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Callus induction, frond regeneration, and genetic transformation of the aquatic plant *Lemna minor* using *Agrobacterium tumefaciens*

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ABSTRACT INFO

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ABSTRACT

Molecular farming involves the large-scale cultivation of plants to produce recombinant proteins for diverse biotechnological applications, including biopharmaceuticals, veterinary products, research tools, and dietary supplements. Members of the Lemnaceae family, small aquatic plants typically found in swamps and lakes, have garnered attention for molecular farming due to their rapid growth, vegetative propagation, and high protein content. In this study, the Iranian ecotype of *Lemna minor* plants was collected from Guilan Province in northern Iran. To induce callus formation, five different media—Murashige and Skoog (MS), 1/2MS, B5, Schenk and Hildebrandt (SH), and Hoagland —were evaluated with various combinations of hormones and sucrose concentrations. The impact of acetosyringone in the co-cultivation medium on transformation efficiency was also examined. The findings revealed that Hoagland and standard MS basal media, combined with 12 mg/L 2,4-Dichlorophenoxyacetic acid (2,4-D), 4.8 mg/L 6-Benzylaminopurine (BAP), and 2% sucrose, significantly enhanced callus induction. For frond regeneration, Hoagland medium supplemented with 2% sucrose, 1.1 mg/L kinetin, 4.5 mg/L indole-3-acetic acid (IAA), and 1 mg/L thidiazuron (TDZ), 4 mg/L IAA showed the highest performance. Additionally, the optimal transformation efficiency was achieved using 200 µM acetosyringone. This study provides an efficient and reproducible protocol for callus induction and frond regeneration in *L. minor*, offering valuable insights for molecular farming and recombinant protein production.

***Key words*:** Callus induction, Frond regeneration, *Lemna minor*, Recombinant proteins, Molecular farming.

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INTRODUCTION

Duckweeds, belonging to the family Lemnaceae, are small, fast-growing aquatic monocotyledons commonly found in stagnant or slow-moving freshwater environments such as ponds and lakes (Li *et al*., 2004). This family consists of 38 species across five genera: *Spirodela, Lemna, Landoltia, Wolffia,* and *Wolfiella* (Wang, 2016). These plants predominantly propagate through vegetative budding from meristematic tissues, resulting in rapid clonal expansion (Chhabra *et al*., 2011). Under axenic culture conditions, Lemnaceae members exhibit remarkably high biomass productivity, often doubling in biomass within 48 to 72 hours (Wang, 2016). Their rapid growth, ease of cultivation, and simple structural characteristics have made duckweeds increasingly significant as sustainable resources for applications in animal feed, biofertilizers, and biofuels. Recently, they have also emerged as promising eukaryotic platforms for producing recombinant bioactive proteins (Yang *et al*., 2021; Diwan, 2023).

The protein content of duckweed varies from 20% to 30%, depending on the species and environmental conditions, surpassing that of cereals (de Beukelaar *et al*., 2019). Because of its high protein content, duckweed serves as an excellent feed option for domestic animals (Putra and Ritonga, 2018; Ujong *et al*., 2025), poultry (Islam, 2002; Paguia *et al*., 2023; Stadtlander *et al*., 2023), and pond fish (Naseem *et al*., 2021; Minich and Michael, 2024; Sosa *et al*., 2024). Furthermore, duckweed shows promise as a sustainable, plant-based protein source for future human food products, offering a healthier and more cost-effective alternative to animal-derived meat (Appenroth *et al*., 2018; Xu *et al*., 2022; Sulaiman *et al*., 2024). Reports indicate that duckweed can achieve a starch content of up to 75% on a dry weight basis (Reid and Bieleski, 1970; Landolt and Kandeler, 1987). This remarkable starch concentration makes duckweed a valuable resource for biofuel production (Guo *et al*., 2023).

The Lemnaceae family possesses several biological traits that establish it as a highly promising platform for the production of foreign proteins, including pharmaceuticals and diagnostic reagents. These traits include rapid growth, a short lifecycle, vegetative propagation, the ability to be cultivated in liquid media or fully controlled bioreactors, and a protein profile with an amino acid composition similar to that of animal proteins (Khvatkov *et al*., 2019). Among its members, *Lemna minor* stands out as the most widely used species for recombinant protein production in both pharmaceutical and veterinary applications (Yang *et al*., 2021). This species has been used to produce a wide range of target proteins, such as antigens of porcine epidemic diarrhea virus (PEDV) (Ko *et al*., 2011), the *M2e* gene from avian influenza virus A/chicken/Kurgan/5/2005 (H5N1), hemagglutinin (HA) (Bertran *et al*., 2015), human monoclonal antibodies (Yamamoto *et al*., 2001; Cox *et al*., 2006), endonuclease E1 (Hobom, 1980), β-glucuronidase and hirudin (Kozlov *et al*., 2019), and human growth hormone (Liu *et al*., 2021).

