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Conservation of an Endangered Medicinal Forest Tree *Oroxylum indicum* (L) Kurz through *in-vitro* Micropropagation-A Review

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Authors' contributions

This work was carried out in collaboration between all authors. Authors ST and RSN designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author SR managed the analyses of the study and the literature searches. All authors read and approved the final manuscript.

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Review Article

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ABSTRACT

The species *Oroxylum indicum* (L) Kurz (Bignoniaceae) is an important medicinal tree being rich in secondary metabolites, employed in various traditional medicinal systems from folk to modern medicine. All the plant parts (root, stem bark, seed, leaf and flower) of the species are known to possess bioactive compounds and the whole plant is over exploited by pharmaceutical industries. In view of the increased anthropogenic activities, destruction of natural habitat, these trees are being rapidly eroded from natural ecosystem. Thus the tree is becoming an endangered, vulnerable and will be facing extinction in near future and is being pushed into the red data book. Hence, there is an urgent need for the development of conservation methodologies through *in-vitro* culture techniques by employing embryo culture, mericlone technology and somatic embryogenesis etc., for large-scale production of this valuable medicinal tree. In the present review we made an attempt to compile and critically analyze the various reports on micro-propagation protocols for the conservation of this endangered valuable medicinal forest tree species.

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1. INTRODUCTION

Plants have long been and continued to be the basis of many traditional medicines worldwide. Asian traditional medicinal systems such as traditional Chinese medicine, Korean Chinese medicine, Japanese Chinese medicine, Ayurveda from India, Jamu from Indonesia are well known [1]. Medicinal plants can provide biologically active molecules and lead structures for the development of modified derivatives with enhanced activity and /or reduced toxicity. The medicinal plants and their derivatives have long been recognized as an important source of therapeutically effective medicines as they contain secondary metabolites which are potential sources of drugs. Plant based medicines are healthier, safer and more reliable than synthetic ones [2]. The medicinal plants appear to be rich in secondary metabolites, widely used in traditional medicine to combat and cure various ailments. The anti-inflammatory, antispasmodic, antianalgesic and antidiuretic activities can be attributed to their high steroids, tannins, terpenoids and saponins.

In view of the tremendously growing world population, increasing anthropogenic activities, rapidly eroding natural habitat a great number of herbs and trees are dwindling. Increasing *per capita* consumption has resulted in unsustainable exploitation of earth's biological diversity, exacerbated by climate change, ocean acidification and other anthropogenic environmental impacts [3].

In view of this to save the unbalanced ecosystem, the plant tissue culture techniques have been increasingly applied to many medicinal plants for their conservation. Thus *in-vitro* culture technology plays a vital role for mass propagation, conservation of germplasm, continuous production of bioactive compounds, and for genetic improvement of the medicinal plants. Plant tissue culture is found to be an attractive alternative approach to the traditional methods of plantations, as it offers a controlled supply of biochemicals independent of plant availability and more consistent product quality [4].

Due to ever growing demand, the availability of medicinal plants to the pharmaceutical companies is not enough to manufacture herbal medicines. The powerful techniques of plant cell, tissue and organ culture, recombinant DNA and bioprocessing technologies have offered mankind a great opportunity to exploit the medicinal plants under *in-vitro* conditions. In clonal propagation, plants are multiplied using shoot tips and nodal segments as explants for developing *true-to-type* of plants. For rapid *in-vitro* clonal propagation of plants, different types of media (MS, B_5, WPM etc) supplemented with various concentrations of auxins and cytokinins and their combinations were used. Shoot number increases logarithmically with each subculture to give greatly enhanced multiplication rates. As this method involves only organized meristems, hence it allows recovery of genetically stable and *true-to-type* progenies [5-8].

The present attempt has been made to review and compile updated information on various micropropagation protocols for the conservation of *Oroxylum indicum* (L) Kurz a plant used in Indian system of medicine for variety of purposes. In view of medicinal importance of the species *O. indicum*, is being over exploited and becoming an endangered species. Due to its demand in pharmaceutical industry, it has been pushed into the list of Indian red data book [9]. Only 30% of the seeds were found to be viable and frequent failure of germination is due to fungal infection of seeds [10,11]. So, there is a need to conserve the species.

The species *O. indicum* Syn*. Bignonia indica* belongs to the family Bignoniaceae. It is distributed in India, Sri Lanka, Malaysia, China, Thailand, Philippines and Indonesia [12]. The species is a native of Indian subcontinent, in the Himalayan foothills with a part extending to Bhutan and Southern China, Indo-China and the Malaysia ecozone. It is visible in the forest biome of Manas National Park in Assam, India. The species lives in relationship with the actinomycetes *Pseudonocardia oroxyli* present in the soil surrounding the roots [13].

