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RESEARCH ARTICLE

Direct *in vitro* organogenesis and plantlet formation from leaf explants of *Oroxylum indicum* (L) Kurz an endangered medicinal forest tree

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Abstract

Direct *in vitro* organogenesis from leaf explants of an endangered medicinally important tree *Oroxylum indicum* has been achieved on MS (Murashige & Skoog's) medium fortified with various concentrations(1.0-5.0mg/L) of cytokinins BAP/Kn alone and also in combination with 0.5mg/L 2,4-D. The *in vitro* developed shoots were transferred to MS medium augmented with 0.5 mg/L GA_3+1.0mg/L BAP for further proliferation and elongation. These elongated micro-shoots were shifted on to ½ strength MS medium fortified with 0.5mg/L-4.0mg/L IAA/IBA/NAA for *in vitro* rooting. Profuse rhizogenesis was observed at 1.0mg/L NAA. *In vitro* rooted plantlets were acclimatized and shifted to green house. These plantlets were identical to their mother plant and the survival percentage was found to be 85%. This protocol can be used for large-scale multiplication of the species.

Introduction

The species *Oroxylum indicum* (Bignoniaceae) is an endangered ethnomedicinally important tree. All the parts of the plant material have active principles although they are mainly encountered in root bark and very well known for their anti-cancerous, anti-inflammatory, antihelminthic, anti-bronchitic, anti-leucodermatic, antirheumatic, anti-anorexic, analgesic properties [1]. Hence it is widely employed in ayurvedic, asian folk, herbal as well as in tribal medicine. Bark of root is the main constituent of ayurvedic preparation *Dasamoola* which is being used as an astringent, bitter tonic, stomachic and anodyne and also in the preparation of *Chyawanaprasha.* It has been reported that the seeds of *O.indicum* contain four flavonoids identified as chrysin, baicalein, baicalein-7-0-glucoside, baiaclein 7-o-di glucoside which inhibit proliferation of cancer cell line *in vitro* via induction of apoptosis [2, 3].

This species is included in the Red Data List and categorized as vulnerable [4] due to over exploitation of whole plant for medicinal uses. Seeds are recalcitrant as they lose their viability very early and have low capacity to regenerate vegetatively. In view of its medicinal importance there is an urgent need of its conservation *in situ* and *ex situ* and mass-scale multiplication. Hence the present protocol reports, the direct organogenesis from leaf explants followed by successful establishment of regenerated plants in *O. indicum.*

Materials and Methods

Plant material

Mature fruits of *O. indicum* were harvested from Kakatiya Arboretum, Forest Department (Research and Development), Warangal circle, Andhra Pradesh, India. The pods were washed thoroughly under running tap water for 10 min to avoid the disinfection, and were air dried. After proper drying, the pods were split open and seeds were collected, which were rinsed thrice in sterile distilled water. These were surface sterilized with 0.1% (w/v) mercuric chloride for 2-5 min followed by three rinses in sterile distilled water under aseptic conditions and soaked in sterile distilled water for 24 hrs. Later the seeds were dried on sterile tissue paper and cultured on MS [5] medium

containing 30 gm/L sucrose. After 3 weeks the tender leaves were excised and used as source of explants from *in vitro* grown seedlings.

Culture media and Culture conditions

The tender leaves were separated and cut into 1.0 cm^2 pieces and cultured on MS medium containing 3% sucrose, supplemented with various concentrations (1.0-5.0mg/L) of cytokinins BAP/Kn alone and also in combination with auxins 2.4-D (0.5-2.0mg/L). The P^H of the medium was adjusted to 5.8 with either 0.1N NaOH or 0.1N HCl and solidified with 0.8% (w/v) Difco- bacto agar (Hi-media, India) and autoclaved at 121ºC for 20 min. All the cultures were maintained at 25±2ºC under a photoperiod of 16 hr with photon flux density of 40-50 µmol $m^{-2}s^{-1}$ supplied by cool-white fluorescent tubes.

Shoot multiplication and elongation

For further shoot multiplication and elongation, the explant along with micro-shoots were subcultured regularly for every 3 weeks on the fresh medium augmented with 0.5mg/L GA_3+1.0mg/L BAP.

