Chapter 10

Effect of Additives on Micropropagation of an Endangered Medicinal Tree *Oroxylum indicum* L. Vent

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Additional information is available at the end of the chapter

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1. Introduction

Sonpatha (*Oroxylum indicum* (L.) Vent.) is a threatened medicinal tree species [1,2] belonging to family Bignoniaceae. It is valued for its antimicrobial, antiarthritic, anticancerous and antihepatitic properties possessed by its various parts. Root extract of this tree has been used for long in ayurvedic preparations like Dashmularisht and Chyawanprash [3,4].This tree possesses a flavonoid viz. Baicalein used to check proliferation of human breast cancer cell line MDA - MB - 435 [5]. Sonpatha grows in India, Sri Lanka, South China, Celebes, Philippines and Malaysia[6,7]. 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 [8].Owing to indiscriminate collection, over exploitation and uprooting of whole plants with roots, this valuable tree has become vulnerable in different states of India like Karnataka, Andhra Pradesh, Kerala, Maharastra, M.P. and Chhatisgarh [9,10]. Hence research towards mass multiplication, conservation and higher production of the active compound under *in vitro* culture conditions is essential [11]. Few reports are available on the *in vitro* regeneration of the species [12,13]. Optimum factors influencing growth and morphogenesis vary with the genotype and types of explants used for micropropagation. [14]. Murashige and Skoog (MS) medium with a high content of nitrate, ammonium and potassium is of widespread use in the successful culture of a wide variety of plants. Sometimes it requires supplementation of additional substances in the medium.

Application of additives is adapted to the cultural needs[15] i.e. objectives of the experimental studies like micropropagation, regeneration, cytodifferentiation, androgenesis, biosynthesis of secondary metabolites and biotransformation of cells as well as the particular plant species taken. In this chapter the importance of some additives like activated charcoal (AC), casein hydrolysate (CH), coconut milk (CM) & silver nitrate ($AgNO_3$) & their impact on the

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direct & indirect *in vitro* multiplication of a threatened medicinal tree Sonpatha (*Oroxylum indicum*) is emphasized.

1.1. Activated charcoal (AC)

Activated charcoal (Carbonized wood) is a fine powdered wood charcoal added to tissue culture media, to bring about changes in the composition of the medium[16]. Being porous, it serves to adsorb toxic & phenolic tissue exudates in culture, which prevents inhibition of growth, promotes embryogenesis/ organogenesis Beneficial effects of addition of activated charcoal to media are highlighted by various researchers [17,18].

Activated charcoal (AC) has a very fine network of pores with large inner surface area on which many substances can be adsorbed & it is often used in tissue culture to improve cell growth and development. It plays critical roles in micropropagation, seed germination, somatic embryogenesis, anther culture, synthetic seed production, protoplast culture, rooting, stem elongation and bulb formation in different plants. The beneficial effects of AC on morphogenesis may be mainly due to its irreversible adsorption of inhibitory compounds in the culture medium and substantially decreasing the toxic metabolites, phenolic exudation and brown exudate accumulation [19]. In addition to this activated charcoal is involved in a number of stimulatory and inhibitory activities including the release of substances naturally present in AC which promote growth and darkening of culture media, adsorption of vitamins, metal ions and plant growth regulators, including abscisic acid and gaseous ethylene [20]. The effect of AC on growth regulator uptake is still unclear but some workers believe that AC may gradually release certain adsorbed products, such as nutrients and growth regulators which become available to plants. This review focuses on various roles of activated charcoal in plant tissue culture and the recent developments in this area.

1.2. Coconut milk (CM)

A natural complex may be used when a defined medium fails to support a particular growth response. Its addition makes a defined medium undefined since variations are to be expected in growth promoting or inhibitory compounds in these complexes [21]. A liquid endosperm such as coconut milk would be a good medium for embryo culture. It was first used successfully for culture of very young embryos of *Datura*[22]. Explants proliferate more readily on CM containing media than that observed with auxin. It enhances the proliferation of tumoral tissues indicating that it contains a stimulating substance different from an auxin. Addition of coconut milk serves to rejuvenate mature and permanent cells into actively dividing cells, promoting cell division & callus formation [23]. The composition of CM has been investigated extensively [24] but the analysis has been complicated by the variability in age of coconuts from which the liquid endosperm was obtained.

