RESEARCH ARTICLE

In-vitro Studies on Callus and Shoot Induction of *Eichhornia crassipes*

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Abstract

Eichhornia crassipes, a monocotyledonous plant species finds various uses including environmental bioremediation, biotechnological and medicinal applications. There are only very few *in-vitro* studies conducted on *Eichhornia* and no successful *in-vitro* callus and shoot regeneration protocols have been developed to date rendering *Eichhornia* as one of the recalcitrant species holding the development of any transgenics and tapping its various biotechnological applications. The present study is the first extensive effort made toward the *in-vitro* callus and shoot regeneration response of *Eichhornia*. We have optimized the sterilization protocol most suitable for the naturally grown *Eichhornia* explants collected from two wet and marshy *ex-situ* locations. Different types and different strengths of salts provided through Murashige and Skoog (MS) and Linsmaier and Skoog (LS media) combinations and different growth regulator combinations such as 2,4-dichlorophenoxyacetic acid (2,4-D) in the presence and absence of naphthalene acetic acid (NAA) (0.5 mg/L) and indole-3-acetic acid (IAA) (1-mg/L) were checked for callus induction from different explants of *Eichhornia*. Similarly, different types and different strengths of salts provided through MS and LS media combinations and growth regulator combinations such as 6-benzylaminopurine (BAP), thidiazuron (TDZ) and zeatin in the presence and absence of naphthalene acetic acid (NAA) were checked for shoot induction from different explants of *Eichhornia*. Alternate approaches such as culturing various explants of *Eichhornia* on two-layered media (lower solid media overlayed with upper liquid media) composed of different strengths (1X, 2X and 4X) of MS and LS combinations along with different combinations of suitable growth regulators were tested for callus and shoot induction responses. Despite trying various salt concentrations, media and different growth regulator combinations as solid or solid:liquid media, no successful callus and shoot regeneration could be achieved from different explants of *Eichhornia*. The present study provides a base to take up further extensive studies in the future to develop first callus and shoot regeneration protocols for *E. crassipes* so far, a recalcitrant monocotyledonous species that holds promise as one of the important biotechnologically and environmentally relevant potential plant species.

Highlights

- The sterilization protocol has been optimized for *Eichhornia* explants.
- Different strengths of MS and LS media combinations with different auxins (2,4-D, NAA and IAA) were checked for callus induction from different explants.
- Different strengths of MS and LS media combinations with different cytokinins (BAP, TDZ and Zeatin) were tested for shoot induction from different explants.
- Alternate approaches such as culturing on two-layered media made of MS and LS combinations with different growth regulators were tested for callus and shoot induction responses.
- Despite trying various salt and growth regulator concentrations, no callus and no shoot regeneration could be achieved from different explants of *Eichhornia*.
- There is a need to take up extensive future studies to generate successful regeneration protocols for *Eichhornia crassipes,* a recalcitrant monocotyledonous species.

Keywords: *Eichhornia crassipes*, callus induction, shoot regeneration, BAP, TDZ, 2,4-D.

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Introduction

Aquatic plants are mostly ornamental and planted in aquaria for beauty and to maintain the water quality. *Eichhornia* is one such plant that is an aquatic, free-floating, monocotyledonous, perennial species from the family Pontederiaceae. It is generally referred to as water hyacinth. *Eichhornia* is a natural plant of South America's Amazon region. In tropical and subtropical areas, *Eichhornia* is widely distributed. Water hyacinth entered the Amazon basin and spread to Africa, Asia, North America, and Australia thanks to human activity (Dagno *et al*., 2012). Water hyacinth was propagated from its origin around the world as an ornamental and botanical garden plant since the nineteenth century (CABI, 2013). Gradually the plant took its way to rivers, lakes, and dams and became an invasive weed (Villamagna & Murphy, 2010). Its widespread presence has been observed in Bangladesh's Sundarbans mangrove forest regions (Biswas *et al*., 2007). In the wetlands of the Kaziranga National Park in India, this has led to heavy siltation.

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Classification

Kingdon - Plantae
Division - Magnoliophyta
Class - Liliopsida
Order - Liliales
Family - Pontederiaceae
Genus - *Eichhornia*
Species - *crassipes* (Mart.) Solms

This aquatic plant comprises of glossy, smooth, circular-shaped lamina and spongy swollen petioles (Fig. 1). Because the petioles contain air, it affects the amount of air in the plant, causing it to float on the surface of the water. Stolons grow horizontally to form daughter plants out of terminal buds. They possess bisexual flowers which can be self-fertilized. Bluish purple-colored flowers are present with a yellow center. The flowers are borne serially on a single spike inflorescence of around 60 cm in length (Julien *et al*.,1999). Roots are often dark in color, fibrous, long and feather-like (Wright and Purcell, 1995).

