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Research article

Callus induction and plant regeneration from cotyledonary leaf explants of *Oroxylum indicum* (L.) Vent. - An endangered medicinal tree

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Abstract: *Oroxylum indicum* is a medicinal tree renowned for the presence of a large number of bioactive compounds and a wide range of healing properties. Several biochemical and molecular studies are ongoing in this plant as per the literature survey. The present study is undertaken to develop a simple and reliable protocol for plant regeneration from callus, so as to exploit the regeneration potential of this tree. The cotyledonary leaf explants from the *in vitro* raised seedlings produced callus in MS medium supplemented with 0.5–2.0 mg l^{-1} Benzylaminopurine (BAP) alone and in combination with auxins- 0.5 mg l^{-1} 2,4-Dichlorophenoxyacetic acid (2, 4-D) and 0.5 mg l^{-1} Indole-3-acetic acid (IAA). Silver nitrate ($AgNO_3$) was used as an additive in a concentration of 1 mg l^{-1}, in all the trials. Callus raised in all combination of growth regulators showed organogenesis in the second cycle of culture. MS medium with the combination 2 mg l^{-1} BAP and 0.5 mg l^{-1} 2, 4-D produced a mean number of 61.33 buds per explants, which was the maximum of the observed results. Direct organogenesis was noted in explants raised in 2 mg l^{-1} BAP supplemented MS medium. The shoot buds were cultured in MS medium fortified with 0.2 mg l^{-1} BAP alone and in combination with 0.5 mg l^{-1} IAA or 0.5 mg l^{-1} Gibberellic acid (GA3) for elongation. GA3 found to be the best combination with BAP for obtaining shoots with better length (6.13 cm) and healthy leaves. Shoots thus obtained were rooted in full or half-strength MS medium with 0.5–1.0 mg l^{-1} IAA or Naphthalene acetic acid (NAA). Half strength MS with 1.0 mg l^{-1} IAA produced 62% rooting, with a mean number of 7.66 roots per shoot. Rooted micro shoots were acclimatized in 1:1 mixture of autoclaved sand and vermiculite, before transferring to the field. About 62% of plantlets survived by this process.

Keywords: Bignoniaceae - Callus proliferation - Direct organogenesis - Morphogenesis.

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INTRODUCTION

Oroxylum indicum (L.) Vent. belonging to family Bignoniaceae is an important medicinal tree native to the Indian subcontinent. It bears a variety of common names, 'Indian Trumpet tree', 'Tree of midnight horror' 'Tree of Damocles' and 'Broken bones tree'. The roots of this tree which is mentioned as 'Syonaka' in Ayurvedic literature (Anonymous 1998) is highly demanded in the herbal drug market. It is an important ingredient of many Ayurvedic formulations including Dasamoolarishta, Narayana Taila, Brahma Rasayana, Dantyarishta and Dhanwanthara Gritha (Gohil *et al*. 2008). Many important flavonoids like, oroxylin-A, baicalin, baicalein, chrysin etc. were reported to be present in various parts of this tree (Sankara *et al*. 1972, Dinda *et al*. 2007). All these compounds proved to have a variety of bioactive properties including anticancerous, antioxidant and antibacterial activity (Roy *et al*. 2007, Mishra *et al*. 2010). The uprooting of the whole plant for root collection,

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added with poor fruit set and seed abortion has resulted in the drastic decline and disappearance of this tree in the natural population (Gunaga *et al*. 2012, Debi & Prakash 2015). Several efforts have been made so far to propagate this valuable tree through axillary shoot proliferation (Dhami *et al*. 2005, Tiwari *et al*. 2007, Parmar & Jasrai 2014). But reports regarding the regeneration of this plant from callus are very limited and are not satisfactory. Jasrai *et al*. (2013) carried out an organogenesis experiment from the leaf explants in auxin supplemented medium. Recently many biochemical studies regarding the *in vitro* production of active principles from this plant have been reported (Gokhale *et al*. 2016, Rami & Patel 2017). The application of molecular techniques for crop improvement or metabolite production highly depends on the morphogenic response of the callus and the production of virus-free clones. However, the regeneration potential of many woody plant species was often found to be recalcitrant due to unknown factors (Bonga 2012). In the present study, a simple and successful protocol was developed for the regeneration of *Oroxylum indicum* plantlets from juvenile cotyledonary leaf explants. The shoot induction medium was slightly modified by adding 1 mg l^{-1} AgNO3 as additive, to enhance shoot growth. Also, the use of an elongation medium after the 3rd passage with 0.5 mg l^{-1} GA3 and 0.2 mg l^{-1} Benzylaminopurine (BAP) as plant growth regulators proved to be very effective in elongating the microshoots.