In India, the tree is indigenous to Eastern and Western Ghats and is also found in North-Eastern regions [14]. It is commonly known as "*Indian Trumpet tree"* due to resemblance of its flower to a trumpet, *Broken bones tree*-when the long leaf and flower bearing stalks dry and fall from the tree, their accumulation beneath the tree resembles a pile of broken bones, *Indian calosanthes, Midnight horror* and *Tree of Damocles*. In India it is called by using

vernacular names: **Bengali:** Sona, Khonha, Paharijora, Kani-Dingi; **Hindi:** Bhut-Vriksha, Dirghavrinta, Kutannat, Manduk (The Flower), Patrorna, Putivriksha, Shallaka, Shuran or Son, Vatuk; **Kannada:** Tattuna; **Konkani:** Davamadak; **Malayalam:** Palaqapayyani, Vashrppathiri, Vellappathiri; **Marathi:** Tayitu, Tetu; **Sanskrit:** Aralu, Shyonaka; **Tamil:** Cori-Konnai, Palai-Y-Utaicci, Puta-Puspam (The Flower); **Telugu:** Manduka-Parnamu, Pampena, Suka-Nasamu. The plant is known for its high medicinal, commercial and economic importance.

1.1 Botanical Description

The species, *O. indicum* is a small to medium sized deciduous tree measuring upto 12 metres in height with light greyish brown, soft, spongy bark having corky lenticels. Leaves are large and bipinnate or tripinnate, upto 1-5 m long, leaflets are ovate or elliptical in shape. Flowers are many, large bell shaped and fleshy with five stamens. The tree is a night bloomer and flowers are adapted to natural pollination by bats (Chiropterophily) [15]. Flowering is from January to March and fruits are produced in April to July. Fruits are capsule, long, up to 1.0 meter, flat, curved downward and resemble the wings of a large bird or dangling sickles or swords in the night, with woody valves enclosing many broad silvery winged seeds which are flat, thin (Fig. 1a-d).

1.2 Medicinal Importance

Several medicinal properties have been attributed to the plant not only in *Ayurveda* but also in *Tribal folk,* and *Unani* system of medicines for the treatment of various ailments [9,16,17]. It is the main ingredient of ayurvedic formulations viz., *Chyavanaprasha* and *Dasamoolam* [18]. Pharmacologically, it has been found to have astringent, antiinflammatory, antihelminthic, antibronchitic, antileucodermatic, antirheumatic, and antianorexic and many other medicinal properties [19]. The fresh root bark is soft and juicy and creamish yellow to greyish in colour. On drying, the bark shrinks, adheres closely to the wood and becomes faintly fissured [20]. This tree possesses a wide range of medicinal properties such as antinflammatory, anticancerous, anti-helminthic, anti-leucodermatic, anti-rheumatic and anti-anorexic, antimicrobial, antibacterial, antioxidant, analgesic and anti-tussive [11,21-24].

Fig. 1a-d. Showing habit of *Oroxylum indicum*. a) Plants growing in Mallur forest, Warangal District, Telangana State; b) Bunch of fruits; c) A single pod about 1.0 m long; d) Seeds with wings

In view of its medicinal importance and over exploitation of the species by pharmaceutical industries, there is a need to conserve this endangered medicinal tree by using *in-vitro* micropropagation technique.

2. MICROPROPAGATION

In-vitro propagation of plants holds tremendous potential for the production of high quality plant-based medicines throughout the year and irrespective of the season [25]. This can be achieved through different methods including micropropagation. Micropropagation has many advantages over conventional methods of vegetative propagation, which suffers from several limitations [26]. The multiplication rate is greatly increased through this technique. It also permits the production of *true-to-type* and pathogen-free plants. The process of micropropagation is usually divided into several stages such as pre-propagation, callus induction, callus subculture and proliferation, shooting, rooting and hardening. Micropropagation of various plant species, including many medicinal plants, has been reported [27-31]. Plant regeneration from shoot and shoot meristems yielded encouraging results in medicinal plants like *Catharanthus roseus, Cinchona ledgeriana; Digitalis sps*, *Rehmannia glutinosa, Rauvolfia serpentina, Isoplexis canariensis, Wrighitia tomentosa* [32-36].

The advantages of *in-vitro* micropropagation of medicinal plants are listed below:

- Higher rate of multiplication.
- Environment can be controlled or altered to meet specific needs of the plant.
- Plant available all year round (independent of regional or seasonal variation)
- Identification and production of clones with desired characteristics.
- Production of secondary metabolites.
- New and improved genetically engineered plant can be produced.
- Conservation of threatened plant species.
- Preservation of genetic material by cryopreservation.

