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Effect of Cytokinin and Auxin on Micropropagation of Oroxylum Indicum (L) Vent: A "Mountain Tree of India"

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***Abstract*: *In vitro* micropropagation of *Oroxylum indicum* (L) Vent was established from nodal explants cultured on MS medium supplemented with varied combinations of BA and TDZ. Nodal explants were inoculated on media supplemented with various cytokinins (BA/TDZ) in combination with or without NAA (0.5 mg/l) and $AgNO_3$ (2 mg/l). Multiple shoot induction with maximum 5 shoots and shoot length of 5 cm was seen on MS medium containing only BA (1 mg/l) and $AgNO_3$ (2 mg/l). For induction of roots, *in vitro* shoots were treated with IBA at various concentrations on half and full strength of MS basal medium. Maximum root induction exhibited on medium containing with IBA (2.5 mg/l) and $AgNO_3$ (2 mg/l). The plantlets so generated were processed through hardening procedure for acclimatization and transfer to the soil.**

***Key words*: Micropropagation, Medicinal forest tree, *Oroxylum indicum*, TDZ, $AgNO_3$**

I. INTRODUCTION

Medicinal plants are defined as those used for human and veterinary application in traditional medicines, galenicals and herbal tisanes phyto-pharmaceuticals, new drugs, intermediates for drug manufacture, industrial and pharmaceuticals auxiliary products and for health foods. Medicinal plants play a vital role in the maintenance of human health throughout the world. In fact, they are of critical importance in poor communities. Medicinal plants also play an important cultural role as well as important economical role. Knowledge of their use is wide spread and their efficacy is trusted, based on a long history of use. India has one of the oldest, richest and most diverse cultural traditions called 'folk tradition' associated with the use of medicinal herbs and it is still a living tradition in India [8], [38], [40].

Oroxylum indicum (L) Vent belonging to family bignoniaceae is a traditional herbal medicine in many Asian countries as a cure of various diseases. *Oroxylum indicum* is derived from a combination of two words. Refers, it as *Oroxylum* means "mountain tree" and *indicum* means from India [26]. *Oroxylum indicum* vernacularly known as Shyonaka or Sonpatha is a small to medium sized deciduous tree with large, flat, sword shaped capsular fruits of many flat and papery seeds with broad silver wings [18]. Every part of this tree possesses medicinal value [25]. This plant is also one of the important ingredients in most commonly used ayurvedic formulation such as Dusamula, Brahmi, Rasayana, Amratarista, Dantyarista, Dhanwantara, Ghrita, Narayana Taila etc [16]. The root bark is used in fever, bronchitis, intestinal worms, leuoderma, asthma, inflammation and troubles etc. The fruit and seeds are used as expectorant, purgative and bitter tonic [42]. Twigs of the tree are traded in India at through a way price of Rs 9/kg but its extract in international market is believed to fetch Rs 500,000/kg [18]. The estimated demand of *Oroxylum indicum* in Southern India is 500 kg per annum [21]. The existence of *Oroxylum indicum* in natural population is highly threatened and has been categorized as vulnerable by the government of India [29].

Destructive and non-sustainable collection methods coupled with low regeneration and habitat destruction have posed serious threat to the survival and availability of this highly useful tree [41]. However, large-scale production is a pre-requisite to meet the pharmaceutical needs and also for the effective conservation of this valuable medicinal plant. Tissue culture techniques can be applied to generate clonal propagules and conservation efforts especially for those species in which either the underground parts or the whole plant is used in drug preparation.

Oroxylum indicum is propagated by seeds. However, the seed set is poor and seed viability is low. Problem related with its natural propagation and indiscriminate exploitation for medicinal purpose (mostly roots) has pushed *Oroxylum indicum* to the list of endangered plant species of India [37]. However, with the growing need for medicinal plants, especially those that are rare and endangered in nature, propagation of these species in large numbers is necessary.