Duckweed presents challenges for direct genetic modification. Since the mid-1970s, researchers have sought to optimize tissue culture techniques such as callus induction and plant regeneration to improve their viability for genetic engineering (Stomp, 2005). Studies have investigated the impact of different plant hormones, such as auxin and cytokinin, treatments in different culture media (Yang *et al*., 2021). Importantly, optimal tissue culture conditions, including hormone concentrations and compositions, vary significantly among different duckweed species, influencing callus induction efficiency and regeneration rates. For example, Chhabra *et al*. (2011) reported successful callus induction in *L. minor* using whole fronds cultured on B5 medium supplemented with 5 µM 2,4-dichlorophenoxyacetic acid (2,4-D) and 50 µM N6-(2-isopentenyl) adenine (2-IP) (or 5 µM TDZ) (Chhabra *et al*., 2011). Similarly, Chang and Chiu (1978) demonstrated that a combination of 10 mg/L 2,4-D and 1 mg/L 2-IP effectively promoted callus formation in *L. gibba*, while regeneration was achieved using 4 mg/L indole-3-acetic acid (IAA) and 1 mg/L kinetin (Chang and Chiu, 1978). Despite these advancements, protocols for tissue culture optimization in native *L. minor* ecotypes, particularly those from regions like Iran, remain limited. Therefore, the present study focuses on establishing efficient protocols for callus induction and frond regeneration in an Iranian-native strain of *L. minor*, which may exhibit distinct hormonal responses compared to previously studied genotypes.

MATERIALS AND METHODS

Collection and sterilization of *L. minor*

Duckweed plants were collected from the Amirkalayi wetland in Langaroud, Guilan province, Iran (37°20’35”N, 50°11’27”E). The whole plants of *L. minor* were used as explants. For surface sterilization, *L. minor* samples were immersed in a 10% w/v commercial bleaching solution under sterile conditions for 5 min with slow agitation, followed by two rinses

with sterile distilled water. The sterile duckweed was then transferred to liquid Hoagland medium (Hoagland and Arnon, 1950) supplemented with 2% sucrose.

Callus induction and regeneration

Callus formation was induced using five different media (MS, 1/2MS, B5, SH, and Hoagland) adjusted to pH 6. Media were solidified with 0.7% w/v agar and supplemented with sucrose as a carbon source at concentrations of 1%, 2%, and 3%. Various combinations of plant growth regulators were applied, including 6-benzyl aminopurine (BAP, 4.8 mg/mL), thidiazuron (TDZ, 0.4 mg/mL), and 2,4-dichlorophenoxyacetic acid (2,4-D, 4 and 12 mg/mL). Ten parental fronds (approximately 2-3 weeks old) were scratched on the back of the leaf and in the meristematic region. These scratched areas were placed in contact with culture media and incubated in darkness at 25±2 °C. Green callus formations were observed at the wounded sites after 2-3 weeks. The efficiency of callus induction was assessed after two months by evaluating the percentage of callus formation and measuring the fresh weight. Calli were subcultured onto fresh medium at three-week intervals. All experiments were conducted in triplicate.

After nine weeks, calli were transferred to regeneration media containing the same basal composition as the callus induction medium, but with varied concentrations of indole-3-acetic acid (IAA, 4.5 and 4 mg/mL) (de Beukelaar *et al.*, 2019), combined with kinetin (KT, 1 mg/mL) and TDZ (1 mg/mL). Calli weighing between 0.4 to 0.6 g were incubated under greenhouse conditions at 25±2 °C with a 16/8 hour light/ dark photoperiod. This experiment was performed in triplicate, and regenerated fronds were counted after two months.

***Agrobacterium* strain and vector**

In this study, to establish an efficient transformation protocol for *L. minor*, the plasmid pBI121 carrying the *GUS* gene was introduced into *Agrobacterium tumefaciens* strain EHA105 for transformation experiments. *Agrobacterium* cultures harboring the plasmids were grown overnight in LB medium at 28 °C with constant shaking at 180 rpm. The overnight culture was centrifuged (3000 rpm, 10 min, 4 °C), and the pellet was resuspended in sterile inoculation medium. The suspension was incubated at 28 °C with shaking at 180 rpm until an OD600 of 0.6 was attained. Sterile calli were aseptically transferred to test tubes with perforated caps containing 5 ml of liquid inoculation medium supplemented with *Agrobacterium*, and subjected to vacuum treatment at 8 kPa for 30 min in a desiccator. Samples were then kept in darkness for 72 hours on solid co-culture medium (MS basic medium, pH 5.8, 3% sucrose, 12 mg/L 2,4-D, and 4.8 mg/L BAP, 0.8% agar) supplemented with acetosyringone at different concentrations (50, 100, and 200 µM). Experiments were conducted in triplicate, with 20 calli placed in a 9 cm Petri dish.

Post co-cultivation, calli were washed three times with distilled water containing 400 µg/mL cefotaxime and transferred to resting medium (Hoagland medium, pH 5.8, 3% sucrose, 0.8% agar, 12 mg/L 2,4-D, 4.8 mg/L BAP) supplemented with 400 µg/mL cefotaxime. The calli were maintained in darkness for one week before being transferred to selective medium (Hoagland medium containing 3% sucrose, 0.8% agar, pH 5.8, 4.5 mg/L IAA, 1 mg/L KT, 400 µg/mL cefotaxime, and 100 µg/mL kanamycin).