2.1 *In-vitro* Conservation Strategies Used for the Propagation

The species *O. indicum* is propagated naturally by seeds, which germinate in the beginning of the rainy season. Seedlings require moderate shade in the early stages. However, the seed set is poor and seed viability is low. The *in-vivo* seed viability of *O. indicum* was found to be 30%. This observation was made by counting the seeds from each pod on the basis of viable (fertile) or non-viable (aborted) nature of the embryo and also sowing the seeds in the research field [36]. Problems related with its natural propagation and indiscriminate exploitation for medicinal purpose has pushed *O. indicum* to the list of endangered plant species of India. According to the report of task force on conservation and sustainable use of medicinal plants, Planning commission, Government of India (2000), the estimated demand of the plant material of *O. indicum* in Southern India is 500 kg per annum [9].

Since the demand of *O. indicum* by pharmaceutical industry is exclusively escalating, the existence of the species in natural population is highly threatened. As an effort towards the conservation and collection strategies for this species, assessed the genetic diversity in different accessions of the species by RAPD [37]. They found that the genetic diversity of this species is very low and depicted the possibility as difficulty in adapting to different environmental conditions [37]. Presently, the following *in-vitro* conservation methods are used for conservation of this endangered forest tree species.

2.2 *In-vitro* Seed Germination

The seeds of *O. indicum* were treated with different plant growth regulators (viz. Benzyl Amino Purine-BAP, Indole-3-acetic acid-IAA, Gibberellic acid-GA_3) and temperatures (cold and hot water treatment) to improve the seed germination percentage in *O. indicum*. Cold water treatment had significantly improved the seed germination as compared to control, hot water treatment and tested plant growth regulators. Cold water at 4℃ for 24 h was found to be optimum, which resulted in more than 80% seed germination response [38]. The study suggested that the seed germination and seedling growth of *Oroxylum* can be improved by using simple and economic method.

We have developed an efficient protocol for *in-vitro* seed germination in *O. indicum* for the first time [39]. The seeds of *O. indicum* were cultured on different types of media such as ½ strength MS, MS, B_5 and WPM media containing 15, 20 & 30 gm/L sucrose. Among the various media used, MS medium containing 20-30 gm/L sucrose showed absolute percentage of germination. Seedlings germinated on these media were found healthy with elongated shoot and root system and the time taken for complete germination was recorded 6-8 days only. Among various types of media used, seed germination was effective (100%) on ½ strength MS and MS media containing different concentrations of sucrose. And the time taken for the germination was also found less (6-7 days) on the MS medium compared to the other types of media used (Fig. 2). The percentage of seed germination was found to be lesser on B_5 and WPM media across the range of sucrose concentrations. The seed germination was delayed up to 25-30 days in these two types of media used. Whereas, the seed germination percentage was found to be 25% and 15% on B_5 and WPM media respectively. These *in-vitro* raised seedlings were hardened and established successfully (Fig. 2). The percentage of survival was recorded as 85%. These acclimatized plantlets were similar to their mother plant. This method of multiplication can be used for conservation of the species [39].

2.3 Zygotic Embryo Culture

An efficient, reproducible *in-vitro* regeneration protocol was developed in *O. indicum* through zygotic embryo culture [40]. The zygotic embryos were inoculated on MS medium containing 30 g/L sucrose and supplemented with different concentrations of BAP/KIN/TDZ (1.0-7.0 mg/L) individually. The multiple shoot bud induction was observed within two weeks of incubation.

Absolute (100%) percentage of response was recorded on MS medium fortified with 5.0 mg/L BAP with maximum number of shoots/explant (20±0.82) in comparision to KIN.

The influence of $AgNO_3$ on *in-vitro* zygotic embryo culture in *O. indicum* was also studied [41]. The zygotic embryos were cultured on MS medium augmented with different concentrations (1.0-7.0 mg/L) of BAP/KIN/TDZ individually and also in combination with 0.1 mg/L $AgNO_3$. More number of multiple shoots/explant (20±0.82) was observed at 5.0 mg/L BAP. Whereas 0.1 mg/L $AgNO_3$+3.0 mg/L TDZ had induced the maximum number of shoots/explant (32±0.02) in comparison to all other PGRs used (Fig. 3). When the explants with multiple shoots subcultured on the same concentration and combination of PGRs showed the enhancement in multiple shoots proliferation. More number of multiple shoots development was observed on MS medium supplemented with $AgNO_3$ + BAP/KIN/TDZ compared to individual PGR in *O. indicum* [41].

2.4 Clonal Propagation

Micropropagation/Clonal propagation technique using shoot tip and nodal segments plays a vital role in mass-scale multiplication and rapid conservation of an endangered or threatened and medicinally important species within short span of time and limited space. The plants produced from this method are *true-to-type*.

