants. The capacity for plant cell, tissue, and organ cultures to develop and accumulate a valuable chemical compounds same as that of parent plant in nature has been recognized in *in vitro* technology. Stable and optimized callus culture is the logical step as the first phase of the production of plant secondary metabolites in suspension cultures. A relatively friable portion of the callus transferred in the liquid medium under proper conditions not only yield chemical

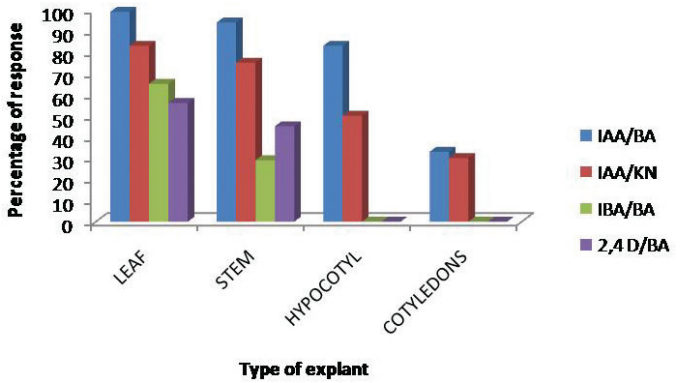

Figure 1: Effect of different explants and growth regulators on callus induction.

Table 1: Effect of different combinations of IAA and BA on callus induction from leaf explants of *V. anthelmintica.*

IAA (mg/l)	BA (mg/l)	Callus induction (%)	Average weight of callus (g)	Number of days required for callus induction
0.5	0.5	70.32 ± 1.75^{d}	1.56 ± 0.33^{c}	16.00 ± 0.66^{cd}
1	0.5	80.60 ± 1.19^{f}	2.20 ± 0.23^{d}	16.00 ± 0.67^{cd}
2	0.5	72.62 ± 1.20^{e}	2.12 ± 0.28^{c}	15.29 ± 0.33^{b}
3	0.5	68.30 ± 0.70^{d}	2.00 ± 0.18^{ab}	15.23 ± 0.31^{bc}
4	0.5	60.00 ± 1.15^{b}	1.68 ± 0.17^{a}	12.66 ± 0.33^{cd}
1	1	82.62 ± 1.00^{f}	1.98 ± 0.18^{ab}	12..68 ± 0.34^{cd}
1.5	**1.5**	**90.99 ± 1.02^{h}**	**3.20 ± 0.16^{e}**	**10.00 ± 0.32^{a}**
0.5	1	73.33 ± 2.85^{g}	2.90 ± 0.20^{bc}	11.00 ± 0.57^{ab}
0.5	2	60.10 ± 2.75^{b}	2.47 ± 0.12^{abc}	13.00 ± 0.37^{de}
0.5	3	56.60 ± 2.84^{b}	1.78 ± 0.12^{a}	14.66 ± 0.33^{f}

Mean values within a column followed by the same letter are not significantly different by Duncan's multiple range test ($p = 0.05$). Values represent mean ± SE of three independent experiments.
The best hormonal concentrations are written in bold.

compounds but also eliminate interfering compounds that occur in the field grown plants [16].

Also, indirect organogenesis from calli leads to somaclonal variations. The formation of organs directly through the callus is an ideal system for selecting somaclonal variants either to enhance the synthesis of secondary metabolite production or to introduce other novel features [17]. Secondary metabolite profiling of callus and somaclones will provide an idea about the metabolite capability of these *in vitro* systems. For this, we need to standardize the protocol for callus induction and plantlet regeneration by indirect organogenesis.

Hence, in the present study, we standardized a protocol for the callus induction, indirect organogenesis, and production of plantlets from leaf explants of *V. anthelmintica*.

