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In-Vitro Optimization Strategies For Micropropagation Of *Elaeocarpus ganitrus* (Rudraksha): Valuable Medicinal Plant

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Abstract

Rudraksha *(Elaeocarpus ganitrus*), is an inheritable shrub and a valuable tree species belonging to the genus Elaeocarpus with significant religious, medicinal, and ecological importance. Irrespective of its high demand, its natural propagation is seriously restricted by low seed germination, persistent dormancy, and poor seed viability. The experiment intended to develop reproducible and an efficient method to optimize and develop *In Vitro* micro propagation protocol for *E. ganitrus* through controlled analysis of explant selection, culture media composition, sterilization procedures, plant growth regulators (PGRs), and environmental conditions. Nodular stem sections were found to be the most responsive, and optimal sterilization was achieved using 0.1% mercuric chloride for 3-5 minutes. Maximum shoot proliferation was observed on Murashige and Skoog (MS) medium supplemented with 1.5 mg/L BAP in combination with 0.5 mg/L 2,4-D. Rooting was best achieved on half-strength MS medium containing 1.0 mg/L IBA. Acclimatization success reached up to 80% under controlled conditions. The optimized protocol provides a reliable and reproducible method for large-scale propagation and conservation of *E. ganitrus.*

Keywords: *In-vitro*, Micropropagation, *Elaeocarpus ganitrus*

1. INTRODUCTION

In order to satisfy the considerable shift in the demand towards natural herbal medicines, food production and plant-derived pharmaceuticals, urgent attention must be given to the conservation of agricultural, economically valuable, rare, and endangered plant species and therefore need to be propagated on a large scale, especially when conventional methods are unsuccessful. These plants play a crucial role in maintaining ecological balance and possess significant medicinal properties (Bhojwani & Razdan, 1996; George et al., 2008). In this context, Plant Tissue Culture (PTC) emerges as a powerful biotechnological tool for rapid, large-scale multiplication and conservation of such plant species within a short duration (Murashige & Skoog, 1962). The application of biotechnology and tissue culture techniques has significantly contributed to rapid multiplication, genetic improvement, and biomass production in tree species (Bajaj, 1986). Tissue culture has also emerged as an important component of forest improvement programmes due to its potential for large-scale clonal propagation of elite genotypes (Haissig et al., 1987).

Plant tissue culture has emerged as a powerful biotechnological tool for the rapid propagation and conservation of economically and medicinally important plant species. Among such species, *Elaeocarpus ganitrus* holds a unique position due to its multifaceted importance. It is widely known for its seeds, commonly called Rudraksha beads or Amritphala (Fruits of nectar) which are used in religious practices and traditional medicine (Bhowmik et al., 2015; Bhatt & Singh, 2012).

By the period of ancient time, Rudraksha finds an important place in Hindu religion. The term "Rudraksha" comes from (Rudra: Refers to “Lord Shiva” and Aksha: means "eyes" or "tears”), roughly translates to "Lord Shiva's tears" or "eyes of Shiva”. Hindu mystics and fakirs wear the nuts. Wearing the Rudraksha amulets are considered useful to guard against evil spirits and ill omens. The species is believed to possess special merits and therefore sells at much higher price. Preliminary observations suggest that the seeds of Rudraksha germinate with great difficulty. Moreover, collection of nuts every year from the forest at large scale to sell them in the market has caused a real threat to natural regeneration of this species (Sharma et al., 2023). The population of Rudraksha is fast depleting in the natural forests due to developmental activities and practice of 'jhum' cultivation by the local people ((Khan et al., 2003 and Garg et al., 2013). In fact Rudraksha has become an endangered species in nature, though it is not included in Red Data Book of Indian flora. Thus, the study on fruit production, dispersal mechanism, germination and seedling fitness of Rudraksha has been taken up with a view to augment its regeneration which would contribute to its conservation in nature (IUCN).

