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**RESEARCH ARTICLE**

# *In vitro* callogenesis from the mature explants of *Croton scabiosus* Bedd. (Euphorbiaceae)

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**ABSTRACT**

*Croton scabiosus* Bedd. (Euphorbiaceae), an endemic tree of southern Andhra Pradesh, India is known to represent a small population and found primarily affected by recurrent fires and secondarily by seed pathology and poor germination in the natural habitat warranting immediate conservation attempts. With an objective to propagate the species *in vitro* through somatic embryogenesis, an attempt has been made to develop a protocol for callogenesis by using different explants collected from natural population. The explants were cultured on different media (MS, $B_5$, and WPM) containing 2% sucrose, 0.8% agar-agar and different concentrations of plant growth regulators. After sterilization callus induction was tested with leaves, internodes and petioles using 2, 4-D, 2, 4 5-T, DICAMBA and NAA in different concentrations. All the plant growth regulators except NAA are found to induce callus proliferation. The maximum callus proliferation by inter nodes was observed on MS medium supplemented with DICAMBA 0.5 mg/l. This is the first successful attempt to establish consistent callus formation from inter nodal segments of *Croton scabiosus* and this procedure can be developed as an *in vitro* regeneration protocol for multiplication of plantlets.



## INTRODUCTION

Most of the plant species do not have potential regenerative capacity in natural conditions because of various extrinsic and intrinsic factors. Different techniques for conservation of plants have been practiced worldwide; most importantly the tissue culture (Parabia *et al*., 2007) in this context, *In vitro* culture of plants has gained importance during recent years, because this technique can be used as *ex situ* conservative measure, by rapid multiplication of plantlets in a limited time boundary. Callus induction from axillary bud segments in *Michelia champaca* was successfully established in this context (Abdelmageed *et al*., 2012). Micropropagation has been a great potential tool to overcome problems related with the field culture for such plant species (Hidaka and Omura, 1989). Members of Euphorbiaceae are well known either for their medicinal value or with the ornamental value. Several workers have been succeeded by working on members of Euphorbiaceae pertaining to micropropagation; Elizabete Catapan *et al*., (2001) on *Phyllanthus stipulatus*, a well known medicinal plant; Wataru Shibata *et al*., (1996) on *Croton sublyratus*. Afaque Quraishi *et al*., (1996) on *Cleistanthus collinus*. *In vitro* propagation of ornamental plants gained importance, and multiplication of such species was successful in *Codieum variegatum*, (Asma Nasib *et al*., 2008) and *Euphorbia pulcherima* through somatic embryogenesis (Yogesh *et al*., 2003). *Croton scabiosus* Bedd., the candidate species for the present study is a small tree known locally with potential medicinal value as an antidote. It is endemic to southern Andhra Pradesh and currently known to represent a small population with scattered distribution in dry deciduous forests (Balakrishnan and Chakrabarthy, 2007). During explorations, our interaction with local herbal healers has revealed that the plant seeds are used as an antidote for snake bite and scorpion sting. Different vegetative parts of the species found to have a diversity of secondary metabolites - alkaloids, coumarins, glycosides, flavonoids, steroids, and Gallic tannins (Sarojini Devi *et al*. 2011). As *Croton scabiosus* is having very attractive silvery or golden foliage (Neginhal, 2004), this species is suitable for cultivation as an ornamental tree in gardens. But the population is found primarily affected by recurrent fires and secondarily by seed pathology and poor germination in the natural habitat warranting immediate conservation attempts. Different techniques for conservation of plants have been practiced worldwide; most importantly the tissue culture (Parabia *et al*., 2007) in this context, *In vitro* culture of plants has gained importance during recent years, because this technique can be used as *ex situ* conservative measure, by rapid multiplication of plantlets in a limited time boundary. Callus induction from axillary bud segments in *Michelia champaca* was successfully established in this context (Abdelmageed *et al*., 2012). With an objective to propagate the species *in vitro* through somatic embryogenesis for augmenting the existing population as well introducing the species in allied habitats we attempted to develop a protocol of callus induction with different explants. The main purpose of this study, callus induction from mature explants was to establish the suitable protocol for the multiplication of plantlets by micro propagation with somatic embryos formed from callus.