Water hyacinth shows strong tolerance to nutrient variation, pH, and temperature conditions which is responsible for its rapid and extensive spread. It has an extremely rapid growth rate that causes social, economic, and environmental damage (Tran *et al*., 2022). It disrupts transportation and hampers fishing by forming thick mats exploiting most of the dissolved oxygen in water and covering the surface of water bodies which ultimately reduces the transmission of sunlight to underwater plants. A recent report published in the year 2017, suggested that the serious impacts of weeds include depletion in biodiversity, trouble in the hydropower sector, rise in crocodile and snake attacks, adverse effects on irrigation systems, tourism, and waterway traffic, rise in epidemics like flukes, cholera and malaria, effect on irrigation system (Gebregiorgis, 2017). The ability of plants to grow in a variety of warm and cool ecosystems such as streams, lakes, canals, ponds, and ditches is challenging the sustainability of plant species.

Despite having limitations, it also has certain social, economic, and environmental values. The plant has an extensive growth rate which produces a yield of 400 tons/ha (Suthar, 2022). This is sufficient to satisfy the demand for 501 biogas production units for cooking, mushroom cultivation, fuel, or silage for ruminant cattle. Water hyacinth juice is added to the pond in alkaline soil that helps raise its pH from 3.2 to 4.5, thereby, leaching more nutrients back into the water and fostering the growth of *Chlorella* algae (Jimenez, 2020). *Eichhornia* accelerates the extraction and absorption of Industrial and Agricultural Wastes and Toxic Metals, making it an effective and economically viable plant for bioremediation under moderate growth conditions. Water hyacinths can remove heavy metals and dissolved ions from wastewater through phytoremediation (Monroy-Licht *et al*., 2024; Placek *et al*., 2015, Odjegba & Fasidi, 2007). The roots, bulbs and leaves have hyperaccumulation ability for heavy metals (Thapa et. al., 2016). *Eichhornia* is very good raw material for vermicomposting. In Zimbabwe, cow dung and water hyacinth were effectively combined to create vermicompost (Deka *et al*., 2012) but yields better when it is used alone as a substrate for earthworms (Álvarez Bernal *et al*., 2016). The compost of *Eichhornia* alone is of better quality than the compost formed by the combination of *Eichhornia*, sawdust, and cow dung (Singh and Kalamdhad, 2015). The compost of water hyacinth prevents the secondary pollutant. Water hyacinth is also used as a raw material in the formulation of fish feed. This could lead to a higher level of crude protein in feed as well as improved digestion (Mohapatra, S.B, 2015). As an alternative feed source for aquaculture, the leaves of *Eichhornia* are used (Polprasert *et al*., 1994). The source of animal feed is a combination of water hyacinth and guinea grass (Mako *et al*., 2016).

The addition of *Eichhornia* in animal feeds increases the production of milk in cattle by up to 20% (Akankali *et al*., 2019). The enhanced bioethanol production was achieved by simultaneous saccharification and fermentation process employing pre-treated substrates sourced from *Eichhornia* (Das *et al*., 2016). *Eichhornia* is used in biogas production due to the presence of a high amount of hemicellulose (Nugraha et. al., 2018). *Eichhornia* has anti-inflammatory, antibacterial, antifungal, and anti-cancerous abilities (Aboul-Enein *et al*., 2014, Ben Bakrim *et al*., 2022), and is used to treat cholera, sore throat, and snake bites (Ayanda *et al*., 2020). Using DPPH radical scavenging assays and DNA inhibition, two dermal creams prepared with ethyl acetate extracts of *Eichhornia* have been tested for antiaging effects (Lalitha & Jayanthi, 2014).

The first *in-vitro* culture of water hyacinth was reported by Hussain *et al*., (2007), wherein different parts of *Eichhornia* i.e., anther, stolon, root, ovules, embryos, leaf were taken as explants and cultured on Murashige & Skoog (MS); Gamborg's B5 salt; and Basal Nutrient Media (BNM). These culture media were used alone as well as supplemented with plant growth regulators such as Kinetin, NAA, BAP, 2,4-D in different concentrations and varying combinations to check for rooting and shooting. No callus induction response was observed in any of the media and growth regulator combinations from any of the kinds of explants tested. However, only the preformed axillary buds

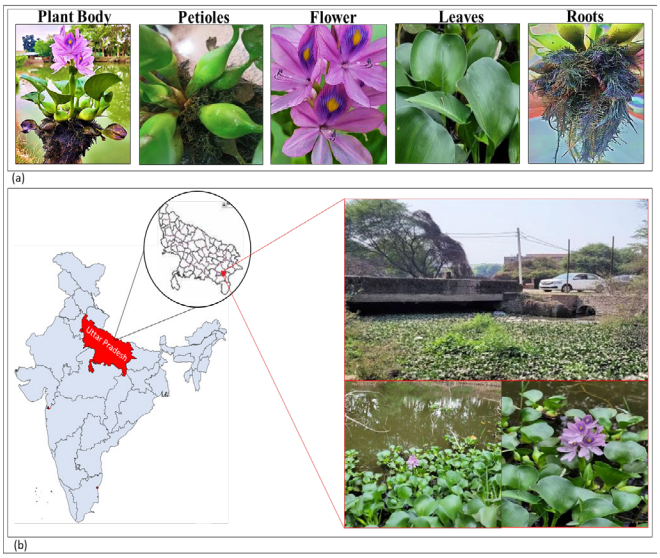

Fig. 1: Habit and site of collection of *Eichhornia crassipes*. (a) Plant body, roots, petioles, leaves, and flower (Upper panel). (b) Map of India showing the state of Uttar Pradesh (UP) highlighted as red area (Lower panel left side and in-set) and actual sites of collection (Lower panel right side)

showed further growth and better response in all culture media. They demonstrated the successful establishment of *Eichhornia* plants under sterile laboratory environments on liquid media or Hoagland's solution suitable for any further research use.