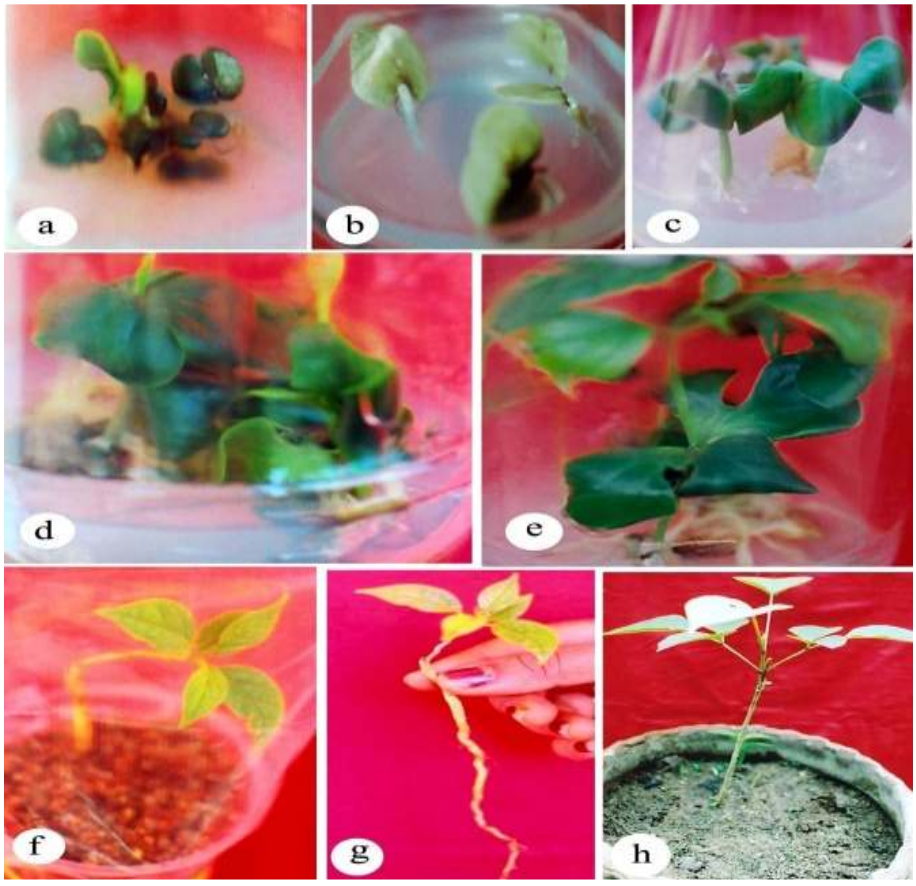

Fig. 2a-h. *In-vitro* seed germination in *O. indicum*
a) Germination of the seeds on WPM+30 g/L sucrose b) Germination of the seeds on B_5+30 g/L sucrose; c) Germination of the seeds on MS medium+30 g/L sucrose; d & e) Development of seedling on MS medium+30 g/L sucrose with fully expanded cotyledonary leaves after 30 days and 45 days of germination respectively; f) Acclimatization of plantlet in soilrite in a plastic pot; g) *In-vitro* raised plantlet showing lengthy root system; h) Acclimatized plantlet in an earthenware pot containing garden soil

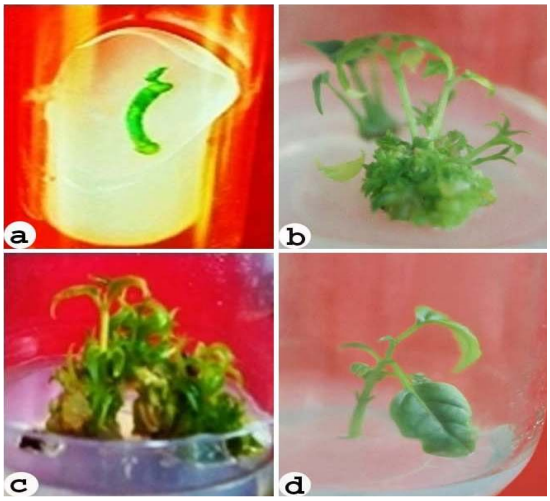

Fig. 3a-d. Influence of $AgNO_3$ on zygotic embryo culture of *O. indicum*
a) Germination of embryo; b) Induction of multiple shoots at 0.1 mg/L $AgNO_3$+3.0 mg/L TDZ; c) Further proliferation of shoots on the same after 1st subculture; d) Shoot elongation on MS+1.0 mg/L GA_3

2.5 Cotyledonary Node Explants

A protocol was reported on *in-vitro* propagation of *O. indicum* using cotyledonary node explants [42]. Among the different types of cytokinins used for culture establishment, 6-benzyladenine (BA) exhibited the best response with higher concentrations (8.87 µM or above) for inducing multiple shoots. Inclusion of IAA (2.85 µM) into BA supplemented medium triggered a high frequency of response as well as a proliferation of shoots. Repeated subculturing of cotyledonary node and *in-vitro* developed nodal segments on MS medium with 6-BA (4.44 µM) at 4-week intervals resulted in continuous mass multiplication of shoots without any evidence of decline. Root induction was best (91.6%) when MS strength was reduced to one-quarter and combined with NAA (2.69 µM) and IAA (5.71 µM), with a high survival rate (70-72%) of plantlets hardened in either soil rite or mixture of soil: sand: soil rite (1:1:2).