MATERIALS AND METHODS

Ripe pods of *Oroxylum indicum* were collected from the Ayurvedic medicinal garden at Kanjirapuzha, Palakkad district. Healthy seeds were selected and were surface sterilized in 70% ethanol for 30 seconds and 1% Sodium hypochlorite for 2 minutes followed by 3 times rinsing in distilled water. The testa is then removed aseptically using sterile forceps and the excised seeds were inoculated in MS basal medium. From this *in vitro* raised seedlings juvenile cotyledonary leaves were excised after 4 weeks, cut into equal size of approximately 2 × 2 cm using sterile scalpel, and were aseptically inoculated in MS medium fortified with 3% sucrose, 0.75% agar and 1 mg l^{-1} $AgNO_3$ along with varying concentrations of plant growth regulators BAP, IAA and 2, 4- D alone and in combinations Before adding agar to the medium, the pH was adjusted to 5.8. The medium was autoclaved at 121ºC at 15 lbs pressure for 15 minutes. Culture bottles were kept in a room temperature of 25±2º C, relative humidity 60 to 70% and were provided with a light intensity of approximately 1500 lux under 16 hour photo period. In every 20–22 days, the explants were transferred to fresh media with same or different composition. The shoot buds obtained from the callus were elongated in MS medium augmented with 0.2 and 0.5 mg l^{-1} BAP, alone and in combination with 0.5 mg l^{-1} GA3 or IAA. For rooting of the micro shoots, half-strength MS medium supplemented 0.5 and 1.0 mg l^{-1} concentration of auxins- IAA and NAA were used.

Statistical analysis

Each treatment consisted of 20 explants and maintained in 3 replicates. The data were analyzed statistically by one way ANOVA. All the values were represented as mean±standard error. The significance of the mean values and the least significant difference (LSD) were calculated by Duncan's Multiple Range Test at level of significance p=0.05, using SPSS computer programme.

RESULTS

Callus induction and shoot bud initiation

MS medium supplemented with all the combinations of growth regulators induced profuse callusing from the 17th day onwards. Cent percent callusing was found in MS medium with 2 mg l^{-1} cytokinin used along with 0.5 mg l^{-1} of auxins- 2, 4-D and IAA. No callusing was found in MS medium without growth regulators. At the initial stage, the nature of the callus was white and friable, which turned to shades of cream to brown in the duration of time. Morphogenic responses were visible in the second cycle, after 35 days of initial culture (Fig. 1B). Cream nodular callus was more responsive than granular callus. It was observed that BAP alone was sufficient for evoking callusing as well as the morphogenic response from the cotyledonary leaf explants of *Oroxylum indicum*. About 99% of the explants produced callus in MS medium supplemented with 2 mg l^{-1} BAP. Of these 65.46% of the callus showed shoot bud initiation, with a mean number of 49.10 shoot buds per explants. When auxins were incorporated along with BAP, callus proliferation as well as morphogeneic response enhanced. MS medium supplemented with 2 mg l^{-1} BAP and 0.5 mg l^{-1} 2, 4-D produced maximum number of 61.33 shoot buds per explants, after the third passage. But more healthy shoots with normal leaves were produced in lower concentrations of BAP (0.5 & 1.0 mg l^{-1}) when used alone and in combination with the two auxins (Fig. 1D). Profuse callusing along with shoot bud initiation prevailed in the second and third cycle. During every sub culturing the calluses or the shoot clumps were divided into small segments and inoculated in