Suh *et al*., (2010) reported culturing of young preformed organs (shoots) of *Eichhornia* on LS media with BA to be more suitable in comparison to TDZ. Increased shoot proliferation was observed in LS media with BA (5 mg/L) supplemented with IAA (1-mg/L). Further, culturing the explants in the above liquid media instead of solid agar media resulted in increased fresh weight with a greater number of shoots.

Tran *et al*., (2022) demonstrated the use of a two-layered MS medium (the upper liquid medium and the lower solid agar medium) containing BA (0.5 mg/L) supporting further the growth of intact organs (buds) of *Eichhornia* and upon culturing on medium containing NAA (0.25 mg/L), root induction was observed.

As per our literature findings, there is only one single proper effort (Hussain *et al*., 2007) made for callus induction from various explants of *Eichhornia crassipes* which was not successful. There is not much *in-vitro* research carried out to understand the physiology and development of water hyacinth so that it can be manipulated to induce callus or shoots. *Eichhornia* appears as one of the recalcitrant monocot plant species for which it has been difficult to develop any successful *in-vitro* tissue culture regeneration protocols so far. Therefore, there is a need to take up research on *in-vitro* propagation of *Eichhornia* to resolve this issue.

In the present study, we intended to develop proper sterilization protocols for *Eichhornia* explants followed by developing *in-vitro* callus induction and shoot regeneration protocols for *E. crassipes*. Once regeneration protocols are established, callus can be used for cell suspension culture for extraction of various useful metabolites. The regenerated *in-vitro* grown plants can be exploited as food, biomass, decoration items, medicinal purposes, or environmental treatment purposes. Conversely, inhibition of growth of *Eichhornia* may be studied to slow its rapid development. In addition, it can be used as small beautiful ornamental plants.

Material and Methods

Plant Material

The naturally grown plants of *E. crassipes* were collected from two sites, one near Vishwakarma hostel, IIT-BHU campus and another one from near Prayagraj Road, Ashokapuram Colony, Dafi, Varanasi, Uttar Pradesh, 221011 (Fig. 1).

Media and Growth Regulators

MS media (Murashige & Skoog, 1962; Himedia-PT099) and LS media (Linsmaier and Skoog, Himedia-PT097) were purchased from HiMedia and the solid and liquid media were prepared as per the manufacturer's instructions. Growth regulators such as 2,4-D, IAA, NAA, BAP, Zeatin, and TDZ were used as per the requirements mentioned in the text. MS and LS media were prepared as per the manufacturer's instructions. Solid and liquid media were prepared by adding and omitting agar from the media respectively. The growth regulators were added as per experimental requirements and mentioned in the respective sections. The pH of the media was set within (5.6-5.8) with the help of 0.1 N HCL and 0.1 N NaOH and the volume was made up with water followed by autoclaving. All the experiments were replicated in triplicates. To avoid any bacterial contamination and to smoothly finish the experimental aims in time, we supplemented media with Augmentin (Glaxo SmithKline Beecham, 75 µg/ml) due to the unavoidable prevailing conditions in and around the Central Plant Tissue Culture room of the School of Biotechnology, BHU.

Culture Conditions

Lighting for 12 hours/day, temperature of 25°C ± 2°C at the Central Plant Culture Room of Banaras Hindu University.

Sterilization Procedure

The explants were rinsed in running tap water for 5 minutes. Explants were washed in a mild detergent such as Labolene (Qualigenes-Q42218) before treatment with any of the two sterilization agents i.e., sodium hypochlorite (HiMedia-AS102, 1% NaOCl, v/v) and mercuric chloride (SRL-25699, 0.1% $HgCl_2$, w/v). Plant materials were washed with running tap water to remove any traces of detergents. The sterilization procedure was enhanced by placing the explants in 70% ethanol for 30 seconds and then washing them again with distilled water to remove traces of ethanol. Later the explants were submerged and agitated in sufficient amount of either NaOCl (1% v/v) or mercuric chloride (0.1% $HgCl_2$, w/v) for various durations such as 5, 10, and 15 minutes; followed by washing with distilled water thrice for 5 minutes each to remove any traces of sterilizing agent used.

Results and Discussion

Standardization of Sterilization Protocol of *Eichhornia* Explants

As the *Eichhornia* plants naturally grow in very contaminated environments, we had to use a combination of multiple sterilizing agents being used serially, like soap solution, 70% ethanol, mercuric chloride (0.1%, w/v) or sodium hypochlorite (1%, v/v) followed by callus culturing on MS medium with 2,4-D (5 and 15 mg/L) to optimize the protocol required for effective sterilization of explants. In our observation, effective sterilization was achieved in all the treatment durations (5, 10 and 15 minutes) with mercuric chloride (Table 1). However, a 15-minute treatment duration with mercuric chloride led to no contamination of explants but explants turned bleached after 1 week of culturing (Fig. 2: (a, b, d, f).

