



REGULAR ARTICLE

SOMACLONAL VARIATION IN *OROXYLUM INDICUM* (L.) VENT- AN ENDANGERED TREE SPECIES

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SUMMARY

Optimum callus formation was observed from apical buds or axillary buds of in vitro grown seedling on Murashige and Skoogs' (1962) medium supplemented with auxins 0.1-1 mg⁻¹ of IBA, IAA, NAA and 2,4-D. Morphogenic callus was cultured on media supplemented with additives (CH & AgNO₃) with BAP (1 mg⁻¹). Maximum shoot regeneration and elongation were achieved upon transferring the callus to medium containing BAP (1 mg⁻¹) with AgNO₃ (2 mg⁻¹). Elongated shoots rooted on ½ MSM supplemented with IBA (mg⁻¹) with AgNO₃ (1 mg⁻¹). Shoots regenerated from this protocol exhibited somaclonal variation in different characters viz. leaf shape, node size.

Key words: Apical bud, Axillary bud, 6-Benzylaminopurine, In vitro, Tissue culture, *Oroxylum indicum* L.

Abbreviations: 2,4-D: 2,4-Dichlorophenoxyacetic acid, MS: Murashige and Skoog (1962), CH: Casien hydrolysate

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1. Introduction

Medicinal plants are important sources of providing health care in India as well as in the world. Extracts of many medicinal tree species have been used for long in ayurvedic preparations. Extract of root & stem bark of *Oroxylum indicum* is a major constituent of Dashmularisht & Chyavanprash (1,2). Shivnak (*Oroxylum indicum*) is an endangered medicinal tree species, possessing several antimicrobial, antiarthritic and antihepatitic qualities in its various parts. This tree possesses flavonoids viz. Baicalein used to check proliferation of human breast cancer cell line MDA - MB - 435 (3).

A small to medium sized tree, *Shivnak* grows in India, Sri Lanka, South China, Celebes, Philippines and Malaysia (4,5). In India, it is distributed throughout the country up to an altitude of 1200 m and found mainly in ravine and moist places in the forests (6).

Owing to the indiscriminate collection, over exploitation & uprooting of whole plants bearing roots this valuable tree has become vulnerable in Karnataka & Andhra Pradesh and endangered in Kerala,

Maharashtra, M.P. & Chhatisgarh (7,8,9,10). Due to severe overexploitation the tree is feared to become endangered in other states too. Hence research involving mass multiplication, conservation and higher production of the active compound under *in vitro* culture conditions is essential. Only a few reports are available on the *in vitro* regeneration of this species (11,12). The present work attempts to obtain regeneration from callus cultures of *Oroxylum indicum* on different PGR concentrations. The present work aims to develop a complete and reliable system for plant regeneration and assess any somaclonal variations associates with regenerants.

2. Materials and Methods

Plant Material and explant preparation

Seeds of *O. indicum* were collected from forest areas in and around Jabalpur. Seeds were germinated on moist filter paper. *In vitro* raised seedlings were given a treatment of 1-2 mins each of 70% ethyl alcohol and 0.1% mercuric chloride. The explants viz. apical buds (ApB) (0.5cm-1cm) and axillary buds (AxB)

(0.7-1cm) were dissected from the 15-20 days old seedling (8cm.). Explants were dried by placing them on sterile filter paper. Explants were inoculated under aseptic conditions on to the sterile culture medium in test tubes on Murashige and Skoog 's medium (MSM) supplemented with 3% sucrose, 0.7 % agar and plant growth regulators viz. auxins (2,4-D, IBA, NAA and IAA) individually. A piece of callus (2-3mm x 2-3 mm) raised on auxin was subsequently used as an explant. The callus was treated with cytokinin (BAP) with additives like CH and AgNO₃. The pH of the media was adjusted to 5.7 before adding agar. Medium dispensed in glass test tube (15x125 mm) was autoclaved at a pressure of 15 psi and a temperature of 121°C for 15 minutes. Before inoculation autoclaved medium was left at 25°C for 24 hrs to check that there was no visible microbial contamination.