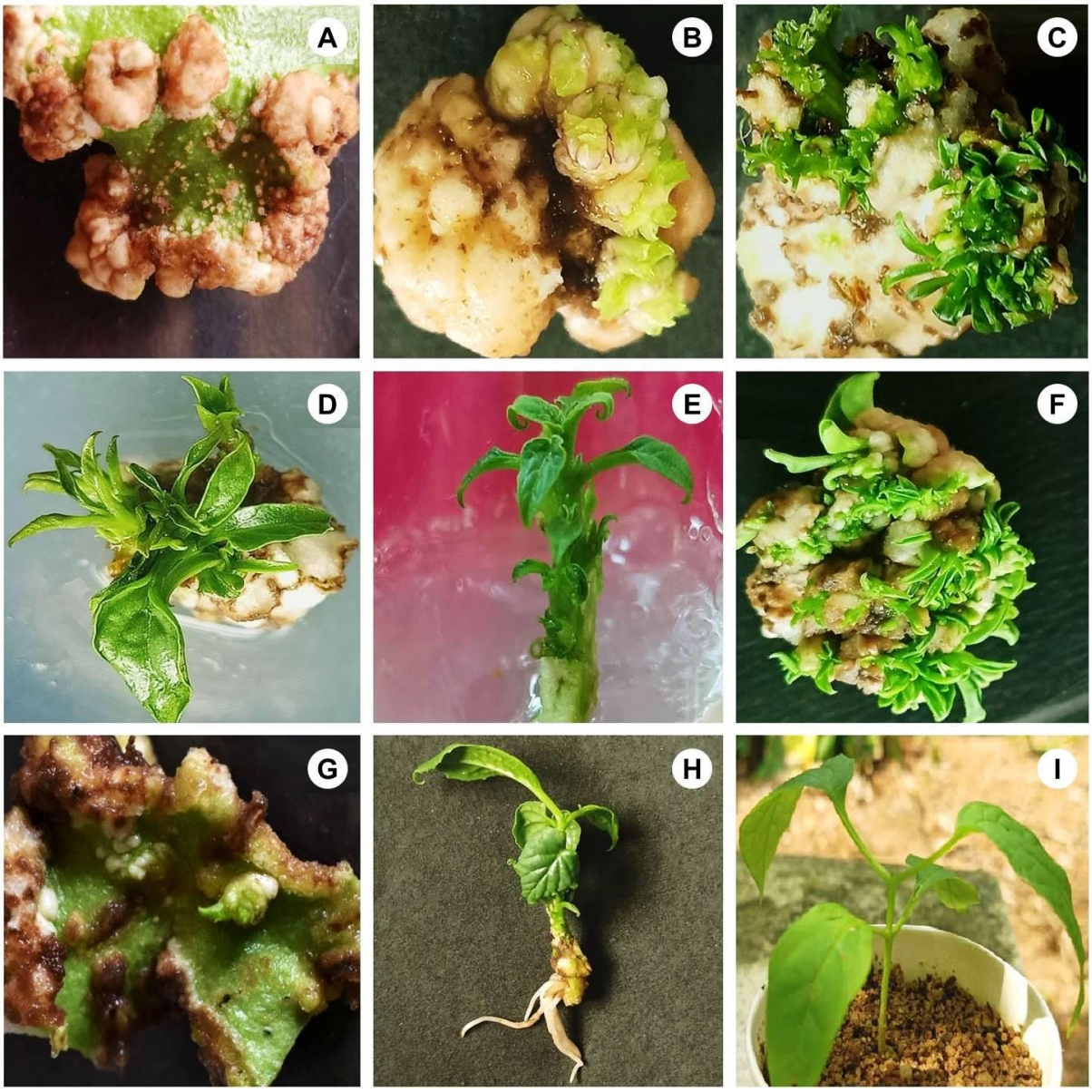

Figure 1. Callus induction and plant regeneration in *Oroxylum indicum* (L.) Vent.cotyledonary leaf explants: **A**, Callus formation in explants; **B**, Shoot bud initiation in creamy nodular callus in 1 mg l^{-1} BAP: **C**, Growth of shoot buds in 1 mg l^{-1} BAP supplemented medium: **D**, Elongation of shoot buds in MS + 0.2 mg l^{-1} BAP + 0.5 mg l^{-1} GA3; **E**, A fully grown shoot transplanted to rooting medium; **F**, Large number of shoot buds in MS+2 mg l^{-1} BAP + 0.5 mg l^{-1} 2,4-D; **G**, Direct organogenesis from the cotyledonary leaves in 2 mg l^{-1} BAP supplemented medium; **H**, A rooted micro shoot; **I**, An acclimatized plant transferred to field, after 2 months.

