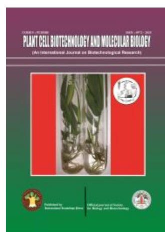

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Effect of Media and Calcium on the Micropropagation of Rudrakhsa (*Eleocarpus ganitrus* Roxb.)

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The Rudraksha tree, scientifically known as *Eleocarpus ganitrus* Roxb., holds significant religious and medicinal value in India, making it a threatened species. This study was conducted to assess the support of the five different media on the *in vitro* micropropagation of *E. ganitrus* using nodal segments as explants. The media; Murashige and Skoog (MS), Lloyd and McCown (WPM) Anderson (AM), Gupta and Durzan (DCR) and Gamborg (B_5) were supplemented with antioxidants (citric acid 10mg/l, ascorbic acid 50mg/l, activated carbon (AC) 500mg/l and polyvinyl pyrrolidine (PVP) 100mg/l), BAP (0.0,0.1, 0.3, 0.5, 1.0, 5.0 mg/l) NAA (0, 1,0, 2 mg/l) to promote plant growth. 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). The calcium content was assessed accordingly. Elevated concentrations of $CaCl_2 \cdot 2H_2O$ in the medium mitigated hyper-

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hydration, with necrosis of the shoot tip notably reduced at 6.5 mM/l $CaCl_2 \cdot 2H_2O$. Healthy shoots were transferred to MS medium supplemented with NAA (1.0 mg/l) to induce root initiation and multiplication. The addition of $CaCl_2 \cdot 2H_2O$ (5.0 mM/l) resulted in the highest recorded root multiplication. Plants that underwent successful acclimatization (80%) exhibited regular growth patterns under favorable environmental conditions.

Keywords: Culture media; Elaeocarpus ganitrus; antioxidants; micropropagation; conservation; browning; recalcitrance.

1. INTRODUCTION

In the Puranic tradition, Rudraksha signifies the tears of "Lord Shiva" ("Rudra" translating to "Shiva" and "Aksh" to "tears"), while botanically it is known as *Elaeocarpus ganitrus* Roxb. (Eleocarpaceae). This seed holds profound significance to Hindus, Buddhists, and Jains since ancient times, revered for its sacredness. Rudraksha beads, which are essentially dried seeds from the tree, stand out as the most expensive and valuable seeds worldwide. This family includes 360 *Elaeocarpus* species, with 25 thriving in India's Gangetic plains and Himalayan regions [1]. In Tamil Nadu alone, researchers have identified eleven *Elaeocarpus* species, eight of which are specifically found in the Western Ghats. Among them, four are steno-endemics: E. blascoi Weibel, *E. gaussenii* Weibel, *E. recurvatus* Corner, and *E. venustus* Bedd [2]. The leaves of *Elaeocarpus ganitrus* have a shiny green upper surface and a dull, leathery underside. Its stem is cylindrical, with fringed petals, and its flowers typically appear in racemes from the axils of fallen leaves, nodding gracefully in white, measuring around 1cm across, and featuring apex-bristled anthers. The fruits are approximately spherical, 1cm in diameter, turning deep blue or mealy when ripe, enclosing a hard bead with eight tubercles [3]. While reproduction primarily occurs through seeds, increased seed collection by local communities has depleted the natural seed bank in the soil, posing a threat to the species' regeneration. *In vitro* propagation of recalcitrant tree species presents unique challenges. While micropropagation methods for such species primarily focus on modifications related to plant growth hormones, standard protocols for nutrients and other factors are still adhered to [4].

Optimizing the condition using the ionic concentration of major and minor nutrients therefore often requires versatile and elegant experimentation, which eventually leads to the identification of the exact requirement of different mineral nutrients for the continuous growth of *In vitro* cultured in tree species [5]. Various factors, including ion interactions, salt reactions and complications, can impact nutrient absorption in *in vitro* tissue cultures, in addition to nutrient milligram concentration. If the concentration of mineral ions in the culture condition is inappropriate, it becomes difficult for the usual metabolism of the plant, and this can be the cause of morphological and growth disorders [6]. Thus, it has been put forward that enhanced levels of major- and minor-nutrients are used for normal physiology, growth and development of *In vitro* tissues [7,8]. Nevertheless, *in vitro* propagation protocols for other *Elaeocarpus* species have been reported [9,10]. The current study aimed to establish a protocol for the propagation of *E. ganitrus* using explants obtained from a tree aged between 8 and 12 years. An initial trial utilizing MS medium with antioxidants, plant hormones, calcium and agar gel revealed inadequate growth response and elongation of axillary shoots formed *In vitro*.

As shown by [11], *in vitro* methods are valuable for germplasm conservation and assimilation of secondary metabolites. Achieving optimal results during micropropagation involves the delicate balance of numerous factors. Key considerations include the selection of appropriate explant types, meticulous control of culture conditions, regulation of growth regulators, and exploration of diverse *In vitro* protocols, as highlighted [12].

Calcium concentration of the culture medium was linked with shoot tip necrosis and hyperhydration, which reduces with increasing calcium content in *Castanea ollissima, C. dentata* and *C. sativa in vitro* medium [13,14]. Calcium is an integral part of the cell wall and plays a salient role in maintaining membrane integrity [15,16]. The study investigated the relationship between tissue calcium concentration in reducing hyperhydricity and shoot tip necrosis.

This study sought to assess the efficacy of antioxidants, various media compositions, plant growth regulators, and calcium chloride in

mitigating tissue browning, shoot necrosis, and promoting *in vitro* regeneration of *E. ganitrus*. Specifically, the emphasis was placed on determining the optimal concentration of these components to minimize explants browning, and shoot necrosis during the *in vitro* propagation of rudraksha.

