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Standardization of micropropagation protocols for *Echinodorus grisebachii*: An aquascape plant

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Abstract

A study was conducted at the College of Agriculture, Vellayani, on *Echinodorus grisebachii,* a common aquatic plant widely used in aquascaping. The study aimed to develop an effective micropropagation protocol for *E. grisebachii*. Eleven treatments were applied for surface sterilization, twenty-six media combinations were tested for the establishment and shoot induction, fourteen treatments were evaluated for root initiation, and six different hardening media were trialed. An effective protocol was successfully developed through these experiments. The stem node explant was successfully sterilized using mercuric chloride 0.05%. The shoot initiation was achieved within a week using MS medium supplemented with 2.0 mg L^{-1} BAP, and the best rooting was achieved in ½ MS medium containing 3.0 mg L^{-1} IBA. Aqua soil was the best hardening media with the highest survival rates and enhanced shoot and leaf production. The developed micropropagation protocol has the potential for large-scale production and can help to satisfy export quality and quantity.

Key words: *Echinodorus grisebachii*, aquascaping, aquascaping plants, micropropagation

Introduction

Echinodorus grisebachii, also known as *Amazon sword,* belongs to *Alismataceae* family. It is an aquatic plant that adapts well in artificial settings like aquariums, making it popular among aquascapers and gardeners. The plants are upright and have long, glabrous green leaves. This species is native to South America and is highly valued by aquarium enthusiasts worldwide as it can be used as background plant aquaria. It is sold under the name Paniculatus. Naturally, it grows by riversides and on the edges of other freshwater streams, where it can grow partially or fully submerged. According to Haque and Ghosh (2019), *Echinodorus spp*. is an aquatic plant commonly used in aquarium decor. The plant is characterized by its wide ensiform leaves, which can develop into exuberant green if optimum light and nutrients are provided. As it is hardy and can thrive in almost all pH and temperature ranges, it has the potential to be used all over the globe by aquascaping enthusiasts. Aquascaping is the art of creating beautiful aqua settings based on aspects of horticulture inspired by landscape under the sea (Baby *et al.*, 2024). Even though *E. grisebachii* is a tall plant of about 60cm and can be maintained in an aquarium as a short plant.

Aquarium plants proliferate primarily by sexual reproduction through seeds or asexually through vegetative reproduction (Shibayama and Kadono 2007). Aquatic plants like *Vallisneria* and *Sagittaria* are propagated through different modes, using runners, whereas plants like Cryptocoryne with rhizomes (Rataj and Horemann 1977). Members of the genus *Echinodorus* typically propagate through rhizomes at a very low rate. *E. grisebachii*, propagates naturally through runners developed when the plant reaches a certain age and the new plantlets develop in their root systems and offshoots.

Tissue-cultured aquatic plants have recently gained significant commercial attention. Many aquatic plants have been successfully propagated using tissue culture techniques (Jabir *et al.*, 2016). Tissue culture is preferred in aquascaping plants due to its potential to produce aseptic plantlets, which is an important factor in maintaining an aesthetic aquarium (Baby *et al.*, 2024). *Anubias barteri* var. nana, *Cryptocoryne wendtii*, *Aponogeton madagascariensis*, and *Nymphoides coreana* are among the ornamental aquatic plants commonly propagated using micropropagation. (Huang *et al.*, 1994; Kane *et al.*, 1990; Ozturk *et al.*, 2004; Smitha *et al.*, 2005; Banerjee and Shrivastava, 2008)

However, there is not much research on the micropropagation of *E. grisebachii*. This research aimed to establish an effective micropropagation protocol for this particular species. The current study focuses on initiating aseptic cultures from stem node explants and optimizing the culture media for shoot proliferation.

Materials and methods

The study was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram from 2023 to 2024. The experiment was laid out in a completely randomized design (CRD). The acquired observations were statistically analyzed using KAU Grapes software.

Plant materials, explant preparation and sterilization: Plants were collected from Srepadma Aqua Flora, an aquatic farm in Aluva, Kerala. Aquatic plants with healthy stems with intact nodes that were free from bacterial or fungal infections were selected. Stem bearing 4 to 5 nodes were cut off from collected plants. They were washed under running tap water for 10 minutes, then with a few drops of Tween 20 solution. Finally, thorough washing was done for several times using sterile distilled water.

