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STANDARDIZATION OF SEASON AND STERILIZATION OF EXPLANTS ON MICROPROPAGATION OF RUDRAKSHA (*ELAEOCARPUS GANITRUS* ROXB.)

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ABSTRACT
The *Elaeocarpous ganitrus* Roxb. species, which is a relative of Rudraksha and belongs to the Elaeocarpaceae family, is endangered in India. To propagate and establish the plants *in vitro*, it is essential to collect shoot tips and nodal segments during the season from April to October. Explants collected during March to April had higher phenolics exudation and more browning than those from juvenile tissue or other months. Surface sterilization is a critical step in explant preparation for micropropagation, and the effectiveness of three sterilizing agents ($HgCl_2$, $Ca(OCl)_2$, and NaOCl) was evaluated. The best results were achieved with 0.1% $HgCl_2$ for 5 minutes, while sterilization with NaOCl and $Ca(OCl)_2$ was not satisfactory. Shoot proliferation was achieved using Murashige and Skoog's media with BAP (2.0 mg/l), Kn (0.5 mg/l), and antioxidants.

Keywords: *Rudraksha, phenolic exudation, contamination, seasonal effect, sterilization, aseptic micropropagation.*

INTRODUCTION
Elaeocarpus ganitrus (Roxb.) is a type of evergreen tree that belongs to the Elaeocarpaceae family. This family includes 360 species of Elaeocarpus, of which 25 are found in India's Gangetic plains and Himalayan regions (Liu *et al*., 2022). Eleven Elaeocarpus species have been identified in Tamil Nadu, and eight are confined to the Western Ghats, including four steno-endemics: *E. blascoi* Weibel, *E. gaussenii* weibel, *E. recurvatus* corner, and *E. venustus* Bedd (Meitei and Khuraijam, 2019). The leaves of *Elaeocarpus ganitrus* are shiny green on the upper side and dull and leathery on the dorsal side. The stem is cylindrical with fringed petals, and the flowers mostly appear in racemes from the axils of fallen leaves, nodding, white, and about 1cm across, with anthers that are bristled at the apex. The fruits are globular and 1cm in diameter, deep blue, or mealy when ripe, and contain a hard bead inside that has eight tubercles (T. Kumar *et al*., 2008). The tree reproduces through seeds, but local people's increased seed collection has resulted in the shrinkage of the natural seed bank in the soil, adversely affecting the species' regeneration. As a result, the tree is currently not listed in the red data book but is being pushed into the threatened category and may even become extinct in the future if immediate conservation measures are not taken (Khan *et al*., 2004).
The population of Rudraksh (*Elaeocarpus ganitrus*) in the tropical wet forest of Arunachal Pradesh is declining due to increased anthropogenic pressure (Baruah *et al*., 2019; Bhuyan *et al*., 2003; Saikia *et al*., 2017). The collection of nuts for beads has led to the reduction of the seed bank in the soil, which has negatively impacted the species' regeneration. Additionally, the frequent disturbances make it difficult for Rudraksha seedlings to survive and grow (Bhuyan *et al*., 2002; Khan *et al*., 2004). The determination of phenolic and flavonoid compounds is essential due to their various biological activities. The plant contains several bioactive compounds that could potentially be used for medicinal purposes (Hardainiyan *et al*.,

2015). Rudraksha has been reported to display multiple biological activities, such as antihypertensive, antidepressant, anti-inflammatory, antimicrobial, analgesic, antidiabetic, and antioxidant activity, suggesting its potential therapeutic applications (Garg *et al*., 2013). There is an increasing interest in the pharmacological evaluation of various therapies used in the Indian traditional system of medicine (Hardainiyan *et al*., 2015). *E. sphaericus* beads, also known as blueberry beads, are composed of different combinations of carbon, hydrogen, oxygen, nitrogen, and trace elements (Pant *et al*., 2013).