2. MATERIALS AND METHODS

2.1 Source and Sterilization of Explants

The Rudraksha explants were harvested from the University garden located at S.I.E.T., Modipuram, Meerut (UP), Bharat. Whole shoots (3-4 cm) from the wet summer season (May-August) were excised, leaves were removed, and 2.0-2.5 cm sections were made from the explants, with 1-2 nodes bearing the axillary buds. Following this, the nodal segment explants underwent a series of washes: firstly, they were rinsed under running tap water for 15 minutes, then submerged in a 1% Tween-20 liquid detergent solution for 10 minutes, and finally rinsed three times in distilled water. Explants Surface was performed in a laminar airflow cabinet using various surface sterilizing agents were employed, encompassing $Ca(OCl)_2$ at concentrations of 9-10%, NaOCl at concentrations of 0.5%, 2.0%, 3.5%, and 5.0%, and $HgCl_2$ at concentrations ranging from 0.01 to 0.10 mg/l, with exposure durations lasting from 3 to 10 minutes. The explants were segmented into pieces measuring 1.5-2.0 cm and placed onto agar (0.8%) substrates across five different media formulations. These media were enriched with differing concentrations of cytokinin, auxin (BA or Kn with or without NAA) and antioxidants.

2.2 Culture Media and Incubation Conditions

Five different media; MS [17], WPM [18], Anderson [19], DCR [20] and B_5 [21] media were prepared, respectively. All media were then augmented with NAA and BAP as plant growth regulators to support explant regeneration. Since explant response and new shoot growth in different media augmented with different concentrations (0.1, 0.3, 0.5, 1.0, 5.0 mg/l) of BAP or Kn or combinations of BA and Kn at different concentrations with NAA (0, 1,0, 2 mg/l). Medium in the absence of growth regulators was used as a control. The pH of the media was adjusted to 5.8 ± 0.02 and then before autoclaving at 121 °C for 15 min. To explore the interplay among BAP, Kn, and NAA, nodal explants were cultured on a medium containing BAP with or without NAA and supplemented with antioxidants such as citric acid (10 mg/l), ascorbic acid (50 mg/l), activated carbon (AC) (500 mg/l), and polyvinyl pyrrolidone (PVP) (100 mg/l). After four weeks of culture, the shoots were subcultured in an optimized medium with a growth regulator added. The cultures were placed in incubation conditions maintained at 26 ± 2°C with a relative humidity of 55–60%. Culture racks were illuminated using cool white fluorescent lamps, providing a photon level density (PFD) of 40-50µmol m-2 s-1 during the 16-hour photoperiod. Following four weeks of incubation, the percentage of response, number of shoots, and average shoot length (cm) were recorded.

2.3 Regeneration and Multiplication of Shoots

Different media were chosen to induce axillary buds on nodal explants, with the addition of 0.5mg/L of BAP. The induction of axillary buds on nodal explants was facilitated by a combination of cytokinins (BAP-1.0-5.0mg/l, Kn-0.2-0.5mg/l) and auxins (NAA-0.1-0.2mg/l), supplemented with agar or without agar (coconut coir) on MS medium. Observations were made frequently for four weeks to identify healthy cultures from non-growing cultures, while infected cultures were discarded. The number of shoots for every explant and shoot length were recorded after 40 days old cultures. Subsequently, the developed shoots were transplanted onto MS medium enriched with BAP (0.5mg/l) and NAA (0.1mg/l), in addition to calcium ($CaCl_2.2H_2O$) at concentrations ranging from 1.25 to 10.0 mM/l, to facilitate shoot regeneration.

2.4 Rooting and Acclimatization

The resulting regenerated multiple shoots were subsequently moved to MS medium augmented with varying concentrations of IAA, IBA, and NAA (0.2-2.0mg/l) with or without various concentration of calcium chloride to root initiation. Observations were recorded after two weeks intervals, rooting percentage, number of roots and average root length were recorded.

Rooted plantlets were rigorously washed with distilled water. Afterward, the rooted plantlets were transferred to garden pots filled with a blend of potting garden soil, sand, and farm yard manure (1:1:2). Transparent polyethylene bags were then used to cover these pots. The plants

were watering 15 days and gradually acclimated to room temperature in the bag until the plants were ready to move to the greenhouse. The survival rate of seedlings grown *in vitro* is recorded.

2.5 Data Analysis

The data is the average of three experiments, each of which was conducted three times. Regular observations were made of the percentage of culture response, the number of shoots for every explant, and the rooting. A mean ± standard error of three replicates was accustomed to illustrate the results.

Induction rate (%) = the number of induced explants/the number of total initial explants ×100%.

Average shoot or root number = the total number of shoots or roots/the number of shoots or rooted plantlets.

Rooting rate (%) = the number of the rooted plantlets/the number of total shoots × 100%.

3. RESULTS

3.1 Effect of Surface Sterilizing Agents

The impact of various surface sterilization agents on nodal explants was assessed, with observations and data collection conducted at two, four, and six weeks post-culture, with values represented as percentages (Fig. 1). Among these sterilization agents, $HgCl_2$ emerged as the most effective method in combating contamination. Contaminants identified within cultured explants primarily stemmed from bacterial or fungus. Generally, contamination became apparent within 5 to 15 days following the culture period (Fig. 1). Notably, NaOCl exhibited the highest contamination rate, resulting in 48% of cultures showing darkening and mortality. Conversely, the use of $HgCl_2$ demonstrated superior efficacy in preventing darkening compared to other sterilization agents. Furthermore, minimal hyperhydration issues were associated with $HgCl_2$, indicating their effectiveness in this regard. Shoot vitrification occurred during the third week of explant culture. Regarding necrosis, NaOCl and $Ca(OCl)_2$ were identified as the least successful sterilization methods, with the highest percentage of necrotic tissue observed. In contrast, the $HgCl_2$ sterilization method resulted in less necrosis and was deemed successful. Necrotic symptoms appeared in shoots between the third and fourth weeks of explant culture.