In vitro micropropagation is an effective means for rapid multiplication of species in which it is necessary to obtain a high progeny uniformity. Great strides have been made in improving herbaceous crops by *in vitro* procedures. In trees, progress has been less pronounced, but for these for benefits of *in vitro* techniques could eventually be greater than for herbaceous crops because improvement through breeding is for more difficult in trees than in herbaceous plants. The long life cycle and the large size of trees prevents breeding on a scale possible with small sized annul crops [12].

The aim of this research was to establish efficient *in vitro* propagation techniques for *Oroxylum indicum* based on investigation of a range of plant growth regulators (PGRs) for maximizing shoot multiplication.

II. MATERIALS AND METHODS

Mature fruits of *Oroxylum indicum* (L) Vent were collected in the month of April from Chorimala village, Bhiloda, Sabarkantha district, Gujarat. The sward shaped woody capsules about 1meter in length was full of many flat and papery seeds with broad silver wings. One capsule contain about 400-450 seeds. The mature seeds were isolated and selected for *in vitro* germination.

The flat, papery wings were cut with scissors. Then, Seeds were treated with 0.5% bavistin (5 minutes) under aseptic conditions in a laminar air-flow cabinet, followed by washing the seeds thoroughly with sterile distilled water for two times. Then, seeds were surface sterilized with 0.05% mercuric chloride ($HgCl_2$) for 30 seconds. The traces of reagent were removed by rinsing the seeds four times with sterile distilled water. Sterile seeds were inoculated on plain MS media. The seeds were germinated in a period of 4-5 weeks.

Single-node, cotyledonary node and shoot tip explants were isolated from *in vitro* generated seedlings (60 days old). Explants (2-3 cm long) were implanted on MS [27] medium containing 3% sucrose and solidified with 0.8% agar-agar (regular grade, SRL; Mumbai, India). Multiple shoot induction from *in vitro* nodal explants was evaluated on medium supplemented with cytokines (BA/TDZ) in combination with or without NAA (0.5 mg/l) and $AgNO_3$ (2 mg/l) (Table 1). The pH of the media was adjusted to 5.8 prior to adding agar. Medium dispensed in culture bottles (10X5 cm) was autoclaved at a pressure of 15 psi and a temperature of 121ºC (15 minutes). The cultures were incubated in a culture room with 25°C temperature and 16 hr photoperiod provided by white fluorescent tubes (55 µmol/m^2/sec).

In vitro generated shoots were separated individually from multiple shoot cultures with scalpel incisions. Such individual shoots were treated with IBA at various concentrations (0.5- 2.5 mg/l) and $AgNO_3$ (2 mg/l) in half and full-strength MS medium for root induction (Table 2). After 15 days, rooting of shoots was observed. After 4 weeks well developed plantlets were observed.

Hardening of well-grown plantlets was followed in greenhouse conditions. The plantlets were removed carefully, washed thoroughly with distilled water to remove agar, dipped in solution of fungicide (Bavistin, 0.1%; w/v) to take care of contamination. The treated plantlets were planted in pots containing a mixture (soil, sand and farmyard manure; 1:1:1). The plantlets were irrigated with ¼ strength MS medium and processed for hardening [20].

III. RESULTS AND DISCUSSION

Oroxylum indicum has very large trifoliate compound leaf and branching pattern is such that availability of axillary bud is less. The size of nodal region is too big and distance between two internodes is less so it is difficult to get enough material for inoculation. Because of these, *in vitro* germinated seedlings (60 days old) were used as source of explants.

During large-scale micropropagation of some plants certain types of slow-growing microbial (bacterial and fungal) contaminants persist even after initial surface sterilization of explants. Such contaminants (*Pseudomonas/ Erwinia/ Bacillus)* may persist for many generations without being noticed and cause extensive loss of cultures [22]. In this study, seeds of *Oroxylum indicum* were subjected to surface sterilization process with 0.5% bavistin (5 minutes) and 0.05% mercuric chloride ($HgCl_2$) for 30 seconds. After each treatment, explants were washed with sterile distilled water (3-4 times).