We have also reported on micropropagation by using cotyledonary node explants [43]. The cotyledonary nodal segments of *O. indicum* were cultured on MS medium supplemented with different concentrations of PGRs viz., BAP/KIN/TDZ alone (Fig.4). The cotyledonary nodal explants cultured on all the concentrations of PGRs, supported the induction of axillary bud break and proliferation of multiple shoots in *O. indicum* [43]. Among the PGRs tested, maximum multiple shoots/explant was found in all the concentrations of BAP in comparison to KIN/TDZ. Maximum percentage of response and more number of multiple shoots (10.0±0.529) formation per explant was found at 3.0 mg/L BAP followed by 4.0 - 5.0 mg/L BAP. Maximum percentage (73%) of response was observed at 3.0 mg/L KIN whereas maximum number of multiple shoots/explant was found at 5.0 mg/L KIN. Less percentage (30%) of response was noted at high concentrations of KIN. It was also found that at 3.0-5.0 mg/L KIN, callus was developed along with the formation of multiple shoots. The cotyledonary nodal segments cultured on MS medium with different concentrations of TDZ showed varied results. Maximum percentage of response (72%) was observed at 0.8 mg/L TDZ, whereas more number of multiple shoots was recorded at 0.6 mg/L TDZ. Low percentage of response with less number of shoots per explant was recorded at low concentrations of TDZ [43]. Among the three types of cytokinins used, the best PGR with maximum percentage of response and increased number of multiple shoots was found to be the BAP as a sole growth regulator followed by KIN and TDZ [44].

2.6 Nodal Culture

A method was reported to regenerate plants from nodal segments [45]. During their experiments, nodal segments of *O. indicum* were cultured on MS medium with and without PGRs and half-strength MS +0.5 mg/L IBA for *in-vitro* rooting. They have successfully established the regeneration and the regenerants have exhibited a normal development.

A protocol was developed for multiple shoot regeneration through axillary bud explants. BAP at 4.43 µM proved better than KIN with highest frequency of number of shoots formation per explant [46]. Half-strength MS medium with 4.92 µM IBA was found to be the best for *in-vitro* rooting. Subsequently these *in-vitro* rooted plantlets were transferred to field. A protocol was developed using axillary seedling buds and cultured on MS medium supplemented with BAP (1.0 mg/L) in combination with different concentrations of $AgNO_3$ [47]. Rooting of the regenerated shoots was achieved on medium (half-strength MS) containing IBA (1.0 mg/L) and $AgNO_3$ (1.0 mg/L).

A protocol was reported by using nodal segments of *O. indicum* in WP medium supplemented with 3.0 mg/l of BA was more effective in bud break and induced maximum (7.6) number of shoots/explant [48]. The shoot elongation was maximum with shoot length of 1.6 cm at 0.5 mg/l BA among various concentrations of growth regulators tried. The best *in-vitro* rooting response (6.0 roots/shoot; 1.8cm root length) was observed on ¼ strength WPM containing α- naphthalene acetic acid (NAA, 0.5 mg/l), though maximum length of root (2.5 cm) was observed at 0.25 mg/L NAA and 0.5 mg/L IAA. The well rooted plants were subsequently hardened and acclimatized with 70% survival rate in the potting mixture having soil, perlite and compost (1:1:1) and when transplanted in field, these plants showed 80% survival after 2 months.

Samatha and Ramaswamy [43] have also reported micropropagation of *O. indicum* using nodal explants. The nodal segments were cultured on MS medium supplemented with different concentrations of BAP/KIN (1.0-5.0 mg/L)/TDZ (0.2 - 1.0 mg/L) as a sole growth regulator. As the concentration of BAP increased the percentage of response and as well as the number of multiple shoots per explant was also found to be enhanced. Maximum percentage of response and more number of multiple shoots (15.0±0.10) formation per explant was found at 5.0 mg/L BAP followed by 3.0-4.0 mg/L BAP. While nodal segments cultured on KIN showed maximum percentage of response at 4.0-5.0 mg/L KIN, and maximum number of multiple shoots was observed at 5.0 mg/L KIN (11±0.10). Less percentage (30%) of response was noted at low concentrations of KIN. It was also recorded that the callus was developed at 4.0-5.0 mg/L KIN along with the formation of multiple shoots. Whereas, at 0.8 mg/L TDZ, maximum percentage of response (72%) with the development of 4.6± 0.042 average number of shoots per explant was recorded. Low percentage of response with less number of shoots per explant was recorded at low concentrations of TDZ. We have observed the superiority of BAP followed by KIN and TDZ in inducing maximum number of shoots per explant in *O. indicum.*

2.7 Mericlone Technology

A simple and reliable protocol for *in-vitro* shoot induction was developed using shoot apical meristems as explants in *O. indicum* [43]. These explants showed significantly high shoot multiplication on MS medium with 2.0 mg/l BAP.