The surviving calli were subcultured weekly onto fresh medium and maintained at a constant temperature of 25 °C until regeneration was fully achieved. After one month of incubation on the selective medium, the calli exhibiting signs of regeneration were monitored closely for one additional week. Upon completing regeneration, the fronds were carefully excised and individually transferred to liquid Hoagland medium (containing 1% sucrose, pH 5.8, 400 µg/mL cefotaxime, and 100 µg/mL kanamycin) to facilitate the establishment of independent transformant lines.

PCR analysis

Genomic DNA of *L. minor* was extracted using the modified cetyl trimethyl ammonium bromide (CTAB) method (Rogers and Bendich, 1989). Non-transformed samples served as negative controls, while putatively transformed samples were analyzed. Polymerase chain reaction (PCR) was performed in a 20 µL reaction volume using Taq DNA polymerase (Sigma, USA). The primers used for amplifying the *GUS* fragment included: *GUS* F: 5'-ATGTTACGTCCTGTAGAAACCCCA-3' and *GUS* R: 5'-TCGTCTGATAGTTGTTGATCCCGC-3'. Each reaction contained approximately 50 ng of genomic DNA, 1 µM of each primer, 50 µM dNTPs, 2X PCR buffer with 1.5 mM $MgCl_2$, and 1 U of Taq DNA polymerase. Amplification was carried out using a Bio-Rad thermal cycler (USA) with the following program: initial denaturation at 94 °C for 10 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 50 s, and extension at 72 °C for 50 s, concluding with a final extension step at 72 °C for 5 min. The amplified DNA fragments, approximately 1600 bp in size, were analyzed on a 1% agarose gel and detected under UV light at 302 nm.

GUS assay

Histochemical *GUS* staining was performed on fronds derived from calli co-cultivated with *Agrobacterium*, following the protocol outlined by Jefferson *et al*. (Jefferson *et al*., 1987). *GUS* activity was determined by incubating the fronds overnight in staining buffer (composed of 100 mM KPO4 buffer at pH 7.0; 5 mM Ethylene Diamine Tetraacetic Acid (EDTA); 0.5 mM K3[Fe(CN)6]; 0.5 mM K4[Fe(CN)6]; and 0.96 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid sodium salt (X-Gluc)), at 37 °C in the dark. The following day, the stained fronds were washed with distilled water and 70% ethanol, then observed under a stereo-microscope.

Statistical analyses

All experimental procedures were performed in a completely randomized design with a minimum of three replicates. Statistical analysis was conducted using one-way analysis of variance (ANOVA) with XLSTAT (Premium v2016.02.28451) and GraphPad Prism 9 software. Normality of percentage data, including callus induction, regeneration, and transformation rates, was assessed using the Shapiro–Wilk test. For data with deviations from normality, the arcsine square root transformation was applied to stabilize variance and meet ANOVA assumptions. Post-hoc mean comparisons were performed with Tukey's multiple comparisons test to identify significant differences among treatment groups.

RESULTS

Callus induction

Statistical analysis revealed significant differences ($P<0.01$) in callus induction percentage and fresh weight among the five tested media (Table 1). The highest rate of callus formation was observed in Hoagland (62.22%) and MS (49.44%) media, while the lowest rates were recorded in B5 (18.88%) and 1/2MS (11.66%) media. Sucrose concentration had a significant effect on callus formation, with 2% sucrose yielding the best results (Figure 1A). The average fresh weight of callus was highest in Hoagland and MS media (0.617 g and 0.68 g, respectively), whereas the lowest fresh weight was observed in 1/2MS medium (0.16 g) (Figure 1B). Callus induction significantly increased in MS and SH media when 2% sucrose was used, with an average of 56.66% and 48.33%, respectively. The best results were achieved using Hoagland (83%) and MS (80%) media combined with 2,4-D (12 mg/mL), BAP (4.8 mg/mL), and 2% of sucrose (Figure 1A and B). Conversely, the combination of sucrose and phytohormones (2, 4-D and BAP) did not show a significant effect on callus induction in B5 and 1/2MS media. However, in SH medium, the same concentration of hormones and carbon source resulted in an average callus induction of 53%. Analysis of callus fresh weight in Hoagland and MS media showed that the increase in sucrose concentration from 2 to 3% could lead to a notable improvement in callus fresh weight, with an average of 0.88 g.

Frond regeneration

Statistical analysis revealed a significant interaction among culture media types, sucrose concentrations, and phytohormone treatments (Table 1). The Hoagland medium supplemented with 2% sucrose yielded the highest regeneration efficiency, with an average frond regeneration rate of 71.66%. The most effective

Table 1. Analysis of variance for the effect of basic culture medium, sucrose, and phytohormones on callus formation and frond regeneration in *Lemna minor*.

Source of variations	df	Mean square (MS)		
		Callus induction (%)	Callus fresh weight	Frond regeneration (%)
Medium	4	7890.556**	0.930**	6844.028**
Sucrose	2	360.000**	0.015**	800.278**
Phytohormones	1	5137.778**	0.388**	22.500^{ns}
Medium×Sucrose	8	440.556**	0.017**	258.611**
Medium×Phytohormones	4	1707.222**	0.077**	81.528*
Sucrose×Phytohormones	2	71.111^{ns}	0.008**	50.833^{ns}
Medium×Sucrose×Phytohormones	8	148.889*	0.008**	99.444**
Error	60	68.889	0.001	27.778
Total	89			

ns, * and **: non-significant, significant at 5% and 1% probability, respectively.