The explants subjected to such pre-treatment showed excellent results. This pre-treatment helped in reducing the rate of contamination and showed maximum control of contamination. About 95% of the explants could survive by this sterilization process. Rate of seed germination was observed to be about 73%. MS medium supplemented with BA (0.67 and 2.02 mg/l) was found to be necessary for bud-break from different types of explants of *Oroxylum indicum* (Table 1). Initial sprouting of axillary bud was noticed on 8^{th} day of inoculation (Fig. 1B). Different types of meristematic tissues were used as explants (Cotyledon node, leaf node and shoot tip). Among them, nodal explants showed 100% bud sprout response (Table 1) on a medium containing BA (1.35 mg/l) (Fig. 1B). The explants cultured on MS medium supplemented with BA (0.67 and 2.02 mg/l) also demonstrated sprouting of buds but percentage of bud sprout and number of shoot multiples not higher than medium supplemented with 1.35 mg/l BA. At higher concentrations of BA, though multiple-shoot production was observed but the shoots so formed were very slow in growth.

Another cytokinin, Thidiazuron (TDZ), a substituted phenylurea (1-phenyl-3- (1,2,3- Thiadiazol-5-yl) urea) is highly used as a synthetic herbicide and a plant growth regulator to stimulate axillary shoot proliferation in many woody plant species. It is highly effective than all adenine type cytokinins in shoot organogenesis [1], [24]. Earlier studies state that TDZ remove the lateral bud dormancy and stimulates shoot formation in wide variety of plant species. It may either induce the synthesis and accumulation of an endogenous cytokinin [5], [11], [24]. TDZ alone and in combination with NAA (0.5 mg/l) demonstrated shoot multiplication with approximate 1 shoot/node. This type of reduction of shoot multiplication may be consistent with its high cytokinin activity. In woody plant species, low levels of TDZ induce the axillary shoot proliferation but higher levels may inhibit it [19].

Oroxylum indicum plant is rich in phenolic compounds that affect the growth of *in vitro* cultures and causes browning of the explant and the surrounding medium. Browning, especially during explant establishment is the main limiting factor. The exudation of phenols (browning) prevents the growth of cultures. To prevent the exudation of phenols, antioxidants such as ascorbic acid and PVP were added in the culture medium. Earlier, similar supplements were reported in *Aloe barbadensis* Mill [6], [23], [31], *Madhuca latifolia* [33] and *Anacardium occidentale* [34] for alleviating the browning problem.

Silver nitrate ($AgNO_3$) is an anti-ethylene compound. Addition of silver nitrate in tissue culture medium inhibits secretion of phenolic compounds from explant, protects the medium from bacterial contamination and allowed the improvement of regeneration process [2], [9]. Shoot regeneration and multiplication in medium supplemented with $AgNO_3$ in combination with cytokinin exhibited better response then medium supplemented without $AgNO_3$. However, MS medium supplemented with BA (1 mg/l) and $AgNO_3$ (2 mg/l) showed maximum response 4 (shoots/explant) in all the combinations studied (Fig. 1C). Under the influence of this chemical, *Oroxylum* explants seldom became brown. Earlier it has been reported that $AgNO_3$ enhance shoot multiplication, without callusing from callus originated from embryonic axis explant in *O. indicum* [17].

Subcultures in the same medium, yielded a cluster of shoots were separated into pieces and each was subculture individually on the fresh medium. Multiplication stage was recycled many time (S5-S6) to produce an unlimited number of shoots. In this study, 6 subcultures were made at four weeks interval. The number of multiple shoots increased with increasing subcultures. In second subculture, highest numbers of shoots (2-3) were obtained (Fig. 1D). After 2^{nd} subculture the shoot multiplication rate demonstrated a declining trend. For elongation growth of *in vitro* generated shoots were transferred on medium supplemented with BA (1 mg/l) and $AgNO_3$ (2 mg/l).