2. MATERIALS AND METHODS

2.1. Explant Preparation and Inoculation

Vernonia anthelmintica plants are identified and voucher specimens were deposited at Regional Herbarium, maintained at St Berchmans College, Kerala, India with accession no. 7636. The explants (leaf, stem, hypocotyls, and cotyledons) were initially washed in running tap water for 10 minutes followed by treating with Tween 20, followed by washing with distilled water three to four times for removing any traces of Tween 20. Then these explants were sterilized with 0.1% mercuric chloride (Sisco Research Laboratories, SRL, India) and 70% ethanol for 2 minutes. Washing with sterilized distilled water was repeated in between these treatments for four or five times. These sterilized explant were cut into small segments (1 cm) and inoculated into Murashige and Skoog (MS) media along with different concentration of growth hormones.

2.2. Callus Culture of *V. anthelmintica*

For all *in vitro* studies, Murashige and Skoog basal medium (Himedia, India) with 30 g l^{-1} sucrose, 8 g l^{-1} agar, and various concentrations of plant growth hormones mainly Indole Acetic Acid (IAA) (0.5–4.0 mg l^{-1}) and BA (Benzyl Adenine) (0.5–4.0 mg l^{-1}), IAA (0.5–4.0 mg l^{-1}) and Kinetin (KN) (0.5–3 mg l^{-1}), 2,4 D (2,4 –Dichlorophenoxy acetic acid) (0.5–8 mg) and BA (0.5–8 mg) were used.

The explants after surface sterilization were inoculated into MS media supplemented with various concentration of hormones mentioned above. After inoculation the tubes were labeled and kept on culture racks. The culture was maintained in the culture room 25 ± 2°C with 70% relative humidity and under 16/8 hours photoperiod at a photosynthetic photon flux density (PPFD) of 45–50 µ mol m^{-2} $second^{-1}$ provided by cool white fluorescent light (40W, Philips, India). The callus initiation and percentage of response were recorded. Best hormonal concentrations were selected for further experiments.

Sub culturing was done at every 30 days using same culture condition and fresh medium. After 4 weeks the frequency of callus development and texture of callus were recorded.

2.3. Indirect Organogenesis and Plantlet Regeneration of *V. anthelmintica*

For regeneration of shoot from callus, healthy friable calli were cut into small pieces and inoculated onto MS medium supplemented with different concentrations and combinations of cytokinins, such as BA and kinetin (1.00–6.00 mg L^{-1}). These cultures were maintained at 24 ± 2°C with 16/8 hours photoperiod at a PPFD of 45–50 µ mol m^{-2} s^{-1} provided by cool white fluorescent light (40W, Philips, India).

The developed micro shoots were aseptically transferred into half strength MS media supplemented with various concentrations of Indole-3-butyric acid (IBA) (0.5–3 mg l^{-1}) for root induction.

The rooted plantlets were removed from the culture and washed with sterile distilled water for removing agar in the plantlets. Then, these rooted plantlets were transferred into pot containing sterile soil and covered with polythene bags in order to maintain humidity. After 3 weeks, the polythene bag was removed and plantlets were placed in green house for 1 month and then directly transferred to field conditions.

3. STATISTICAL ANALYSIS

Each experiment consisted of thirty tubes containing thirty explants and each experiment was repeated thrice. Data was expressed as mean ± SE for three replicates. One-way analysis of variance analysis followed by Duncan's multiple range test was used to compare the means. All statistical analyses were performed using SPSS Ver.20.