***In vitro* Rooting and Plantlet Establishment**

For *in vitro* rooting, the micro-shoots developed were transferred on to ½ strength MS medium containing 15 g/L sucrose supplemented with various concentrations (0.5-4.0 mg/L) of auxins viz., IAA/IBA/NAA. The *in vitro* rooted plantlets were washed in sterile distilled water to remove remains of agar and transferred to plastic cups containing sterile garden soil:soilrite (3:1) and covered with polythene bags to maintain the RH (85-90%). These were incubated in the culture room for 4 weeks. Later these acclimatized plants were transferred to the research field and maintained under shady conditions.

Data Analysis

Data on the time taken for the shoot initiation, multiple shoot number per explant, number of roots/microshoot and shoot and root lengths were recorded periodically. Maximum of 30 replicates were maintained for each experiment and each experiment was repeated atleast thrice. The data were analysed statistically following the method of Pillai and Sinha[6].

Results and Discussion

The present investigation has been under taken to find out the efficiency of explant and also the growth regulator concentration and combination for direct regeneration in *O. indicum.* The leaf explants were cultured on MSO and MS medium supplemented with different concentrations of cytokinins BAP/Kn individually and also in combination with 0.5 mg/L 2, 4-D. The results showed that the morphogenesis was absent on MS medium without growth regulators (MSO). The shoot bud initiation was observed from cut ends of the explants after three weeks of culture on MS medium fortified with all the concentrations of BAP/Kn and also in combination with 0.5 mg/L 2,4-D (Tables.1-2).

Adventitious shoots were induced after two weeks of incubation in all the concentrations of BAP and Kn used as a sole PGR (Fig.1). Absolute percentage (100%) of response with maximum number of shoots formation (45±0.04) per explant was found at 5.0 mg/L BAP in comparison to Kn (20±0.13). As the concentration of the growth regulators increased there was gradual enhancement in the shoot bud induction from 2.0-5.0mg/L BAP/Kn (Table-1). Whereas less average number of shoots per explant were recorded beyond to the concentration of 5.0mg/L BAP/Kn.

To know the efficacy of auxin on enhancement for the multiple shoot induction 2, 4-D (0.5 mg/L) was added along with different concentrations (1.0-4.0mg/L) of BAP/Kn (Table-2). Shoot formation per explant was enhanced in all the concentrations of BAP/Kn along with 2, 4-D used. Maximum number of shoots per explant (52±0.04) was observed at 0.5 mg/L 2, 4-D+5.0 mg/L BAP followed by 4.0 mg/L BAP (Table 2). Absolute percentage of response was also recorded at the same concentration of BAP/Kn. BAP alone and also in combination with 2, 4-D had shown superiority in inducing more number of shoots/explant (Figs. 2, 3).

Elongation of shoots

The explant with multiple shoots were cultured on MS medium supplemented with MS+0.5 mg/L GA_3+1.0 mg/L BAP for further proliferation and elongation of shoots. Repeated subculture of explants with shoot buds on fresh shoot proliferation medium helped to achieve continuous production of healthy elongated shoot buds.

***In vitro* rooting and plantlet establishment**

The healthy elongated micro-shoots (4.0cm) were transferred onto ½ strength MS medium supplemented with 1.0-4.0mg/L IAA/ IBA /NAA for *in vitro* rooting. *In vitro* rooting was observed in all the concentration of IAA/IBA/NAA used (Table-3). Maximum percentage of response was observed at 1.0-2.0mg/L NAA with profuse rhizogenesis at 1.0mg/L NAA. More number of roots was recorded in all the concentrations of NAA compared to

IAA/IBA. After *in vitro* rooting the plantlets were acclimatized in culture room. After 4 weeks of acclimatization these plantlets were shifted to earthenware pots containing garden soil and maintained under shady conditions in the research field. These plantlets were identical to their mother plant with 85% survival.