Explants sterilization: Surface sterilization of explants was carried out inside a laminar air flow chamber just before inoculation. The explants were treated with different concentrations of mercuric chloride and sodium hypochlorite for different periods as given in Table 1. After treatment with the sterilizing agents, the explants were thoroughly rinsed four to five times with sterile distilled water to remove any residual chemicals, critical for preventing toxic residues that could hinder growth. Following sterilization, the percent survival of the explants was determined:

$$\text{Plant survival (\%)} = \frac{\text{Number of explants surviving}}{\text{Total number of explants}} \times 100$$

Standardization of shooting media: The kind and quantity of growth regulators added to the tissue culture medium is vital in shoot development (Kunene *et al.*, 2018). To standardize tissue culture media for establishment and shoot induction, explants were treated with various combinations of growth regulators. These treatments included different concentrations of cytokinins (BA and kinetin) and the auxin (NAA) in both full-strength and half-strength MS medium (Murashige and Skoog, 1962). The treatments were replicated three times. Each replication had ten explants. The treatments tried to assess the effect of plant growth regulators on culture establishment and shoot induction of *E. grisebachii* are given in Tables 2. After four weeks of shoots, development plants were removed from the culture bottle inside the laminar air flow chamber, under aseptic condition. Shoots with excess growth of callus were subcultured after removal of callus and dead region using a sterilized blade. Then multiple shoots were separated and transferred to the culture bottles with various treatments. Bottles were placed on the rack, and subsequent subculturing was done at intervals of 21 days. Observations were recorded on various parameters *viz*., number of shoots, shoot initiation percentage, days to shoot emergence, shoot length (cm), days to emergence of first leaf, number of leaves.

Standardization of rooting media: The healthy shoots regenerated *in vitro* were then subcultured onto rooting media to encourage the establishment of strong root systems. This technique used two types of media: full-strength Murashige and Skoog (MS) medium and half-strength MS medium. Both mediums were supplied with varied amounts of auxins, notably indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA), well-known plant hormones promoting root development. The treatments tried for *in vitro* rooting of shoots are presented in Table 3. Fourteen treatments were tried and were replicated three times. Observations were recorded on days to root initiation and root initiation percentage.

Standardization of hardening media: Once the plantlets had grown sufficient roots in the culture jars, they were carefully removed. This step signalled the transition from a controlled *in vitro* setting to more natural surroundings. After being removed from the culture tubes, the plants were thoroughly cleansed to remove any remaining growth medium. To do this, the plantlets were gently cleaned with running tap water for a prolonged period. This method efficiently eliminated all traces of nutrient residues, including any agar or other compounds that may have stuck to the roots or plant surfaces during the culturing process. After being removed from the tissue culture medium, aquatic plants were placed in netted pots filled with substrates such as gravel or aquatic soil as shown in Table 4 and submerged in water-filled containers. The plants are gradually introduced to submerged settings, which include controlled water levels and light exposure, allowing them to transfer carefully. After hardening for one month, they were carefully transferred to the aquariums. Six different treatments were used and were replicated five times. Observations on the percentage survival of plantlets, number of roots, length of roots (cm), number of shoots, length of shoots (cm), number of leaves were recorded after one month.

Results and discussion

Effect of different sterilizing agents on explants: Surface sterilization is an essential step in tissue culture by using different sterilizing agents such as mercuric chloride, calcium hypochlorite, ethanol hydrogen peroxide and silver nitrate (Mihaljevic *et al.*, 2013). The sterilizing agent was used according to the morphological characters of explant used. The surface sterilization was done to reduce contamination (Srivastava *et al.*, 2010).

Among the different concentrations of $HgCl_2$ and $NaOCl_2$ used, the highest percentage of survival rate was recorded when the explants were surface sterilized with 0.05% $HgCl_2$ for 10 minutes. Stem nodes showed about 98% survival after two weeks of inoculation (Table 1). Plants host a range of bacteria and fungi in their natural environment. These microbes can potentially cause systemic infections by infiltrating the plant tissues. Consequently, cultures originating from these tissues are at risk of contamination. In the study, $HgCl_2$ (0.05%) treatment for 5 minutes was found to be most effective for surface sterilization. Similarly, in *Bacopa chamaedryoides,* mercuric chloride was found to be an effective sterilizing agent (Haque and Gosh, 2013).