In the case of treatment with sodium hypochlorite, some explants showed contamination within a 5-minute duration of treatment. No contamination was observed in the 10 and 15-minute duration of treatment but some explants turned bleached within a 10 minutes duration and all explants got bleached upon the 15-minute duration of treatment with sodium hypochlorite observed after one week of culturing (Table 2) (Fig. 2: (c, e, g, -h)). Therefore, a 5-minute treatment of mercuric chloride was chosen as the best and most effective dose of sterilization for *Eichhornia* explants.

Table 1: Optimization of a duration of treatment of mercuric chloride (0.1% w/v) to achieve effective sterilization on *Eichhornia* explants

Time duration	*No. of explants used for sterilization*	*No. of explants showing contamination*	*No of explants without any contamination*
5 minutes	16	-	16
10 minutes	16	-	16
15 minutes	12	-	12

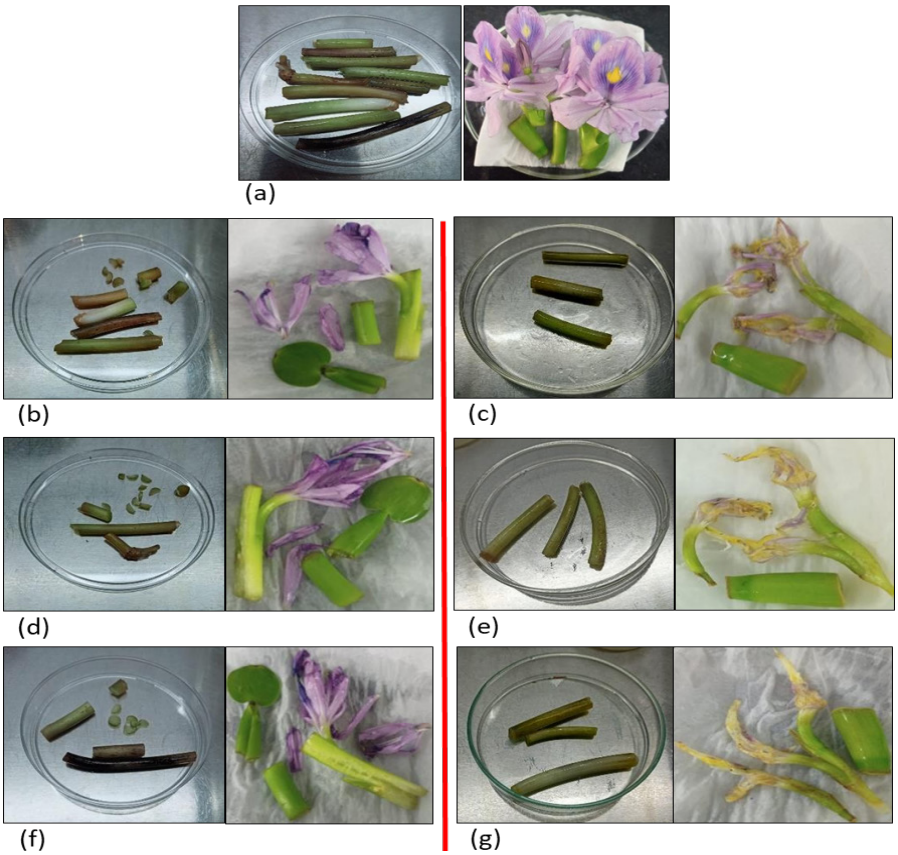

Fig. 2: Before and after status of explants treated with different durations of treatment with two sterilizing agents. (a) Explants before sterilization. (b), (d), (f) After sterilization with 0.1% mercuric chloride ($HgCl_2$) for 5,10 and 15 Minutes respectively. (c), (e), (g) (h) After sterilization with 1% sodium hypochlorite (NaOCl) for 5, 10, and 15 Minutes respectively

Callus induction studies from various explants of *E. crassipes*

Investigation of the effect of various 2, 4-D concentrations with different culture media in the presence or absence of NAA for callus induction in *E. crassipes***.**

To achieve callus induction, we tried different concentrations of salts for callus culture provided through full-strength Murashige & Skoog (MS) and Linsmaier and Skoog (LS) media supplemented with increasing concentrations of 2,4-D in the presence and absence of NAA (Table 3). However, no callus induction response was observed irrespective of the concentration of growth regulator and media combinations used even after a culture period of 4 weeks (Fig. 3). Both low as well as increased levels of 2,4-D failed to induce any callus induction response when used alone or in combination with NAA from the leaf, petiole, stolon, flower, and root explants of *Eichhornia*.

Investigation of the effect of IAA growth regulator on callus induction of *Eichhornia* explants cultured on MS media containing 2,4-D and NAA

Upon culturing on MS medium supplemented with 2,4-D (20 mg/L) and NAA (0.5 mg/L) in the presence of IAA (0.5 mg/L), the explants survived but no callus induction response was observed from various explants of *Eichhornia* (Table 4, Fig. 4).