The *Elaeocarpacae* family is predominantly found in tropical and subtropical regions of South and Southeast Asia. Rudraksha, its seeds are extensively used in spiritual practices and traditional medicine systems. However, its natural regeneration is limited due to several biological and environmental constraints. The hard seed coats impose mechanical dormancy, resulting in delayed and poor germination rates. Furthermore, overexploitation and habitat degradation have significantly reduced its natural populations (Sharma et al., 2014; Singh & Sharma, 2018). The medicinal significance of Rudraksha underscores the importance of developing efficient propagation and conservation strategies for its sustainable utilization (Hardainiyan et al., 2015). Therefore, optimization of *In Vitro* micropropagation techniques offers a promising alternative for large-scale multiplication and conservation of this valuable species.

Fig.1. Seven Years old plant of Rudraksha at Shobhit University, Gangoh, Saharanpur.

Conventional propagation methods, such as seed-based propagation and vegetative cuttings, are proved to be inefficient and unreliable for its conservation. In this context, Plant Tissue Culture is a biological technique used to regenerate new plantlets from a small part of the mother plant, (known as an explant) *In Vitro* (in a controlled environmental conditions in lab) (Khan et al., 2005). This process involves the *In Vitro* cultivation of plant cells, tissues, or organs under sterile conditions on a nutrient medium with carefully controlled physical and chemical parameters (Murashige & Skoog, 1962; George et al., 2008). Plant tissue culture offers a promising alternative for mass propagation under controlled conditions. However, successful *In Vitro* regeneration requires careful optimization of several interacting factors, including explant type, sterilization methods, nutrient media, plant growth regulators, and environmental parameters (Kant et al., 2010).

Despite its advantages, several technical and biological problems reduce its success rate. Understanding these challenges and implementing appropriate solutions is critical for optimizing outcomes (Bhojwani & Razdan, 1996). Contamination is a major challenge in tissue culture, and it may be caused by both physiological and pathological factors. The common contaminants found in cultures include bacteria, fungi, and yeast (Leifert, 1994). Although it is difficult to identify a single cause of contamination, maintaining strict laboratory practices at every stage helps reduce this problem (Pierik, 1997).

For successful plant tissue culture, several important steps must be followed. These include maintaining aseptic (sterile) conditions, selecting plant material that is free from microbes, ensuring proper sterilization of tools and media, and providing the correct balance of nutrients. Additional challenges such as browning of tissues (caused by oxidation of phenolic compounds) and somaclonal variation (genetic changes in cultured cells) must also be managed carefully. After plants are successfully grown *In Vitro*, they cannot be directly transferred to the field. They must first undergo hardening (acclimatization) in a greenhouse, where they gradually adapt to natural environmental conditions. This step is crucial for improving their survival when moved to the field (Gamborg & Phillips, 1955).

The current manuscript focuses on developing a standardized and optimized micropropagation protocol for *E. ganitrus*, which can be utilized for both commercial cultivation and conservation programs. The study also highlighted the feasibility of optimizing culture conditions for enhanced regeneration, providing a

foundation for large-scale micropropagation of medicinally important Rudraksha species (Khan & Ahmad, 2005 and Arshad & Kumar, 2006).

2. OPTIMIZATION OF MICROPROPAGATION PROTOCOL

2.1 Plant Material and Explant Source

The first and most important step in plant tissue culture is explant selection. The commonly used explants include leaves, stems and roots however, possibly the seeds, embryos, organs, and more can also be used as an explants depending on the purpose of the culture (George, Hall & De Klerk, 2008). During the peak vegetative season nodal sections and shoot tips were collected from actively growing branches to ensure high morphogenetic potential. Successful regeneration protocols have been developed using various explants such as cotyledons, nodal segments, shoot tips, and leaves in several woody species. In *Elaeocarpus robustus*, cotyledon explants exhibited high regeneration potential under *In Vitro* conditions (Rahman et al., 2004), while rapid clonal propagation was achieved through tissue culture techniques using juvenile explants (Rahman et al., 2003).

For this study, healthy and disease-free leaves and nodal segments were sampled from a 7-year-old *E. ganitrus* mother plant at Shobhit University, Gangoh Saharanpur, UP, India (Fig 1) to serve as the source of the explant. Leaf explants of approximately 1–2 cm in length whereas nodal explants usually consist of stem segments 10 to 20 mm in length, 2 to 3 mm in diameter, with one or two preformed buds (depending on the arrangements of the leaves), were excised and immediately transported to the laboratory under sterile conditions.