Culture conditions

The cultures were maintained in culture tubes and conical flasks and were kept in the culture room at a temperature of 25±2°C, relative humidity (RH) of 60-70% and a light intensity of approx. 1500 lux provided by cool, white, fluorescent tubes under a photoperiod of 16/8 hr (light/dark).

Histological studies

Histological investigations were carried out on microshoots formed on the explant &/or calli. Light microscopic (LM) technique employing Formalin - Aceto - Alcohols (FAA), Safranin and Fast Green (13,14) was followed by for the purpose.

Plant regeneration

For multiple shoot induction, calli were transferred to MSM supplemented with BAP alone and with additives like CH and AgNO₃. The % culture showing regeneration criteria viz. frequency of shoot initiation from callus

(FSI), shoot number (SN) and shoot length (SL) were recorded after each passage.

The effect of continuous supplementation of PGRs on indirect shoot regeneration was observed up to three subculture passages each of 20-22 days. Shoot buds from *in vitro* raised shoots for indirect multiplication, from I passage were used as explants for II and III passage. The data on various parameters was statistically analyzed for the analysis of variance (ANOVA), 'F' test for significance and least significant difference (LSD) was calculated at p=0.05 (LSD_{0.05}). All computations were done employing computer programme SPSS (15).

All experiments were completely randomized and repeated at least twice. Each treatment consisted of 20-25 replicates.

3. Results and Discussion

Shoot Initiation and multiplication from Callus-

The multiplication rate was high on BAP (5mg l⁻¹) supplemented media. Maximum shoot induction obtained after 20 days of inoculation on MSM supplemented with BAP (5 mg⁻¹). But healthy shoots with better length were formed on BAP (1 mg l⁻¹) (Table-1). The superiority of BAP for shoot bud initiation also reported earlier (16). By using this concentrations (BAP 1 mg l⁻¹ with MSM) as selected media (SM), addition of different concentrations of CH and AgNO₃ was applied to callus (Table 2&3). Most of the transformation procedures have relied on inducing long term morphogenic potential after repeated subculture and this is critical for successful transformation. With this view the callus was multiplied on same concentration of PGR on which shoots got initiated from 1-3 subcultures.

Table 1-Effect of BAP on shoot number (SN) and shoot length (SL) in *O. indicum* (After 22 days of inoculation)

BAP (mg l ⁻¹)	Number of shoots per explant (SN)			Shoot length (SL)		
	Passage			Passage		
	Primary culture	Subculture I	Subculture II	Primary culture	Subculture I	Subculture II
0	0	0	0	0	0	0
0.1	2.8	2.5	3.4	0.73	0.5	0.5
0.5	4.5	3.4	3.6	0.8	0.8	0.8
1	12.4	16.4	21.6	1.5	1	1
5	20.7	17.4	23.5	0.5	0.5	0.5
10	0	0	0	0	0	0
F value	1.41			0.36		
P value	<0.001			<0.001		

Table 2: Effect of AgNO₃ and Casein hydrolysate with SM on SN and SL in *O. indicum*. SM (Selected Medium)

