RESEARCH ARTICLE

Indirect Organogenesis from Stem Derived Callus of Dregea Volubilis (L.F) Benth

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Abstract

In vitro propagation methods offer a powerful tool for germplasm maintenance and multiplication. This report established the methodology for callus regeneration of *Dregea volubilis* from stem explants. The effect of various concentrations and combinations of IAA, NAA, 2,4-D, BAP, KIN and IBA were tested for *in vitro* callus induction, plant regeneration, and rooting of *D. volubilis*. The maximum number of greenish, organogenic callus was achieved on the MS medium containing 1.5 mg/L 2,4-D+ 0.5 mg/L KIN and then subsequent shoot regeneration was obtained on MS medium supplemented with 3.0 mg/L KIN from stem derived callus. The highest frequency of shoot regeneration (81.93%) with the maximum number of shoots (10.56 ± 0.44) was achieved in this medium. *In vitro* raised micro shoots showed a 90% response with the maximum number of roots (5.31 ± 0.66) per shoot on MS medium supplemented with 2.5 mg/L IBA after 15 days. Then *in vitro* rooted plantlets were moved to mud pot bearing soil and vermiculite mixture in 1:1 ratio for hardening and hardened plantlets showed 90% survivability in field conditions. Using this protocol, it is possible to clonally produce viable, uniform and healthy plants with a maximal survival rate that can be used for large scale cultivation, genetic transformation and biopharmaceuticals applications.

Keywords: Callus, *Dregea volubilis*, Hardening, *In-vitro* organogenesis.

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Introduction

Medicinal plants and herbs have been used for healing purposes and maintaining good health. Herbal healing has been sustained over a long period since ancient times. There are numerous evidences that herbs and plants have been used to treat diseases and revitalise body systems in all ancient civilizations like Indian, Chinese, Roman etc., (Shawl and Qazi, 2004). About 90% of medicinal plants used by the industries are collected from the wild. Many medicinal plant species are disappearing at an alarming rate due to rapid agricultural and urban development, uncontrolled deforestation and indiscriminate collection. Hence, the conservation of these valuable genotypes is imperative (Nalawade and Tsay, 2004). Plant tissue culture technology holds great promise for conservation and enhancement of valuable secondary plant products' natural levels and to meet pharmaceutical demands and reduce the *in situ* harvesting of natural forest resources. There are sufficient reports available for *in vitro* micropropagation of many threatened medicinal species (Mendelsohn and Balick, 1994).

Dregea volubilis (L.F) Benth is an essential medicinal woody climber belonging to the family Asclepiadaceae. It is broadly used in Indian conventional remedy and the leaf paste to treat rheumatic pain, cough, fever and severe cold (Muthu *et al.,* 2006 and Rajadurai *et al.,* 2009); leaf paste is taken along with pepper to treat dyspepsia (Pandikumar *et al.,* 2007); bark paste, mixed with hot milk is used internally for treating urinary troubles (Silija *et al.,* 2008) and leaf powder is taken orally along with cow's milk have antidiabetic activity (Ayyanar *et al.,* 2008). The stems and leaves contain a pigment taraxerol, a triterpenoid, kaempferol, a glycoside of kaempferol and saponins (Sauer *et al.,* 1965).

Traditionally, *D. volubilis* propagates through seeds, but poor seed viability is the most important natural breeding restriction and vegetative stem cuttings are too difficult. Hence, it is necessary to device a method for the development of a potentially large scale multiplication protocol for the commercial production of this endangered species. *In vitro* propagation methods offer a powerful tool for germplasm maintenance and multiplication. Earlier, several plant parts of *D. volubilis* have been investigated for their chemical constituents (Reddy *et al.,* 2002; Sahu *et al.,* 2002; Yoshimura *et al.,* 1983) and only limited progress has been made on *in vitro* regeneration. In a previous report, we have described the effect of explants source on axillary shoot multiplication of *D. volubilis* using nodal explants (Yogananth *et al.,* 2011). Other regeneration protocols also reported nodal explants in *D. volubilis* (Chakradhar and Pullaiah, 2006; Arulanandam *et al.,* 2011; Vinothkumar *et al.,* 2011) and leaf (Chakradhar and Pullaiah, 2014). There is no published information on the stem callus regeneration of *D. volubilis*.

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Hence, this report established the methodology for callus regeneration of *D. volubilis* from stem explants.