Direct organogenesis was successfully established in *O. indicum* from leaf explants cultured on MS medium supplemented with different concentrations (1.0-5.0 mg/L) of cytokinins BAP/Kn individually and also in combination with 0.5 mg/L 2, 4-D. However, the shoot buds proliferation was found to be maximum (52±0.04) at 0.5mg/L 2,4-D+5.0mg/L BAP among all other growth regulator combinations and concentrations used which also showed superiority in inducing maximum frequency number of shoots per explant in *O. indicum.*

Repeated subculture of leaf segments on fresh shoot proliferation medium helped to achieve continuous production of healthy shoot buds and shoots at least through three to four subculture cycles in *O. indicum*. A similar phenomenon was also obtained in other forest tree species such as *Aegle marmelos*[7] and *Dalbergia sissoo*[8].

Regenerative shoot organogenic ability depends on the genotype of the donor tissue, source of explant and growth regulators such as auxins or auxin-cytokinin combinations and also the culture conditions during incubation period. The influence of these factors was reported in various tree species [9].

Morphogenic response of explants dependent on many factors including balancing of exogenous and endogenous auxin-cytokinin levels [10]. Effect of these growth substances on morphogenesis *in vitro* was well documented [11]. Endogenous hormones are triggered in explants by exogenous supply which interact with each other and lead to the establishment of some sort of balance to achieve regeneration [12]. Thus the morphogenic expression in a particular direction is manifested as a result of the cumulative effect of many factors viz., growth substances, nutrient medium, temperature, humidity, photoperiod. These are the important morphogenic tools which can control the internal milieus of the cell [13].

Thus direct *in vitro* organogenesis and plantlet establishment from leaf explants of an endangered medicinally important tree *O. indicum* has been achieved successfully. Achievement of direct organogenesis is a break- through in tree species like *O. indicum.*

Table -1
Effect of BAP/Kn on shoot induction from leaf explants of *O. indicum*

Concentration of PGR(mg/L)	Percentage of cultures responding	Average No. of shoots/explant±SE[a]	Average length of shoot(cm) ±SE[a]
MSO	-	-	-
BAP			
1.0	76	15±0.02	0.5±0.03
2.0	80	25±0.08	1.0±0.12
3.0	89	34±0.06	1.1±0.06
4.0	96	39±0.13	1.0±0.04
5.0	100	45±0.04	1.5 ±0.05
6.0	78	32±0.16	1.1 ±0.04
7.0	70	26±0.13	0.6 ±0.06
Kn			
1.0	80	8.0±0.05	0.4±0.01
2.0	85	15±0.10	0.6±0.03
3.0	90	17±0.03	0.5±0.02

4.0	97	19±0.16	0.5±0.07
5.0	100	20±0.13	0.8±0.01
6.0	80	17±0.06	0.5 ±0.06
7.0	84	12±0.13	0.4 ±0.04

[a]SE=Mean±Standard Error

Table – 2

Effect of 2, 4-D (0.5mg/L)+BAP/Kn on multiple shoot induction from leaf explants of *O. indicum*

Concentration of PGR(mg/L)		**Percentage of cultures responding**	**Average No. of shoots/explant±SE[a]**	**Average length of shoot(cm) ±SE[a]**
2,4-D	**BAP**			
0.5	1.0	80	21±0.07	0.5±0.06
0.5	2.0	85	35±0.12	1.3±0.02
0.5	3.0	90	42±0.04	1.5±0.05
0.5	4.0	96	48±0.15	1.3±0.06
0.5	5.0	100	52±0.04	1.8±0.08
0.5	6.0	87	40±0.05	1.2±0.05
0.5	7.0	73	31±0.12	0.9±0.04
2,4-D	**Kn**			
0.5	1.0	78	09±0.01	0.5±0.02
0.5	2.0	87	17±0.09	0.6±0.09
0.5	3.0	90	18±0.13	0.5±0.01
0.5	4.0	92	21±0.06	0.7±0.02
0.5	5.0	100	23±0.13	0.8±0.04
0.5	6.0	84	18±0.10	0.6±0.02
0.5	7.0	77	13±0.09	0.5±0.02

[a]SE= Mean ± Standard Error

Table -3

Effect of auxins on *in vitro* rooting of shoots developed through organogenesis from leaf explant *O. indicum*