For centuries, *Elaeocarpus ganitrus* has been used in Ayurvedic medicine and there have been several investigations into its potential therapeutic uses. Future research could focus on identifying the active components of the extracts and their mechanisms of action through *in vitro* and *in vivo* studies (Arivu and Muthulingam, 2017). Recent studies have shown that *Elaeocarpus ganitrus* exhibits immune-stimulatory, anti-inflammatory, antimicrobial, antihypertensive, anxiolytic, anti-ulcerogenic, antidepressant and antioxidant properties (Hule *et al*., 2011; Kakalij *et al*., 2014; Kumar *et al*., 2008).

Elaeocarpus ganitrus leaf extracts have also been found to possess significant antioxidant activity in various *in vitro* methods. The ethanolic extracts were found to exhibit significant total antioxidant activity, reducing power potential, metal chelating activity, and ABTS scavenging activity. Moreover, a positive correlation between total phenolic content and antioxidant capacity, as well as between total flavonoid content and antioxidant activity, was reported (Kumar *et al*., 2008).

Plant tissue culture technology has become of major industrial importance in recent years, as it allows for large scale plant multiplication and is useful in plant propagation, disease elimination, plant improvement, and production of secondary metabolites. Micropropagation technology has a vast potential to develop commercially important improved varieties and is rapid, allowing for virus-free plant production (Akin-Idowu *et al*., 2009; Brown and Thorpe, 1995; García-Gonzáles *et al*., 2010).

In this study, the impact of the season of explants collection on contamination and phenolic content was investigated. The correlation between phenolic content and browning, which affects explant survival during *in vitro* culture establishment, was also examined. Given that the production of planting stock is essential for any plantation program, biotechnological methods can be employed for mass propagation of this plant species to prevent it from being classified as a threatened plant. Moreover, the extensive use of various *Elaeocarpus* species for medicinal and timber purposes has placed several species in the Red Data List of threatened plants, highlighting the urgent need to conserve them (Saklani *et al*., 2015).

MATERIALS AND METHODS

The study aimed to standardize the season for explant collection in *Elaeocarpus ganitrus* Roxb. from the SIET Rudraksha arboretum in Meerut. The plant material used in the study included shoot tips and nodal segments of field-grown plants collected in different months of 2018 and 2019. The explants collected in July-August 2018 were further categorized based on their location on the stem. Nodal segment explants were also excised in August of both 2018 and 2019. Additionally, shoot tips and nodal segments explants were excised from 3-month-old shoots that sprouted after the pruning for the first explants collection in August 2019. Finally, explants 1.0-2.0 cm long were excised from nine-week-old new sprouted apical buds. The study determined the phenolic content of explants and correlated it with browning, which affects explant survival during *in vitro* culture establishment. The standardization of the season for explants collection is important for the mass propagation of this plant species and its conservation, especially given its listing in the Red Data List of threatened plants.

Initially, the shoot tips and nodal segments were washed with tap water and then subjected to different surface sterilizing agents, including $CaOCl_2$ (9-10%), NaOCl (0.5, 2.0, 3.5, and 5.0%), and $HgCl_2$ (0.01-0.10 mg/l), for 3-10 minutes to determine the most effective sterilization method. The explants were subsequently washed with autoclaved double distilled water five times in a laminar airflow to eliminate any residual surface sterilizing agents.

The medium was prepared using double distilled water and stock solutions of nutrients, organic constituents, antioxidants, and growth regulators. Sucrose and myo-inositol were added to the medium, which was then

gelled with 0.8% bacteriological grade agar and poured into various vessels. The pH of the medium was adjusted to 5.8 using NaOH and HCl, and it was sterilized by autoclaving at 121°C and 15 lb/in^2 pressure. Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with different concentrations and combinations of auxins (α-NAA) and cytokinins (BAP, kinetin, and 2iP) with antioxidants (PVP, ascorbic acid, and citric acid) was used for the experiments. The cultures were incubated at 25±2ºC in a culture room with a light intensity of 2000-3000 lux, using cool white and yellow fluorescent tubes for 16/8 hours daily light/dark cycles.