3.2 Effect of Antioxidants and Media

The secretion of phenols and the browning of the substrate were overcome by adding citric acid 10 mg/l, ascorbic acid 50 mg/l, activated carbon (AC) 500 mg/l and polyvinylpyrrolidine (PVP) 100 mg/l to the medium, which reduced the passage quite often by growing subcultures and placing the cultures in a dark condition for a few days. A successful *in vitro* propagation method requires the correct size and physiological state of tissues, which play a pivotal role in regeneration. The explants lost their regenerative potential, causing tissue darkening and death after 3-4 days.

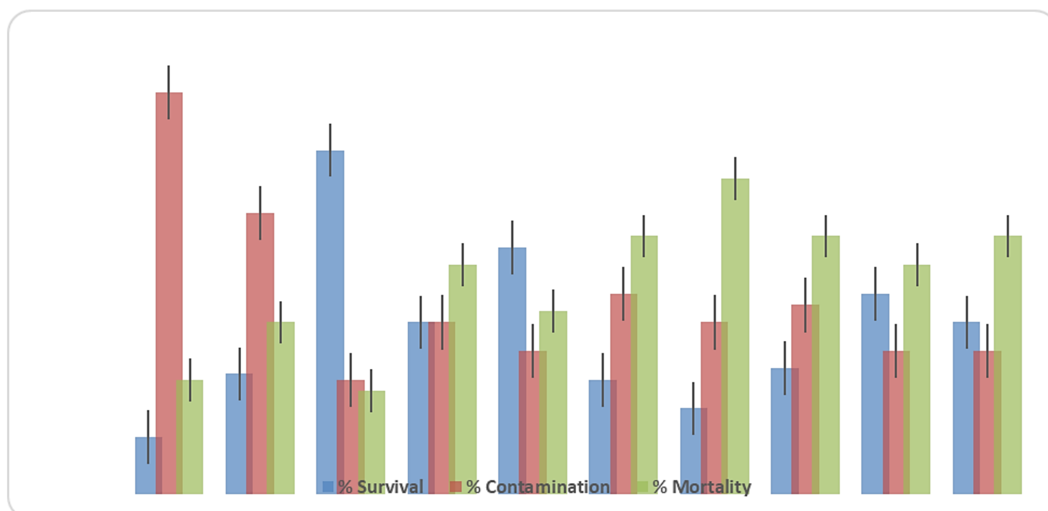

Fig. 1. Effect of Sterilizing agents on nodal segment explants of *Elaeocarpus ganitrus*

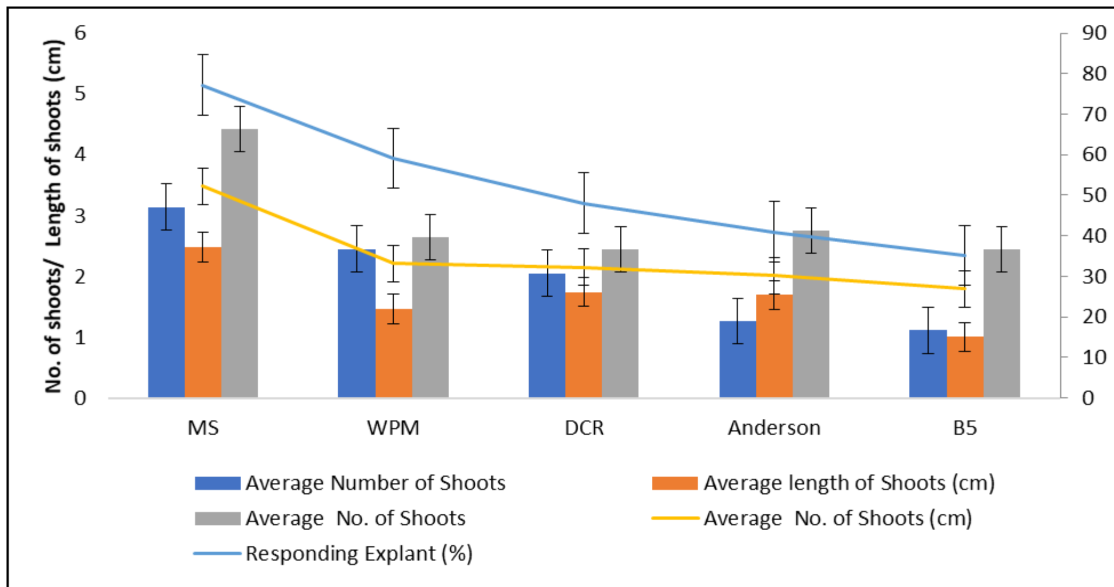

Fig. 2. Effect of distinct media supplemented with 0.5mg/l BAP on initiation of axillary buds on nodal explants and multiplication of *in vitro* raised shoots of *Eleocarpus ganitrus*. (Data recorded after 28 days of culture)