Concn. of PGRs (mg/L)	% of response	Average no. of roots/shoot(±SE)[a]	Average length of roots (cm) (±SE) [a]
IAA			
0.5	40	2±0.28	6±0.35
1.0	48	2±0.29	6±0.35
2.0	56	4±0.33	3±0.47
3.0	63	3±0.26	2±0.38
4.0	68	4±0.22	2±0.25
IBA			
0.5	70	3±0.29	6±0.47
1.0	66	3±0.34	8±0.66
2.0	66	3±0.21	8±0.32
3.0	60	2±0.16	4±0.21
4.0	60	2±0.13	2±0.11
NAA			
0.5	80	5±0.35	4±0.28
1.0	86	10±0.68	4±0.23
2.0	86	8±0.37	2±0.15
3.0	73	6±0.26	2±0.23
4.0	66	2±0.18	2±0.14

SE[a] Mean±Standard Error

Fig. 2: Effect of BAP/Kn on shoot induction from leaf explants in *O. indicum*

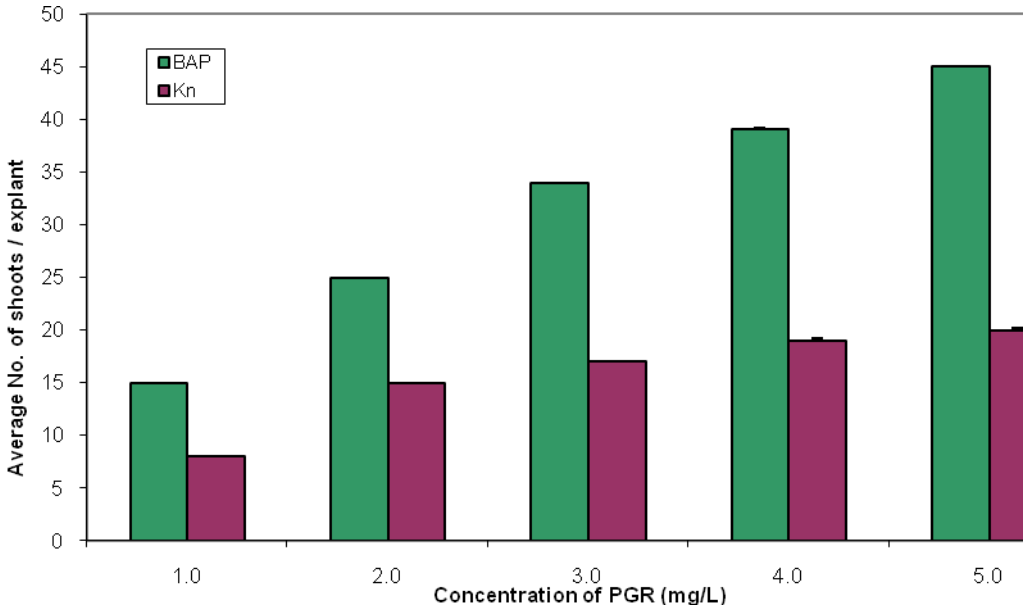


Fig. 3 : Effect of 2, 4-D (0.5 mg/L) + BAP/Kn on shoot induction from leaf explants in *O. indicum*

