Brazilian Journal of Biology®

ISSN 1519-6984 (Print)
ISSN 1678-4375 (Online)

Original Article

# First report of an efficient *in vitro* regeneration protocol via callus-mediated organogenesis for *Limnophila rugosa*

## Primeiro relato de um protocolo eficiente de regeneração *in vitro* via organogênese indireta mediada por calo em *Limnophila rugosa*

N. T. M. Phuoc[a], T. M. Duc[b], T. N. Thang[b], N. T. Q. Trang[a], N. D. N. Tan[b] and P. Thanh[a]*

[a]Hue University, University of Education, Hue City, Vietnam
[b]Hue University, University of Agriculture and Forestry, Hue City, Vietnam

**Abstract**

*Limnophila rugosa* is a valuable medicinal plant listed in the Vietnam Red Data Book and threatened by overexploitation and habitat loss, highlighting the urgent need for effective propagation and conservation strategies. This study aimed to establish an efficient in vitro regeneration protocol via callus-mediated organogenesis using leaf and petiole explants. Explants were cultured on Murashige and Skoog (MS) medium supplemented with different plant growth regulators (PGRs), and callus induction, shoot regeneration, and rooting responses were evaluated. The highest shoot regeneration was achieved on MS medium supplemented with 0.3 mg/L TDZ and 0.1 mg/L IAA, resulting in a regeneration frequency of 60.00 ± 7.70% and 14.25 ± 1.51 shoots per explant. Rooting was optimal on MS medium containing 0.1 mg/L IBA, with a rooting frequency of 64.00 ± 6.78%. To our knowledge, this study represents the first report of an efficient callus-mediated regeneration protocol for *L. rugosa*. The established protocol provides a promising platform and has the potential to support large-scale clonal propagation, germplasm conservation, and future biotechnological applications of this threatened medicinal species.

**Keywords:** *Limnophila rugosa*, *in vitro* propagation, callus-mediated organogenesis, TDZ, IBA, medicinal plant.

**Resumo**

*Limnophila rugosa* é uma planta medicinal de elevado valor, incluída no Livro Vermelho da Flora do Vietnã e ameaçada pela exploração excessiva e pela perda de habitat, o que evidencia a necessidade urgente de estratégias eficazes para sua propagação e conservação. Este estudo teve como objetivo estabelecer um protocolo eficiente de regeneração *in vitro* por organogênese indireta mediada por calo, utilizando explantes foliares e de pecíolo. Os explantes foram cultivados em meio Murashige e Skoog (MS) suplementado com diferentes reguladores de crescimento vegetal (PGRs), sendo avaliadas a indução de calos, a regeneração de brotos e o enraizamento. A maior regeneração de brotos foi obtida em meio MS suplementado com 0,3 mg/L de TDZ e 0,1 mg/L de AIA, alcançando uma frequência de regeneração de 60,00 ± 7,70% e uma média de 14,25 ± 1,51 brotos por explante. O melhor enraizamento foi obtido em meio MS contendo 0,1 mg/L de AIB, com frequência de 64,00 ± 6,78%. Até onde é de nosso conhecimento, este estudo representa o primeiro relato de um protocolo eficiente de regeneração mediada por calo para *L. rugosa*. O protocolo estabelecido constitui uma plataforma promissora e apresenta potencial para apoiar a propagação clonal em larga escala, a conservação de germoplasma e futuras aplicações biotecnológicas dessa espécie medicinal ameaçada.

**Palavras-chave:** *Limnophila rugosa*, propagação in vitro, organogênese mediada por calo, TDZ, AIB, planta medicinal.

## 1. Introduction

*Limnophila rugosa* (Roth) Merr. is an important medicinal plant widely distributed in Southeast Asia and has long been used in traditional medicine because of its antimicrobial, anti-inflammatory, diuretic, hypotensive, and antioxidant properties (Yu and Cheng, 1986; Liu et al., 1991; Linh and Thach, 2011; Phong et al., 2022; Hota et al., 2023). In Vietnam, *L. rugosa* is listed in the Vietnam Red Data Book, and its natural populations have declined considerably owing to excessive harvesting and habitat degradation. Consequently, the development of effective propagation strategies is essential to ensure sustainable utilization, ex situ conservation, and the long-term preservation of its genetic resources.