We have developed the protocol for shoot induction of multiple shoots from shoot tip explants of *O. indicum*. These were cultured on MS medium supplemented with different concentrations of BAP/KIN (1.0-7.0 mg/L)/TDZ (0.2 - 1.0 mg/L) as a sole growth regulator and also on MSO. It was observed that the number of shoots per explant and the mean length of the shoots were found to be varied with the growth regulators and their concentrations used. Maximum number of multiple shoots (12.0±1.078) was recorded at 3.0 mg/L BAP. At higher concentrations of BAP, low percentages of response as well as the number of shoots per explant was found to be reduced. At higher (5.0 mg/L) levels of KIN, less number of multiple shoots and also callus formation was found. While TDZ showed less percentage of response compared to BAP and KIN. Maximum percentage (60%) of response was observed at 0.4 mg/L TDZ, and high frequency number of shoots development per explant (4.0±0.084) was found at the same concentration of TDZ. At higher concentration of TDZ less percentage of response and also low number of shoots per explant were noted. Shoot tip explants cultured on MS medium without growth regulators (MSO) showed the elongation of only single shoot.

2.8 Callus Induction

A simple and reliable protocol for *in-vitro* callus induction was developed using leaf midrib explants of *O. indicum* [48]. Among the different types of growth regulators used for culture establishment, BAP and 2, 4-D exhibited the best response for inducing callus. MS medium with 4 mg/l 2, 4-D was found to be more effective for callus induction from leaf midrib explants in *O. indicum.*

Callus induction from leaf and cotyledonary leaf explants was observed on MS medium containing 30gm/L sucrose and supplemented with different concentrations (1.0-5.0 mg/L) of auxins (IAA/IBA/IBA/2,4-D) [11]. Callusing response was found to be varied from auxin to auxin and also with explant to explant. Morphogenic response (morphology and texture) of callus was found to be different in leaf and cotyledonary leaf explants cultured on MS medium fortified with different PGRs in *O. indicum* (Fig. 5a-d). Callusing response was higher in cotyledonary leaf explants in all the

auxins and concentrations used except at high and low concentrations of PGRs. Maximum frequency of responding cultures and high amount of callusing were recorded at 2.0 mg and 3.0 mg/L auxins tested. It is evident from the results that among the auxins, IBA and 2, 4-D were found to be potent for callus development followed by IAA/NAA. Proliferation of callus was faster and very high yield of callusing mass was achieved within 4 weeks on IBA, 2, 4-D followed by IAA/NAA. Among the explants tested cotyledonary leaf explants were highly efficient for inducing the callus production in *O. indicum* [11].

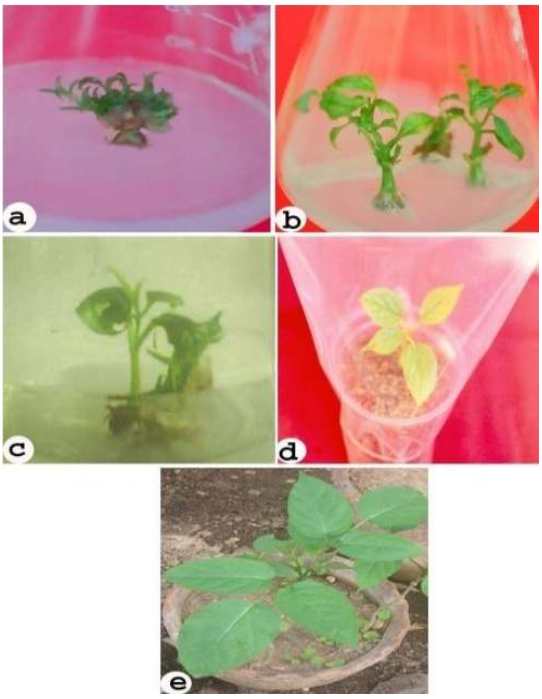

Fig. 4a-e. Showing *in-vitro* micropropagation in *O. indicum.* a) Initiation of multiple shoots formation from cotyledonary nodal segments on MS+3.0 mg/L BAP after 4 weeks of culture; b) Further multiplication and elongation of healthy shoots on MS+3.0 mg/L BAP+1.0 mg/L GA_3; c) *In-vitro* rooting on ½ MS+1.0 mg/L IBA; d) Hardening in the culture room; e) Healthy and established *in-vitro* raised plantlet in an earthenware pot containing garden soil

Callus cultures are extremely important in medicinal plant biotechnology. Manipulation of the auxin to cytokinin ratio in the medium can lead to the development of shoots, roots or somatic embryos from which whole plants can subsequently be produced. The whole plant can be regenerated in large numbers from callus tissue through manipulation of the nutrient and hormonal constituents in the culture medium which is known as organogenesis or morphogenesis. Similarly, callus can be induced to form somatic embryos which can give rise to whole plant. Callus tissue is good source of genetic or karyotypic variability, so it may be possible to regenerate a plant from genetically variable cells of the callus tissue. Cell suspension culture in moving liquid medium can be initiated from callus culture. Callus culture is very useful to obtain commercially important secondary metabolites which can be used in preparation of biopharmaceuticals. Callus induced from explants such as a leaf/root of a medicinally important plant species, the secondary metabolites or drugs can be directly extracted from these callus tissues without sacrificing the whole plant.