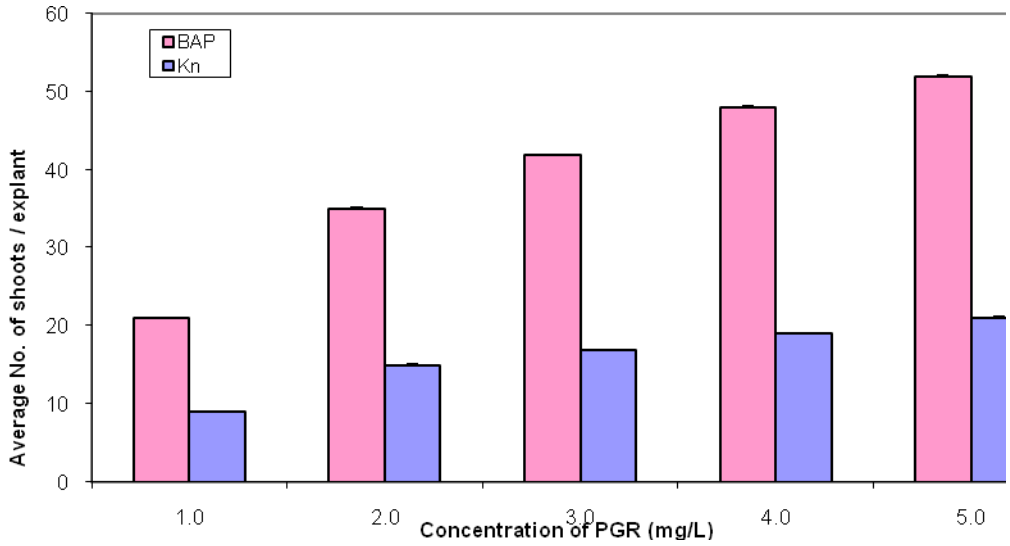
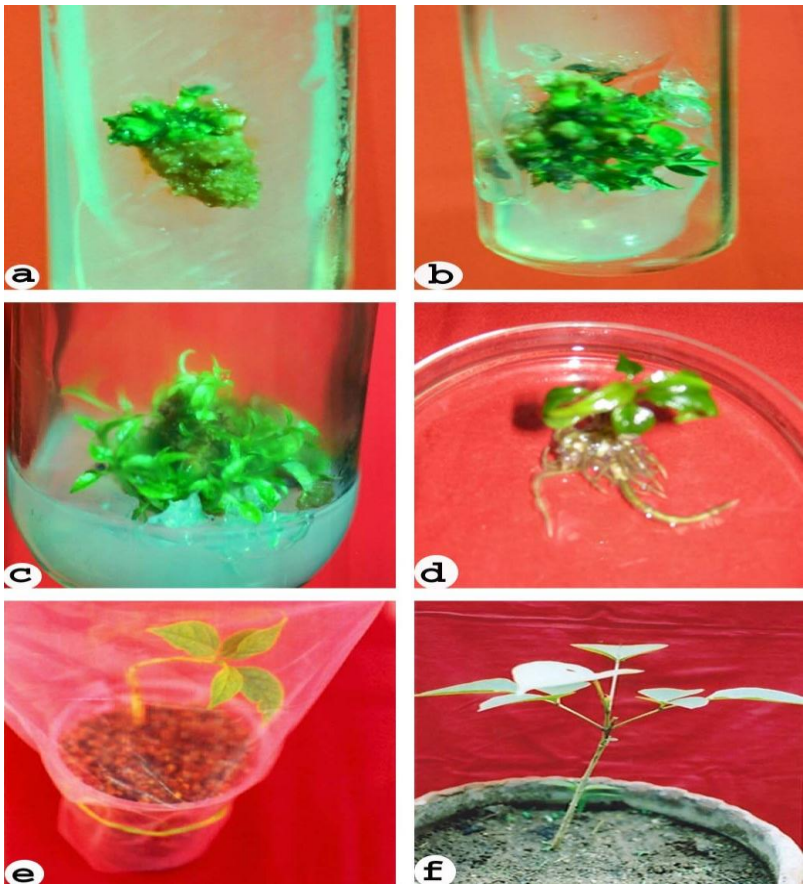


Fig. 1(a-f): Direct shoot organogenesis from leaf explants in *O. indicum*.

a) Formation of adventitious shoot buds on MS+1.0 mg/L BAP.
b) Multiple shoot induction on MS+5.0 mg/L BAP.
c) Development of multiple healthy shoots on MS+0.5 mg/L 2, 4-D+5.0 mg/L BAP after 4 weeks of inoculation respectively.
d) *In vitro* rooted plantlet on ½ MS+1.0 mg/L NAA.
e) Hardening in the culture room.
f) Healthy and established *in vitro* raised plantlet



Conclusion

In conclusion, the results presented here describe an efficient protocol for *in vitro* multiplication of an endangered medicinal forest tree *O. indicum* through direct organogenesis from leaf explants. This protocol can be used for its conservation and rapid multiplication of the species.

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