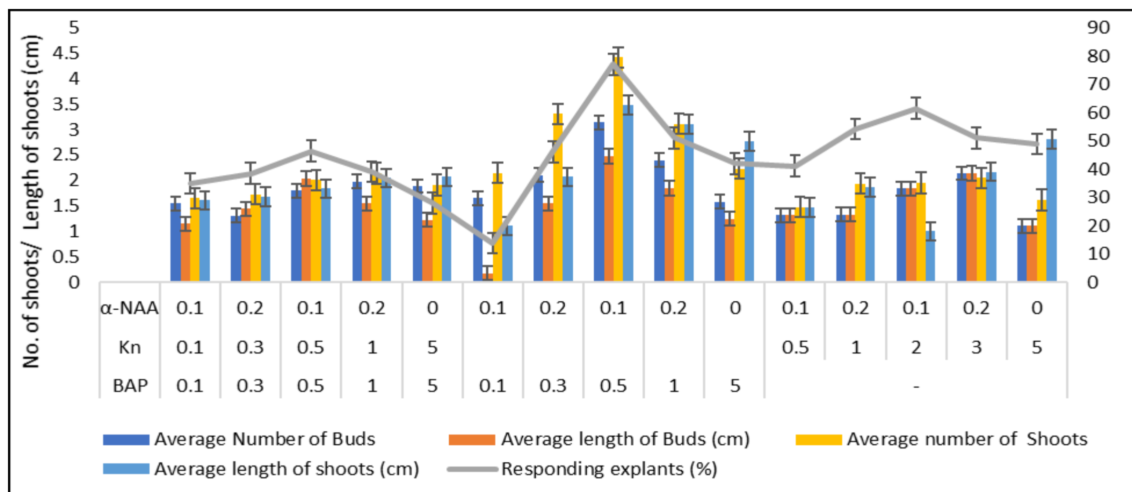

Fig. 3. Effect of cytokinins and auxins combination supplemented in MS medium on induction of axillary buds on nodal explants and multiplication of *in vitro* raised shoots of *Eleocarpus ganitrus.* Data recorded after 42 days of culture

The best potency of the medium was observed MS in all parameters recorded throughout *in vitro* supplementation. Shoot induction of nodal segments of *E. ganitrus* in MS was determined to be 77%. However, 59%, 48%, 41% and 35% were determined in WPM, DCR, AM and B_5 medium, respectively. Utmost shoot proliferation was taken in plants grown in complete MS medium with BAP (0.5 mg/l), average shoot number and length of 4.4 and 3.48 cm in MS medium. However, this mean shoot number and length decreased in WPM (2.6 and 2.2 cm), DCR (2.45 and 2.15 cm), Anderson (2.75 and 2.05 cm) and B5 media (2.45 and 1.79 cm) (Fig. 2).

3.3 Effect of Plant Growth Regulators on Shoot Induction and Multiplication

The nodal segment explants were found best for shoot initiation with agar-based MS medium. The cultures of *E. ganitrus* nodal explants were detected to be ~85% infection free. Five different media were used for the selection of the best medium for the induction of axillary buds on

nodal explant supplemented with 0.5mg/L BAP. Commencements of shoots were perceived after 6 weeks on MS medium. Shoot initiation, elongation, and multiplication were significantly enhanced, reaching a peak of 77.20% when utilizing a BAP concentration of BAP 0.5mg/l along with 0.1mg/l NAA in an Agar-based medium. In the induction phase targeting shoot apices, an average of 3.14 shoots was observed, with an accompanying average shoot length of 2.48 cm. Subsequently, during the multiplication phase, an average of 4.42 shoots was recorded, considering both the number and length of shoots, which averaged at 3.48 cm (Fig. 3).

Afterwards, the initiated shoots were moved to liquid MS medium; having sterile coconut coir and the multiplication of shoot and elongation of shoots were observed the best results were observed at BAP 0.5mg/l along with 0.1mg/l NAA.

The essential role of the MS medium in plant tissue culture as a fundamental substrate is investigated in this study. Specifically, we focus on sub-culturing shoot buds derived from explants on MS medium, in both liquid and agar-based forms, supplemented with BAP and NAA. The primary goal is to direct the most effective medium for optimal outcomes. Favorable conditions for the proliferation and elongation of shoot buds were identified in a culture medium comprising MS with varying combinations of BAP (0.1-0.5mg/l) and NAA (0.1mg/l). The most favorable shoot growth occurred with the combination of 0.5 mg/l BAP and 0.1 mg/l NAA, which resulted in vigorous shoot growth characterized by the absence of defoliation or callus. Over a six-week period, the average number of shoots reached 4.2, with an average shoot length of 3.54 cm (Fig. 4).

3.4 Effect of BAP and NAA along with Calcium ($CaCl_2.2H_2O$) on Shoot Multiplication

Proliferated shoots were moved to a culture medium based on liquid MS Medium having sterile coconut coir, augmented with BAP at a concentration of 0.5mg/l and NAA at 0.1mg/l, together with the addition of calcium ($CaCl_2.2H_2O$). The influence of varying concentrations of calcium ($CaCl_2.2H_2O$) ranging from 2.5 to 10 mM/l on the *in vitro* regeneration of *E. ganitrus* was examined through five different combinations of calcium with BAP (0.5mg/l) and NAA (0.1mg/l).

Among the combinations tested on MS media with BAP, NAA and calcium ($CaCl_2.2H_2O$) at a concentration of 6.25 mM/l recorded to be the most effective. This particular combination yielded optimal results, leading to the highest number of shoots and the greatest shoot length. After an eight-week period, notable outcomes were observed, including a significant increase in the number of shoots to 3.14, a shoot length of 2.57 cm, and improvements in the texture of cultures (Fig. 5).