Materials and Methods

The young stem was collected from a mature field grown healthy plant of *Dregea volubilis* maintained in the green house at JJ College of Arts and Science, Pudukkottai, Tamil Nadu, India and thoroughly washed with running tap water. Next, the explants treated with a few drops of Tween-80 for 10 minutes with constant shaking, followed by washing with sterile distilled water until the explants completely free from Tween -80. Subsequently, the explants were sterilized with 70% ethanol for few seconds and washed with sterile distilled water for 3-4 times. Next, the explants were brought to the inoculation chamber and disinfected with 0.1% $HgCl_2$ for 2 minutes and washed with sterile distilled water 3-5 times. Then, the sterilized explants were injured all over the surface and used for further study.

MS basal medium was used for shoot proliferation and adventitious shoot regeneration and half strength of MS was used for *in vitro* rooting. All media were supplemented with 30 g/L sucrose, 8.0 g/L agar and dispensed into 15 × 150 mm culture tubes. The pH of the media was adjusted to pH 5.8 before autoclaving at 121°C for 20 min. All the inoculated cultures were incubated in the growth room in controlled conditions at a temperature of 25 ± 2°C, 16 h light/8 h dark photoperiod and continuous illumination provided by cool white fluorescent tubes 3000 lux

For callus induction, the excised explants were cultured on MS (Murashige and Skoog, 1962) medium augmented with different concentrations of auxins like IAA, NAA and 2, 4-D (1.0, 1.5, 2.0, 2.5 and 3.0 mg/L) used alone along with one of cytokinins i.e., KIN or BAP (0.50 mg/L). The well-developed calli were transferred to shoot induction MS basal medium supplemented with various concentrations of BAP (1.0 to 5.0 mg/L) or KIN (1.0 to 5.0 mg/L). Well grown shoots were transferred to half- strength MS medium supplemented with IBA (1.0 to 3.0 mg/L) or hormone-free medium for root induction. Regeneration plantlets were transferred to pots containing autoclaved soil, sand and manure mixture in the ratio of 1:1:1 for 30 days under green house conditions and subsequently transferred to potting soil. All experiments were performed in triplicate. The data obtained were analysed based on one way analysis of variance test (ANOVA) and the means compared using Duncan's multiple range test (DMRT).

Results

The young stem explants collected from mature field grown healthy plant of *D.volubilis* were surface sterilized and inoculated on callus induction medium containing various auxins like IAA, NAA, and 2, 4–D (1.0-3.0 mg/L used alone in combination with KIN or BAP (0.50 mg/L). When the young stem explants cultured on callus induction medium, the callus induction frequency and callus growth varied the auxins and cytokinins treatments. In stem explants, callus formation was first initiated within 7 days.

Effect of IAA with KIN or BAP on callus induction

The highest percentage of callus induction (83.09 ± 1.46 %) was observed in MS medium containing 2.0 mg/L of IAA+0.50 mg/L KIN and the lowest percentage of callusing (64.53 ± 0.54 %) found in 1.0 mg/L IAA and 0.50 mg/L BAP combination. The highest callus growth in terms of fresh and dry weight (2.99 ± 0.37 g and 0.26 ± 0.04 g) was obtained in MS medium fortified with 2.0 mg/L of IAA+ KIN 0.50 mg/L. The minimum growth rate of 1.26 ± 0.39 g fresh weight and 0.12 ± 0.03 g dry weight was obtained in 1.0 mg/L IAA and 0.50 mg/L BAP combination (Fig. 1).

Effect of NAA with KIN or BAP on callus induction

The highest percentage of callus induction (78.70 ± 0.92 %) was observed in MS medium containing 1.50 mg/L of NAA+0.50 mg KIN. The highest callus growth in terms of fresh and dry weight (2.62 ± 0.33 g and 0.25 ± 0.03 g) was observed in MS medium fortified with 2.50 mg/L of NAA+ BAP 0.50 mg/L. The lowest growth rate of fresh weight and 0.11 ± 0.03 g dry weight was obtained in 3.0 mg/L NAA and 0.50 mg/L KIN combination (Fig. 2).

Effect of 2, 4-D with KIN or BAP on callus induction

Among the different concentrations of 2, 4-D used along with KIN or BAP combination, maximum callusing (85.03 ± 0.17 %) was observed in 2,4-D 2.50 mg/L and KIN 0.50 mg/L concentration. Whereas the minimum callus response (72.10 ± 1.05 %) was obtained in 2, 4-D 1.0 mg/L used along with KIN 0.50 mg/L combination. The maximum fresh and dry weight (3.47 ± 0.50 g and 0.28 ± 0.01 g, respectively) was found in basal medium supplemented with 2, 4-D 2.50 mg/L and KIN 0.50 mg/L. Minimum fresh and dry weight was obtained in 1.0 mg/L 2, 4-D and 0.50 mg/L BAP combination (Fig. 3 and Fig 4).