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## MATERIALS AND METHODS

Lab experimentation was carried out in Plant Tissue Culture Laboratory, Department of Botany, Sri Krishnadevaraya University, Anantapur, and Andhra Pradesh-India.

### Materials

*Croton scabiosus* Bedd. belongs to family Euphorbiaceae (APG III, 2009). It is a small deciduous tree; grow up to 5-7m; characteristically, leaves on both surfaces and inflorescence clothed

with subsilvery lepidote scales; stamens 8-12. capsules globose or sub-globose, usually 3- lobed.

**Explants source**

The source explants for *in vitro* callogenesis were collected from the representative population in Chinna Palli forest (N - 14° 10' 40", E - 70°13' 23.6"; elevation 570m-648m) of Anantapur district, Andhra Pradesh State, India.

**Callus induction media**

Along with 2, 4-D, (various concentrations), we have employed different concentrations (varying from 0.5 mg/l to 4 mg/l) of 2, 4 5-T, NAA, and DICAMBA for callus induction from different explants of *Croton scasbiosus* Bedd. MS basal medium (Murashige and Skoog, 1962) was prepared by adding 2% of Sucrose (Merck, India) as stable carbon source. The $P^H$ was adjusted to 5.6-5.8, and then 0.8% of agar-agar (Hi-media, India) was added and melted at $100^0$ c for 2-3 minute. Then the media was poured into the culture tubes and autoclaved at $121^0$c for 15 minute at 15 lbs pressure. After the sterilization of media, the culture tubes were placed in a cool chamber for solidification.

**Surface sterilization of explants**

Fresh and healthy growing juvenile plant parts were collected for explants source. The tender leaves, petioles, inter-nodes were taken for surface sterilization. The plant material was thoroughly washed with running tap water for more than 15 minutes. After adding 2-3 drops of Labolien as surfactant, the material was washed thoroughly with running tap water and then with double distilled water. The remaining sterilization procedure was carried out in Laminar Air flow cabinet. Then explants material was washed with sterilized double distilled water in order to remove all the remnants of the surfactants from the surface of the explants. Sterilization of plant material was tested with three different sterilants: Mercuric Chloride ($Hgcl_2$) at 0.1%, 0.3%, 0.5%, 1.0% concentrations; Sodium Hypochlorite (Naocl) at 5%, 10%, 20%, w/v and Hydrogen Peroxide ($H_2O_2$) 10% and 15%, 30%. Among the sterilants used, Mercuric Chloride at 0.1% showed best results in preventing microbial contamination and hence this concentration was used for sterilization. Since the source material was collected from natural population, the contamination rate may be high though the sterilant is used. Hence 70% alcohol was used for complete sterilization for about 60 seconds. Finally the plant material was rinsed with autoclaved double distilled water for more than 4-5 times.

**Inoculation and incubation**

The plant material was taken out from Erlin Meyer's bottle and transferred on to the sterilized Petri plates having autoclaved filter papers. Leaves, internodes, and petioles selected as explants. The explants were trimmed into small pieces in 2 cm diameter with sterilized forceps and surgical blades, and the excised explants were inoculated on to the full strength MS medium (Murashige and Skoog, 1962) in culture tubes (Borosil Make). The culture tubes were incubated at $25^0$c±$1^0$c with 16 hrs photoperiod and 8 hrs of darkness at 2000 lux light intensity of cool white fluorescent light (Philips Make). All the experiments were repeated for 4 times and the results were recorded after 30 days of inoculation.