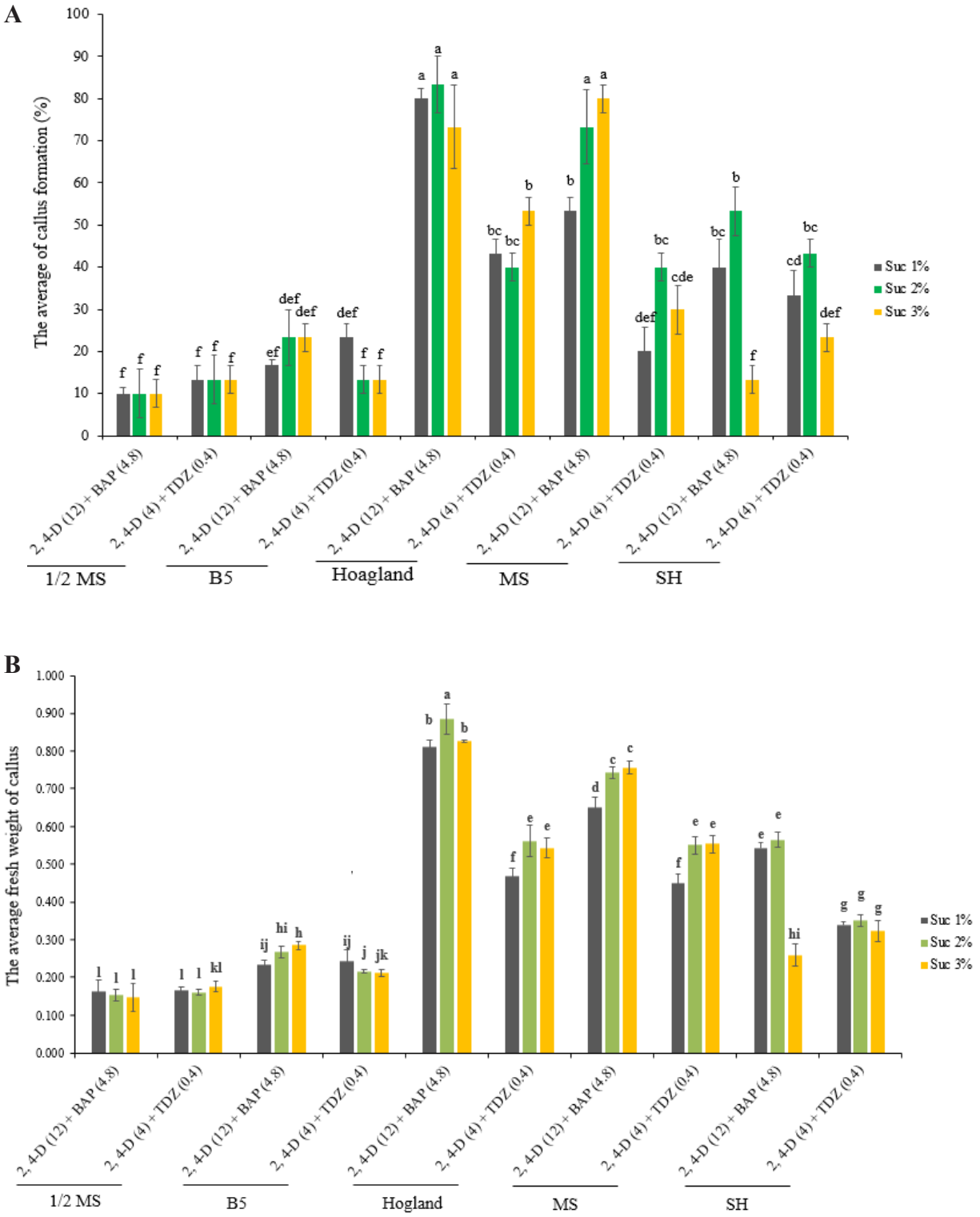

Figure 1. The impact of various culture media (MS, 1/2MS, B5, SH, Hoagland), sucrose concentrations (1%, 2%, and 3%), and phytohormone combinations (2,4-D and BAP at specified concentrations) on **A:** the percentage of callus formation and **B:** the fresh weight of callus in *Lemna minor*. Callus induction was evaluated after two months of culture under dark conditions at 25±2 °C. Data represent the mean±standard deviation of three replicates (N=60). Statistically significant differences among treatments were observed (P<0.01).

treatment was observed with the Hoagland medium enriched with 2% sucrose, combined with KT (1 mg/L) and IAA (4.5 mg/L), resulting in a peak regeneration rate of 78.33%. Additionally, plantlets in this medium successfully developed a root system within two weeks. In comparison, the MS medium supplemented with 1% sucrose, TDZ (1 mg/L), and IAA (4 mg/L) achieved a moderate regeneration rate of 43.33% (Figure 2D). Similarly, the SH medium treated with 2% sucrose, TDZ (1 mg/L), and IAA (4.5 mg/L) exhibited a regeneration rate of 45%. No statistically significant differences were detected between the 1/2MS and B5 media, both of which showed the lowest regeneration performance among the evaluated media (Figure 3).