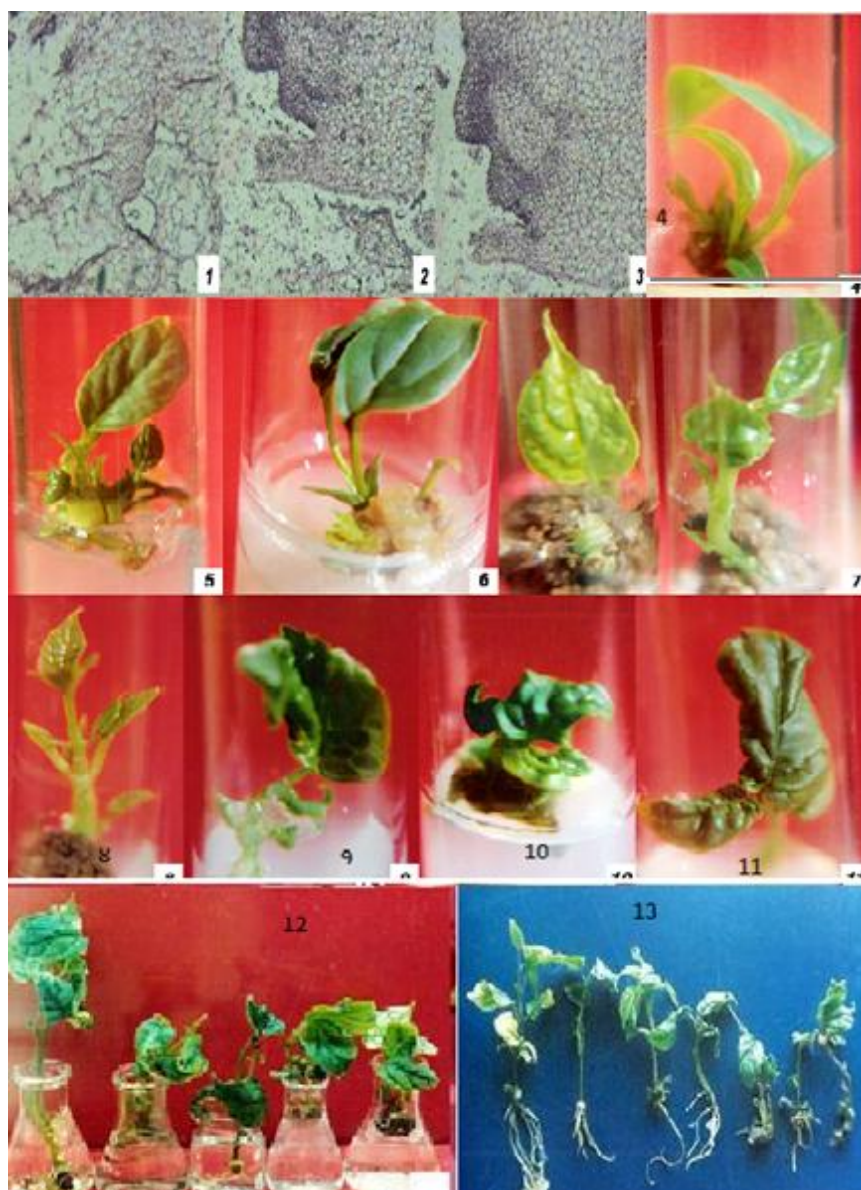
AgNO ₃ (mg l ⁻¹)	Number of shoots per explant (SN)			Shoot length (SL)		
	Passage			Passage		
	Primary culture	Subculture I	Subculture II	Primary culture	Subculture I	Subculture II
0	12.4	16.4	21.6	1.5	1	1
1	10.8	15.4	12.6	2	2.3	2.5
2	18.9	26.7	27.9	5.2	4	6
4	6.3	8.3	3.1	1	0.5	0.7
F value	3.01			0.99		
P value	<0.001			<0.001		
CH (mg l ⁻¹)						
1	11	9.8	13.7	0.5	0.7	0.5
2	12.8	16.4	20.3	1.2	1.5	1
4	2.3	3.1	2.5	0.5	0.5	0.5
F value	2.39			0.53		
P value	<0.001			<0.001		

Table 3: Effect of IBAAgNO₃ on Frequency of rooting (FR) Root number (NR) & Root length (RL)

AgNO ₃ (mg l ⁻¹)	FR			NR			RL		
	IBA (mg l ⁻¹)			IBA (mg l ⁻¹)			IBA (mg l ⁻¹)		
	0.1	0.5	1	0.1	0.5	1	0.1	0.5	1
0	30.24	57.84	80.17	3	9.2	12.5	1	2.3	4
0.1	43.17	62.14	62.43	4.21	9	10.42	1.5	2.5	4.5
1	47.57	67.34	91.73	3.17	15.24	17.41	1.8	3	5.2
2	54.32	73.26	73.62	2.35	7.45	21.36	1	2	5.5
4	39.42	50.47	0.00	2.24	3.50	0.00	1.00	1.50	0.00
F value	5.77				2.98				1.02
P value	<0.001				<0.001				<0.001

Indirectly regenerated normal & abnormal shoots of *Oroxylum indicum*:

Figs.1-3 Histological analysis showing meristemoids of indirect regeneration, Figs 4-6 Normal shoots in different subculture passages. Figs. 7-11 Regenerated shoots with abnormal shape of leaves
Figs. 12-13 Rooted & hardened plants in water



In present study multiple shoot proliferation as well as elongation of shoots was enhanced efficiently on SM (BAP 1mg/l) with AgNO₃ (2mg/l). Shoots attained a length of 5 cm within 22 days.