2.9 Organogenesis

Organogenesis is the process by which cells and tissues are forced to undergo changes which lead to the production of a unipolar structure, a shoot or root primordium whose vascular system is often connected to the parent tissue.

For the first time indicated that the organogenesis could be chemically controlled [49]. He observed root initiation (rhizogenesis) and shoot inhibition (caulogenesis) after addition of auxin to the medium. Further, Skoog and co-workers gave the concept of regulation of organogenesis by a balance between cytokinin and auxin. They demonstrated that a high ratio of auxin: cytokinin stimulated the formation of root in tobacco callus, but a low ratio of the same induced shoot formation [50].

Organogenesis means the development of adventitious organs or primordia from undifferentiated cell mass by the process of differentiation. In organogenesis, shoots and/or roots are induced in plant tissue culture. Organogenesis (i.e. development of organs) starts with stimulation caused by the chemicals of medium, substances carried over from the original explants and endogenous compounds produced by the culture [51,52].

Direct organogenesis bypasses the need for a callus phase. Direct *in-vitro* organogenesis from leaf explants of *O. indicum* has been achieved on MS medium fortified with various concentrations

(1.0- 5.0 mg/L) of cytokinins BAP/KIN alone and also in combination with 0.5mg/L 2, 4-D [53]. The *in-vitro* developed shoots were transferred to MS medium augmented with 0.5 mg/L GA_3+1.0 mg/L BAP for further proliferation and elongation (Fig. 5a-h). These elongated micro-shoots were shifted on to ½ strength MS medium fortified with 0.5 mg/L-4.0 mg/L IAA/IBA/NAA for *in-vitro* rooting. Profuse rhizogenesis was observed at 1.0 mg/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.

2.10 Somatic Embryogenesis

Reproducible regenerative protocol through somatic embryogenesis for the first time has been developed for conservation of the species *O. indicum* [54]. Cotyledonary leaf explants were cultured on MS medium fortified with various concentrations of BAP (0.25-0.75 mg/L) alone and also in combination with 0.5 mg/L IAA/2, 4-D. Direct somatic embryogenesis was observed from all the concentrations and combinations of PGRs used. Maximum percentage (95%) of response in inducing somatic embryos was found at 0.5 mg/L 2, 4-D+0.75 mg/L BAP. Maximum number of somatic embryoids/explant was also developed at the same concentration and combination of PGRs. More percentage of somatic embryo conversion into bipolar embryos was also found at the same concentration of PGRs used. Highest percentage of bipolar somatic embryos was found to be germinated and converted in to plantlets at 0.2 mg/L IAA+0.25 mg/L BAP. These regenerated plantlets were acclimatized in the culture room and successfully transferred into the field. Thus, somatic embryogenesis and plantlet formation has been successfully achieved in *O. indicum* (Fig. 6). This protocol can be used for developing synseeds, conservation and also for rapid multiplication of *true-to-type* of the species in a short period [54].

The *in-vitro* regeneration of tree species remains difficult due to high degree of callusing, high phenolic exudation into the medium and consequent blackening of explants. Fortification of culture media with different PGRs i.e. auxins and cytokinins is not enough to regenerate the plant with high efficiency. These type of cultures in some cases may be improved by incorporation