Conventional propagation of *L. rugosa* relies primarily on seeds and vegetative cuttings; however, these methods are constrained by low multiplication rates, seasonal

*e-mail: phamthanh@hueuni.edu.vn
Received: June 9, 2026 – Accepted: August 7, 2026
Editor: Takako Matsumura Tundisi

This is an Open Access article distributed under the terms of the Creative Commons Attribution license (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

dependence, and limited availability of planting materials. Plant tissue culture offers an efficient alternative for the rapid production of genetically uniform plantlets while simultaneously providing a valuable platform for germplasm conservation and future biotechnological applications (Kumar and Singh, 2023). Among the available in vitro regeneration approaches, callus-mediated indirect organogenesis is particularly valuable because it enables high multiplication efficiency and provides a regeneration pathway suitable for genetic transformation, mutation breeding, and phytochemical improvement.

Considerable progress has been achieved in developing regeneration systems for several species within the genus *Limnophila*. In *Limnophila aromatica*, successful in vitro shoot regeneration has been obtained from nodal and internodal explants using TDZ-containing media, demonstrating the high morphogenic potential of this cytokinin for shoot induction (Karataş and Aasim, 2015; Dogan et al., 2016). Subsequent studies further showed that regenerated shoots could be successfully rooted and acclimatized, and that the regeneration capacity of this species was influenced by environmental factors such as salinity (Dogan, 2020). Similarly, rapid micropropagation protocols have been established for *Limnophila sessiliflora*, in which combinations of cytokinins and auxins promoted efficient shoot multiplication and rooting (Gu et al., 2008). Collectively, these studies demonstrate that species of the genus *Limnophila* respond favorably to in vitro culture; however, the regeneration strategies reported thus far have focused mainly on direct shoot regeneration or nodal culture systems, with marked differences in explant sources, plant growth regulator combinations, and regeneration efficiency.

Studies on other aquatic medicinal plants have likewise demonstrated that regeneration efficiency depends largely on the optimization of cytokinin–auxin interactions. In particular, TDZ has been consistently reported as one of the most effective cytokinins for inducing morphogenic competence and shoot organogenesis, whereas IBA generally provides superior rooting responses compared with other auxins (Karataş and Aasim, 2014; Chung and Ouyang, 2021; Faisal et al., 2023; Fatima et al., 2024). These findings provide an important physiological basis for optimizing regeneration protocols in medicinal aquatic species but also suggest that regeneration responses remain highly species-specific and therefore require individual optimization.

Despite the increasing number of regeneration studies in related *Limnophila* species, knowledge regarding *in vitro* regeneration of *L. rugosa* remains extremely limited. In particular, no study has established a complete regeneration system encompassing callus induction, indirect shoot organogenesis, and rooting from vegetative explants. Consequently, the optimal explant source, plant growth regulator combinations, and regeneration pathway for this threatened medicinal species remain unresolved. To the best of our knowledge, this study represents the first report describing an efficient callus-mediated indirect organogenesis protocol for *L. rugosa*. The establishment of such a regeneration system is expected to provide an essential platform for rapid clonal propagation, germplasm conservation, and future applications in genetic improvement and phytochemical production.

Therefore, the objectives of the present study were to optimize callus induction, shoot regeneration, and root formation from leaf and petiole explants of *L. rugosa*, and to establish a reproducible callus-mediated regeneration protocol that can facilitate the conservation and sustainable utilization of this valuable medicinal species.

## 2. Materials and Methods

### *2.1. Plant materials*

Healthy and disease-free plants of *L. rugosa* were collected from natural populations in A Luoi, Hue city, Vietnam, and maintained in the greenhouse of the Faculty of Biology, Hue University of Education, Hue University (Figure 1). Young and vigorously growing leaves, petioles, and nodal segments were excised from the donor plants and used as explant sources.

Four explant types, including shoot tips, nodal stem segments, leaves, and petioles, were collected from healthy donor plants and used to evaluate the effectiveness of surface sterilization. Based on the results of the sterilization experiment, leaf, nodal stem segment, and petiole explants were subsequently selected for callus induction experiments.

Based on the morphogenic responses obtained in this preliminary evaluation, leaf- and petiole-derived calli, which exhibited superior callus induction and regenerative potential, were selected for the subsequent optimization of shoot regeneration and rooting. Therefore, unless otherwise stated, all regeneration experiments described below were conducted using calli derived from leaf and petiole explants.