Investigation of the effect of 2-layered media (Solid bottom with Liquid top) on the callus induction response from various explants in *E. crassipes*

In line with recent reports (Tran *et al*., 2022), we used 2-layered media for culturing explants on the surface of solid media overlayed with another liquid medium. Here we used different strengths (1X, 2X, and 4X) and different combinations of MS and LS medium supplied as solid:liquid medium supplemented with high conc. of 2,4-D (40 mg/L) with or without NAA (0.5 mg/L)

Table 2: Optimization of a duration of treatment of sodium hypochlorite (1% v/v) to achieve effective sterilization on *Eichhornia* explants

Time duration	*No. of explants used for sterilization*	*No. of explants showing contamination*	*No of explants without any contamination*
5 minutes	16	5	11
10 minutes	16	-	16
15 minutes	12	-	12

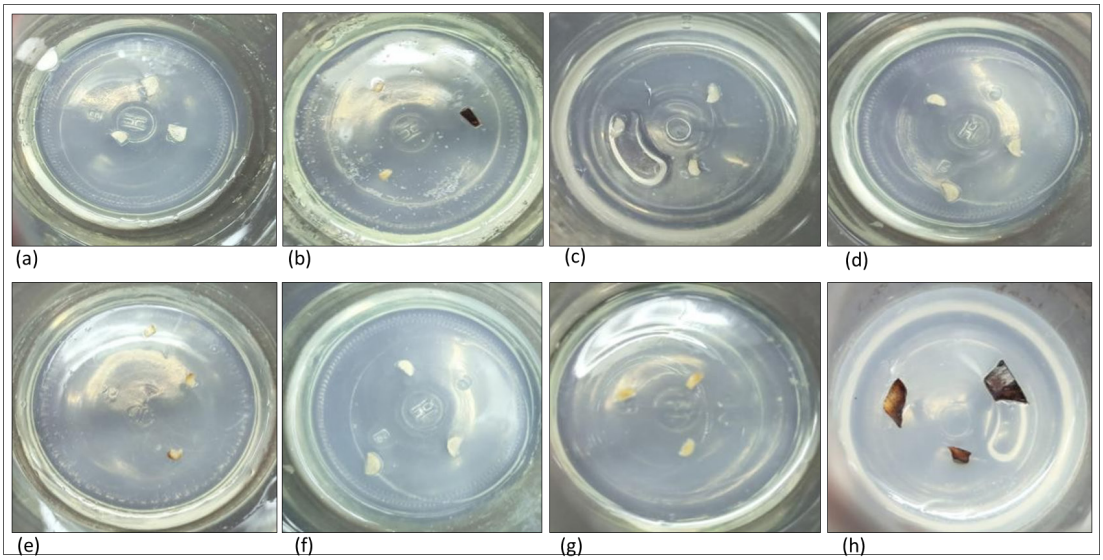

Fig. 3: *Eichhornia* explants cultured on two different media (MS and LS) with increasing levels of 2,4-D. Explants after three weeks of culturing with 2,4-D levels of 20, 30, 40, and 50 mg/L respectively on MS medium (a, b, c, d) and with same levels on LS media respectively (e, f, g, h)

Table 3: Investigating the effect of increasing levels of 2,4-D with two different media (MS and LS media) in the presence and absence of NAA on callus induction response in *Eichhornia*

Media	*Growth regulator concentration*	*No. of explants*	*Results*
MS media	2,4-D (5 mg/L)	50	Not responded
	2,4-D (15 mg/L)	50	Not responded
	2,4-D (20 mg/L)	50	Not responded
	2,4-D (20 mg/L) +NAA (0.5 mg/L)	50	Not responded
	2,4-D (30 mg/L)	50	Not responded
	2,4-D (30 mg/L) +NAA (0.5 mg/L)	50	Not responded
	2,4-D (40 mg/L)	50	Not responded
	2,4-D (40 mg/L) +NAA (0.5 mg/L)	50	Not responded
	2,4-D (50 mg/L)	50	Not responded
	2,4-D (50 mg/L) +NAA (0.5 mg/L)	50	Not responded
LS media	2,4-D (20 mg/L)	50	Not responded
	2,4-D (20 mg/L) +NAA (0.5 mg/L)	50	Not responded
	2,4-D (30 mg/L)	50	Not responded
	2,4-D (30 mg/L) +NAA (0.5 mg/L)	50	Not responded
	2,4-D (40 mg/L)	50	Not responded
	2,4-D (40 mg/L) +NAA (0.5 mg/L)	50	Not responded
	2,4-D (50 mg/L)	50	Not responded
	2,4-D (50 mg/L) +NAA (0.5 mg/L)	50	Not responded

Table 4: Checking the effect of IAA in the presence of 2,4-D and NAA on callus induction from various explants of *Eichhornia*

Media	*Growth regulator concentration*	*No. of explants used*	*Results*
MS media	2,4-D (20 mg/L) + NAA (0.5 mg/L) + IAA (0.5 mg/L)	25	No response
MS media	2,4-D (30 mg/L) + NAA (0.5 mg/L) + IAA (0.5 mg/L)	25	No response