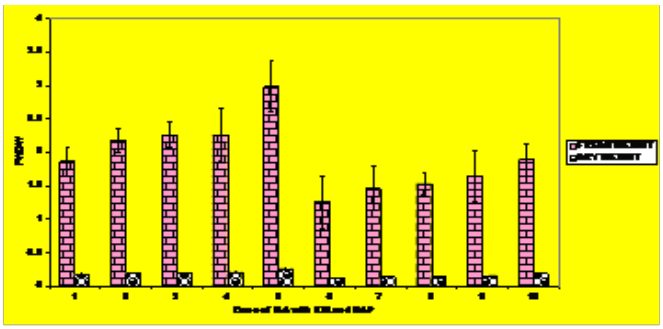

Fig. 1: Effect of IAA on Callus induction, Callus Growth of young stem explants of Dregea volubilis (L.F.) in combination with KIN and BAP

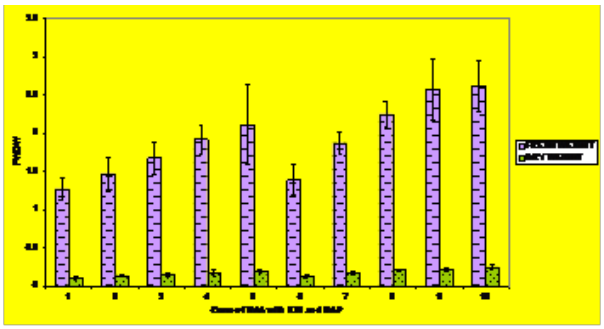

Fig. 2: Influence of NAA on Callus induction, Callus Growth of young stem explants in combination with KIN and BAP

Effect of BAP and KIN on Shoot Induction

The multiple shoot buds can be regenerated when calli were incubated on MS medium supplemented with different concentrations of either of KIN (1.0 – 5.0 mg/L) or BAP (1.0 – 5.0 mg/L) in various concentrations. Initially two or three shoot buds per callus were emerged within 15 days after inoculation and gradually the number of shoot buds per explant increased up to 10.56 ± 0.44 on MS medium fortified with 3.0 mg/L KIN alone. The percentage of response was varied from 55.40 ± 0.89^{a} – 72.79 ± 0.31^{e} % in different concentration of BAP. Nevertheless, the same tendency was highly influenced by varying concentrations of KIN. The maximum percentage of response was recorded in 3.0 mg/L of KIN (81.93 ± 0.62%). The highest number of multiple shoots (10.56 ± 0.44^{e}) was noticed in KIN used at the concentration of 3.0 mg/L followed by 8.53 ± 0.28 shoots in BAP 3.0 mg/L. Among the various concentrations of the BAP (1.0 - 5.0 mg/L) the lowest average number of shoots 2.26 ± 0.35^{a} was recorded in 1.0 mg/L of BAP followed by 3.29 ± 0.48^{a}shoots in 1.0 mg/L KIN. Further increase in the concentrations of KIN and BAP did not show any improvement in term of multiple shoot induction (Table 1, Fig. 5a,b,c).

Effect of IBA on Root Induction

The simultaneous root induction was observed in few cultures of callus derived shoots. After 30 days of incubations, well developed shoots were transferred to root induction medium containing half strength MS medium supplemented withB5 vitamins and IBA (1.0 – 3.0 mg/L). Maximum percentage of root induction was observed in 2.50 mg/L of IBA (Table 2 and Fig 5d). Further increase in the concentrations of IBA in rooting media did not show any improvement in rooting and tend to produce poor quality rooting.

Hardening and Field Establishment

The well developed rooted plantlets were hardened and grown in potted soil. For hardening and initial growth, plantlets were first grown in a mixture autoclaved garden soil, sand and manure in the ratio of 1:1:1 each for 30 days under controlled greenhouse conditions and then transferred to the field for further growth. Plantlets derived after *in vitro* rooting showed 90% survival in field conditions. The plantlets transferred revived growth after 9 days and grew well in field (Figure 5e). The field established plants were identical in morphology and growth pattern to that of mother plant.