### *2.2. Culture conditions*

The basal medium consisted of Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 30 g/L sucrose and 8 g/L agar. The pH was adjusted to 5.8 before autoclaving at 121°C for 20 min.

All cultures were maintained at 25 ± 2°C under a 16-h photoperiod provided by cool-white fluorescent lamps with a light intensity of 40 µmol $m^{-2}$ $s^{-1}$. Subcultures were performed at four-week intervals unless otherwise specified.

### *2.3. Surface sterilization*

Leaf, petiole, and nodal stem segment explants were washed thoroughly under running tap water, treated with a few drops of liquid detergent for 15 min, and rinsed with distilled water. The explants were then transferred to a laminar airflow cabinet and surface-sterilized according to the procedure described below.

Explants were washed with detergent for 15 min, followed by immersion in 70% ethanol for 30 s and 0.1% $HgCl_2$ for 2 min, then rinsed 3–4 times with sterile distilled water.




**Figure 1.** Morphological characteristics of *Limnophila rugosa*. (A) Whole plant. (B) Mature leaf. (C) Flower.

*2.4. Callus induction*

Leaf, petiole, and nodal stem segment explants were cultured on Murashige and Skoog (MS) basal medium supplemented with TDZ (0.1–0.7 mg/L) or 2,4-D to evaluate their callus induction responses. Callus induction frequency, callus morphology, and growth characteristics were recorded after the culture period.

Based on the results of the preliminary explant screening, leaf and petiole explants showing superior callus induction responses were selected for the subsequent shoot regeneration experiments.

*2.5. Shoot regeneration*

Calli derived from leaf and petiole explants were excised into approximately equal-sized pieces and transferred to MS regeneration media supplemented with different combinations of combinations of TDZ with either IAA or IBA. Shoot regeneration frequency, the number of shoots per explant, and shoot length were recorded after the designated culture period.

*2.6. Rooting*

Regenerated shoots obtained from leaf- and petiole-derived calli were individually transferred to rooting media containing different concentrations of IAA or IBA. Rooting percentage, number of roots per shoot, and root length were recorded after the culture period.

*2.7. Experimental design and statistical analysis*

Experiments were arranged in a completely randomized design with three replicates, each consisting of 10 explants. Data were subjected to one-way analysis of variance (ANOVA), Statistical analyses were performed using SPSS version 19.0 (IBM Corp., Armonk, NY, USA). Values are presented as mean ± standard error (SE) and mean comparisons were performed using Duncan's multiple range test at $p \leq 0.05$.

## 3. Results

### 3.1. Establishment of aseptic cultures

The effectiveness of surface sterilization was significantly affected by the duration of $HgCl_2$ treatment (Table 1). Among the evaluated treatments, immersion in 0.1% $HgCl_2$ for 2 min resulted in the highest explant survival rate (68.33%), accompanied by a relatively low mortality rate (9.33%). Increasing the sterilization period progressively reduced contamination; however, prolonged exposure adversely affected explant viability, leading to a marked decline in survival to 13.33% after 5 min of treatment.

The response also varied according to explant type. Leaf explants exhibited the highest survival rate (58.00%), followed by nodal stem segment explants (56.00%), whereas petiole explants showed the lowest contamination rate (13.33%). Based on these results, leaf and petiole explants were selected for subsequent callus induction experiments.

### 3.2. Callus induction

Callus formation was successfully induced from leaf and petiole explants cultured on MS medium supplemented with TDZ or 2,4-D (Tables 2 and 3). No callus formation was observed on growth regulator-free medium.

Among the TDZ treatments, the highest callus induction frequency was obtained on MS medium containing 0.1 mg $L^{-1}$ TDZ. Petiole explants exhibited the greatest response, with a callus induction frequency of 40.00 ± 3.58%, followed by leaf explants (33.33 ± 3.58%). Increasing the TDZ concentration above 0.1 mg $L^{-1}$ resulted in a gradual reduction in callus formation in all explant types.

Compared with TDZ, media supplemented with 2,4-D produced substantially lower callus induction frequencies. The maximum callus formation rate achieved with 2,4-D was 26.67 ± 2.39% in petiole explants cultured on MS medium containing 0.1 mg $L^{-1}$ 2,4-D, whereas only limited responses were observed in leaf and nodal explants.

Distinct morphological differences were observed between calli induced by TDZ and those induced by 2,4-D. Calli produced on TDZ-containing media were compact to semi-compact, cream to light green in color, and showed vigorous growth (Figure 2A). In contrast, calli induced on media supplemented with 2,4-D were generally smaller and exhibited weaker growth.