1.3. Casein hydrolysate (CH)

Casein hydrolysate (CH) is an organic nitrogen supplement containing a mixture of amino acids. Being a good source of reduced nitrogen it has been widely used as an additive to embryo culture media [25,26]. It has proved superior to the combined effect of the amino

acid mixture. It has been thought that nitrogen deficiency can cheaply be fulfilled by its addition [27,28] presumably it contains some stimulatory factors yet unidentified.

1.4. Silver nitrate ($AgNO_3$)

Effect of nitrate supplementation in media has been well established in tissue culture [29,30] so as to enhance shoot multiplication and somatic embryogenesis. Several researchers assume that NO_3 /NH_4^+ ratio acts as buffering stabilization of medium pH resulting *in vitro* organization by adventitious shoot (apical meristem) and NO_3 subsequently promotes extension growth by these meristems into full fledged shoots.

Silver nitrate works as an inhibitor of ethylene activity [31] through the Ag^{2+} ions by reducing the receptor capacity to bind ethylene [32-35].With these observations, water solubility and lack of phytotoxicity at effective concentration led to its application in tissue culture [36].

2. Materials and methods

2.1. Plant materials and culture conditions

Seeds of *O. indicum* were collected from forest areas in and around Jabalpur (M.P.), India. Seeds were germinated on moist sterilized filter paper under *in vitro* conditions. Fifteen to twenty days old seedlings were given a treatment of 1 minute each of 70% ethyl alcohol and 0.1% mercuric chloride followed by sterilized water washing (3-4 times) and excess water was blotted on sterile filter paper. The explants viz. apical buds (ApB) (0.5-1cm) , axillary buds (AxB) (0.7-1cm) and embryonic axis (Ea) (0.4-0.6 cm) were excised and explants were inoculated under aseptic conditions in test tubes on Murashige and Skoog's (MS) medium [37] supplemented with 3% sucrose, 0.8 % agar and different concentrations (0.1-10 mgL^{-1}) of plant growth regulators viz. auxins (2,4-Dichloro phenoxy acetic acid (2,4-D), Indole butyric acid (IBA), Naphthalene acetic acid (NAA) and Indole acetic acid (IAA)) individually. The pH of the media was adjusted to 5.7 before adding agar. Medium (8-10 ml) was dispensed in glass test tubes (15x125 mm) and autoclaved at a pressure of 15 psi and a temperature of 121ºC for 15 minutes. Before inoculation autoclaved medium was left at 25ºC for 24 hrs to check that there was no visible microbial contamination. A piece of callus (2-3mm x 2-3 mm) raised on auxin was subsequently used for indirect organogenesis. The cultures were maintained in culture room at a temperature of 25±2ºC, 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).

2.2. Callus induction, plant regeneration and rooting

Apical bud, axillary bud and embryonic axis explants were inoculated on MS medium supplemented with different concentrations of plant growth regulators to induce multiple shoots & callus. Calli were subcultured onto fresh medium every 20-22 days for further proliferation on suitable medium. Regenerated shoots were elongated up to approx 2 cm, excised and transferred to MS medium fortified with different (0.1, 0.5, 1.0, 5.0 mgL^{-1}) concentrations of NAA, IBA and IAA for root induction.