Explant material collected in early morning before afternoon and from plants grown under greenhouse conditions is generally preferred because it is cleaner and less exposed to environmental stress, leading to a higher survival rate in culture (Bhojwani & Razdan, 1996). In addition, collecting explants during the dry season can improve survival because there is a lower risk of microbial contamination during this period (Thorpe, 2007). The plant parts favorably regarded (leaves, axillary buds or shoot tips and internodes) are often considered the best sources of explants because they are actively growing, easier to sterilize, and have a high capacity for regeneration (Kataky & Handique, 2010). These tissues also show better growth and development in culture compared to many other plant parts (Dar et al, 2012).

2.2 Protocol for establishing aseptic cultures from explants

Most of the scientific articles reported a sterilization technique involving three steps: pretreatment, treatment and post treatment. Following the collection of plant material, the next important step is to sterilize its surface. Surface sterilization is a very critical but crucial step in plant tissue culture, as contamination can severely affect culture establishment.

When plant tissues are cut they release phenolic compounds. These get oxidized (in presence of oxygen and enzymes like polyphenol oxidase), forming brown or black pigments (Fig-2). This inhibits tissue growth, preventing callus formation that results in failure of tissue culture. These strategies help to reduce oxidative damage (Thomas, 2008). The explants were subjected to the following sterilization procedure:

1. Explants were thoroughly washed under running tap water for about half an hour to remove surface debris like dust particles.
2. Treated with a few drops of liquid detergent (0.1 % v/v Tween-20) for 10-15 minutes
3. Post-treatments are typically followed by rinsing 4–5 times with sterilized water.
4. The explants were dissected into small pieces under laminar air flow and carefully transferred to the culture tubes.
5. Lastly under the laminar airflow chamber surface sterilization was done using 70% ethanol for 30

seconds and 0.1% mercuric chloride ($HgCl_2$) for varying durations (2, 3, 5 and 7 minutes)

6. Rinsed 4–5 times with sterilized distilled water to remove traces of sterilants.

Effective sterilization and aseptic handling are essential for successful plant tissue culture. Poor sterilization can lead to contamination by bacteria and fungi, reducing explant survival and culture establishment. Contamination usually occurs due to inadequate disinfectant concentration, insufficient exposure time, improper washing, or highly contaminated plant material. To minimize these problems, explants should be thoroughly washed and treated with suitable sterilizing agents such as ethanol (70% for 10 sec) as it can be harmful to plant cells (phytotoxic), sodium hypochlorite (0.1% for 2-5 min), calcium hypochlorite, or mercuric chloride (5% for 20 min) at appropriate concentrations and durations. Since excessive exposure to sterilants can damage plant tissues, protocols must be carefully optimized for each plant species to achieve effective disinfection without harming the explant (Khasdeo et al., 2014).

A **B** **C**

Fig-2: A- Browning of explant, B - Contamination of media, C- Bacterial growth in explant

2.3 Culture Media Preparation

For all experiments Murashige and Skoog's (1962) (MS) medium was best used as the basal medium. The medium was prepared with the following components:

- Formulated MS media containing all essential vitamins and nutrients w/o sucrose and agar
- 3% Sucrose (30 g/L) as a carbon source was added to the medium
- 0.8 % Agar (8 g/L) as a gelling agent
- IAA 1.0 mg/L and 2, 4-D 0.5 mg/L and
- The medium was dispensed into culture tubes (15 ml/ tube) and the pH was adjusted at 5.8 $^{+}$– 0.5 before autoclaving at 15 psi (121 °C) for 20 minutes

Careful optimization of sterilization procedures, combined with proper aseptic techniques, is essential to minimize contamination and ensure overall culture efficiency. To evaluate the effect of nutrient strength, both full-strength and half-strength MS media were tested during different stages of culture. Higher content of agar also leads to the loss of nutrient content and also the microbial growth in the culture.