**Usage of auxins for callus induction**

The employment of plant growth regulators (auxins) is very much needy in callogenesis of any plant species (Vietez and San-jose, 1996). Hence for assessing the effect of auxins on callus induction, in the present study, the explants was cultured on full strength MS basal medium supplemented with different concentrations of NAA ( 0.5, 1, 2, 3, 4 mg/l), 2, 4-D (0.5, 1, 2.5, 3.25,4.5, mg/l) 2, 4 5-TP (0.5, 1, 2, 3, 5 mg/l), DICAMBA ( 0.5, 1, 2, 3, 4 mg/l). Concentration of the plant growth regulator was selected by the general consideration that high levels of auxins usually promotes the root induction where as the low levels of auxins promotes callus formation.

## RESULTS

The leaves, petioles, internodes cultured on MS basal medium fortified with different concentrations of auxins showed various results (Table 1).

***Leaves*** were culture on MS medium supplemented with 2, 4-D resulted rare proliferation of callus (Table 2). Whereas incase of 2, 4 5-T and NAA, the callus formation was totally suppressed. Leaf explants showed good proliferation DICAMBA 1 mg/l. The response was more than 60 %. Both leaf and petiole explants showed brown colored callus (Table 2). Lima *et al*., (2008) has got callus induction from leaf segments of *Croton urucurana* with 2, 4-D 3 mg/l. But in the case of *Croton scabiosus* the leaf segments have not shown good response with 2, 4-D.