Table 1. Influence of different concentrations of plant growth regulators on callus induction and organogenesis from cotyledonary leaf explants of *Oroxylum indicum* (L.) Vent.

Concentration of PGR (mg l^{-1})			% of callus induction	Type of callus	% of callus showing organogenesis	Number of shoot buds after passage 3
BAP	**2,4-D**	**IAA**				
0.0	0.0	0.0	-	-	-	-
0.5	-	-	78	White granular	27.43 ± 1.73^{g}	15.66 ± 0.45^{f}
1.0	-	-	92	Creamy granular	53.50 ± 1.66^{d}	33.31 ± 0.23^{d}
2.0	-	-	98	Creamy nodular	65.46 ± 2.02^{c}	49.10 ± 0.54^{b}
0.5	0.5	-	86	Creamy nodular	43.43 ± 1.36^{e}	27.65 ± 0.32^{d}
1.0	0.5	-	99	Creamy nodular	76.33 ± 1.76^{b}	47.33 ± 0.99^{b}
2.0	0.5	-	100	Light brown nodular	83.35 ± 2.01^{a}	61.33 ± 1.07^{a}
0.5	-	0.5	82	Creamy nodular	33.67 ± 1.45^{f}	24.36 ± 0.34^{e}
1.0	-	0.5	98	Creamy nodular	74.66 ± 1.73^{b}	40.66 ± 0.55^{c}
2.0	-	0.5	100	Light brown nodular	77.39 ± 1.45^{b}	58.66 ± 0.76^{a}

Note: values represent means ± standard error. Column values with different superscripts differ significantly ($p<0.05$).

fresh medium, to ensure better availability of nutrients. Direct organogenesis (which was not reported earlier in this plant) was observed occasionally in the explants raised in 2 mg l^{-1} BAP (Fig. 1G). However, these buds did not show healthy growth due to profuse callusing around the buds. The observations obtained on parameters like percentage of callusing, morphogenic response of the callus and bud induction in different medium compositions was summarized in table 1.

Shoot elongation and Rooting

The shoot buds when maintained in the same medium did not show much elongation. So the shoots were transferred to MS medium containing 0.2 mg l^{-1} BAP, alone and in combination with 0.5 mg l^{-1} IAA or GA3 for elongation. MS medium supplemented with the combination of 0.2 mg l^{-1} BAP and 0.5 mg l^{-1} GA3 found to be the optimum for shoot elongation. In this medium the micro shoots attained a mean length of 6.13 cm with a mean number of 6.81 leaves (Table 2). Shoots which acquired 2–3 cm length were transferred to full or half strength MS medium with different concentrations of auxins- NAA and IAA for rooting. The rooting response was evident after 30 days. Roots were formed with a little amount of callus at the base of the shoots (Table 3). Half strength MS medium augmented with 1 mg l^{-1} IAA found to be optimum for better-rooting response, which rooted about 62.42% of the shoots. A mean number of 7.66 roots were produced in this medium with mean length of 4.34 cm. The roots formed were white and without lateral branches (Fig. 1H). The overall rooting response found to be poor.

Table 2. Influence of plant growth regulators in enhancing the length of shoot buds of *Oroxylum indicum* (L.) Vent.

Concentration of PGR (mg l^{-1})			Shoot length (cm)	Leaf number
BAP	**IAA**	**GA3**		
0.2			3.91±0.04^{b}	4.61±0.04^{b}
0.5			2.46±0.31^{c}	2.16±0.05^{c}
0.2	0.5		4.46±0.22^{b}	6.26±0.04^{a}
0.2		0.5	6.13±0.13^{a}	6.81±0.32^{a}

Note: values represent means ± standard error. Column values with different superscripts differ significantly p<0.05).

Table 3. Influence of medium strength and different auxin concentrations on rooting response of the micro shoots of *Oroxylum indicum* (L.) Vent.