2.4 Plant Growth Regulator (PGRs) Treatments

PGRs in the correct proportion or combination of such as auxins and cytokinins, which control morphogenesis in cultured tissues is important as the media or explant. *Pinus roxburghii* regeneration *In Vitro* was significantly influenced by the type and concentration of plant growth regulators as well as the pH of the culture medium.The findings emphasized that optimization of growth regulator combinations and medium pH is crucial for achieving efficient shoot regeneration and micro propagation (Arya et al., 2012). Similar findings have been reported in Litchi *(Litchi chinensis)*, where optimized cytokinin concentrations promoted multiple shoot induction and plant regeneration (Khan & Ahmad, 2005).

Efficient clonal propagation through appropriate explant selection and growth regulator combinations has

also been demonstrated in *Boerhavia diffusa* (Kumar et al., 2013). Likewise, embryogenic callus formation and somatic embryogenesis in *Commiphora wightii* were strongly influenced by plant growth regulator treatments (Kumar et al., 2003). The balance between these hormones determines the developmental pathway of the cells. For instance, a higher concentration of auxins relative to cytokinins generally promotes root formation, whereas a higher concentration of cytokinins encourages shoot development.

Different concentrations and combinations of cytokinins and auxins were tested. Callus induction and rooting were best achieved from dormant buds and young leaves on MS medium with different hormones. MS medium containing IAA 1.0 mg/L and 2, 4-D 0.5 mg/L, and GA_3 0.2 mg/L was effective for callus induction and subculture. For root induction, MS medium supplemented with NAA and sucrose (30 g/L) gave good results. Hence, maintaining the correct PGR ratio is essential for successful organogenesis and plant regeneration in addition to developing shoot Induction and Multiplication 6-Benzylaminopurine (BAP) 2.2 µM in the combination with Kinetin 2.2 µM Kn used. Different combinations were tested to determine optimal shoot induction.

Furthermore, to avoid incorrect PGRs (plant growth regulator) ratios is really about precision**,** timing, and restraint. Here's how you can manage it effectively in practice:

1. Different crops respond very differently to PGRs so always follow crop-specific recommendations and standard protocols.
2. Do not copy practices from other crops or regions blindly.
3. Applying the wrong PGR at the wrong time is a common mistake. Each growth stage has specific hormonal needs like Vegetative stage (more auxins/cytokinins), Flowering & fruiting (gibberellins) etc.
4. Don't mix multiple PGRs to prevent

2.5 Culture Conditions: All cultures were then incubated in the growth chamber under controlled environmental conditions:

- Temperature: 25 ± 2°C
- Photoperiod: 16 hours light / 8 hours dark
- Light intensity: 2000–3000 lux
- Relative humidity: 60–70%

FACTORS AFFECTING SUCCESS

Factor	Effect
Explant age	Younger explants respond better
Medium composition	Determines growth rate
PGR balance	Controls morphogenesis
Sterilization	Prevents contamination
Light intensity	Affects photosynthesis
Subculture frequency	Enhances multiplication

Environmental factors such as temperature, light intensity, and humidity greatly influence *In Vitro* morphogenesis. Plant responses to abiotic stresses, particularly heat stress, involve complex physiological and molecular mechanisms that may affect culture establishment and growth (Kotak et al., 2007).

2.6 Sub Culturing

Generally the subculturing is done after 45 days of an incubation period to prevent death of the callus as the time delays there is lack of nutrition that slows the growth. The positive results are clearly seen in the fig 3 (A, B, C, D and E). To maintain culture viability and promote growth, explants were subcultured onto fresh media with the same composition at intervals of 3–4 weeks. Subculturing helped prevent nutrient depletion and accumulation of toxic metabolites. *Elaeocarpus grandiflorus* callus cultures exhibited differential growth responses on MS medium supplemented with varying concentrations of plant growth regulators.The study demonstrated that optimization of auxin and cytokinin levels is essential for enhancing callus proliferation, providing valuable insights for *In Vitro* propagation and conservation of Elaeocarpus species (Habibah et al., 2019).