Discussion

Generally, the plant tissue culture media containing high auxin and relatively low cytokinin concentrations promote cell proliferation resulting in callus formation (Slater *et al.,* 2003). In present callus induction study, the cytokinin concentration used was restricted to 0.50 mg/L because the higher concentrations of cytokinin minimized the callus formation and leads to shoot

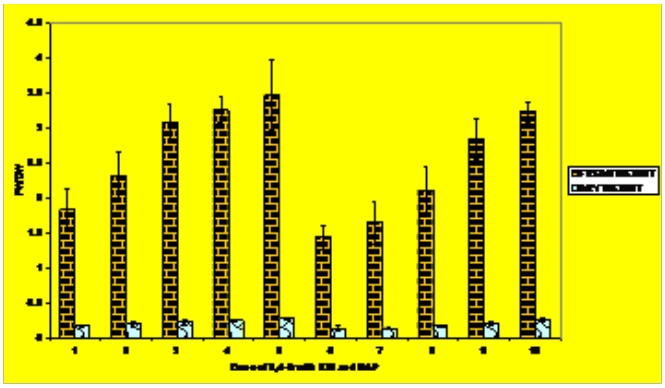

Fig. 3: Role of 2, 4-D on Callus induction, Callus growth of young stem explants in combination with KIN and BAP

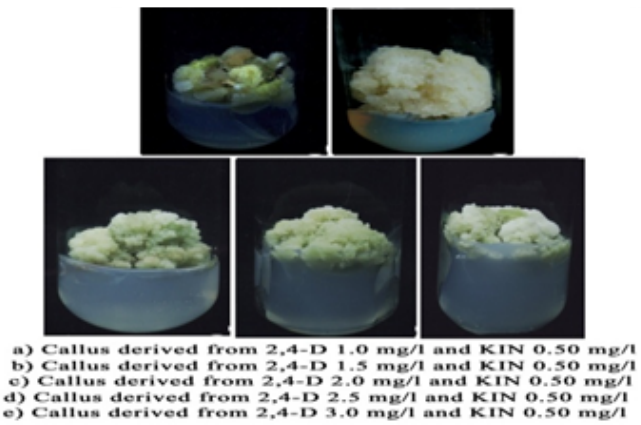

Fig. 4: Effect of 2, 4-D with KIN on callus induction from stem exp.

Table 1: Effect of KIN and BAP on multiple shoot induction (Values with the same superscript are not significantly different at P> 0.05 level according to the DMRT test)

Hormones mg/L	*% of response*	*No of shoots/callus*
	KIN	
1.0	56.96 + 0.76^{a}	3.29 + 0.48^{a}
2.0	72.38 + 0.19^{b}	5.80 + 0.12^{b}
3.0	81.93 + 0.62^{e}	10.56 + 0.44^{e}
4.0	76.33 + 0.78^{d}	8.02 + 0.15^{d}
5.0	70.72 + 0.17^{c}	7.24 + 0.51^{c}
	BAP	
1.0	55.40 + 0.89^{a}	2.26 + 0.35^{a}
2.0	61.46 +0.65^{b}	4.83 + 0.15^{b}
3.0	72.79 + 0.31^{e}	8.53 + 0.28^{e}
4.0	68.93 + 0.44^{d}	6.15 + 0.56^{d}
5.0	65.03 + 0.28^{c}	5.82 + 0.34^{c}

Table 2: Effect of IBA on root induction (Values with the same superscript are not significantly different at P> 0.05 level according to the DMRT test)

IBA (mg/L)	*% of response*	*No of roots/shoot*
1.0	52.86 + 0.57^{a}	2.12 + 0.36^{a}
1.5	60.33 + 0.14^{b}	2.90 + 0.24^{b}
2.0	70.90 + 0.34^{c}	3.69 + 0.40^{c}
2.5	75.33 + 0.14^{e}	5.31 + 0.66^{e}
3.0	72.96 + 0.12^{d}	4.76 + 0.29^{d}