### 3.3. Shoot regeneration

Adventitious shoot regeneration occurred through indirect organogenesis from callus tissues. During the early stage of regeneration, green nodular structures developed on the callus surface and subsequently differentiated into multiple shoots (Figure 2B).

Shoot regeneration was significantly influenced by the interaction between TDZ and auxin type (Table 4). Among the tested treatments, MS medium supplemented with 0.3 mg $L^{-1}$ TDZ in combination with 0.1 mg $L^{-1}$ IAA produced the highest shoot regeneration frequency (60.00 ± 7.70%) and the greatest number of shoots per explant (14.25 ± 1.51). Lower regeneration frequencies and fewer shoots were obtained at lower TDZ concentrations.

Replacing IAA with IBA reduced both the regeneration frequency and shoot multiplication capacity at all TDZ concentrations. Under the optimal regeneration medium, regenerated shoots developed into dense shoot clusters suitable for further multiplication (Figure 2C).

### 3.4. Rooting

Root initiation was observed within two to three weeks after regenerated shoots were transferred to rooting media (Table 5). Significant differences were detected among auxin treatments with respect to rooting frequency, root number, and root length.

IBA was more effective than IAA in promoting root formation. The highest rooting percentage was obtained on MS medium supplemented with 0.3 mg $L^{-1}$ IBA (66.00 ± 5.10%), whereas the greatest number of roots per shoot (8.40 ±

**Table 1.** Effects of sterilization duration and explant type on the survival, contamination, and mortality of *Limnophila rugosa* explants after 14 days of culture

| Factor | Survival (%) | Contamination (%) | Mortality (%) |
|---|---|---|---|
| Sterilization time | | | |
| 1 min | 46.67 ± 7.64$^{a}$ | 30.83 ± 9.87 | 22.67 ± 6.84$^{b}$ |
| 2 min | 68.33 ± 7.64$^{a}$ | 45.00 ± 9.87 | 9.33 ± 6.84$^{b}$ |
| 3 min | 61.67 ± 7.64$^{a}$ | 40.00 ± 9.87 | 13.00 ± 6.84$^{b}$ |
| 4 min | 50.00 ± 7.64$^{a}$ | 20.83 ± 9.87 | 24.67 ± 6.84$^{b}$ |
| 5 min | 13,33 ± 7.64$^{b}$ | 15.00 ± 9.87 | 57.33 ± 6.84$^{a}$ |
| Explant type | | | |
| Shoot tip | 36.00 ± 6.83 | 36.00 ± 8.83$^{ab}$ | 28.00 ± 6.12 |
| Nodal stem segment | 56.00 ± 6.83 | 46.67 ± 8.83$^{a}$ | 25.33 ± 6.12 |
| Leaf | 58.00 ± 6.83 | 25.33 ± 8.83$^{b}$ | 16.67 ± 6.12 |
| Petiole | 42.00 ± 6.83 | 13.33 ± 8.83$^{b}$ | 27.33 ± 6.12 |

Values are presented as mean ± SE of three biological replicates. Means followed by different lowercase letters within the same column are significantly different according to Duncan's Multiple Range Test ($P < 0.05$).

**Table 2.** Effect of TDZ concentration on callus formation from different explant types

| Explant | MS + TDZ (mg/L) | Callus formation rate (%) |
|---|---|---|
| Leaf | 0 | 0.00 ± 3.58$^{c}$ |
| | 0.1 | 33.33 ± 3.58$^{ab}$ |
| | 0.3 | 13.33 ± 3.58$^{bc}$ |
| | 0.5 | 13.33 ± 3.58$^{bc}$ |
| | 0.7 | 6.67 ± 3.58$^{c}$ |
| Nodal stem segment | 0 | 0.00 ± 3.58$^{c}$ |
| | 0.1 | 13.33 ± 3.58$^{bc}$ |
| | 0.3 | 13.33 ± 3.58$^{bc}$ |
| | 0.5 | 6.67 ± 3.58$^{c}$ |
| | 0.7 | 6.67 ± 3.58$^{c}$ |
| Petiole | 0 | 0.00 ± 3.58$^{c}$ |
| | **0.1** | 40.00 ± 3.58$^{a}$ |
| | 0.3 | 26.67 ± 3.58$^{b}$ |
| | 0.5 | 0.00 ± 3.58$^{c}$ |
| | 0.7 | 0.00 ± 3.58$^{c}$ |

Values are presented as mean ± SE of three biological replicates. Means followed by different lowercase letters within the same explant type indicate significant differences according to Duncan's Multiple Range Test ($P < 0.05$).