Effect of auxin and cytokinin in shoot initiation in *E. grisebachii*: The results showed that the treatment with 2.0 mg L^{-1} BAP (T_6) was the most effective, achieving 100% shoot initiation, the shortest time to shoot initiation (8.43 days), the highest number of shoots (9.83), and the greatest shoot length (10.33 cm), along with the highest leaf count (31 leaves) (Table 2). Similar results were reported by Sheeja *et al.* (2015) in *Anubias barteri* var. Nana Petite.

Effects of IBA and IAA on root initiation in *E. grisebachii*: The results (Table 3) indicate that treatment T_7 (½ MS + 3.0 mg L^{-1} IBA) was the most effective, achieving 100% root initiation

Table 1. Response of *E. grisebachii* for surface sterilization with various concentrations of $HgCl_2$ and $NaOCl_2$

Treatment	Treatment details	Explant survival (%)
T1	Control	0.00 (0.02) c
T2	0.03% $HgCl_2$ 5 min	6.66 (0.22) b
T3	0.05% $HgCl_2$ 5 min	98.00 (1.44) a
T4	0.08% $HgCl_2$ 5 min	0.00 (0.02) c
T5	0.08 % $HgCl_2$ 7 min	3.33 (0.12) bc
T6	0.1 % $HgCl_2$ 5 min	0.0 (0.02) c
T7	0.1 % $HgCl_2$ 7 min	0.00 (0.02) bc
T8	1% NaOCl 10 min	3.33 (0.12) bc
T9	1% NaOCl 15 min	3.33 (0.12) bc
T10	1.5% NaOCl 10 min	1.00 (0.07) bc
T11	1.5% NaOCl 15 min	1.00 (0.07) bc
CD (*P*=0.05)		0.196

Table 2. Effect of auxin and cytokinin in shoot initiation in *E. grisebachii*

No	Treatment	Shoot initiation (%)	Days to shoot initiation	No of shoots	Shoot length (cm)	Days Leaf emergence	No of leaves
T_1	MS	10(0.32)^{i}	34.07^{b}	0.87^{u}	0.57^{uv}	34.07^{b}	1.70^{uv}
T_2	MS+(0.2 mg L^{-1}) BAP	100(1.52)^{a}	17.92^{kl}	8.17^{e}	8.07^{e}	17.92^{kl}	24.20^{e}
T_3	MS+(0.5 mg L^{-1}) BAP	100(1.52)^{a}	15.59^{m}	8.57^{d}	8.43^{d}	15.59^{m}	25.30^{d}
T_4	MS+(1 mg L^{-1}) BAP	100(1.52)^{a}	14.25^{n}	8.87^{c}	9.10^{c}	14.25^{n}	27.30^{c}
T_5	MS+(1.5 mg L^{-1}) BAP	100(1.52)^{a}	11.59^{o}	9.23^{b}	9.73^{b}	11.59^{o}	29.20^{b}
T_6	MS+(2.0 mg L^{-1}) BAP	100(1.52)^{a}	8.43^{p}	9.83^{a}	10.33^{a}	8.43^{p}	31.00^{a}
T_7	½ MS	10(0.32)^{i}	35.50^{a}	0.17^{v}	0.43^{v}	35.50^{a}	1.30^{v}
T_8	½ MS+(0.2 mg L^{-1}) BAP	100(1.52)^{a}	19.74^{k}	6.73^{i}	5.10^{i}	19.74^{k}	15.30^{i}
T_9	½ MS+(0.5 mg L^{-1}) BAP	100(1.52)^{a}	18.60^{k}	7.07^{h}	5.83^{h}	18.60^{k}	17.50^{h}
T_{10}	½ MS+(1.0 mg L^{-1}) BAP	100(1.52)^{a}	16.90^{l}	7.33^{g}	6.03^{h}	16.90^{l}	18.10^{h}
T_{11}	½ MS+(1.5 mg L^{-1}) BAP	90(1.25)^{b}	17.48^{l}	7.83^{f}	6.67^{g}	17.48^{l}	20.00^{g}
T_{12}	½ MS+(2.0 mg L^{-1}) BAP	93(1.34)^{b}	13.30^{n}	8.00^{ef}	7.63^{f}	13.30^{n}	22.90^{f}
T_{13}	MS+(0.2 mg L^{-1}) NAA	33(0.61)^{fg}	29.00^{d}	3.00^{k}	1.67^{r}	29.00^{d}	5.00^{r}
T_{14}	MS+(0.5 mg L^{-1}) NAA	33(0.61)^{fg}	27.83^{e}	3.45^{p}	2.03^{k}	27.83^{e}	6.10^{k}
T_{15}	MS+(1.0 mg L^{-1}) NAA	40(0.68)^{ef}	27.00^{ef}	3.63^{op}	2.43^{p}	27.00^{ef}	7.30^{p}
T_{16}	MS+(1.5 mg L^{-1}) NAA	50(0.79)^{dc}	26.43^{f}	3.80^{n}	2.83^{o}	26.43^{f}	8.50^{o}
T_{17}	½ MS+(0.2 mg L^{-1}) NAA	13.33(0.37)^{i}	33.67^{b}	1.67^{t}	0.77^{tu}	33.67^{b}	9.70^{n}
T_{18}	½ MS+(0.5 mg L^{-1}) NAA	16.66(0.42)^{h_i,j}	31.33^{c}	2.00^{s}	1.00st	31.33^{c}	11.00^{m}
T_{19}	½ MS+(1.0 mg L^{-1}) NAA	23.33(0.50)^{rgh}	27.87^{e}	2.17^{s}	1.13^{s}	27.87^{e}	12.00^{l}
T_{20}	½ MS+(1.5 mg L^{-1}) NAA	26.66 (0.54)^{gh}	27.67^{e}	2.67^{p}	1.50^{r}	27.67^{e}	13.60^{k}
T_{21}	MS + (1 mg L^{-1}) Kinetin	80.00 (1.11)^{c}	23.42^{h}	5.70^{k}	3.23^{n}	23.42^{h}	14.40^{zk}
T_{22}	MS + (3 mg L^{-1}) Kinetin	86.66 (1.25)^{b}	22.10^{i}	6.00^{j}	3.67^{m}	22.10^{i}	15.00^{ic}
T_{23}	MS + (5 mg L^{-1}) Kinetin	90.00 (1.29)^{b}	19.87^{t}	6.23^{j}	4.53^{k}	19.87^{t}	3.00st
T_{24}	½ MS + (1 mg L^{-1}) Kinetin	53.33 (0.82)^{d}	23.33^{h}	5.07^{l}	4.80^{zk}	23.33^{h}	3.40^{s}
T_{25}	½ MS + (3 mg L^{-1}) Kinetin	56.66 (0.85)^{d}	23.93^{gh}	4.73^{m}	5.00^{ic}	23.93^{gh}	4.50^{r}
T_{26}	½ MS + (5 mg L^{-1}) Kinetin	60 (0.89)^{d}	24.68^{f}	4.10^{n}	4.00^{l}	24.68^{f}	2.30^{tu}
	CD (*P*=0.05)	0.134	1.106	0.267	0.273	1.106	0.82