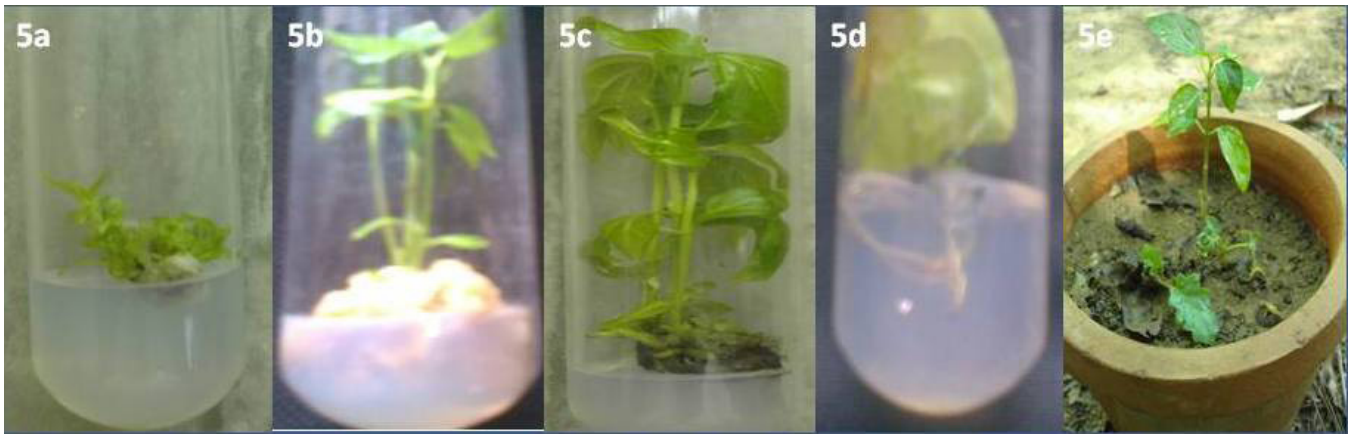

Fig 5: Stagewise development of multiple shoot induction from stem callus of D. volubilis
(5a) Shoot bud development; (5b) Multiple shoots in earlier stage; (5c) Well developed multiple shoots(5d) Rooted plantlets; (5e) Hardening.

initiation. The callus culture was established from shoots taken from field grown plants of *D. volubilis* by varying concentrations of auxins like IAA, NAA and 2, 4-D used alone in combination of KIN or BAP. Similarly, callus induction through auxin–cytokinin combination has been reported in several other Asclepiadaceae plants, including *Tylophora indica* (Singh *et al.,* 2009), *Pergularia daemia* (Vinothkumar *et al.,* 2010), *Sarcostemma brevistigma* (Palanivel *et al.,* 2013), and *Oxystelma esculentum* (Devimaliga and Yogananth, 2016). In our study, the 2, 4-D with KIN was more effective for callus induction than IAA and NAA as a source of auxin. Results of effects of various combinations of 2, 4-D and KIN on callus growth as measured by fresh weight and dry weight of callus of *D. volubilis* showed increasing effect of 2,4-D as its concentrations increases which is in agreement with prior reports (Brunakova *et al.,* 2004; Karimian *et al.,* 2014).

In addition, the regeneration capacity through indirect shoot organogenesis from stem derived callus was also investigated. The young stem derived calli were highly viable, whereas the callus derived from the leaf was soft and could not be maintained beyond a second or third sub cultures as reported earlier (Rao and Narayanaswamy, 1972; Patil, 1998). In this study, the shoot induction was initiated at all concentrations of KIN and BAP used alone. The KIN was found more effective than BAP for the induction of multiple shoots from stem callus of *D. volubilis*. An earlier showed that MS medium supplemented with 3.0 mg/L of kinetin produce 80 % shoot bud initiation response after 9 days of inoculation (Ali *et al.,* 2013). However, Tadhani *et al.,* 2006 reported that 4.0 mg/L of kinetin showed maximum shoot formation response.

IBA is a synthetic auxin widely used to initiate root growth in many commercial species (Ludwig-Muller *et al.,* 2005). It may be due to it's decelerate interchange and degradation which assist its localization near the site of application and thus find its ability in root induction (Nickell and KirkOthmer, 1982). In present study, rooting of the young microshoots was achieved on MS medium fortified with 1.0- 3.0 mg/L IBA. Roots were initiated from the base of the shoots after 3 weeks of culture. A high rate (75%) with the highest number of roots (5.3) was obtained on 2.5mg/L IBA after 15 days of inoculation. Similar *in vitro* root responses was reported in *Hemidesmus indicus* (Sreekumar *et al.,* 2000), *Ceropegia candelabrum* (Beena *et al.,* 2003), *Tylophora asthmatica* (Mukundan *et al.,* 2002) and *Wattakaka volubilis* (Vinothkumar *et al.,* 2011).

The plantlets with well-proportioned shoots and roots were transferred from *in vitro* conditions to autoclaved soil, sand and manure mixture (1:1:1) containing mud pots which were initially kept in the controlled culture room conditions for hardening and finally move to the natural environment in the field conditions. The survival rate of rooted plants were 90% and do not produce any visible morphological variation or any change of growth characteristics when grown in field conditions.

Conclusion

To our knowledge this is first report of plant regeneration *via* indirect organogenesis of *D. volubilis* from callus derived from stem explant. We also demonstrated a simple and reliable efficient protocol for callus induction and plantlet regeneration of *D. volubilis* from stem callus with successful acclimatization of *in vitro* rooted plantlets. Using this protocol, it is possible to produce viable, uniform and healthy plants with a maximal survival rate that could be used for large scale cultivation, genetic transformation and biopharmaceutical applications.

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