During the season and sterilization experiments, several parameters were recorded, including the contamination rate, mortality, and survival rate. These were calculated by dividing the number of contaminated explants, the number of non-contaminated but dead explants, and the number of uncontaminated and surviving explants, respectively, by the total number of inoculations and multiplying by 100. In the experiments for bud induction in nodal segment and shoot tip explants, the frequency of axillary bud induction was calculated by dividing the number of segments that induced axillary buds by the total number of inoculated stem segments and multiplying by 100. Additionally, the length of each shoot was recorded.

RESULTS AND DISCUSSION

This study investigated the challenges of contamination and explant browning that can hinder the successful establishment of *Elaeocarpus ganitrus* Roxb. *in vitro* cultures. Although all types of explants could be used for culture initiation, the success of the process was dependent on effective sterilization of the explants and the prevention of phenolic-induced browning. Nodal segments and shoot tips collected during different times of the year had varying responses to *in vitro* culture. Those collected in January-February and November-December did not proliferate, while those obtained in May-June proliferated without predominant shoot initiation. However, most of the shoots that formed from these explants ultimately turned brown after reaching a length of about 15 mm (Table 1

Table **2**) (Figure 1).

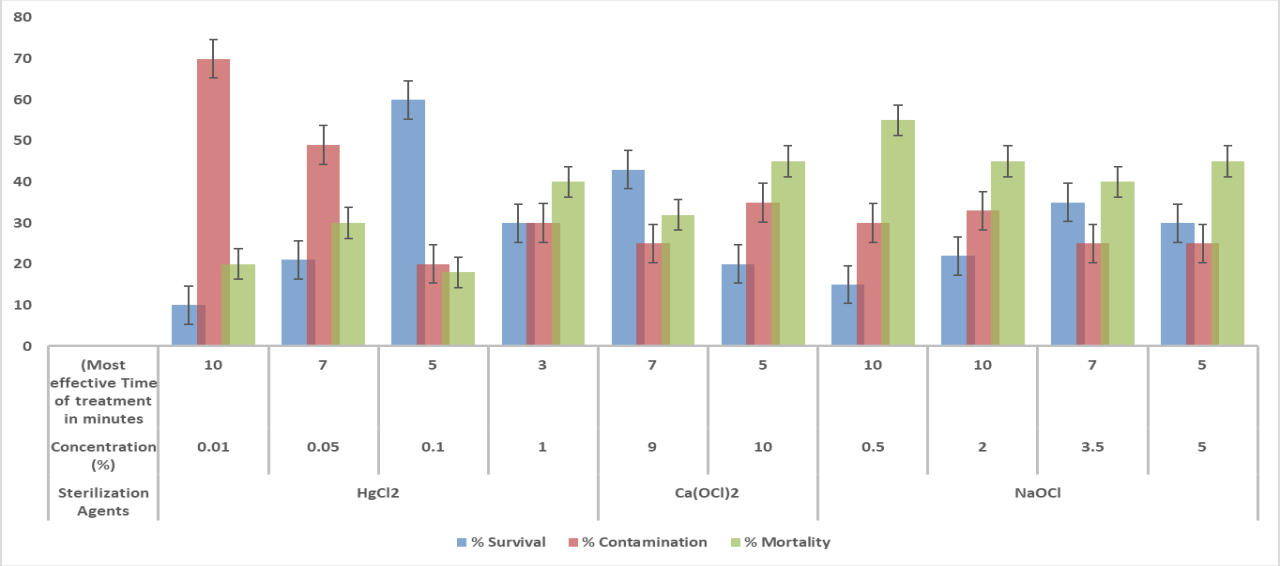
In the current study, it was observed that the release of phenolics into the medium and explant browning was more pronounced during November-February, and decreased gradually after March. The use of MS media supplemented with antioxidants partially controlled explant and medium browning, but the problem persisted. Adult shoot explants released phenolics into the medium at a higher percentage than young field-grown shoot explants. The season of explant collection has been reported to affect the browning of apple shoot tip explants (Baydar, 2006; Thomas and Ravindra, 2015), as well as the survival of mango explants due to lower phenolic content (Thomas and Ravindra, 1997).