2.3. Fortification of additives into the media

Out of different plant growth regulators (auxins and cytokinins) used , the frequency of shoot initiation, rate of multiplication and shoot length was significantly high on BAP ($1mgL^{-1}$) supplemented media (Selected medium SM) in both *in vitro* regeneration routes (both directly i.e. from explants ApB, AxB & Ea as well as indirectly from callus. Addition of different concentrations of additives (CH, CM, AC & $AgNO_3$) was studied on different explants & calli in SM. Most of the cultures have been established to study their morphogenic potential up to three subcultures. With this view the shoot buds regenerated indirectly were multiplied on the same concentration of PGR on which shoots got initiated up to 3 subcultures (one sub culture Passage of 20-22 days). Although the three auxins (IBA, NAA, IAA) induced roots in *in vitro* raised shoots of *Oroxylum indicum* IBA turned out to be the best for all parameters of rooting. Shoots with the highest frequency of root induction and maximum number of elongated roots were developed on MS medium containing IBA ($1mg\ L^{-1}$). MS medium with IBA ($1mg\ L^{-1}$) was selected to apply additives for further rooting experiments (data are not shown for effect of CM & CH on rooting).

2.4. Hardening and acclimatization

Approximately four-month-old plants bearing a well-developed root system were washed carefully to remove traces of agar. The plantlets (5 month old) remained fresh when transferred to conical flask with root system immersed in distilled water (4 days) followed by ordinary water (4 days). Such vigorously growing regenerated plantlets were then transferred to pots containing soil: sand (1:1) mixture for 15 days. Approx 82 % of the hardened plants survived in the pot.

2.5. Data recording:

To test the efficiency of direct shoot regeneration, frequency of shoot induction (FSI) directly from different explants was noted. While for indirect shoot regeneration, frequency of shoot regenerated from callus was calculated. Same parameters were calculated for rooting. The effect of continuous supplementation of plant growth regulators on indirect shoot regeneration was observed up to three subculture passages each of 20-22 days. Shoot buds obtained from I subculture passage were subsequently used as explants for II and III subculture passages. All experiments were completely randomized and repeated twice. Each treatment consisted of 25 replicates.

3. Results and discussion

3.1. Activated charcoal

Activated charcoal has been reported to inhibit heavy leaching of phenolics [38]. In the present work AC successfully overcame this problem during regeneration resulting in shoots with good shoot length. However, the shoots formed in the presence of the AC were rather weak with small leaves (Plate 1, Figs. 1a-1c). Even at low concentration it (activated charcoal)