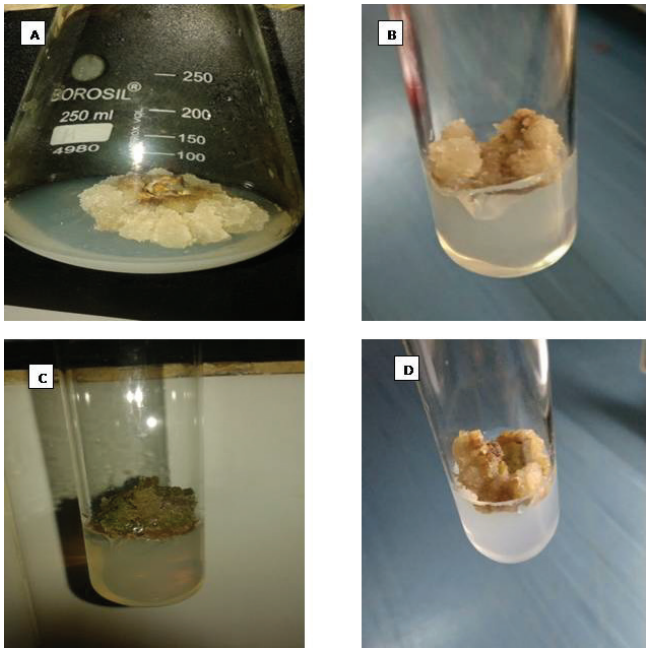

Figure 2: Callus induction in *V.anthelmintica*. (a) Callus formation from leaf explants on medium containing IAA (1.5 mg) and BA (1.5 mg). (b) Callus formation from leaf explants on MS medium containing IAA (4 mg) and Kn (0.5 mg). (c) Callus formation from stem explants on MS medium containing IAA (1.5 mg) and BA (1.5 mg). (d). Green compact calli were obtained from axillary bud on medium containing 3 mg l^{-1} IBA and 3 mg l^{-1} BA.

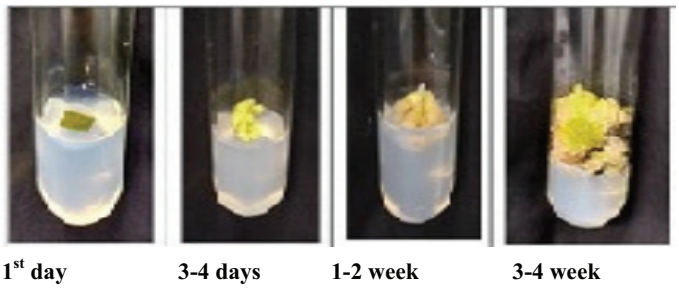

Figure 3: Stages of Callus development.

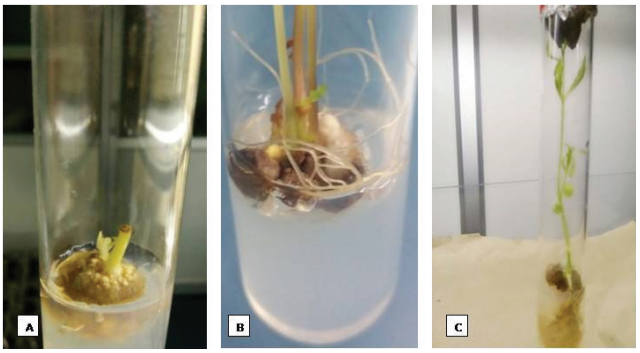

Figure 4: Shoot induction obtained from callus. (A) Shoot induction was observed in 4 mg l^{-1} BA and 6 mg l^{-1} kinetin. (B) Rooted shoots on MS medium containing 2 mg l^{-1} IBA. (C) Plantlet regenerated from callus of *V.anthelmintica.*

4. RESULTS

4.1. Callus Induction from Explants

Explant started callusing after 2 weeks and leaf, stem, hypocotyledon and cotyledon segments were able to produce callus with different concentrations of IAA, IBA, BA, and KN. Leaf explants responded well within 2 weeks of inoculation in comparison to other explants and gave better results. The color and nature of the callus depended on the explant type and plant growth regulators added in the medium. Callus formation occurred at the cut ends and then spread to the entire surface of the leaf explant. The percentage of callus induction was very low in cotyledon and hypocotyl segments. In case of IAA and BA combinations about 99% of the leaf explants showed callus formation followed by stem explant showing 83% callus induction.