The season of collection has been observed to have an impact on the behavior of *Elaeocarpus ganitrus* explants, with shoot tips and nodal segments showing no response to the season of excision. Similar observations have been made with other foliage plants, indicating the importance of the season of explant collection over the selection of appropriate explants and nutrient media (Chaturvedi, 1985). Maximum shoot bud response (80%) and number of proliferating shoots (4.7) occurred during May-June, while a 50% shoot response was recorded during July-August. The minimum number of sprouted shoot buds was observed during November-February, where phenolic exudation was higher, and shoot growth was slower. This finding is consistent with previous studies conducted on *Tridax procumbens* (Malik and Wadhwani, 2009). Bud breaking and shoot growth of *Elaeocarpus ganitrus* decreased during November-December, with less browning of spouted shoot tips and nodal segments during this season. In the spring season, the main constraints for axenic establishment of cultures were observed. The maximum bud breaking percentage of the rudraksha was recorded during May-June, and contamination was better controlled by collecting explants from new vegetation between April and August (Table 1 and

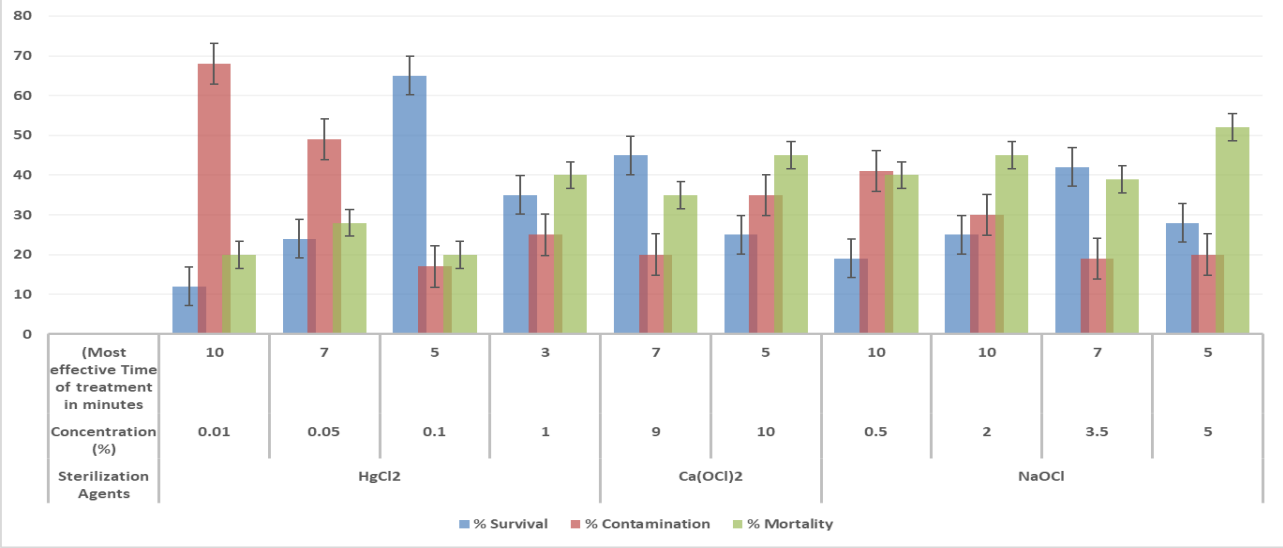
Table 2). The season of explant collection has also been reported to affect the survival of mango explants due to microbial contamination, with reduced phenolic exudation observed in explants collected during this season (Thomas and Ravindra, 1997). During the monsoon season, heavily contaminated cultures were

observed even after 3-4 weeks, and previous studies have reported the influence of seasonal rainfall patterns on axillary bud-break and fungal contamination in bamboo (Ramanayake and Yakandawala, 1997; Saxena and Bhojwani, 1993).