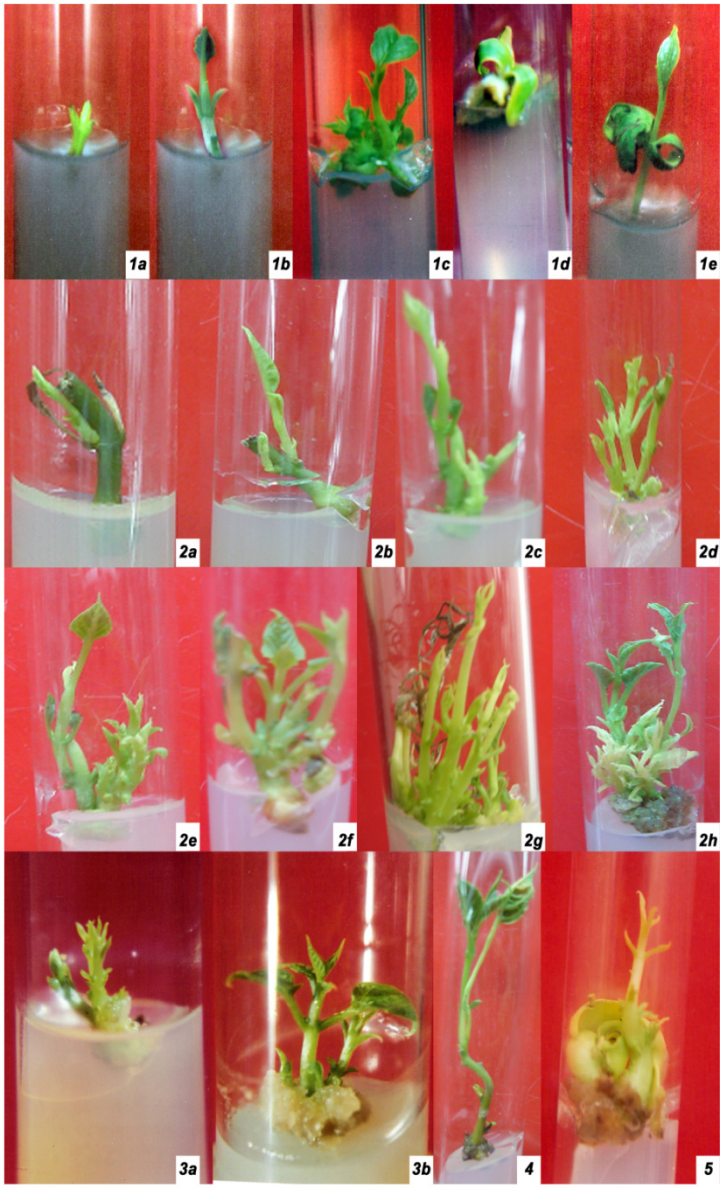

Plate 1. Effect of additives on multiple shoot regeneration from ApB and AxB explants of *O. indicum* (L.) Vent. (1a-1c: Effect of AC on multiple shoot regeneration from AxB explants, 1d-1e: Effect of AC on multiple shoot regeneration from ApB explants, 2a-2d: Effect of $AgNO_3$ on multiple shoot regeneration from AxB explants in I subculture passage 2e-2f II subculture passage, 2g-2h: III subculture passage, 3a-3b: Effect of $AgNO_3$ on multiple shoot regeneration from ApB explants, 4: Elongated shoot, 5: Regeneration from embryonic axis explant

inhibited multiple shoot formation (direct) (Graphs 1, 2) as well as callusing at the base of regenerated shoots. Inhibition of multiple shoot formation after AC supplementation was also observed in *Ficus carica* [39]. Rooting of *in vitro* regenerated shoots initiated after 20-25 days of inoculation on IBA (1mgL^{-1}) supplemented medium containing AC. Only low and moderate (2, 4 %) concs. of AC were effective, while high conc. (6%) failed to induce rooting. Roots developed in *in vitro* regenerated shoots on this rooting media were long, thin, unbranched and too weak to be transferred for hardening process.

3.2. Coconut milk

The explants remained totally unresponsive when cultured on MS medium fortified with CM. Significant reduction in frequency of shoot initiation, shoot number (SN) and shoot length (SL) was found. Mostly shoots developed with only one leaf at a node. Leaves were small in size and light green in colour (Plate 2, Figs. 6a-7b). Increment in basal callusing was observed after 6-8 days of inoculation. Callus was fresh, light brown in colour and non-regenerative in nature. Coconut milk supplementation as an additive in combination with BAP did not support multiplication of shoot either directly or indirectly in *O. indicum*. This observation supports the past reports [40, 41].

3.3. Casein hydrolysate (CH)

Casein hydrolysate (CH) supplementation to culture medium successfully overcame inhibition of regeneration from explants directly as well as indirectly. The number of shoots was found to be enhanced on CH (20 mgL^{-1}) supplemented medium resulting in 9.34 fold increase over control. Higher concs. of CH (30 and 40 mgL^{-1}) resulted in no further increase in the number of shoots (Graphs 1-3). CH has also been found useful in *Anogeissus pendula* & *A. latifolia* [42]. Induction of healthy shoot formation has been reported in *Crataeva nurvala* [43] using CH.

Compared to shoot number (SN) shoot length (SL) was adversely affected at all concs. of CH enhancing only 0.5 to 2 cm shoot length in apical meristem derived shoots and 0.7 –1.0 cm in axillary bud derived shoots respectively (Plate 2, Figs. 1a-5). CH (500 mg L^{-1}) supplemented medium did not support shoot growth as a consequence of which shoots remained compact and stunted. In some cases reduced concentrations of CH induced elongation of shoots [44]. CH was unable to induce indirect multiple shoot formation from calli as efficiently as directly from the explants (Graph 4).