Transformation efficiency

Preliminary confirmation of transformation was conducted using PCR analysis (Figure 4), followed by final validation via *GUS* assays for all collected lines (Figure 5). A one-way ANOVA showed a significant effect of acetosyringone concentration on regeneration percentage ($F(2,6)=27.52$, $p=0.0010$) (Table 2). The average transformation efficiency at acetosyringone concentrations of 50, 100, and 200 µM were 11.24±2.43%, 25.7±2.11%, and 31.03±3.52%, respectively (Figure 6). Statistical analysis revealed that the highest transformation efficiency was achieved at 200 µM acetosyringone. While no statistically significant difference was observed between the transformation efficiencies at 100 µM and 200 µM acetosyringone, significant differences were detected between 50 µM and 100 µM, as well as between 50 µM and 200 µM.

DISCUSSION

In the field of *Lemna* tissue culture, foundational studies have emphasized the pivotal role of auxin–cytokinin interactions in facilitating both callogenesis and regeneration. Early research by Chang and Chiu (1976) identified the effectiveness of 2,4-D and 2IP in promoting callus formation, while IAA and KT were found to enhance regeneration in *L. gibba*. Since then, numerous tissue culture protocols have been developed for various *Lemna* species, including *L. aequinoctialis* (Wang *et al*., 2021), *L. minor* (Stefaniak *et al*., 2002), and *L. turionifera* (Yang *et al*., 2022). Despite these advancements, optimization remains highly dependent on species and genotype. Among plant growth regulators, 2,4-D has consistently demonstrated efficacy in callus induction, with its performance often improving when combined with cytokinins (Mayerni *et al*., 2020).

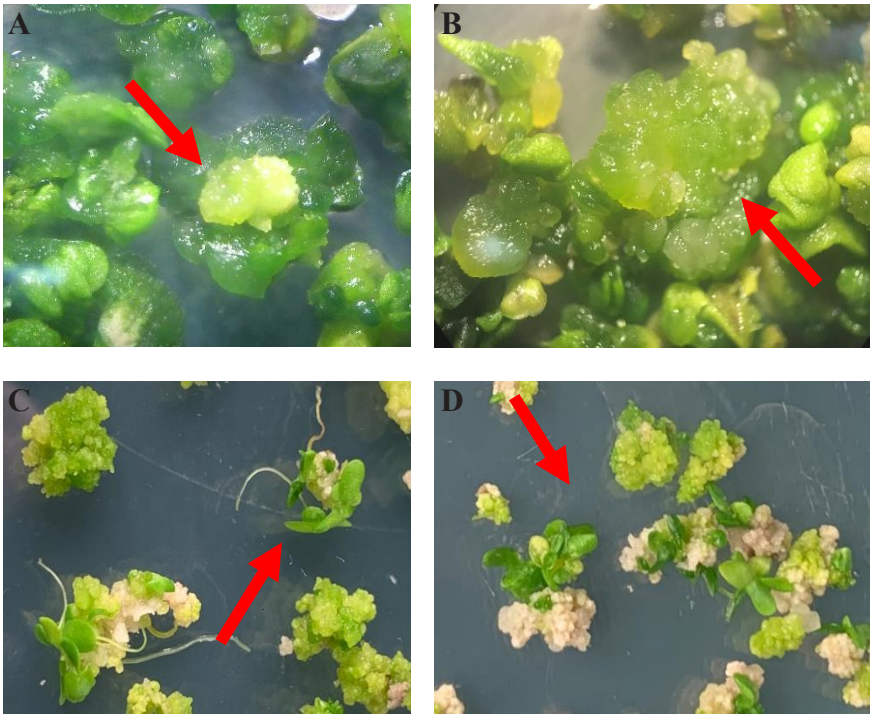

Figure 2. Callus induction and frond regeneration in *Lemna minor*. **A:** Callus induction on MS medium supplemented with sucrose 3%, 2,4-D (12 mg/mL) and BAP (4.8 mg/mL). **B:** Callus induction in Hoagland medium with sucrose 2% , 2,4-D (12 mg/mL) and BAP (4.8 mg/mL). **C:** Frond regeneration on Hoagland medium supplemented with sucrose 2%, IAA (4.5 mg/mL) and KT (1 mg/mL). **D:** Frond regeneration on Hoagland medium supplemented with sucrose 2%, IAA (4 mg/mL) and TDZ (1 mg/mL).