In order to determine the most efficient procedure for initiation of tissue culture of *Elaeocarpus ganitrus* using nodal segment and shoot tips explants, various surface sterilizing agents were used at different concentrations and durations (Graph 1andGraph 2). The effectiveness of the sterilizing agents, Mercuric chloride ($HgCl_2$), Calcium hypochlorite $Ca(OCl)_2$, and Sodium hypochlorite (NaOCl), was evaluated by observing the inoculated explants for 21 days for contamination, tissue mortality, and survival of culture. The results indicated that increasing the time and concentration of sterilizing agents significantly reduced contamination but adversely affected the explants. Among the three sterilizing agents, $HgCl_2$ was found to be the most effective surface sterilization treatment for nodal segments and shoot tips.



Graph 1: Effect of Sterilizing agents on nodal segment explants of *Elaeocarpus ganitrus*



Graph 2: Effect of sterilizing agents on shoot tips explants of *Elaeocarpus ganitrus*.

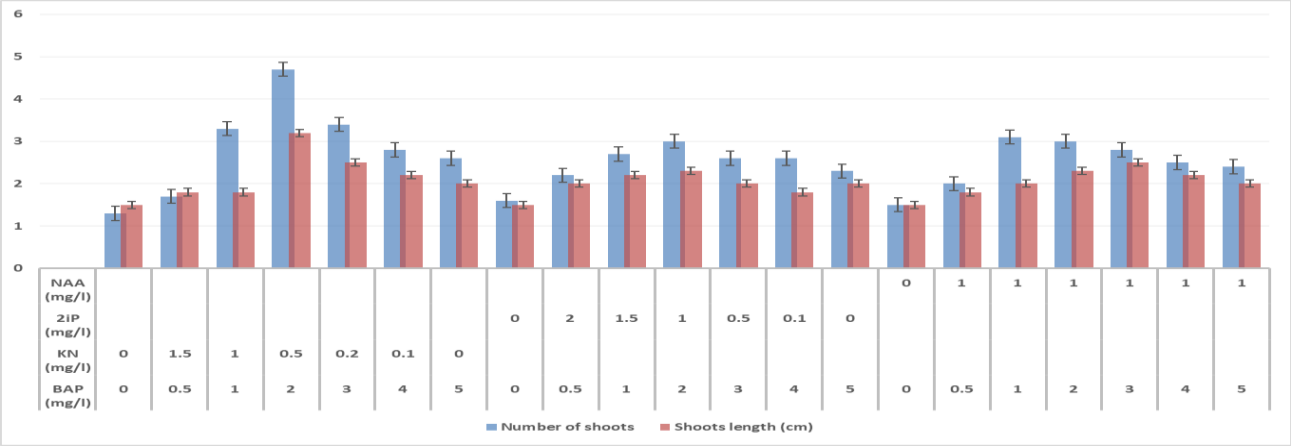
Table 1: Effect of season of collection of shoot tips from field-grown plants of *Elaeocarpus ganitrus* on their proliferation *in vitro* in the treatment containing MS Media 2.0 mg/l BAP and 0.5 mg/lKn

Period of collection	Percent explants responded	No. of shoots regenerated per explants	Percent explants Phenolic exudation	callusing	Remark
Jan.-Feb.	10	2.2±0.50	70	+	Regenerated shoots with callus turned brown
March-April	20	4.0±1.33	50	+	Regenerated shoots grew
May-June	80	4.9±1.56	30	+	Regenerated shoots grew normally
July-Aug.	50	4.2±1.4	45	++	Explants along with callus turned brown
Sept.-Oct.	30	3.0±1.0	48	-	Explants along with callus turned brown
Nov.-Dec.	05	1.1±1.0	65	-	Explants turned brown

Table 2: Effect of season of collection of nodal segments from field-grown plants of *Elaeocarpus ganitrus* on their proliferation *in vitro* in the treatment containing MS Medium with 2.0 mg/l BAP and 0.5 mg/lKn