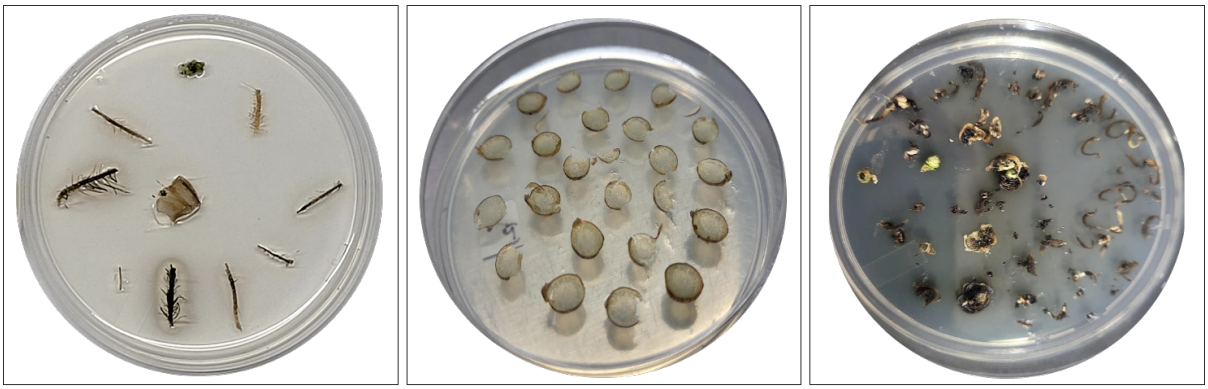

Fig. 4: No callus induction response observed after culturing different explants of *Eichhornia* on MS medium supplemented with 2,4-D levels (5, 15 and 20 mg/L) in presence of NAA (0.5 mg/L) and IAA (1 mg/L)

(Table 5). Further, we also checked the effect of increased salt concentrations on callus induction efficiency from various explants. After 2 weeks of culture, we could not observe any positive callus induction from any of the combinations tested. However, we observed that the explants survived but did not respond for a prolonged period when cultured on low salt concentrations such as 1X and 2X media whereas the explants cultured on high salt concentrations (4X media) failed to survive with no callus response (Fig. 5).

Shoot regeneration studies from various explants of *E. crassipes*

Investigating the effect of different concentrations of cytokinins (BAP and TDZ) and different media (MS and LS) on shoot regeneration response of *Eichhornia* explants

No response could be obtained from culturing different explants (stolons and petioles) on MS medium containing BAP alone in increasing concentrations of 10, 20, and 30 mg,

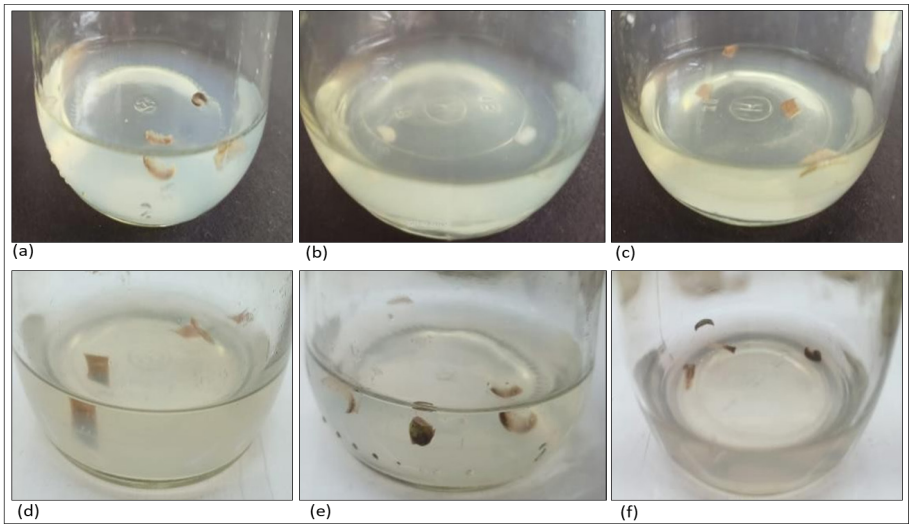

Fig. 5: No callus induction response obtained post culturing on 2-layered media (Lower Solid-Upper liquid) with combinations of increasing MS and LS salts. Explants after 3 weeks of culturing: a) represents stolon cultured in MS solid (1x) and MS liquid (1x), b) represents petiole cultured in LS solid (1x) and MS liquid (1x), c) represents leaf cultured in LS solid (4x) and MS liquid (4x), d) represents leaf cultured in LS solid (2x) and MS liquid (2x), e) represents petiole cultured in LS solid (4x) and MS liquid (4x), and f) represents petiole cultured in MS solid (4x) and MS liquid (4x) respectively

Table 5: Investigating the effects of 2-layered media combinations (Lower solid-upper liquid media) with increasing salt concentrations of MS and LS salts on callus induction from *Eichhornia* explants