Medium strength	Concentration of PGR (mg l^{-1})		% of rooting response	Number of roots	Root length (cm)
	IAA	NAA			
1/2 MS	0.5	-	44.61±1.43	3.61±0.01^{c}	2.36±0.01^{c}
1/2 MS	1	-	62.42±1.46	7.66±0.03^{a}	4.34±0.009^{a}
1/2 MS	-	0.5	32.54±1.08	2.35±0.04^{d}	1.65±0.01^{cd}
1/2 MS	-	1	54.56±1.17	5.66±0.02^{b}	3.52±0.02^{b}
MS	1	-	26.45±0.91	2.26±0.01^{d}	1.12±0.008^{d}
MS	-	1	19. 87±0.46	1.26±0.009^{e}	0.63±0.008^{d}

Note: values represent means ± standard error. Column values with different superscript differ significantly (p<0.05).

Hardening

Rooted plants were washed thoroughly in tap water and transferred to plastic pots containing 1:1 autoclaved mixture of sand and vermiculite. The plantlets were covered with transparent polythene sheets and kept in the culture room for two weeks to maintain high humidity. After that, they were transferred to the garden and placed in the shade for 3 weeks to protect them from direct sunlight. About 62% of field survival was observed in these hardened plants (Fig 1I)

DISCUSSION AND CONCLUSION

The requirement for overcoming recalcitrance and attainment of morphogenesis relies mainly on the selection of explants and the maintenance of optimum culture conditions and these conditions varies from species to species (De Fossard 1977, Mc Cown 2000). Benson (2000) recommended the use of juvenile explants or juvenile flushes from mother plants for micropropagation in tree species. In the present study, the selection of juvenile cotyledonary leaf explants produced healthy and responsive callus, free of contamination. The superiority of BAP on callus proliferation and shoot multiplication was well documented in many literatures (Thiyagarajan & Venkatachalam 2012). Here also it was evident that BAP alone can produce a sufficient number of shoots from the callus. But the better callusing and morphogenetic response was observed in the explants, when BAP is used in combination with lower concentration (0.5 mg l^{-1}) of auxins. A higher concentration of BAP (2.0 mg l^{-1}), both alone and in combination with auxins are not recommended for this plant, as it produces micro shoots with curled leaves and abnormal shoots, which were probably somaclonal

variations. Similar reports of somaclonal variations exist for this plant (Gokhale & Bansal 2010) and also in other species like Banana (Bidabadi *et al*. 2010) when higher concentrations of BAP were used. However it was observed in the present study that the *in vitro* raised shoots with noticeable morphological variations are not surviving in the culture. An inverse correlation between shoot length and shoot number was observed in the present experiment. Shukla *et al*. (2012) also reported that, in *Stereospermum personatum* (Hassk.) Chatterjee belonging to the same family Bignoniaceae increased number of shoot bud and callus proliferation lead to suppression of apical dominance. Hence, in the present experiment, an elongation medium with 0.5 mg l^{-1} concentration of GA3 or IAA along with very low concentration of BAP (0.2 mg l^{-1}) was employed for the elongation of microshoots, which proved to be much effective. GA3 was also used successfully for shoot elongation in other species like *Tectona grandis* L. f. (De Gyves *et al*. 2007) and *Morus australis* Poir. (Patnaik *et al*. 1996). $AgNO_3$ at a concentration of 1 mg l^{-1} was used as an additive in all the shoot induction medium, based on previous reports of micropropagation in *Oroxylum indicum* (Bansal & Gokhale 2012), and this produced better growth of the shoots, as well as prevented the exudation of phenolics into the medium. The orientation of the explants in the medium seems to be insignificant in the callusing response. However, it was observed that explants with their cut end fully immersed in the medium exhibited rapid callusing and bud initiation.

Thus, it can be concluded that *Oroxylum indicum* plantlets can be regenerated from the cotyledonary leaf explants with high frequency. 1 mg l^{-1} BAP in combination with 0.5 mg l^{-1} 2,4-D or IAA can produce maximum morphogenic callus. The shoot buds need to be cultured in elongation medium augmented with either 0.5 mg l^{-1} GA or IAA along with 0.2 mg l^{-1} BAP. For rooting of the microshoots 1 mg l^{-1} IAA seem to be the single best medium. This protocol can be used effectively for callus induction and plant regeneration in this tree. Callus can be used for carrying out further molecular studies of crop improvement and also for the *in vitro* production of secondary metabolites.

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