Hypocotyl and cotyledon explants showed very low callus induction. IAA and KN combination induced callus in 94% in leaf explant, whereas stem showed only 75% and hypocotyl and cotyledon region showed 29% and 45% callus induction respectively. Plant growth hormones IBA and BA induced callus in 83% of the leaf explants and 50% in stem explants. However, hypocotyl and cotyledon explants failed to develop callus in theses hormonal combinations. In case of 2, 4 D, and BA combinations, leaf and stem explants exhibited very low percentage of callus induction (33% and 30%, respectively). Here also, cotyledon and hypocotyl region failed to show any callus induction (Fig. 1).

Medium containing IAA and BA combinations produced highly proliferating callus. Best callusing was observed in a MS medium containing IAA (1.5 mg l^{-1}) and BA (1.5 mg l^{-1}) which produced friable creamish white callus (Table 1). IAA and Kinetin combination also produced callus in 4 mg l^{-1} IAA and 0.5 mg l^{-1} Kinetin (Table 2). It was observed that IAA and BA combinations are more suitable for callus induction in this plant (Fig. 2). Stem explants produced loose creamish brown callus. Also, when leaves are used as explants the callus growth was faster compared to other explants, namely, stem, cotyledon, hypocotyl and root.

The texture, type, and stages of callus depended on explants, plant growth regulators, and their various combinations. In this study, two different types of calli were obtained. One is highly proliferating, yellow whitish, soft, friable calli which is designated as Type 1 and another one is green compact calli represent Type II calli (Fig. 3). Leaf segments predominantly produced soft friable type 1 callus.

4.2. Shoot Induction

The callus obtained from the leaf explants were transferred to shoot inducing medium and observed the percentage of shoot proliferation and total number of shoots per culture. The highest shoot induction was observed in MS medium containing 4 mg l^{-1} BA and 6 mg l^{-1} kinetin (Fig. 4 and Table 3).

4.3. Root Induction from Callus

Callus was rooted on MS medium supplemented with various concentration of IBA (Fig. 5). Significant rooting was reported on MS medium with 2 mg l^{-1} IBA (Table 4). Roots appeared to be white long and tuberous.

4.4. Rooting and Acclimatization

Once the microshoots were produced, they were separated and transferred to the rooting medium. Various concentrations of

Table 2: Effect of different combinations of IAA and Kinetin on callus induction from leaf explants of *V. anthelmintica.*

IAA	KN	Callus induction (%)	Average weight of callus (g)	No. of days required for callus induction
0.5	0.5	60.66 ± 1.40^{e}	1.72 ± 0.09^{bcd}	16.33 ± 0.88^{bc}
1	0.5	65.20 ± 1.20^{f}	1.89 ± 0.09^{d}	16.32 ± 0.89^{bc}
2	0.5	71.66 ± 1.45^{e}	2.00 ± 0.16^{e}	15.00 ± 0.57^{cd}
3	0.5	80.00 ± 1.52^{d}	2.90 ± 0.17^{a}	14.32 ± 0.88^{d}
4	**0.5**	**89.73 ± 1.52^{g}**	**3.30 ± 0.26^{f}**	**12.00 ± 0.33^{a}**
1	1	79.66 ± 2.02^{e}	2.24 ± 0.18^{de}	14.32 ± 0.66^{d}
1.5	1.5	72.33 ± 1.30^{e}	2.08 ± 0.08^{cde}	13.32 ± 0.33^{e}
0.5	1	65.32 ± 0.88^{f}	1.52 ± 0.41^{ab}	15.62 ± 0.66^{cd}
0.5	2	60.20 ± 1.15^{b}	1.54 ± 0.30^{ab}	16.66 ± 0.33^{d}
0.5	3	56.66 ± 1.10^{a}	1.39 ± 0.16^{a}	17.00 ± 0.58^{d}

Mean values within a column followed by the same letter are not significantly different by Duncan's multiple range test (p = 0.05). Values represent mean ± SE of three independent experiments.