Salt strength	*Type of media/ consistency*	*Growth regulators*	*No. of explants used*	*Results*
1X	MS Solid LS Liquid	2,4-D (40 mg/L) 2,4-D (40 mg/L) +NAA (0.5 mg/L)	40	Explants survived but not responded
1X	LS Solid MS Liquid	2,4-D (40 mg/L) 2,4-D (40 mg/L) +NAA (0.5 mg/L)	40	Explants survived but not responded
1X	MS Solid MS Liquid	2,4-D (40 mg/L) 2,4-D (40 mg/L) +NAA (0.5 mg/L)	40	Explants survived but not responded
1X	LS Solid LS Liquid	2,4-D (40 mg/L) 2,4-D (40 mg/L) +NAA (0.5 mg/L)	40	Explants survived but not responded
2X	MS Solid LS Liquid	2,4-D (40 mg/L) 2,4-D (40 mg/L) +NAA (0.5 mg/L)	40	Explants survived but not responded
2X	LS Solid MS Liquid	2,4-D (40 mg/L) 2,4-D (40 mg/L) +NAA (0.5 mg/L)	40	Explants survived but not responded
2X	MS Solid MS Liquid	2,4-D (40 mg/L) 2,4-D (40 mg/L) +NAA (0.5 mg/L)	40	Explants survived but not responded
2X	LS Solid LS Liquid	2,4-D (40 mg/L) 2,4-D (40 mg/L) +NAA (0.5 mg/L)	40	Explants survived but not responded
4X	MS Solid LS Liquid	2,4-D (40 mg/L) 2,4-D (40 mg/L) +NAA (0.5 mg/L)	40	Explants not survived
4X	LS Solid MS Liquid	2,4-D (40 mg/L) 2,4-D (40 mg/L) +NAA (0.5 mg/L)	40	Explants not survived
4X	MS Solid MS Liquid	2,4-D (40 mg/L) 2,4-D (40 mg/L) +NAA (0.5 mg/L)	40	Explants not survived
4X	LS Solid LS Liquid	2,4-D (40 mg/L) 2,4-D (40 mg/L) +NAA (0.5 mg/L)	40	Explants not survived

Table 6: Effect of different cytokinins (BAP and TDZ) and media (MS and LS) on shoot regeneration response from *Eichhornia* explants

Media	*Cytokinins*		*NAA (mg/L)*	*No. of explants used*	*No. of explants responded*
	BAP (mg/L)	*TDZ (mg/L)*			
MS	10	-	-	30	No response
	20	-	-	27	No response
	30	-	-	28	No response
	10	10	-	30	No response
	20	20	-	30	No response
	30	30	-	30	No response
LS	10	-	0.5	30	No response
	20	-	0.5	30	No response
	30	-	0.5	30	No response
	10	10	0.5	30	No response
	20	20	0.5	30	No response
	30	30	0.5	30	No response

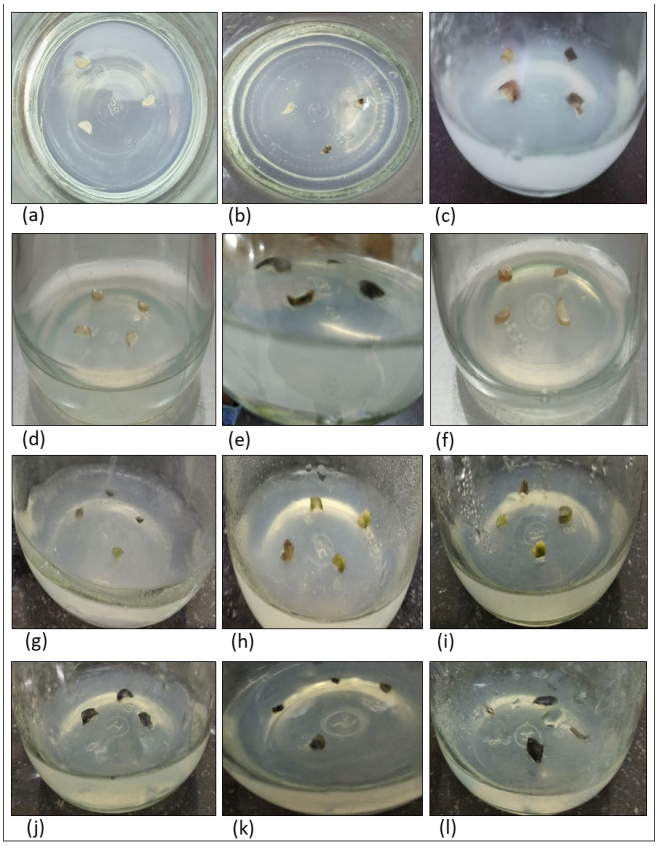

Fig. 6: Explants upon culturing on two different salt concentrations present in MS and LS shoot induction mediums containing different cytokinin levels (BAP and TDZ)

respectively. After 30 days of culturing, no callusing or shoot bud induction was observed, however, the explants remained viable and thereafter, they turned black. Further, we checked the response of *Eichhornia* explants on MS medium containing a combination of two different cytokinins i.e., BAP (10, 20, 30 mg/L) with TDZ (10, 20, 30 mg/L) (Table 6). However, no shoot regeneration response was observed even after culturing with high and low doses of two different cytokinins (BAP and TDZ) (Fig. 6). We also tested the combinatorial effect of supplementation of Zeatin (1-mg/L) along with different increasing concentrations of BAP in presence and absence of NAA (1-mg/L) in full-strength MS media (Data not shown). However, there were no regeneration response was observed after 1.5 months of culturing.