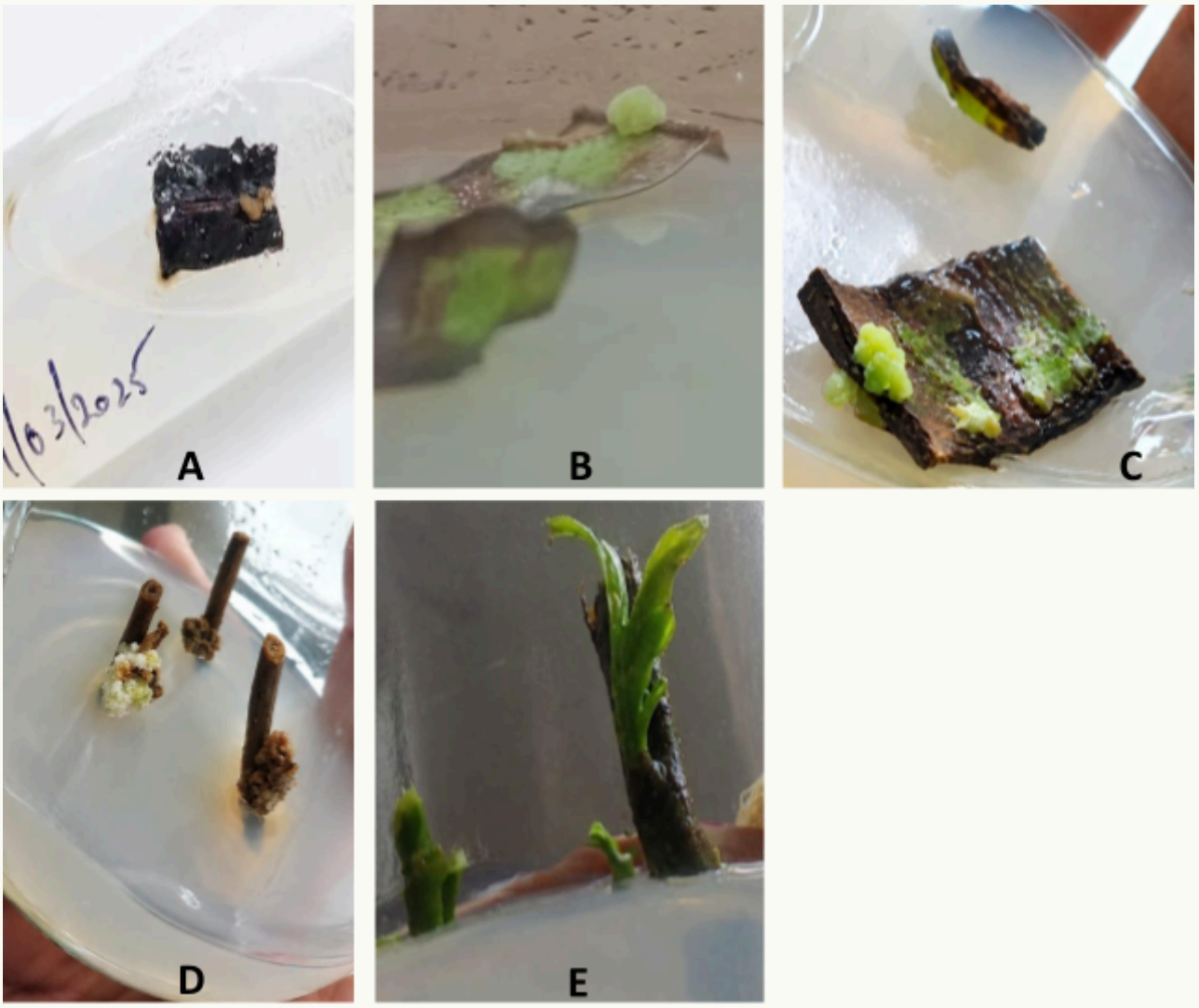

Fig.-3. A : Callus induction, B : Callus Proliferation, C & D: Bud Breaking & Shoot Initiation for Proliferation, E : Callus formation in Shoot

Fig.-3 (A-E): *In Vitro* **Leaf & Shoot Regeneration of *Elaeocarpus ganitrus.***

3. RESULTS

In Vitro callus formation and culture have been reported for *E. ganitrus* in about 45 days. Moreover among the tested treatments, (BAP 1.5 mg/L + Kn 0.5 mg/L) showed the best results in nodal cultures, new buds and shoots also continue to arise in the axils of leaves and new shoots, often without elongation, resulting in dense clumps of buds while the optimal proliferation of shoots was attained by combining BAP (0.5

mg/l) and NAA (0.1 mg/l) in the MS medium, with or without $CaCl_2 \cdot 2H_2O$ (ranging from 1.25 to 10.0 mM/l) reported by Chaudhary et al., 2024.