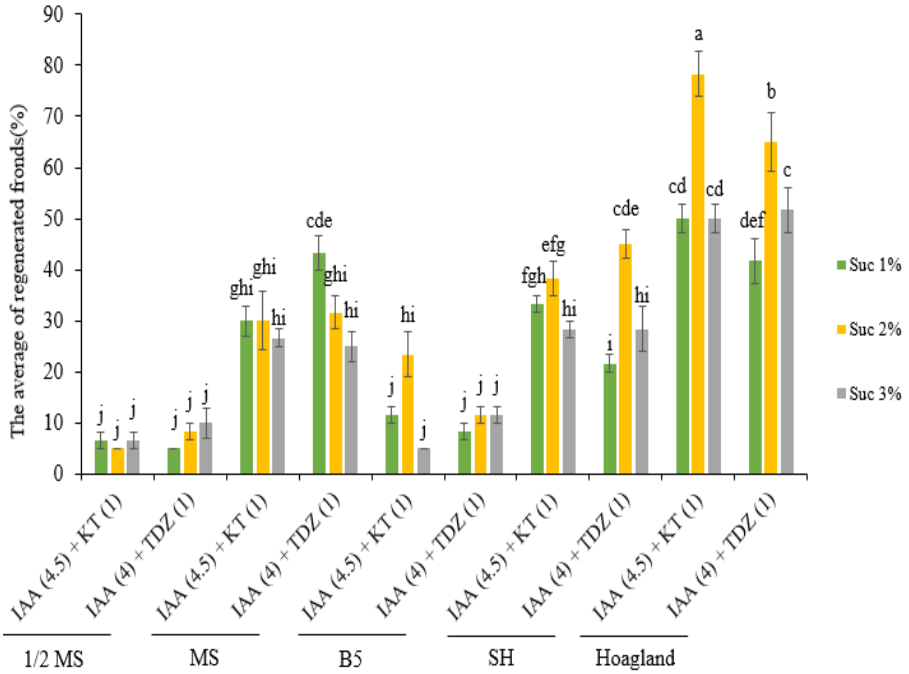

Figure 3. The impact of various culture media (MS, 1/2MS, B5, SH, Hoagland), different sucrose concentrations (1%, 2%, and 3%), and phytohormone treatments (KT, IAA, TDZ at specified concentrations) on frond regeneration percentage in *Lemna minor*. Regeneration rates were recorded after two months of culture under controlled conditions (25±2 °C, 16/8 h light/dark photoperiod). The data are represent as the mean±standard deviation from three replicates (N=60). Statistically significant differences among treatments were analyzed (P<0.05).

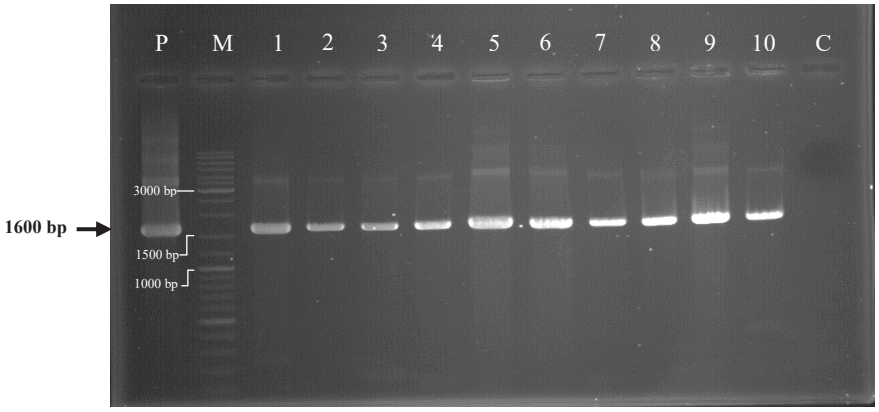

Figure 4. Molecular confirmation of transformed *Lemna minor* by PCR. P: Plasmid containing the *GUS* fragment as a positive control.; M: DNA ladder marker; (1-10) PCR amplification of the *GUS* fragment in transgenic plants; C: Wild-type plant as a negative control.

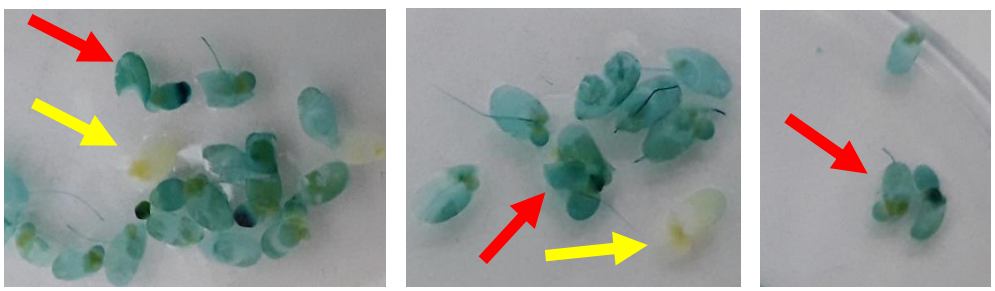

Figure 5. Assessment of GUS activity in fronds derived from *Agrobacterium*-transformed calli (indicated by red arrows). The wild-type (non-transformed) group was included as a negative contro (indicated by yellow arrows).

Table 2. Analysis of variance of the effect of different acetosyringone concentrations on regeneration percentage in *Lemna minor*. A one-way analysis of variance (ANOVA) was performed to evaluate this effect, and a statistically significant difference was observed among the groups ($p<0.05$).