Since no shoot regeneration response was obtained in previous media combinations, LS media with different cytokinin concentration combinations was checked in combination with NAA as suggested by previous literature. Similar to the previous reports (Hussain *et al*., 2007, Suh *et al*., 2010), our explants survived for 20 to 30 days on medium, after which they turned brown and died (Fig. 6). The leaf explants could not survive long and turned black within a week.

Investigation of shoot regeneration response of 2-layered (solid bottom with liquid top) media consisting of LS and MS salts in increasing concentrations with BAP (10 mg/L) & NAA (0.5 mg/L)

As per the latest literature reports (Tran *et al*., 2022), a 2-layer media (solid bottom and liquid top) was found to support the growth of preformed intact organs of *Eichhornia*. We cultured different explants of *Eichhornia* by using increasing concentrations (1X, 2X and 4X) of MS and LS salt combinations as solid media at the bottom overlayed with liquid media at

Table 7: Shoot regeneration response on 2-layered media (as solid bottom with liquid top layer) combinations with increasing MS and LS salts with BAP (10 mg/L) & NAA (0.5 mg/L)

Salt Strength	*Media*		*BAP*	*NAA*	*No. of explants*	*Response observed*
	LS solid	LS liquid			25	No response
1X	MS solid	MS liquid			25	No response
	MS solid	LS liquid			25	No response
	LS solid	MS liquid			25	No response
	LS solid	LS liquid	10 mg/L	0.5 mg/L	25	No response
2X	MS solid	MS liquid			25	No response
	MS solid	LS liquid			25	No response
	LS solid	MS liquid			25	No response
	LS solid	LS liquid			25	No response
4X	MS solid	MS liquid			25	No response
	MS solid	LS liquid			25	No response
	LS solid	MS liquid			25	No response

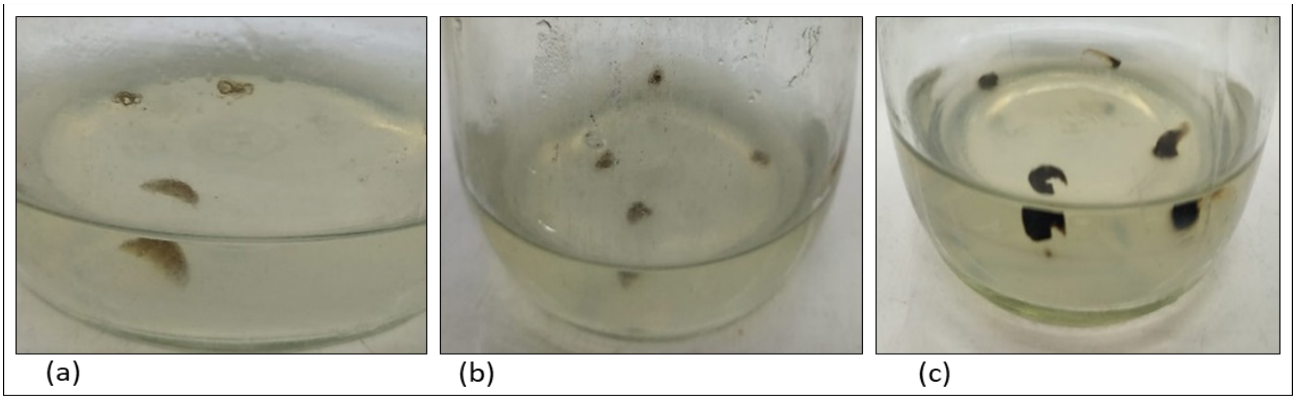

Fig. 7: Explants showing no shooting response upon culturing on 2-layered media (Lower Solid-Upper liquid) using a combination of increasing concentration of MS and LS salts with BAP (10 mg/L) and NAA (0.5 mg/L). a) MS and LS solid-liquid media with 1X concentration, b) MS and LS solid-liquid media with 2X concentration, c) MS and LS solid-liquid media with 4X concentration

the top. Both the media were enriched with BAP (10 mg/L) and NAA (0.5 mg/L) as shown in (Table 7). However, in our studies, no shoot regeneration response was observed from different explants of *Eichhornia* in any of the media and growth regulator combinations (Fig. 7).

Conclusion

The present study investigated *in-vitro* development of callus and shoot induction for water hyacinth using somatic tissues such as stolon, petiole, root and leaf as explants and an improved sterilization protocol was standardized with $HgCl_2$ (0.1%). Explants were cultured on MS and LS media supplemented with auxins like 2,4-D and NAA to induce callus while BAP, TDZ and Zeatin were used to induce shoots. Further, two-layered media techniques with different growth regulator combinations were also tested for regeneration. However, despite trying various media and growth regulator combinations, no callus or shoot induction response was observed.

The present *in-vitro* study provides an improved protocol for the sterilization of explants and a future direction for *in-vitro* studies in *Eichhornia*. A further detailed study using various supplements like coconut milk, malt extract, potato extract, *etc*. along with different media is required to be undertaken for successful callus induction and shoot regeneration of *E. crassipes*.

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Author Contributions

Both the authors: VS and AB contributed equally to this article. DD conceived the research work. DD and KL designed the experiments. VS, AB and AO performed the experiments. DD acquired the funding. All the authors contributed in writing and approving the paper draft for publication.

Conflict of Interest

The authors declare that they have no conflicts of interest.

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