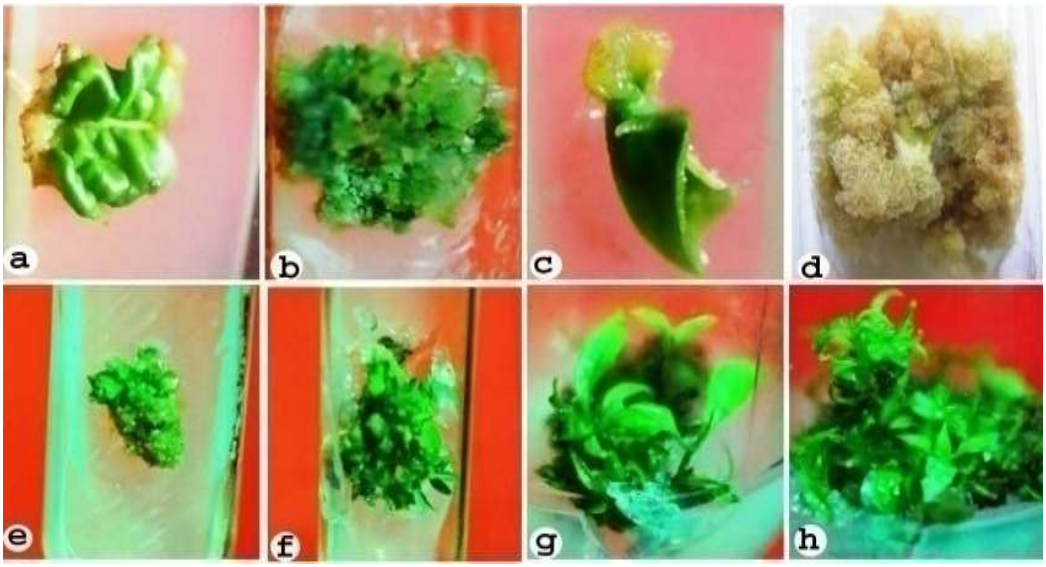

Fig. 5a-h. Callus induction and direct regeneration from leaf explants of *O. indicum.* a & b) Callus induction & Proliferation of callus from Leaf expalnts at 3.0 mg/L 2, 4-D respectively; c & d) Callus induction and high amount of friable callus development from cotyledonary leaf explant at 2.0 mg/L 2, 4-D respectively; e) Formation of adventitious shoot buds on MS+1.0 mg/L BAP from leaf explants; f) Multiple shoot induction on MS+5.0 mg/L BAP; g & h) Development of multiple healthy shoots on MS+0.5 mg/L 2, 4-D+5.0 mg/L BAP after 4 & 6 weeks of culture respectively

of additives in the media due to their growth and development promoting activities. According to Additives [55] like Casein hydrolysate (CH), Activated Charcoal (AC), Coconut milk (CM) and Silver nitrate ($AgNO_3$) were used to induce *in-vitro* regeneration of an important endangered medicinal tree species *O. indicum*. Among all the additives used CH and $AgNO_3$ acted positively for multiple shoot regeneration from different explants 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 $AgNO_3$ has turned out to be the best additive for regeneration of *O. indicum* [55]. Whereas Samatha and Ramaswamy [41] have used $AgNO_3$ as an additive in the medium along with other PGRs and it was found that the enhancement in regeneration efficiency in zygotic embryo culture of *O. indicum.*

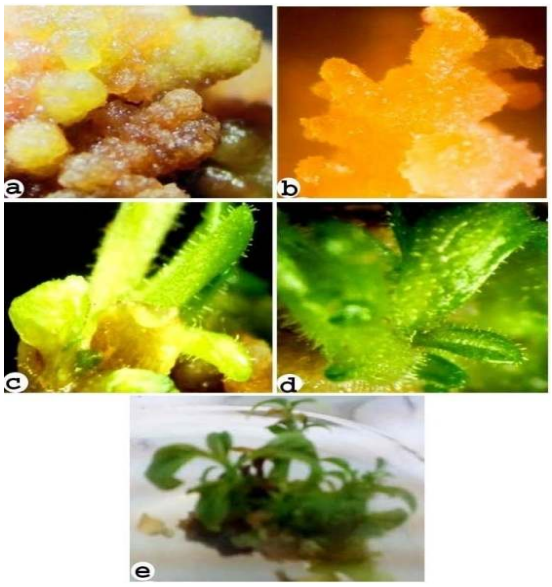

Fig. 6a-e. Somatic embryogenesis and plantlet development in *O. indicum.*
a) Induction of somatic embryos on MS+ 0.5 mg/L 2, 4-D+0.75 mg/L BAP; b) Development of different types of embryoids; c) A group of bipolar embryos; d-e) Germination of somatic embryo and complete plantlets respectively

The production, consumption and international trade in medicinal plants and phytomedicine have grown and are expected to grow further in the future. To satisfy growing market demands, surveys are being conducted to unearthen new plant sources of herbal remedies and medicines and at the same time to develop new strategies for better yield and quality. This can be achieved through different methods including micropropagation [56,7]. It may help in conserving many medicinally valuable tree species in the process and may open new vistas in the medicinal plant biotechnology.

3. CONCLUSION

In conclusion, it is mentioned that the effectiveness of plant regeneration depends on the type of used growth regulators and source of explants. Thus characteristics of the responded explants depend on the type of explant, culture medium and the micropropagation protocols developed or standardized including our research work for the plant regeneration *and thus* opens new prospective that could facilitate conservation and mass multiplication of vulnerable and valuable medicinal forest tree species *O. indicum*. Various problems related with conventional propagation and high demand of planting material of medicinal plants can be addressed by efficient and economical *in-vitro* propagation in a short span of time.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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