Source of variation	df	Sum of squares (SS)	Mean square (MS)	F-value	P-value
Between groups (Treatment)	2	629.80	314.90***	27.52	0.0010
Within groups (Error)	6	68.66	11.44		
Total	8	698.40			

***: significant 0.1% probability.

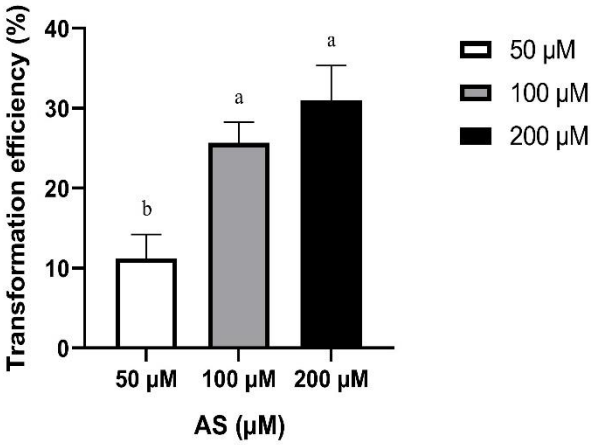

Figure 6. The effect of different acetosyringone (AS) concentrations on plant regeneration efficiency of *Lemna minor* in the co-cultivation medium.The error bars represent the standard deviation. Different letters (a, b, c, etc.) indicate statistically significant differences among treatment groups based on Tukey's multiple comparisons test at $p<0.05$. N=3.

For instance, Moon and Stomp (1997) reported limited success in callus induction (~10%) in *L. gibba* using MS medium with 2,4-D alone. While MS medium remains the most widely used basal medium in duckweed tissue culture, alternative media such as SH and B5 have demonstrated promising potential, though direct comparative studies are limited.

In the present study, MS (full- and half-strength), SH, B5, and Hoagland media were assessed to compare their effects on callus formation and regeneration processes. The primary objective was to identify the most effective culture medium for optimizing duckweed growth and propagation under *in vitro* conditions. Results demonstrated that both Hoagland and MS media significantly enhanced callus induction in *L. minor* under *in vitro* conditions. Specifically, Hoagland medium achieved a callus induction rate of 62.22%, significantly higher than the 49.44% observed with MS medium ($P<0.01$).

Fresh callus weight serves as a vital parameter for assessing the impact of culture media on callogenesis, complementing the callus induction rate. Heavier calluses typically signify a greater number of metabolically active cells, which actively grow and divide, contributing to increased callus mass. These metabolically active cells are crucial for subsequent processes such as plant regeneration and secondary metabolite synthesis (Mostafa *et al*., 2020). In this study, both MS and Hoagland media exhibited optimal performance in terms of average fresh callus weight. While MS medium achieved an impressive 80% callus induction rate, the calluses produced were slightly lighter compared to those grown in Hoagland medium. This suggests that the ionic composition and nutrient balance of Hoagland medium, originally developed for hydroponic systems, may better support *L. minor* tissue proliferation under in vitro conditions. The observed quantitative difference underscores the superior ability of Hoagland medium to enhance callogenic responses compared to MS medium, establishing its potential as a more effective culture medium for *L. minor* tissue culture.

In contrast, earlier findings by Chhabra *et al*. (2011) demonstrated the induction of hard, nodular calluses in *L. minor* using B5 medium supplemented with 1% sucrose, 50 µM 2,4-D, and 5 µM TDZ; however, the average fresh callus weight remained relatively low. Similarly, our study found that B5 medium performed poorly in terms of average fresh callus weight. Heavier calluses generally possess a larger volume and higher cell count, with an increased likelihood of containing metabolically active cells. These cells utilize energy and nutrients more efficiently, promoting robust growth and division. Given their differentiation potential, heavier calluses are typically of superior quality for processes such as regeneration and secondary metabolite production (Mostafa *et al*., 2020). In this study, callus fresh weight—a direct indicator of cell proliferation and metabolic activity—was significantly higher in MS and Hoagland media compared to B5 medium. Our findings align with those of Chhabra *et*

al. (2011), as B5 medium yielded the lowest average callus fresh weight among the tested media, indicating its limited efficacy in supporting callus growth relative to MS and Hoagland media.

Callus initiation predominantly occurs in the meristematic regions of fronds (Moon and Yang, 2002; Moon and Stomp, 1997). As highlighted by Stefaniak *et al*. (2002), surgical incision of fronds is a critical step for inducing callus formation, significantly enhancing callus production. To optimize callus formation in the meristematic regions, all fronds in this study were scratched. Wounding triggers a cascade of transcriptional changes, starting with rapid stress responses and progressing to the activation of metabolic pathways and protein synthesis, ultimately regulating cell cycle-related genes. Gene ontology data further reveal that wounding influences the expression of genes involved in hormone biosynthesis and response pathways (Ikeuchi *et al*., 2017).

Several studies underscore the influence of environmental factors, particularly light and temperature, on callus induction in *Lemna* species. Frick (1991) reported successful callus formation in *Lemna minor* under continuous light, while Moon and Stomp (1997) highlighted light as a critical factor for callus induction in *Lemna gibba*. Conversely, Stefaniak *et al*. (2002) found that elevated temperatures (25 °C) combined with darkness were more conducive to callogenesis in *Lemna* fronds. Subsequent research has reinforced the role of darkness in accelerating callus induction in duckweeds by promoting cellular dedifferentiation and suppressing photomorphogenic signals (Abdelwahed, 2016; Huang *et al*., 2018; Yang *et al*., 2022). In agreement with these findings, our results demonstrated the highest callus induction rates (62.22% and 49.44% in Hoagland and MS media, respectively) under dark conditions at 25 °C.