with the shortest time to root initiation (5.2 days). Following this, T_8 (½ MS + 5.0 mg L^{-1} IBA) also performed well, with 96.33% root initiation in 7 days. Among the treatments with IAA, T_9 (MS + 0.5 mg L^{-1} IAA) showed relatively high effectiveness, with an 85.17% root initiation rate within 11.83 days. This demonstrates that IBA at higher concentrations, particularly in a ½ MS medium, is optimal for promoting rapid and high-percentage of root initiation. Similar results were reported by Kumar and Rao (2007) in *Heliotropium indicum*.

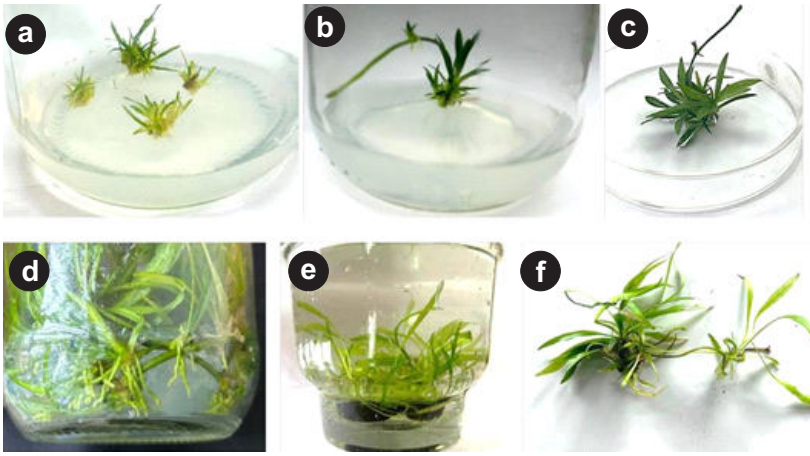

Fig. 1. *In vitro* propagation and hardening stages: (a. Shoot initiation; b. Shoot emergence after two weeks; c. Plant taken out for subculture; d. Root emergence; e. Hardening; f. Hardened plants after one month)