Period of collection	Percent explants responded	No. of shoots regenerated per explants	Percent explants Phenolic exudation	callusing	Remark
Jan.-Feb.	12	2.5±0.10	80	+	Regenerated shoots with callus turned brownish white
March-April	40	3.5±1.33	65	+	Regenerated shoots grew no callus
May-June	76	4.2±1.66	35	+	Regenerated shoots grew normally few callus
July-Aug.	44	3.8±1.4	49	++	Explants along with callus turned brown
Sept.-Oct.	30	2.8±1.88	48	-	Explants along with callus turned brownish black
Nov.-Dec.	05	1.4±2.66	68	-	Explants turned brown

The study evaluated the efficiency of different surface sterilization agents and their concentrations and exposure times for initiating tissue culture of *Elaeocarpus ganitrus* using nodal segments and shoot tips explants (Graph 1andGraph 2). After 21 days of observation for contamination, tissue mortality, and culture survival, it was found that increasing sterilant concentration and exposure time significantly reduced contamination but negatively affected the explants. Among the three sterilization agents tested, namely Mercuric chloride ($HgCl_2$), Calcium hypochlorite ($Ca(OCl)_2$), and Sodium hypochlorite (NaOCl), $HgCl_2$ was the most effective on both nodal segments and shoot tips. The optimal treatment for nodal segments was found to be 0.1% $HgCl_2$ for 5 minutes, resulting in 20% contamination, 18% tissue mortality, and 60% survival (Graph 1). The optimal treatment for shoot tips was 0.1% $HgCl_2$ for 5 minutes, resulting in 17% contamination, 20% tissue mortality, and 65% survival (Graph 2).



Graph 3: Morphogenesis response of *Elaeocarpus ganitrus* at different concentration and combination of growth regulators on MS media with antioxidants (nodal segments).

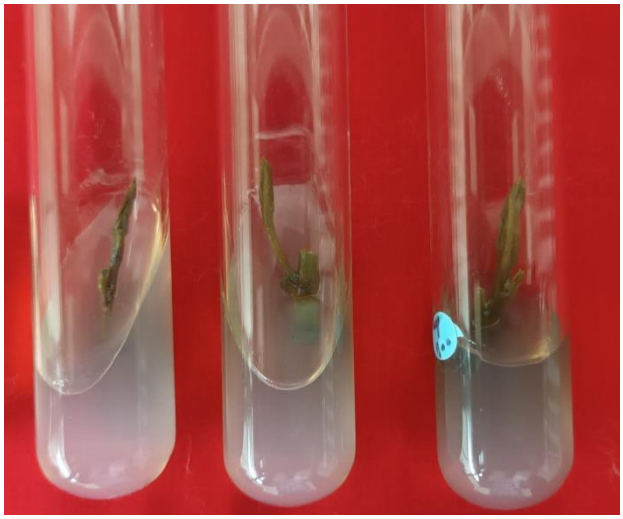


Figure 1: Phenolic exudation of shoot tips explants of *Elaeocarpus ganitrus* (3 days old culture)

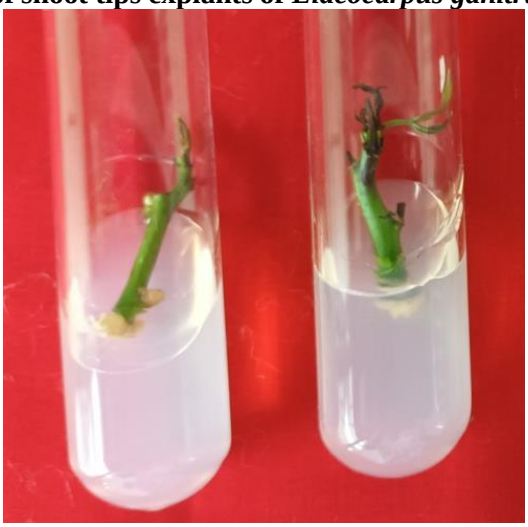


Figure 2: Induction of shoots from shoot tips explants of *Elaeocarpus ganitrus* with antioxidants

The maximum contamination rate was observed with 10% Calcium hypochlorite for 10 minutes (Graph 1andGraph 2). The present study suggests that sterilization with Calcium hypochlorite is less efficient compared to the other two disinfectants.