Well grown *in vitro* generated micro-shoots were transferred for root induction on half-strength and full-strength MS supplemented with IBA at various concentrations (0.5- 2.5 mg/l). Poor root induction was observed on micro-shoots of *Oroxylum indicum.* The maximum number of roots per shoot (3-4) with mean length 3.58 cm were obtained on half strength MS medium supplemented with IBA (2.5 mg/l) and $AgNO_3$ (2 mg/l) after four weeks of culture (Table 2). Hundred percent shoots showed rooting in this medium. Length of roots and number of roots were increased with the

duration of culture. They were whitish in colour and 1-6 cm long (Fig. 1E). In full strength MS basal medium root formation was also observed, but frequency of root induction was very poor, only 1-3 roots formed per shoot and roots were very thin.

In vitro propagation of *Oroxylum indicum* has been reported earlier on MS medium supplemented with 6-benzyladenine (8.87µM) and indole-3-acetic acid (2.85µM) [10]. Bansal and Gokhale (2012) studied effect of different additives like Casein hydrolysate, Activated Charcoal, Coconut milk and Silver nitrate for regeneration of *Oroxylum indicum* [4]. Silver nitrate was found to be the best additive for regeneration of *Oroxylum*. Earlier reports on tropical trees belonging to the family bignoniaceae exemplified the shoot proliferation on MS medium supplemented with BA and rooting on MS medium supplemented with IBA can achieved [32], [36], [39].

Addition of $AgNO_3$ in culture media supported multiple shoot formation and root induction has been reported in different plants viz. *Vanilla* [15], *Decalepis hamiltonii* [3], [30], *Rotula aquatica* Lour [35], *Coffea* sp [13],[14], Zinnia [2],[9] and *Brassica* sp [7],[28].

The *in vitro* raised plantlets were acclimatized well in the greenhouse conditions (Fig. 1F). Developed and hardened plants were transferred to the earthen pots and then to the soil. The mortality rate was 40% and the regenerants were morphologically very similar to the plants raised through seeds.

IV. CONCLUSION

MS medium containing only BA (1 mg/l) and $AgNO_3$ (2 mg/l) is best formulation to achieve shoot multiplication of *Oroxylum indicum*. Though, TDZ reported highly effective than all adenine type cytokinins to stimulate axillary shoot proliferation in many woody plant species. It reported not so effective for shoot multiplication of *Oroxylum indicum*. Further study needed for standardization of TDZ concentration for *Oroxylum indicum* shoot multiplication. Very much low concentration of TDZ (less than 0.5 mg/l) alone and in combination with other types of auxins may be effective for *Oroxylum indicum* shoot multiplication.

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APPENDIX-A

TABLES AND FIGURES:

Table 1: Influence of cytokinins (BA/ TDZ), auxin (NAA) and $AgNO_3$ on shoot multiplication of *Oroxylum indicum* through nodal explant

Medium No.	Plant hormones (mg/l) BA	TDZ	NAA	$AgNO_3$ (mg/l)	Shoot multiples Average ±S.E.	Response %
1	1.35	-	-	2	3.75 ± 1.16	100
2	1	-	-	2	4.75 ± 1.37	66.66
3	-	0.5	-	-	1.66±0.32	50
4	-	1	-	-	0	0
5	-	1.5	-	-	0	0
6	-	2	-	-	0.6±0.24	60
7	-	2.5	-	-	0.33±0.2	33.33
8	-	3	-	-	0.2±0.19	20
9	-	0.5	-	2	0.5±0.22	50
10	-	1	-	2	0.66±0.2	50
11	-	1.5	-	2	0.59±0.3	40
12	-	2	-	2	0.66±0,2	66.66
13	-	2.5	-	2	0	00
14	-	3	-	2	0	0
15	-	0.5	0.5	2	1±0	66
16	-	1	0.5	2	0.5±0.22	50
17	-	1.5	0.5	2	0	0
18	-	2	0.5	2	1.2±0.19	83

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Table 2: Root induction on in vitro generated shoots of *Oroxylum indicum*