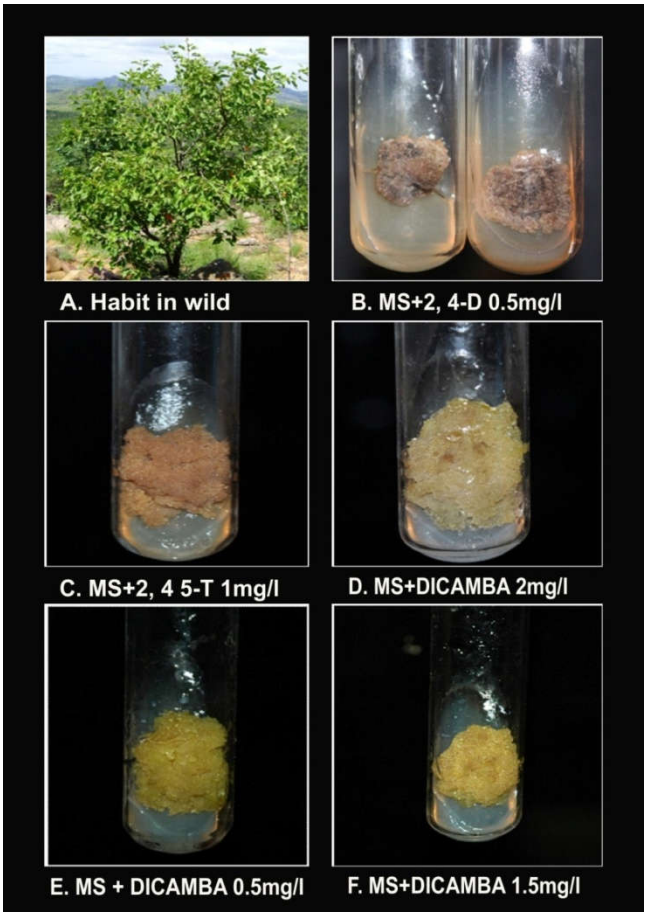


**Figure 1. A- Habit of *Croton scabiosus*, B-F showing callogenesis of *Croton scabiosus* with various auxins**

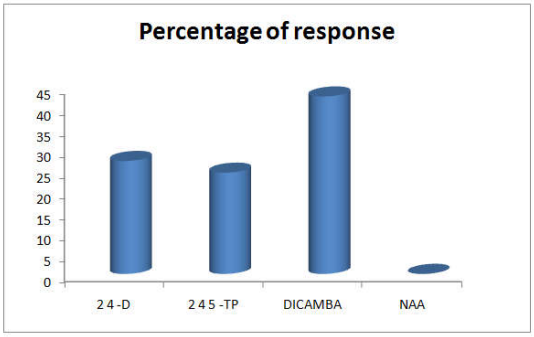


**Figure 2. Rate of response of explants with different auxins**

**Table 1. Types of callus on different concentrations of auxins**

| Name of Explant | 2 4 -D | | | | | DICAMBA | | | | | 2, 4 5 –T | | | | | NAA | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.5 | 1 | 2.5 | 3.25 | 4.5 | 0.5 | 1 | 2 | 3 | 4 | 0.5 | 1 | 2 | 3 | 4 | 0.5 | 1 | 2 | 3 | 4 |
| Leaf | * | ++ | + | * | - | + | ++ | + | * | - | - | - | - | - | - | - | - | - | - | - |
| Petiole | - | - | - | - | - | + | * | ++ | - | - | * | - | + | - | - | - | - | - | - | - |
| Inter node | * | + | +++ | + | - | ++++ | +++ | ++++ | + | - | + | ++ | +++ | +++ | - | - | - | - | - | - |

1. * = Dark brown tough callus
2. + = Brown callus
3. ++ = Brown friable callus
4. +++ = Cream colored callus
5. ++++ = Green friable callus.
6. - = No response

**Table 2. Effect of different concentrations of 2, 4 -D on Callus induction from different explants**

| S.No | Name of Explant | Conc. of PGR used (mg/l) | No. of explants inoculated | No. of explants responded | % of Callus induction |
|---|---|---|---|---|---|
| 1 | Leaf | 0.5 | 40 | 17 | 42.5 |
| | | 1 | 40 | 18 | 45 |
| | | 2.5 | 40 | 21 | 52.5 |
| | | 3.25 | 40 | 11 | 27.5 |
| | | 4.5 | 40 | No response | ….. |
| 2 | Petiole | 0.5 | 40 | No response | ….. |
| | | 1 | 40 | No response | ….. |
| | | 2.5 | 40 | No response | ….. |
| | | 3.25 | 40 | No response | ….. |
| | | 4.5 | 40 | No response | ….. |
| 3 | Inter node | 0.5 | 40 | 22 | 55 |
| | | 1 | 40 | 28 | 70 |
| | | 2.5 | 40 | 32 | 80 |
| S.No | Name of Explant | Conc. of PGR used (mg/l) | No. of explants inoculated | No. of explants responded | % of Callus induction |
| | | 3.25 | 40 | 14 | 35 |
| | | 4.5 | 40 | No response | ….. |

**Table 3. Percentage of response of various explants on different auxin formulated media**

| S. No | Name of explant | Con. of PGR used(mg/l) | No. of explants inoculated | 2, 4 5 –T | | DICAMBA | | NAA | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | No. of explants responded | % of Callus induction | No. of explants responded | % of Callus induction | No. of explants responded | % of Callus induction |
| 1 | Leaf | 0.5 | 40 | No response | ….. | 26 | 65 | No response | … |
| | | 1 | 40 | No response | ….. | 19 | 47.5 | No response | …. |
| | | 2 | 40 | No response | ….. | 16 | 40 | No response | …. |
| | | 3 | 40 | No response | ….. | 14 | 35 | No response | … |
| | | 4 | 40 | No response | ….. | No response | …. | No response | …. |
| 2 | Petiole | 0.5 | 40 | 19 | 47.5 | 22 | 55 | No response | …. |
| | | 1 | 40 | No response | … | 23 | 57.5 | No response | … |
| | | 2 | 40 | 20 | 50 | 27 | 67.5 | No response | … |
| | | 3 | 40 | No response | ….. | No response | …. | No response | … |
| S. No | Name of explant | Conc. of PGR used(mg/l) | No. of explants inoculated | 2, 4 5 –T | | DICAMBA | | NAA | |
| | | | | No. of explants responded | %of Callus induction | No. of explants responded | % of Callus induction | No. of explants responded | % of Callus induction |
| | | 4 | 40 | No response | …. | No response | …. | No response | … |
| 3 | Internodes | 0.5 | 40 | 24 | 60 | 35 | 87.5 | No response | … |
| | | 1 | 40 | 29 | 72.5 | 29 | 72.5 | No response | … |
| | | 2 | 40 | 26 | 65 | 26 | 65 | No response | … |
| | | 3 | 40 | 28 | 70 | 19 | 47.5 | No response | … |
| | | 4 | 40 | No response | …. | No response | … | No response | … |