Irrespectively, dormancy and senescence of explants are major obstructions to the establishment of cultures. Sterilization procedures may evoke a wound response in addition to that caused by excision. In woody plants, phenolic compounds are produced in response to wounding and these can be deleterious to growth *In Vitro*. The oxidation of phenolic compounds in the tissue and explant exudates is evident by browning of the medium, or the explant or both. The strategies used to overcome this problem:

- Addition of antioxidant to treat explant such as ascorbic acid (vitamin C) or citric acid
- Use of adsorbents like activated charcoal or Polyvinylpyrrolidone (PVP)
- Careful handling of explant during inoculation and frequent transfer to fresh media also prevent this.

Studies on *Elaeocarpus robustus* further demonstrated that rapid regeneration and clonal multiplication can be achieved through optimization of explant source and culture conditions (Rahman et al., 2003 & 2004).

5. DISCUSSION

The present study highlights the importance of systematic optimization in plant tissue culture. The success of micro propagation in *E. ganitrus* depends on multiple interacting factors. The importance of selecting the appropriate explants of *Elaeocarpus* species for the initiation of *In Vitro* cultures should receive more attention. *In Vitro* techniques have been employed to rejuvenate tissues of mature *Elaeocarpus* over the past 25 years, and there exists the potential for mass production of selected material. However, the techniques are not yet sufficiently refined for commercial application. A number of large-scale field trials with *In Vitro* derived clones are in progress. Clonal hedges can be established through micro propagation to provide shoot material for conventional propagation by cuttings.

6. LIMITATIONS

Despite its advantages in conservation and mass scale production, the technique has certain limitations slow growth and low multiplication rate, risk of microbial contamination, phenolic exudation and browning, somaclonal variation in long-term cultures, vitrification (hyperhydricity), requirement of specialized infrastructure, and sometimes required a high initial cost. The success of plant tissue culture depends on careful management of biological and environmental factors. Each challenge requires specific strategies, and often a combination of approaches is necessary. Advances in molecular biology and improved culture techniques have helped overcome many of these limitations, yet species-specific responses remain a challenge.

7. FUTURE PROSPECTS:

The techniques can also be used for the *In Vitro* selection of cell lines possessing selected properties. *In Vitro* techniques are presently being applied to *Elaeocarpus* to achieve genetic transformations. These techniques are likely to play a vital role in future tree-improvement programs. Future research can focus on genetic fidelity testing using molecular markers, automation using bioreactors or integration of artificial intelligence for optimization. Pharmacognostic investigations have highlighted the medicinal importance of Rudraksha and its diverse therapeutic applications, thereby increasing the need for sustainable propagation approaches (Garg et al., 2013).

The ecological studies conducted on Rudraksha regeneration, seedling establishment, and forest disturbance effects provide strong justification for adopting micropropagation as a complementary

conservation strategy for this threatened species (Khan et al., 2003; Khan et al., 2004; Khan et al., 2005).

8. CONCLUSION:

The study successfully developed an optimized protocol for the *In Vitro* micropropagation of *Elaeocarpus ganitrus*. Among the various factors studied, key factors influencing successful culture initiation and regeneration included the explant type, plant growth regulators, and nutrient media strength played crucial roles in determining the success of culture establishment and regeneration. The protocol demonstrated high efficiency in terms of shoot proliferation, rooting, and acclimatization. It provides a reliable and scalable method for the conservation and commercial propagation of this valuable species. Additionally, it offers a dependable and scalable approach for both conservation and large-scale propagation of this important species.

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