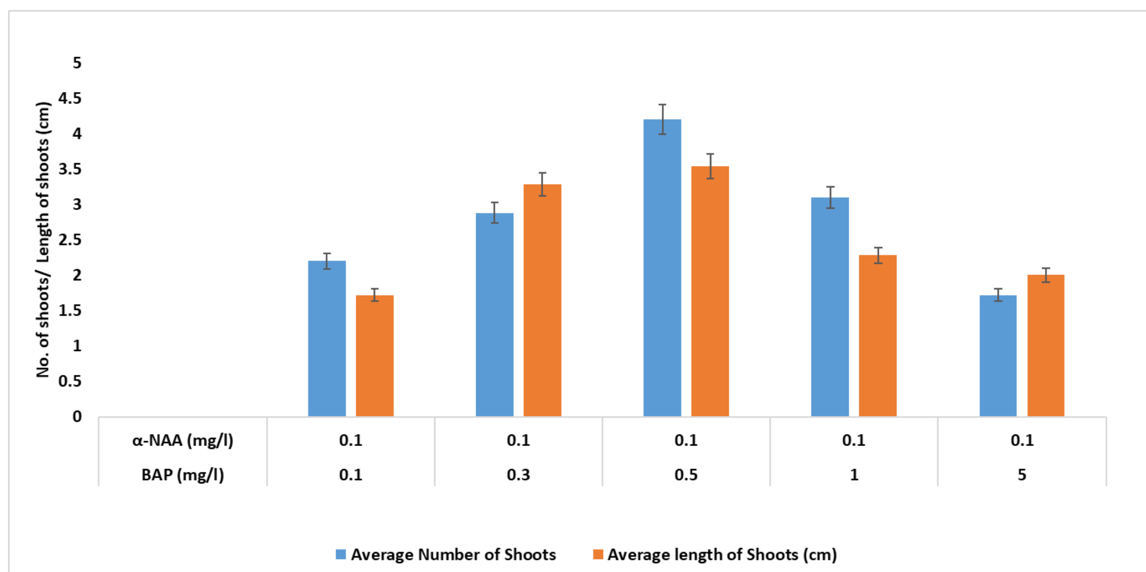

Fig. 4. Effect of plant growth regulator on multiplication of shoots on MS Media (Data recorded after six weeks)

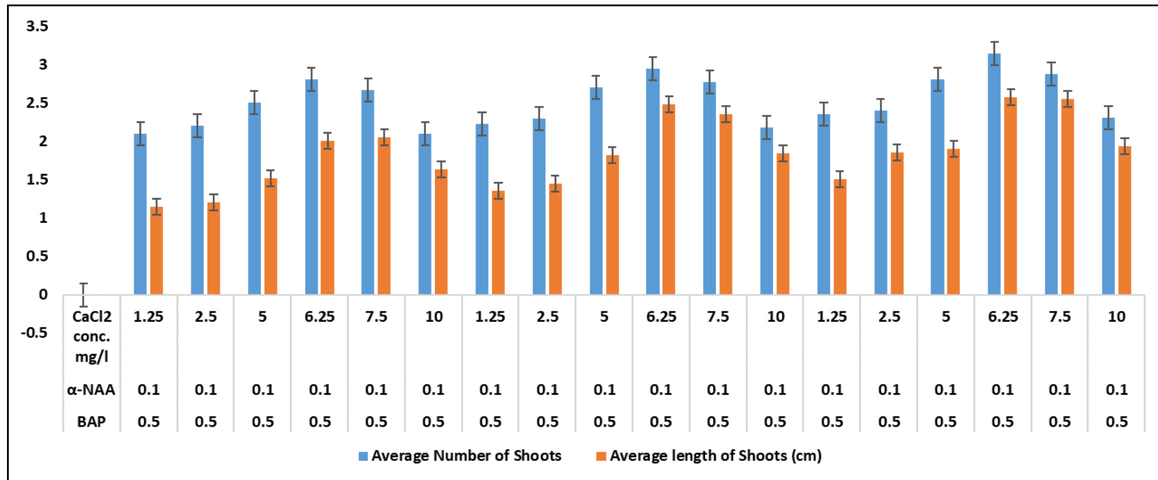

Fig. 5. Effect of calcium concentrations on multiplication of shoots on MS media with BAP (0.5 mg/l) and NAA (0.1mg/l) of *Eleocarpus ganitrus*

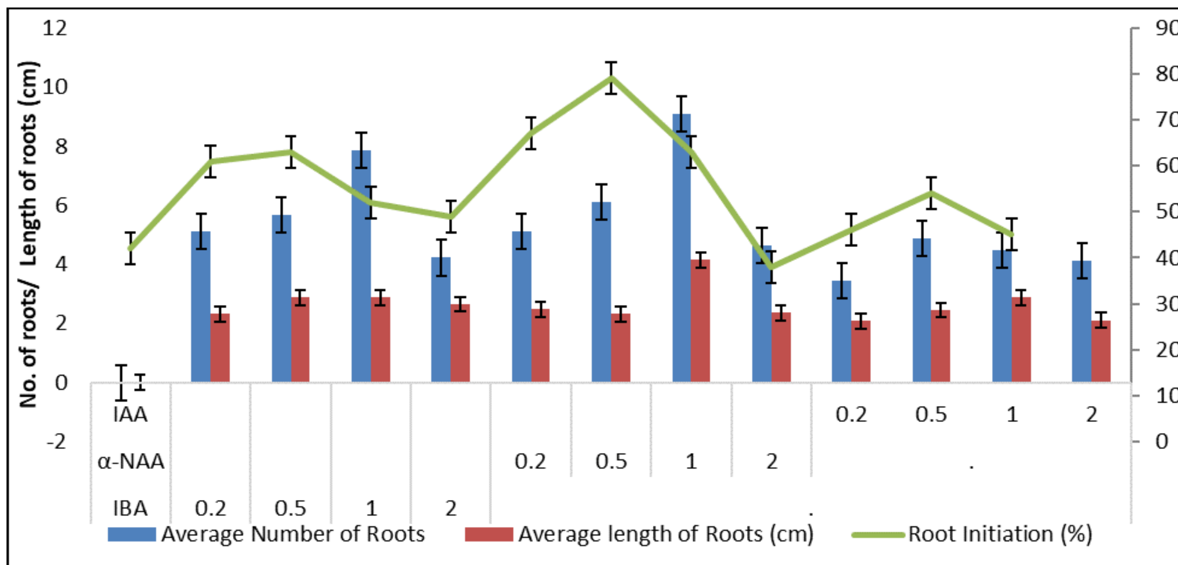

Fig. 6. Effect of various concentrations of IAA, IBA and NAA supplemented in MS basal medium on root initiation from in vitro raised shoots of *Eleocarpus ganitrus*