Auxins, key regulators of callus formation, degrade when exposed to light, making darkness crucial for maintaining high auxin concentrations, preserving their activity, and facilitating somatic embryogenesis (Wahyuni *et al*., 2020; Di Pauli *et al*., 2021). Consistent with Stefaniak *et al*., our study confirmed that dark conditions were essential for callus formation in *L. minor*.

Sucrose is widely recognized as a vital carbon source in duckweed regeneration protocols. Jingjing Yang *et al*. (2018) examined the effects of various sugars—such as sucrose, sorbitol, maltose, and glucose—on *Spirodela polyrhiza* regeneration and found that 1% sucrose significantly enhanced regeneration efficiency. Building on these findings, our study tested sucrose concentrations ranging from 1% to 3% as potential carbon sources.

In addition to its role in osmotic regulation (Ćosić *et al*., 2021), sucrose is more stable than other sugars, resisting degradation and maintaining its efficacy in culture media. Furthermore, sucrose acts as a signaling molecule, stimulating critical processes like cell division and ribosome biosynthesis (Horacio and Martinez-Noel, 2013). However, the effects of sugar type and concentration on callus induction and regeneration in duckweeds are species-dependent (Li *et al*., 2004; Khvatkov *et al*., 2015). While sucrose is the most commonly used carbohydrate source for *L. minor* cultures, alternative sugars have been explored with varying success (DeKock *et al*., 1979; Frick, 1991; Li *et al*., 2004).

Our findings revealed that 2% sucrose was optimal for both callus induction and frond regeneration. Increasing the concentration to 3% led to a decline in these parameters, likely due to higher osmotic pressure impairing nutrient uptake and cellular metabolism. This inhibitory effect was most pronounced in MS and SH media supplemented with phytohormones, where 3% sucrose reduced callus fresh weight. However, in other media, increasing sucrose concentration to 3% caused no significant changes in average callus weight, suggesting a medium-specific interaction between carbon source concentration and tissue response.

Huang *et al*. (2016) demonstrated that the optimal medium for *Landoltia punctata* regeneration was MS supplemented with 0.5% sucrose, 1% sorbitol, and 1.0 mg/L 6-BA, achieving a frond regeneration rate of approximately 90%. In our study, successful plant regeneration and root development were observed after two months in solid Hoagland medium supplemented with 1 mg/L kinetin (KT), 4.5 mg/L indole-3-acetic acid (IAA), and 2% sucrose. The synergistic interaction between IAA and nitrogen in the medium likely acted as signaling molecules to promote root initiation and elongation, enhancing resource acquisition. Cytokinins and auxins are critical for regulating root growth, vascular differentiation, and gravitropic responses, while their interplay with ethylene influences lateral root formation (Aloni *et al*., 2006).

Previous studies have shown that acetosyringone (AS) enhances transformation efficiency across various plant species, including Arabidopsis (Sheikholeslam and Weeks, 1987), cotton (Afolabi-Balogun *et al*., 2014), rice (Sahoo *et al*., 2011), and wheat (Raja *et*

al., 2010). Several reports highlight that incorporating AS into the co-cultivation medium significantly boosts T-DNA transfer by Agrobacterium tumefaciens (Pandey *et al*., 2010; Manfroi *et al*., 2015; Nakano, 2017). In this study, we assessed the impact of varying AS concentrations on transformation efficiency in *Lemna minor* to optimize T-DNA delivery and explant regeneration.

Our findings indicate that AS concentrations of 100 µM and 200 µM in the co-cultivation medium resulted in statistically similar transformation efficiencies. These results align with Yang *et al*. (2018), who identified 100 µM AS as optimal for Spirodela polyrhiza. Notably, the transformation efficiency achieved at 100 µM AS in our study (27.3±2.11%) was substantially higher than 13±1% and 13±4% reported by Yang *et al*. (2018) for TCS::GUS and DR5::GUS constructs, respectively. This disparity may be attributed to genotype-specific responses, as the Lemna minor genotype used in this study is native to Iran, underscoring the critical role of genetic background in transformation success.

Additionally, factors such as growth conditions of source material (Caswell *et al*., 2000; Delporte *et al*., 2001), culture medium composition, greenhouse environment (Saharan *et al*., 2004), and explant genotype substantially influence in vitro culture outcomes. The pivotal role of genotype in tissue culture and regeneration has been extensively documented in monocots such as *Triticum aestivum* (Arzani and Mirodjagh, 1999; Zale *et al*., 2004), rice (*Oryza sativa*) (Hoque and Mansfield, 2004), and herbaceous plants like *Primula* spp. (Schween and Schwenkel, 2003). Our results reaffirm that explant type and genotype are critical determinants of tissue culture success, likely extending to duckweed species.

CONCLUSION

This study assessed callus induction rate, callus fresh weight, and regeneration capacity in *Lemna minor*, demonstrating the efficacy of Hoagland medium for in vitro culture. Among the treatments tested, the combination of 2,4-D (12 mg/L), BAP, IAA (4 mg/L), and TDZ significantly enhanced both callus formation and regeneration. These findings emphasize the strong potential of solid Hoagland medium for tissue culture applications in *L. minor*. Furthermore, the addition of acetosyringone (AS) to the co-cultivation medium—particularly at concentrations of 100 µM or 200 µM—substantially improved transformation efficiency.

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