The best hormonal concentrations are written in bold.

Table 3: Influence of BA and Kinetin on shoot induction from callus culture of *V. anthelmintica*.

BA	KN	Percentage of callus induction	Total number of shoots
1	1	20	2.2 ± 0.40^{a}
1	2	23	2.6 ± 0.41^{ab}
1	4	25	2.6 ± 0.42^{ab}
1	6	30	3.3 ± 0.79^{b}
2	1	35	4.4 ± 1.60^{c}
2	4	42	6.2 ± 0.88^{bc}
2	6	50	7.2 ± 0.20^{d}
3	2	58	8.6 ± 1.15^{e}
3	4	60	9.2 ± 0.88^{e}
3	6	55	6.1 ± 0.98^{cde}
4	2	63	7.6 ± 0.52^{abc}
4	4	72	6.8 ± 0.98^{cd}
4	**6**	**87**	**12.2 ± 1.27^{f}**
5	2	75	7.9 ± 0.88^{bc}
5	4	72	6.4 ± 0.51^{abc}
5	6	35	4.2 ± 1.70^{cde}

Mean values within a column followed by the same letter are not significantly different by Duncan's multiple range test (p = 0.05). Values represent mean ± SE of three independent experiments.

The best hormonal concentrations are written in bold.

IBA were tested for their effect on root induction. The medium supplemented with 1.5 mg l^{-1} of IBA induced optimum root induction within 20 days (Fig. 4c and Table 5). *In vitro* developed plantlets were acclimatized and transferred to soil with a survival rate of 14%–15% (Fig. 6).

5. DISCUSSION

Plants generally contain many pharmaceutically important molecules: but, large-scale production of these molecules is a real challenge. Here, *in vitro* culture comes to our aid. Tissue culture helps to rapidly multiply, conserve, and use the medicinal plants in a sustainable manner. Development of callus can pave way to secondary metabolite production. Also, it will help to develop a protocol for indirect organogenesis for the development of elite somaclones with better biosynthetic ability. Furthermore, it can serve as a starting point for suspension culture.

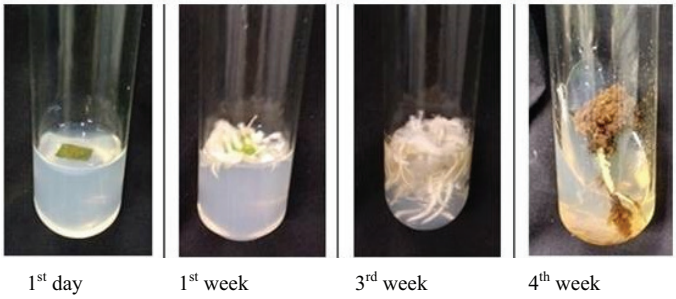

Figure 5: *In vitro* rooting on MS medium.

Table 4: Influence of IBA on root induction from callus.

IBA	Percentage of root induction	Number of roots	Nature of root
0.5	48.32 ± 0.71^{c}	6.61 ± 0.32^{a}	White tuberous
1	50.33 ± 0.88^{c}	6.62 ± 0.33^{a}	"
1.5	77.66 ± 1.00^{g}	12.66 ± 0.88^{c}	"
2	**83.33 ± 0.88^{h}**	**18.66 ± 0.33^{d}**	"
2.5	70.31 ± 1.8^{f}	10.00 ± 0.58^{b}	"
3	63.32 ± 1.20^{d}	7.00 ± 0.57^{c}	"

Mean values within a column followed by the same letter are not significantly different by Duncan's multiple range test (p = 0.05). Values represent mean ± SE of three independent experiments.

The best hormonal concentrations are written in bold.

Table 5: Influence of various concentration of IBA on root induction from shoots of *V. anthelmintica*.