3.4. Silver nitrate ($AgNO_3$)

Maximum frequency of shoot intiation has been observed on $AgNO_3$ supplemented medium in both types of shoot regeneration systems (direct and indirect) among all the additives attempted (Graph1). Explants when treated with different concs. of $AgNO_3$ (0.1, 1, 2, 4 mgL^{-1}) with BAP (1mgL^{-1}) resulted in the formation of healthy shoots bearing large dark green leaves (Plate 1, Figs. 2a-5). Silver nitrate has produced positive effect on all the shoot

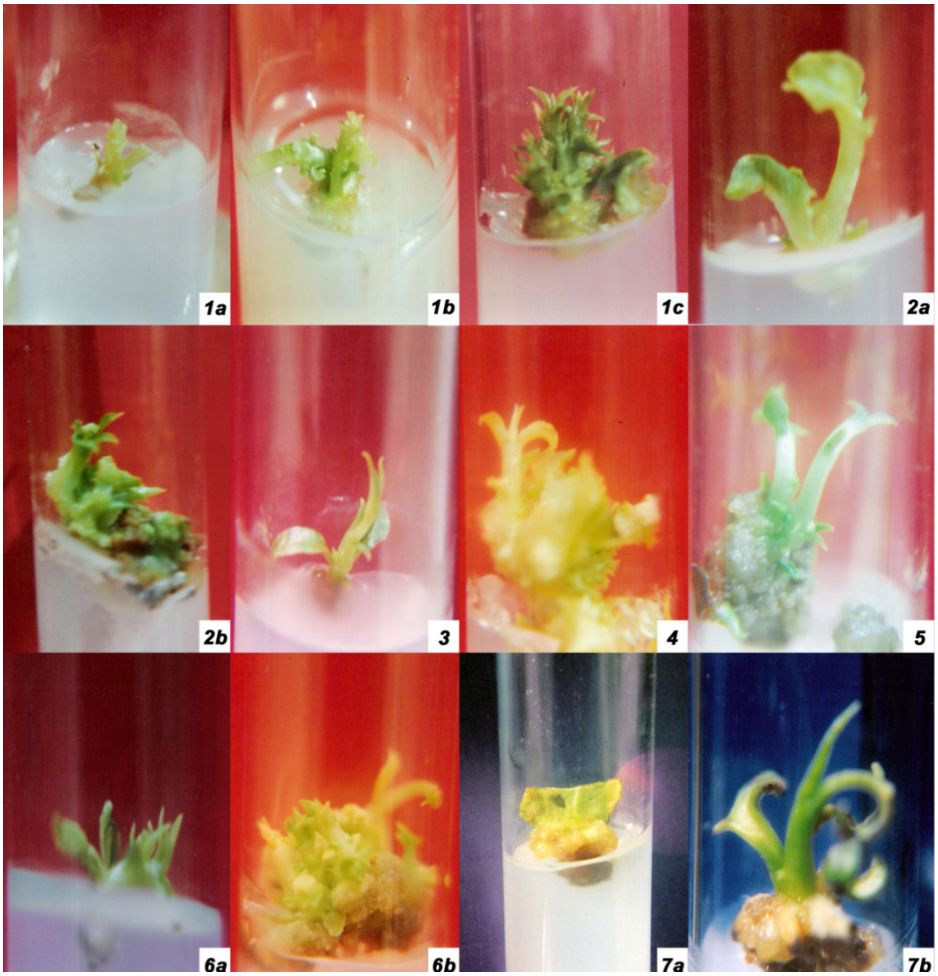

Plate 2. Effect of additives on multiple shoot regeneration from ApB and AxB explants of *O. indicum* (L.) Vent. 1a-2a: Effect of CH on multiple shoot regeneration from ApB explants, 2b-5: Effect of CH on multiple shoot regeneration from AxB explants , 6a-6b: Effect of CM on multiple shoot regeneration from AxB explants, 7a-7b: Effect of CM on multiple shoot regeneration from ApB explants.