Table 3. Effects of IBA and IAA on root initiation in *E. grisebachii*

	Treatment	Days to root initiation	Root initiation (%)
T_1	MS	25.92 ^{a}	13.67 (0.38) ^{k}
T_2	MS+(1.0 mg L^{-1}) IBA	23.66 ^{b}	26.67 (0.54) ^{j}
T_3	MS+(3.0 mg L^{-1}) IBA	17.33 ^{d}	35.33 (0.64) ^{i}
T_4	MS+(5.0 mg L^{-1}) IBA	19.33 ^{c}	46.33 (0.75) ^{h}
T_5	½ MS	9.83 ^{g}	51.67 (0.80) ^{h}
T_6	½ MS+(1.0 mg L^{-1}) IBA	8.40 ^{h}	91.67 (1.28) ^{c}
T_7	½ MS+(3.0 mg L^{-1}) IBA	5.20 ^{j}	100.00 (1.52) ^{a}
T_8	½ MS+(5.0 mg L^{-1}) IBA	7.00 ^{i}	96.33 (1.41) ^{b}
T_9	MS+(0.5 mg L^{-1}) IAA	11.83 ^{f}	85.17 (1.18) ^{d}
T_{10}	MS+(1.0 mg L^{-1}) IAA	12.80 ^{f}	77.33 (1.07) ^{e}
T_{11}	MS+(1.5 mg L^{-1}) IAA	14.43 ^{e}	69.67 (0.99) ^{f}
T_{12}	½ MS+(0.5 mg L^{-1}) IAA	16.80 ^{d}	57.67 (0.86) ^{g}
T_{13}	½ MS+(1.0 mg L^{-1}) IAA	16.47 ^{d}	61.67 (0.90) ^{g}
T_{14}	½ MS+(1.5 mg L^{-1}) IAA	16.87 ^{d}	62.67 (0.91) ^{g}
	CD (*P*=0.05)	1.37	0.06

Effect of different hardening media on *E. grisebachii*: The data (Table 4) indicates that Aqua soil (T_2) was the most effective substrate, achieving a 100% survival rate, the highest number of shoots (17.46), the longest shoot length (20.43 cm), the greatest number of leaves (35.4), the highest root count (19.70), and the longest root length (14.86 cm). Coco Fibre (T_6) also performed well, with a survival rate of 93.4% and strong values for shoot and root growth, making it the next best substrate. In comparison, LECA (T_3) showed the lowest performance across all parameters, with a 67% survival rate and minimal shoot and root growth. This highlights aqua soil as the superior substrate for optimal plant survival and development.

This research developed an effective micropropagation protocol and suitable hardening medium for *E. grisebachii*. The best results were achieved with MS medium supplemented with 2.0 mg L^{-1} BAP for optimal establishment and shoot induction. For root induction, ½ MS medium with 3.0 mg L^{-1} IBA was the most effective. Aqua soil emerged as the superior hardening medium, providing the highest survival rate and promoting robust shoot and root development. This protocol offers a reliable method for the propagation and acclimatization of *E. grisebachii*.

Table 4. Effect of different hardening media on *E. grisebachii*

Treat-tment	Treatment	Percentage survival	No of shoots	Length of shoots (cm)	No of leaves	No of roots	Length of root (cm)
T_1	Soil	77.0 (1.07)^d	2.86^d	2.88^e	9.0^e	5.74^d	5.00^d
T_2	Aqua soil	100.0 (1.52)^a	17.46^a	20.43^a	35.4^a	19.70^a	14.86^a
T_3	LECA	67.0 (0.96)^e	1.00^e	0.90^f	7.2^f	3.12^f	3.00^e
T_4	Sand	85.2 (1.18)^c	5.18^c	7.26^c	23.0^c	11.76^c	6.86^c
T_5	Gravel	79.8 (1.11)^d	2.94^d	4.96^d	15.2^d	3.96^e	4.64^d
T_6	Coco fibre	93.4 (1.33)^b	5.98^b	14.16^b	29.6^b	14.36^b	8.38^b
CD (*P*=0.05)		0.071	0.380	0.392	1.685	0.534	0.634

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