**Table 3.** Effect of 2,4-D concentration on callus formation from different explant types

| Explant | MS + 2,4-D (mg/L) | Callus formation rate (%) |
|---|---|---|
| Leaf | 0 | 0.00 ± 2.39$^{b}$ |
| | 0.1 | 6.67 ± 2.39$^{b}$ |
| | 0.3 | 0.00 ± 2.39$^{b}$ |
| | 0.5 | 0.00 ± 2.39$^{b}$ |
| | 0.7 | 0.00 ± 2.39$^{b}$ |
| Nodal stem segment | 0 | 0.00 ± 2.39$^{b}$ |
| | 0.1 | 13.33 ± 2.39$^{ab}$ |
| | 0.3 | 6.67 ± 2.39$^{b}$ |
| | 0.5 | 0.00 ± 2.39$^{b}$ |
| | 0.7 | 0.00 ± 2.39$^{b}$ |
| Petiole | 0 | 0.00 ± 2.39$^{b}$ |
| | 0.1 | 26.67 ± 2.39$^{a}$ |
| | 0.3 | 6.67 ± 2.39$^{b}$ |
| | 0.5 | 0.00 ± 2.39$^{b}$ |
| | 0.7 | 0.00 ± 2.39$^{b}$ |

Values are presented as mean ± SE of three biological replicates. Means followed by different lowercase letters within the same explant type indicate significant differences according to Duncan's Multiple Range Test ($P < 0.05$).

1.29) was achieved on medium containing 0.1 mg $L^{-1}$ IBA. Plantlets cultured on these media developed well-formed root systems with average root lengths of 1.52–2.45 cm. Increasing the IBA concentration above 0.3 mg $L^{-1}$ markedly reduced rooting frequency as well as root number and length.

In contrast, IAA showed considerably lower rooting efficiency across all tested concentrations. The highest rooting percentage obtained with IAA was only 14.00 ± 2.45% at 0.1 mg $L^{-1}$, accompanied by fewer and shorter roots than those produced on IBA-containing media.