***Petioles*** cultured on MS basal medium fortified with NAA (0.5, 1, 2, 3, 4 mg/l), 2, 4-D (0.5, 1, 2.5, 3.25, 4.5, mg/l) had never shown any morphogenetic response. The morphogenetic response was delayed but more than 50 % of response was recorded on 2, 4 5-T. The response was rare but more than 60 % of the explants showed morphogenetic response on MS medium with DICAMBA (0.5, 1, 2, 3, 4 mg/l).

***Inter-nodal explants*** cultured on MS medium with 2, 4-D shown brown callus in all the concentration except in 2.5 mg/l. The explants showed best morphogenetic response on 2, 4 5 -T and DICAMBA; brown friable, cream friable, green colored callus formed (Figure-1). On MS full strength medium fortified with 2, 4 5-T 2 mg/l, and DICAMBA 0.5mg/l green friable callus was luxuriantly formed (Table 3). DICAMBA showed maximum rate of response from all the explants inoculated in all the concentrations (Figure-2). Inter nodal segments were found to be the best source of explants for callus induction that showed healthy and profuse callus naturally having the capacity of regeneration. By using inter nodal segments as explants on 2, 4-D 2 mg/l, Fazil Azim *et al*. (2011) have got 68±2 % of callus in Malta. This is somewhat nearer to the results obtained I.e. 76.6% (data not shown) in the present study using 2, 4-D 2.5 mg/l.
NAA showed no response at any kind of concentration with any sort of explants (Table 3). Phenolic exudation was suppressed by recurrent transformation of callus. All the results were calculated by using Micro soft excel soft ware.

## DISCUSSION

*Croton scabiosus*, an endemic of southern Andhra Pradesh warrants immediate conservation measures as the populations in the natural habitat are primarily threatened by forest fires and poor germination. With this backdrop, we attempted *in vitro* propagation with different explants for production of somatic embryos through callogenesis which will be used for multiplication of plantlets to augment existing natural population. During the examination of standardizing sterilant which can prevent the microbial growth during *in vitro* cultures with three sterilants, it was noted that 0.1 % $HgCl_2$ showed maximum disinfection during the callus proliferation. Hence it was understood that the requirement of sterilant for inhibition of contaminant of this species is low. Among the three explants used inter-nodes was more suitable and responsive to the provided nutrients. Healthy green colored callus was obtained from internodes cultured on MS medium supplemented with DICAMBA 0.5mg/l. After the initiation, the green friable callus gradually covered the entire surface of the explants. In this present study all the explants of *Croton scabiosus* did not shown any callus induction on any concentrations of NAA, and even in some of the concentrations of 2, 4 5 –T. This may due to the provided concentration of plant growth regulators may not be in optimum range for callus induction. From all the calli obtained with inter nodal explants, green friable callus induced in the medium having DICAMBA 0.5 mg/l may be selected for further studies I.e. formation of somatic embryos from this calli. The results here indicate that exogenously supplied auxin DICAMBA by itself was enough to promote a hormonal balance capable of inducing the highest callus formation from internodes of *C. scabiosus*. At the earlier stages of callus proliferation, in some of the culture tubes, phenolic exudation was observed and to arrest this, the callus was immediately transferred to freshly prepared MS basal medium having the same concentration of auxin as the previous one was. Since the plants have a diversity of secondary metabolites and as it also observed exuding some phenolic compounds in unorganized cell mass, effective *in vitro* mass production of callus may be beneficial for high yield of alkaloid production. Hence there is a scope for evaluating a protocol for extraction and commercialization of the particular secondary metabolites especially the alkaloids. To the best of our knowledge no report is available about micro propagation of *Croton scabiosus*, and this is the first attempt of standardizing the consistent callus induction protocol from the inter nodes.

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