Medium Strength	Growth regulator IBA (mg/l)	$AgNO_3$ (mg/l)	No. of Roots per shoot Mean ±S.E.	Root length (mg/l) Mean ±S.E.	Response %
½ MS	0.5	2	0	0	0
	1	2	1± 0.89	5.25±1.24	66.66
	1.5	2	1±0.44	1.1±0.43	33.33
	2	2	1±0.44	2.8±1.12	33.33
	2.5	2	3.33±0.95	3.58±1.09	100
MS	0.5	2	0±0	0±0	0
	1	2	1±0.48	2.25±0.59	66.66
	1.5	2	1.5±0.17	0.73±0.17	0
	2	2	3±0.60	0.96±0.32	33.33
	2.5	2	3±0.73	1.43±0.24	66.66

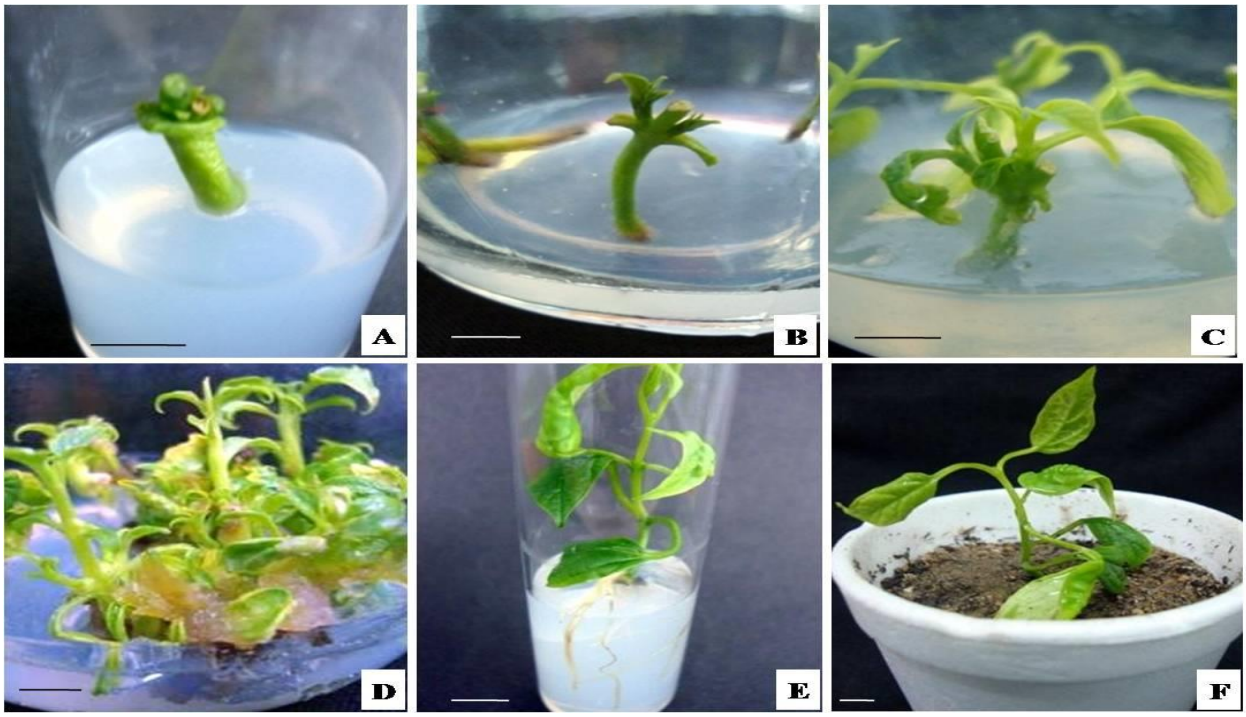

Fig. 1- Micropropagation of *Oroxylum indicum* (L) Vent. A, Nodal explant; **B & C,** Sprouting of nodal buds on MS medium supplemented with BA (1.35 mg/l) and BA (1 mg/l) in combination with $AgNO_3$ (2 mg/l) subsequently; **D,** Multiple shoots in 2nd subculture; **E,** Rooted shoots on ½ MS medium supplemented with IBA (2.5 mg/l) and $AgNO_3$ (2 mg/l); **F,** Hardening of *Oroxylum* plantlets. **Horizontal bar in each photograph is equal to 1 cm.**