Indirect multiple shoot formation was noticed in many plants earlier (17). Effect of NO₃ supplementation in media has been well established in tissue culture (18,19). Silver nitrate has been previously reported to inhibit callusing during multiplication (20). Recently it has been used to enhance multiplication of shoot, without callusing.

Shoot regeneration from callus originated from embryonic axis explant was studied previously in *O. indicum* (21)

Histological evidences

The meristematic structures showed a broad base and a protruding dome shaped apex with vigorously dividing cells from callus. In the parenchymatous mass of large cells, distinct regions of small isodiametric cells with thin cell wall, large nuclei and densely staining cytoplasm. These structures termed as meristemoids later developed into adventitious shoot buds.

Rooting

In the present study addition of ½ MSM with AgNO₃ (1mg/l) and IBA (1 mg/l) produced efficient healthy root systems with proper shoot growth. Incorporation of AgNO₃ along with IBA prevented callus formation and produced healthy, strong, root system (20). It also act as root promoting agent in apple, chicory plants and *Dacalepis hamiltonii* when used in micro-molar concentrations (22,23). Complete plantlets were obtained 40 days after the regenerated shoots transferred to this medium (Fig. 4).

Acclimatization

The plantlets were covered with polythene bags to maintain high humidity and to prevent excessive loss of water from the leaves cuticle possessing wax (24). Interestingly in *Oroxylum indicum* a middle stage was required by *in vitro* raised plantlets before transferring them to soil. For this the plants were initially kept in distilled/tap water in flasks covered with beaker for approx 8 days (4 days in dw and 4 days in tap water respectively). The beakers were removed once for 1-2 hours duration during each day. Eventually the plantlets were transferred to soil:sand (1:1) cups.

Formation of new roots was initiated in water treatments, which resulted in the rate of survival of *in vitro* raised plantlets to 82%.

Cytokinin stimulates stomatal opening Although *in vitro* raised plants showed less frequency of stomata. In *Oroxylum indicum* stomata are present only on the abaxial surface of the leaves. Wide stomata in *in vitro* plants of *Oroxylum indicum* was reported (25) are incapable of controlling water loss.

Somaclonal variations

Shoots with leaves of different shapes and abnormal nodes as compared to normal ones were obtained in *in vitro* regeneration of *O. indicum* (Fig. 1-14). After hardening and acclimatization, rate of survival of abnormal plants was 23%. Although their growth, root system was as good as normal plants. But they lost survival capacity after transferring in to field.

It was reported that sometimes due to supplementation of the higher concentration

of BAP, the shape of the leaves on shoots was altered becoming more acuminate in *Garrya elliptica* (26). Greater amount of hyperacidity were found in culture containing higher concentration of BAP, with higher incidence of fasciations and leaf distortion.

Various stresses, as experienced during *in vitro* culture can further enhance replication errors & thus mutation. The spontaneous mutation rates vary from 10-4 to 10-7 per locus. Certain genes are more mutable than others. Therefore heritable changes in cultured plant cells and tissues are to be expected (27), such heritable changes are a limitation for *in vitro* culture. At the same time, tissue culture induced variations provides a new tool for enhancing genetic variability.

It affects many important characters and shows promise for improvement of varieties particularly those with single defects. Application of this technology to many crops can lead to selection of clones with resistance to diseases, increased yield etc. (28,29).

4. Conclusion

A large number of shoots can micropropagated from callus of *O. indicum*, after Supplementation of AgNO₃ with BAP. Occurrence of somaclones in indirect regeneration of *O. indicum* is an interesting feature. Further research in this area may open some vistas.

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