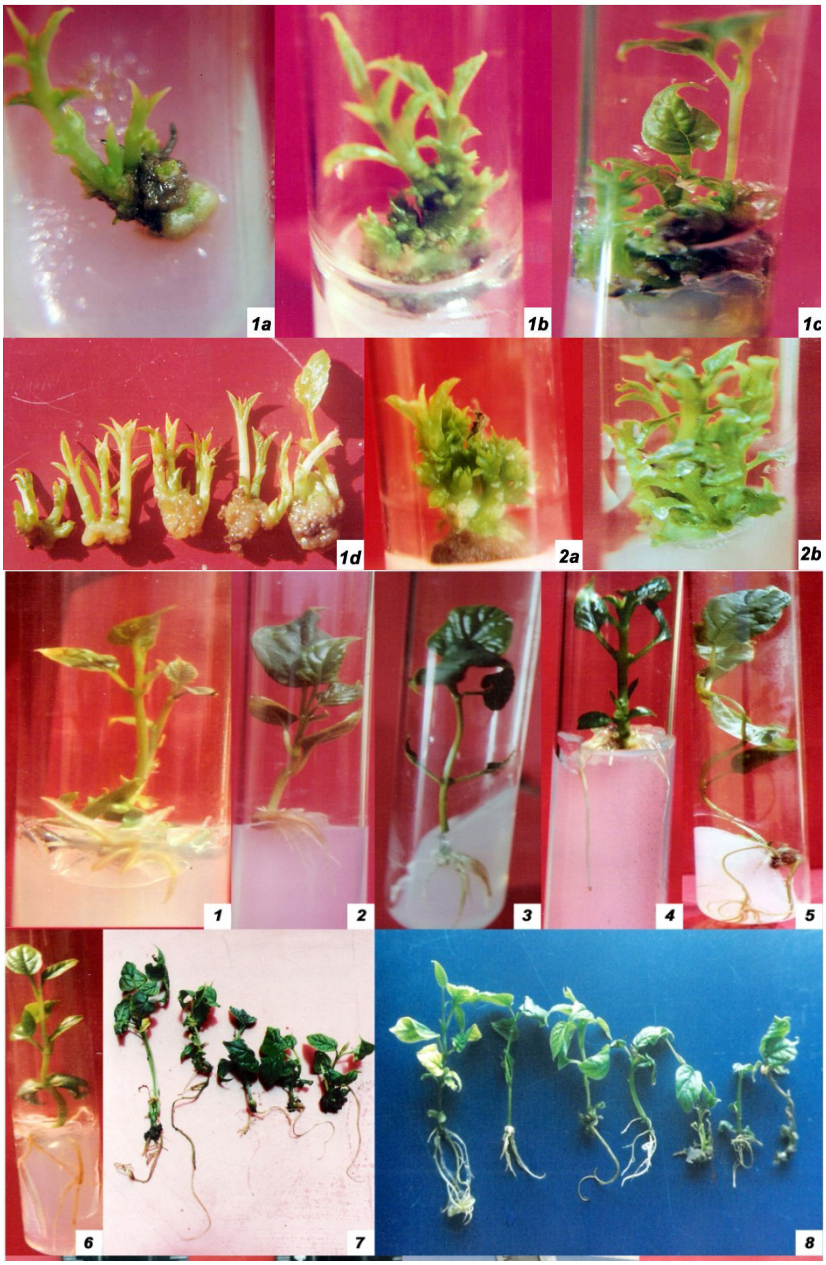

Plate 3. Effect of additives on multiple shoot regeneration (indirect) and rooting from in *O. indicum* (L.) Vent. 1a-2b: Effect of silver nitrate AgNO3 (2mgL^{-1}) on indirect multiple shoot formation (1a-b:I subculture passage, 1c-d: II subculture passage, 2a-2b: III subculture passage), 1-8: Rooting in *in vitro* regenerated shoots on IBA (1mgL^{-1})+ AgNO3 (2mgL^{-1}) +MS medium