3.5 Effect of Auxins on Rooting

Root commencement was observed after 4–6 weeks on agar based media. Subculture for root commencement was performed after 4 weeks of initial subculture where explants from which shoots were initiated and moved to MS medium augmented with root hormones. The effectiveness of different auxins on rooting of in vitro grown shoots in MS medium was analyzed with IAA (0.2-2.0 mg/l), IBA (0.2-2.0 mg/l) and NAA (0.2-2.0 mg/l). The foremost result was obtained when 1.0 mg/l (NAA) was added to the MS medium. Root induction was recorded in 79% of cases, with an average of 5.09 roots per shoot (Fig. 6). Additionally, the average length of these roots was measured to be 4.14 cm.

3.6 Effect of Calcium Chloride on Rooting and Acclimatization

When the MS medium charged with three different auxins at different concentration IAA(0.2-2.0mgL^{-1}), IBA(0.2-2.0mg/l) and NAA(0.2-2.0mg/l) along with calcium ($CaCl_2.2H_2O$) range of 2.5 to 7.5mM/l, the best result was shown when MS medium supplemented with 1.0mg/l (NAA) + 5mM/l calcium ($CaCl_2.2H_2O$). Root initiation was observed in 82% of cases, with an average of 6.8

roots per shoot. Additionally, the average length of these roots was measured to be 5.8cm (Fig. 7).Well-rooted plants grown in culture were removed and washed rigorously with running tap water to remove media attached to the roots, and planted in plastic pots carrying sterilized potting mix (garden soil, sand, and farm yard manure (1:1:2). A transparent plastic cover was put over each pot during curing to maintain adequate humidity. Watering was done every 15 days and the plastic covering the pots was removed after a month; and then the plants were allowed to grow in a greenhouse under natural light, temperature and humidity.

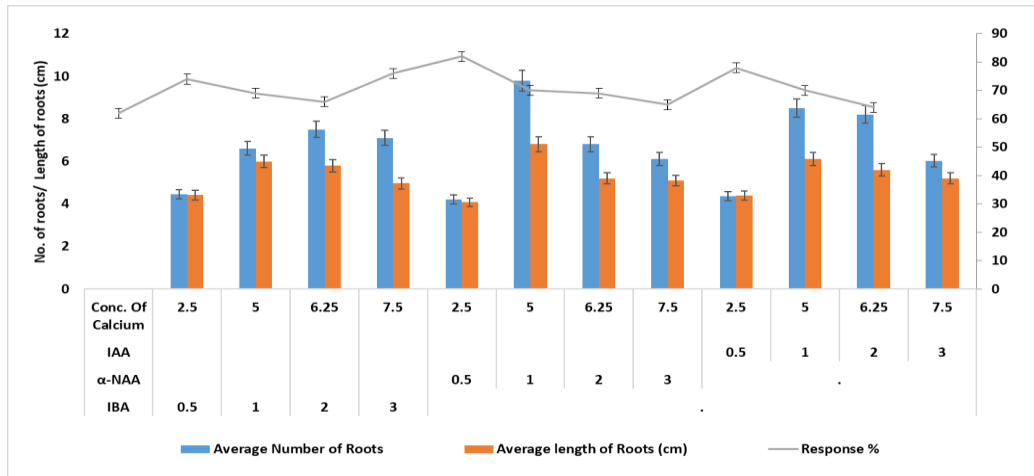

Fig. 7. Effect of differentconcentrations of IAA, IBA and NAA along with calcium supplemented in MS basal medium on root initiation from *in vitro* raised shoots of *Eleocarpus ganitrus*