It was observed that increasing the exposure time and concentration of sterilant above a certain limit resulted in loss of explants due to the oxidizing effect of the chemical ingredient killing the plant tissue (Danso *et al*., 2011). Suma *et al*. (2008) reported that a combination of 1% (w/v) $HgCl_2$ for 3 minutes followed by 0.1% streptomycin for 1 minute was effective in avoiding bacterial contamination. Several studies have reported the use of $HgCl_2$ for surface sterilization in plant tissue culture (Anburaj *et al*., 2011; Preethi *et al*., 2011; Raja Naika and Krishna, 2008). In contrast, Calcium hypochlorite ($Ca(OCl)_2$) treatment showed unsatisfactory results, with a high rate of contamination and low survival rate of explants.

In various studies, sodium hypochlorite (NaOCl) has been found to be a highly effective surface sterilizing agent against different bacterial strains, with even micro-molar concentrations proving effective in significantly reducing bacterial populations (Nakagawara *et al*., 1998). In the present study, a 3.5% concentration of NaOCl for 7 minutes was found to be effective in sterilizing both nodal segment and shoot tip explants of *Elaeocarpus ganitrus*. Similar findings were reported by Hamirah *et al*. (2010) for micropropagation of red ginger. Sathyagowri and Seran (2011) also found NaOCl to be an efficient treatment for removing microbes. The use of NaOCl as a surface sterilizing agent for plant explants from various sources has been widely reported in the literature (Maina *et al*., 2010; Miche and Balandreau, 2001; Vejsadová, 2006).

Shoot tips and nodal segments explants of *Elaeocarpus ganitrus* were cultured on MS medium supplemented with different concentrations of BAP (0.5-5.0 mg/l) with Kinetin (0.1-1.5 mg/l) or 2iP (0.1-2.0mg/l) or NAA (1.0mg/l). The explants responded better in the presence of BAP (2.0mg/l) with Kn (0.5mg/l) compared to higher concentrations of 2ip, Kn and BAP. When NAA (1.0 mg/l) was used in combination with Kn or BAP, more callusing and less proliferation of shoots were observed. The effectiveness of 2iP in all the concentrations used was less than Kn at other concentrations for both shoot proliferation and callusing. According to the results, it was expected that 2.0mg/l BAP and 0.5mg/l Kn would induce more shoot proliferation in two months old cultures (Graph 3) (Figure 2).

The off-shoots differentiated from a shoot tip explant in the optimum treatment containing BAP and Kn, when subcultured in the same medium, developed into normal and vigorously growing shoots in eight weeks. Previous studies have also shown the beneficial effect of BAP and Kn combination for shoot proliferation in different plant species (Aruna *et al*., 2012; Tang *et al*., 2004; Zahara *et al*., 2018).

In summary, the collection season of explants had a significant effect on phenolic exudation and browning, which posed a challenge during the establishment of *in vitro* culture of *Elaeocarpus ganitrus*. The season of explant collection not only impacted contamination and browning but also had a direct influence on shoot proliferation. Therefore, for an efficient micropropagation protocol for rudraksha, it is advisable to use nodal segment explants obtained during May-June months from juvenile sprouted shoot arboretum-grown plants. Among all the treatments used, 0.1% $HgCl_2$ for 5 minutes was found to be the most effective for controlling contamination and tissue damage of *Elaeocarpus ganitrus* Roxb. It can be concluded that sterilization requirements vary depending on the tissue type, age, nature, and season of the explants used for proliferation. However, further research is needed to develop a quick regeneration and transformation protocol for rudraksha and to gain a better understanding of the actual mechanism.

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