regeneration parameters FSI (Frequency of Shoot Initiation), MNS (Mean Number of Shoots), MSL (Mean Shoot Length) for direct and indirect regeneration, Graph 1-4) and rooting parameters FR (Frequency of Rooting), MNR (Mean Number of Roots), MRL (Mean Root Length) (Graph 5) tested for regeneration of *O. indicum* by developing healthy plantlets (Photoplate1:2a-5; Photoplate 3). Silver nitrate has been used previously to prevent callus formation in tree and woody species viz. *Garcinia mangostana* [45], *Albizia procera* [46] and *Manihot esculanta* [47] as well as in other plants viz. *Vanilla planifolia* [48]. In the present study multiple shoot proliferation & elongation of shoots (Table-1) were enhanced efficiently on selected medium (SM) by $AgNO_3$ (2 mgL^{-1}). Such silver nitrate supported multiple shoot formation has been reported in different plants viz. *Coffea* sp. [49, 50] and *Brassica* sp. [51, 52].Supplementation of $AgNO_3$ in culture media caused significant positive effect on shoot number with its best response being observed at 2 mgL^{-1} in subculture passage-III. In some plants, the regeneration potential of cultured cells and tissues has been reported to decrease with increasing cycle of subcultures [53]. Incorporation of $AgNO_3$ in the culture media in the present species enhances shoot multiplication up to sub culture passage III (Graph 4) by retaining the regeneration potential as reported in *Albizzia julibrissin* & *Nicotiana plumbaginifolia* [15].

Addition of $AgNO_3$ in culture media resulted in maximum rooting frequency and root length (Graph 5) of *in vitro* regenerated shoots in the present study. Silver nitrate induced rooting has been reported in *Vanilla* [48], *Decalepis hamiltonii* [54, 55] and *Rotula aquatica* Lour. [56].

4. Conclusion

The *in vitro* regeneration of some plants remains difficult due to high degree of callusing, high phenolic excretion into the medium and consequent blackening of explants. Fortification of culture media with different plant growth regulators i.e. auxins and cytokinins is not enough to regenerate the plant with high efficiency. This type of cultures in some cases may be improved by incorporation of additives in the media due to their growth and development promoting activities. In the present work additives used were Casein hydrolysate (CH), Activated Charcoal (AC), Coconut milk (CM) and Silver nitrate ($AgNO_3$) to induce *in vitro* regeneration of an important endangered medicinal tree species *Oroxylum indicum* (L.) Vent. Among all the additives used CH and $AgNO_3$ acted positively for multiple shoot regeneration from different explants (ApB, AxB and Ea) directly as well as indirectly by overcoming inhibition during regeneration. Whereas more conspicuous role of Casein hydrolysate (CH.) in *O. indicum* seems to be on the number of shoots induced while that of $AgNO_3$ was mainly on shoot lengths besides the number of shoots produced. Also $AgNO_3$ favors efficient rooting from *in vitro* regenerated shoots when supplemented in combination with auxin IBA. Overall Silver nitrate has turned out to be the best additive for regeneration of *O. indicum*. The production of secondary metabolites from *in vitro* regenerated vis-a-vis nature grown tissues is expected to provide useful information in future.

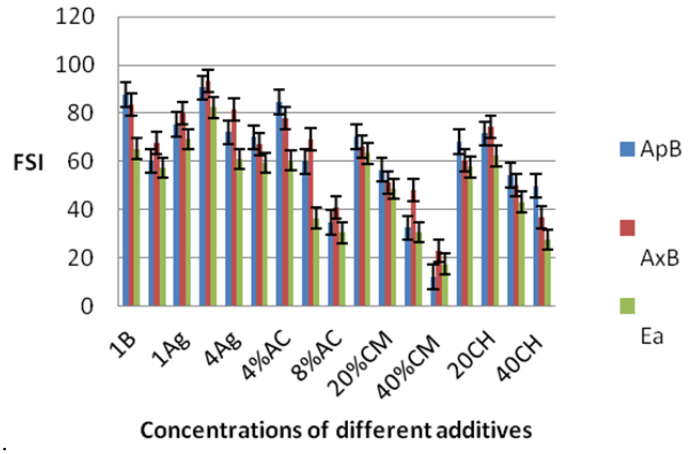

Figure 1. Effect of BAP +Additives on FSI from different explants of O. indicum (L.) Vent.