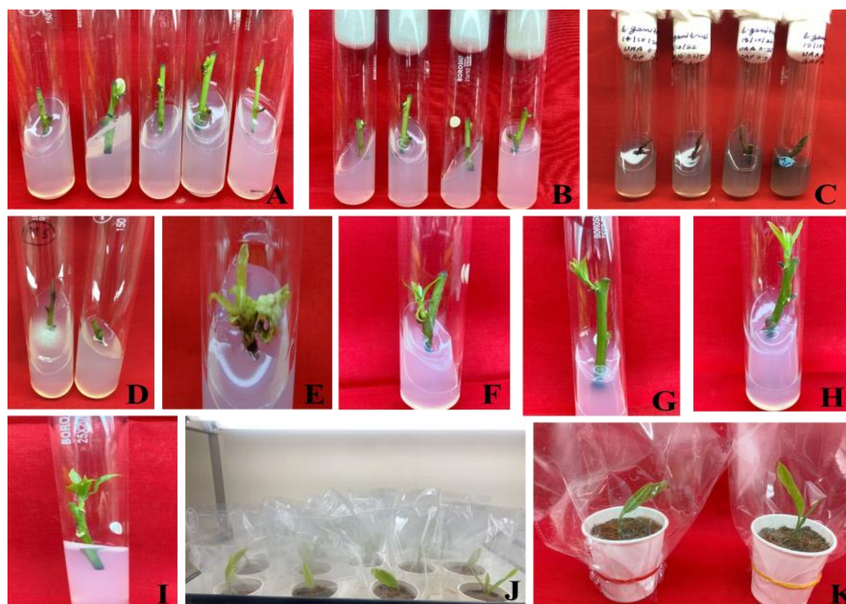

Fig. 8. *In vitro* culture of *E. ganitrus* from nodal segment explants. A) Induction of shoots on different media with BAP, NAA and antioxidants. B) Initiation of shoots on MS media with BAP, NAA and antioxidants. C) The symptoms of browning of shoots and leaching of phenolic exudation in media without antioxidants. D) *In vitro* fungal and bacterial contamination. E) Multiple shoots showing hyperdydration and shoot tip necrosis. F and G) Shoots induction and multiplication on MS medium supplemented with BAP and NAA. H) Shoots initiation and multiplication on MS medium supplemented with BAP, NAA and calcium chloride. I) Root induction from regenerated shoots on MS medium supplemented with 1.0mg/l NAA. J and K) Healthy plantlets transferred to plastic pot filled with a mixture of garden soil, sand and farm yard manure (1:1:2) after 8 weeks of acclimatization

4. DISCUSSION

4.1 Effect of Surface Sterilizing Agents

Increasing exposure time and sterilization concentration beyond a certain limit resulted in loss of explants due to the oxidative action of the plant tissue-killing chemical ingredient [22]. In our previous studies 0.1% (w/v) $HgCl_2$ for 3 minutes followed was effective in preventing bacterial and fungus contamination [23]. Some studies have reported the use of $HgCl_2$ for surface sterilization in plant tissue culture [24,25,26]. In contrast, treatment with calcium hypochlorite ($Ca(OCl)_2$) gave unsatisfactory results with high contamination and low tissue survival rates. Different studies found sodium hypochlorite (NaOCl) to be a very effective surface sterilizer. In addition to several antibiotics, even micromolar concentrations have been found to be effective in significantly decreasing bacterial populations [27]. In this study, a concentration of 0.1% (w/v) $HgCl_2$ for 3 minutes was found to be efficacious in sterilizing the nodal and shoot explants of *Elaeocarpus ganitrus* (Fig. 8-A,B and D).

4.2 Effect of Antioxidants

Browning of explants, or phenolic browning, is a phenomenon that naturally results from the enzymatic oxidation of polyphenolic compounds, which are well known explant inhibitors (Fig. 8-A and B). Phenols liberated from damaged or excised explants are then oxidized to quinones by polyphenol oxidases (PPO) and peroxidases (POD), which also cause browning of tissues and media [28]. These quinones secured to cell proteins or polymerize upon drying, causing interruption of cell metabolism, inhibition of growth, and ultimately death of explants [29]. Tissue browning can be decreased by timing the collection of explants, augmenting the culture medium with antioxidants such as citric acid, ascorbic acid, activated carbon (AC) and polyvinylpyrrolidine (PVP), unescorted or in combination, or using liquid culture or semi solid media [30,31,32]. Different compounds known to inhibit browning, such as citric acid (10 mg/l), ascorbic acid (50 mg/l), activated charcoal (500 mg/l), and PVP (100 mg/l), were added to the growth medium. Notably, antioxidants emerged as the most potent inhibitors, demonstrating a remarkable 80% reduction in shoot browning, thus effectively preventing and mitigating this phenomenon.

4.3 Effect of Media

Following 42 days of culture, the difference in shoot recovery appeared that the increase in initial nodal parts depends on several factors, the type of medium tested and the plant growth regulators and their combinations. The proliferation was higher in MS medium with 0.5 mg/l BAP and 0.1 mg/l NAA (43.7% ± 0.66) compared to WPM media (38.33% ± 0.44) and other media, while DCR, Anderson and B_5 had the lowest percentage of regeneration. Shoot proliferation varied between media and union of auxin and cytokinin (Fig. 8-A). In general, it is observed that MS and DCR media had a larger number and shoot multiplication rate than WPM. Genotypic outcomes have also been demonstrated in other *Prunus* species [33,34]. In this study, MS medium was more acceptable for *E. ganitrus* shoot growth than WPM and DCR medium with phytohormones. Previous studies have revealed that the composition of the medium also affected the shoot proliferation. In fact, explants inoculated on MS or WPM medium produced significantly more multiple shoots than explants grown on other media. Similar instead outcomes were obtained from wild cherry *in vitro* culture [35]. Growth regulators significantly improved shoot reproduction. Cytokinin-supplemented MS medium supported the proliferation of multiple shoots from nodal segment explants.

4.4 Effect of Plant Growth Regulators

MS medium augmented with 0.5 mg/L BAP was optimal for inducing higher proliferation, while MS medium recorded the maximum number of shoots per explants (Fig. 8-F and G). Analytical data revealed that the use of 0.5 mg/L BAP and 0.1 mg/L NAA added to all studied media was beneficial to elevate the multiplication of shoot; average shoot number for every explant and average shoot length were crucial for different platforms. Similarly, BAP at a concentration of 2 mg/l proved to be the foremost in regenerating a higher number of shoots that showed maximum shoot length from the axillary bud explants and a significant number of shoots in other plant species such as *Morus* species [36]. Other concentrations of cytokinins were not tested in this study, as BAP at higher concentrations has been detailed to reduce shoot length and thus negatively affect shoot proliferation in rudraksha Moreover, an elevated concentration of cytokinin in the surroundings led to hyperhydricity, necrosis, and diminished shoot quality and

viability [37]. Notably, the combination of BAP at 0.5 mg/l and NAA at 0.1 mg/l demonstrated a marked increase in shoot formation, with an average of 6 shoots ± 0.1, particularly those cultured on MS medium, yielding shoots longer than 2 cm. To promote shoot growth, an attempt was made to test the outcome of TDZ and KIN at 0.1-2.0 mg/l MS medium. The percentage of recovery was that much affected by the number and length of shoots with TDZ. Since the main functions of cytokinin come from its role in DNA synthesis, cell division, shoot growth, furthermore the mechanism responsible for the formation of the mitotic spindle, which is the regulation of protein synthesis [38].