IBA	Percentage of root induction	Number of roots	Nature of roots
0.5	51.32 ± 0.87^{ab}	5.66 ± 0.32^{a}	White tuberous
1	61.60 ± 1.30^{c}	11.60 ± 0.80^{c}	White tuberous
1.5	62.61 ± 1.29^{c}	11.65 ±0.88^{c}	White tuberous
2	**77.45 ± 0.88^{d}**	**15.66 ± 0.88^{d}**	White tuberous
3	55.63 ± 1.46^{a}	9.00 ± 0.57^{a}	White tuberous

Mean values within a column followed by the same letter are not significantly different by Duncan's multiple range test (p = 0.05). Values represent mean ± SE of three independent experiments.

The best hormonal concentrations are written in bold.

Plant growth regulators play an important role in tissue culture for the development of callus and organ formation [18]. From this study it was clear that leaf explants show better callus response compared to other explants. As expected auxin and cytokinin combinations produced callusing and high concentration of cytokinin and low concentration of auxins resulted in shoot induction. Similarly, high concentration of auxins in the media resulted in root induction.

Callus morphology (white friable and green compact) and texture of callus depend on type of growth regulators added in the culture media. Different hormonal combinations produced different types of callus. In *Allium chinense*, it was found that different hormone combinations produced morphologically different callus [19]. In the present study, among the various hormonal combinations

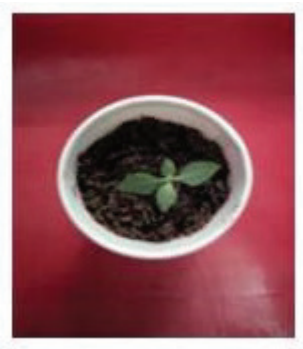

Figure 6: *In vitro* raised plants. Rooted plants growing in plastic cup with soil and sand.

tested IAA and BA and IAA and KN were found to be optimum for inducing calli within 10 days. Also, IAA and BA were able to produce white friable callus which can be further exploited for suspension culture.

Low IAA and high BA was found to be favorable for shoot development. Several studies reported that BA is good for shoot development [20–22]. IAA and Benzyl adenine (BA) combination resulted in shoot regeneration in *Zehneria scabra* [23]. Here also, it was observed that BA and kinetin helped in shoot induction. BA was also found to be effective in shoot multiplication. In other plants like *Musa* species, BA helped in the induction of shoot buds [24]. Also, in *Helicteres isora,* plant regeneration *via* shoot organogenesis from callus culture has been established using nodal explants cultured on MS medium containing BA and Kinetin [25].

On transferring the elongated shoots to half strength, MS Medium supplemented with IBA rooting was observed. IBA acts as the best rooting hormone in a number of plant species. Maximum number of roots and maximum percentage of rooting was observed with IBA. IBA showed best rooting in *Quercus leucotrichopora* [26]. An increased frequency of roots and number of roots per shoot were observed in *Vigna radiata* when the medium was supplemented with IBA [27]. Also, in *Citrus reticulata* the combination of IAA and IBA showed highest frequency of rooting [28].

After 4 weeks, the regenerated plantlets were washed to remove the adhering culture medium and successfully hardened in the culture room with sterilized planting substrates for 3 weeks, and transferred to soil. No previous reports on callus development from leaf explant and plantlet regeneration from callus was reported in this plant. Callus development and organogenesis are efficient and alternative methods for the production of valuable secondary metabolites with pharmaceutical relevance. Phytochemicals can be directly extracted from the calli without sacrificing the entire plant. Also, callus development helps to protect rare and endangered plant species by producing secondary metabolites *in vitro*. Also, callus can be converted to single cell suspension cultures in order to produce the desired secondary metabolites. In this work, we established a protocol for generating friable calli from *V. anthelmintica*; for setting up cell suspension culture and indirect organogenesis for the production of elite somaclones.

CONFLICT OF INTEREST

Authors declare that they do not have any conflicts of interest.

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