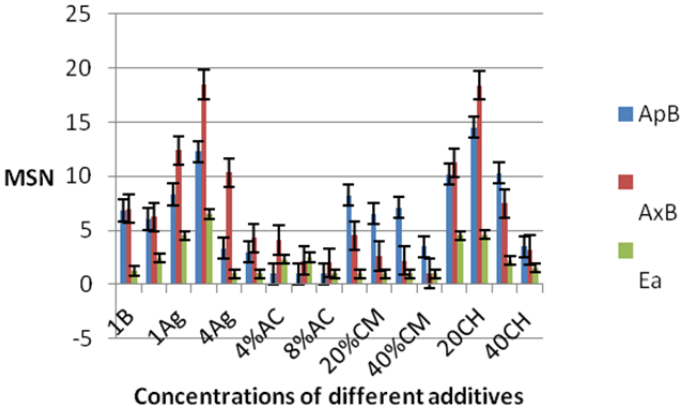

Figure 2. Effects of BAP +Additives on MSN from different explants of O.indicum Vent.

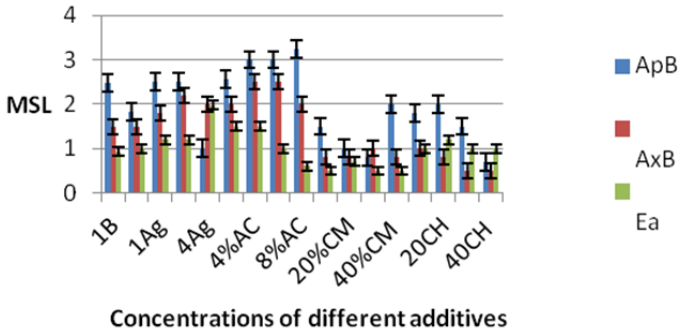

Figure 3. Effect of BAP +Additives on MSL from different explants of O. indicum (L.) Vent.

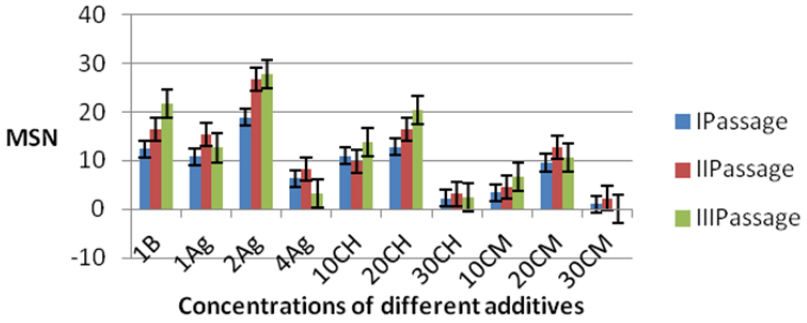

Figure 4. Effect of BAP +Additives on MSN (indirect) in O.indicum (L.) Vent

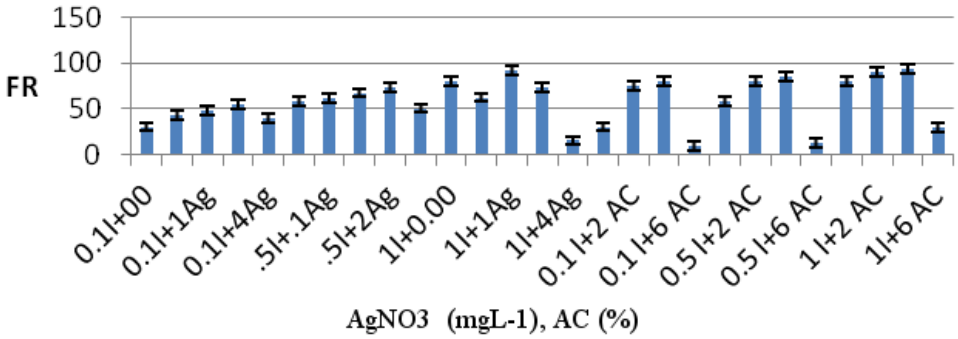

Figure 5. Effect of AC and $AgNO_3$ with IBA on rooting from *in vitro* regenerated shoots of *O. indicum*

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