4.5 Effect of Calcium

In the initiation of shoots in *E. ganitrus,* plant growth regulators played a crucial role. Cytokinins, a major class of growth regulators, were identified as key contributors to *in vitro* shoot induction . Previous studies by [39,40,41] investigated the use of BAP and Kn, with BAP demonstrating superior efficacy in promoting axillary bud proliferation. However, our research showed that the most favorable axillary shoot proliferation occurred on MS medium with varying concentrations of BAP (0.5mg/L) and NAA (0.1mg/L) with $CaCl_2.2H_2O$ (6.5mM/l). Notably, the combination of 0.5 mg/L BAP, NAA with calcium resulted in the generation of multiple shoots from explants derived from *in vitro* shoots (Fig. 8-H). Rahman *et al.,*2004 previously established an effective plant tissue culture protocol for *E. robustus.* For plant regeneration, our research identified that MS medium containing 0.5 mg/L BAP with 0.1 mg/L NAA and calcium ($CaCl_2.2H_2O$) 6.5 mg/l was suitable, similar findings reported by [42].

In Roy et al.'s 1998 study, positive outcomes were documented for *E. robustus* using 1 mg/l IBA and 0.5 mg/l IAA. Similarly, in another experiment involving *E. sphaericus*, maximum root initiation was observed with 1 mg/L IBA, despite the inclusion of NAA in their investigation [43]. In our study, *E. ganitrus* found that the foremost outcome for root initiation was achieved with 1 mg/L NAA and also when supplemented with 5mM/l calcium ($CaCl_2.2H_2O$). Additionally, it was observed that 1.0 mg/L IBA exhibited a positive response for root multiplication. However, the growth rate of rooting was slow on agar-based MS medium, as evidenced by results reported after eight weeks, a finding consistent with [44], who also observed effective rooting with NAA.

4.6 Rooting and Acclimatization

Rooting of micropropagated shoots has been reported to involve a short root induction phase of one-two weeks, often using IBA, followed by a longer phase of various weeks, with NAA being the most usual auxin supplied by the root growth medium. In addition, the optimal auxin for rooting depended on the plant species. Therefore, auxin selection is a very pivotal issue for woody plants that have difficulties with *in vitro* rooting. IBA was found to be commonly used instead of indole-3-acetic acid (IAA) and NAA as the most efficacious auxin to stimulate root induction in tree species such as apple and *Cassia angustifolia* Vahl. ; While 1,2-benzisoxazole-3-acetic acid (BOA) had a very poor effect on woody plant root induction [32].

It was also perceived that *in vitro* hardening after long-term incubation in culture (12 weeks) was useful for improving the survival rate of ruhraksha plants in tissue culture-grown plantlets. The lowest survival was observed in 6-week-old plants, while 84% survival was obtained after long incubation in culture (12 weeks), i.e., after in vitro treatment. In general, except in some cases, the green microshoots used in the plantations varied from 0.5 to 0.7 mm in diameter with 4 to 5 leaves (Fig. 8-J and K). A previous study showed that microshoots with a green stem are most suitable for rooting with almost 100% efficiency [45]. In this experiment, researchers noted enhanced growth in tissue culture plants following extended incubation periods. These plants exhibited accelerated secondary stem growth, leading to the development of semi-hard stems conducive to survival post-planting in soil. Typically, the transition of plantlets into soil took approximately 8-10 weeks, as indicated by the emergence of new shoots. The findings highlight how minor adjustments in field conditions substantially enhance the viability of *in vitro* propagated rudraksh plants during their transfer from laboratory to soil environments. Furthermore, plants cultivated in tissue culture displayed morphological characteristics akin to those of naturally grown specimens, both in leaf structure and overall growth patterns.

5. CONCLUSION

Among all the treatments used, 0.1% $HgCl_2$ for 3 minutes was ascertained the most effective in controlling contamination and tissue damage of *Elaeocarpus ganitrus* Roxb. It can be concluded

that the sterilization requirements vary depending on the tissue type, age, nature and season of the explants used for propagation. Nutrient medium plays an pivotal role in plant propagation by tissue culture. The results showed that MS medium gives a better response to shoot and root growth of *E. ganitrus* compared to DCR, WPM, Anderson and WPM medium. Nodal segment explants were utilized in *in vitro* culture to assess their proliferative capacity in Murashige and Skoog (MS) medium containing various combinations of auxins and cytokinins. The highest proliferation rate was achieved with 0.5 mg/L BAP and 0.1 mg/L NAA, while the lowest response rate, shoot number, and length were observed in the presence of TDZ, Kn, and NAA. This indicates the efficacy of the developed method for the micropropagation of elite rudraksha germplasm. Additionally, increasing the level of $CaCl_2.2H_2O$ (6.5mM/l) in the culture led to elevated calcium content in the explants and reduced hyperhydration. Moreover, shoot tip necrosis was mitigated in the medium containing 6.5 mM/l $CaCl_2.2H_2O$, but not in other subcultures. The highest rooting rate was attained in MS medium supplemented with 1.0 mg/l NAA along with $CaCl_2.2H_2O$. Morphological evaluation of hardened plantlets in the field confirmed the effectiveness of the developed method for the micropropagation of selected *E. ganitrus* germplasm. Subsequently, shoots and roots-bearing plantlets were transplanted into plastic containers and successfully acclimatized to the greenhouse environment.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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