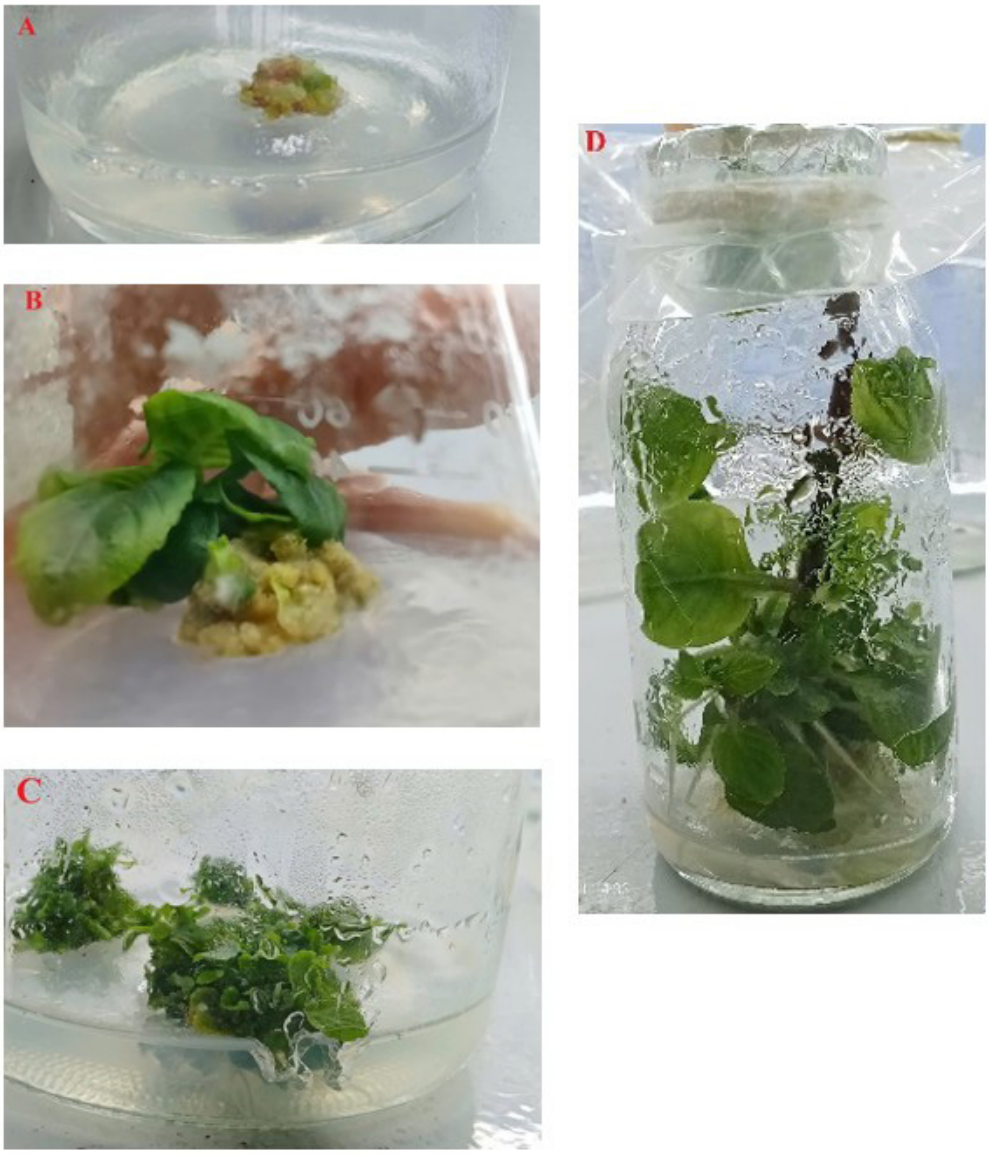



**Figure 2.** Stages of in vitro regeneration of *Limnophila rugosa* via callus-mediated rganogenesis. (A) Callus formation from petiole explants cultured on MS medium supplemented with 0.1 mg/L TDZ. (B) Differentiation of somatic embryos and subsequent shoot organogenesis from callus tissues. (C) Multiple shoot proliferation on MS medium containing 0.3 mg/L TDZ in combination with 0.1 mg/L IAA. (D) Root induction and development of complete plantlets on MS medium supplemented with 0.1 mg/L IBA.

**Table 4.** Effect of TDZ combined with IAA or IBA (0.1 mg/L) on shoot formation

| Auxin (0,1 mg/L) | TDZ (mg/L) | Shoot formation rate (%) | Number of shoots |
|---|---|---|---|
| IAA | 0.1 | 26.67 ± 7.70$^{b}$ | 5.50 ± 1.51$^{b}$ |
| | 0.2 | 46.67 ± 7.70$^{ab}$ | 8.90 ± 1.51$^{b}$ |
| | **0.3** | **60.00 ± 7.70**$^{a}$ | **14.25 ± 1.51**$^{a}$ |
| IBA | 0.1 | 26.67 ± 7.70$^{b}$ | 4.50 ± 1.51$^{b}$ |
| | 0.2 | 13.33 ± 7.70$^{b}$ | 1.17 ± 1.51$^{c}$ |
| | 0.3 | 26.67 ± 7.70$^{b}$ | 1.83 ± 1.51$^{c}$ |

Values are presented as mean ± SE of three biological replicates. Means followed by different lowercase letters within the same column are significantly different according to Duncan's Multiple Range Test ($P < 0.05$).

**Table 5.** Effect of IAA and IBA concentrations on rooting of shoots

| Auxin | Concentration (mg/L) | Rooting rate (%) | Number of roots/ shoot | Root length (cm) |
|---|---|---|---|---|
| IBA | 0.0 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| | **0.1** | **64.00 ± 6.78**[a] | **8.40 ± 1.29**[a] | **1.52 ± 0.07**[a] |
| | 0.3 | 66.00 ± 5.10[a] | 7.40 ± 0.81[a] | 2.45 ± 0.16[a] |
| | 0.5 | 18.00 ± 3.74[b] | 2.60 ± 0.51[b] | 0.20 ± 0.02[b] |
| | 0.7 | 8.00 ± 3.74[b] | 0.80 ± 0.37[c] | 0.10 ± 0.04[b] |
| IAA | 0.0 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| | 0.1 | 14.00 ± 2.45[a] | 2.00 ± 0.45[a] | 0.21 ± 0.04[a] |
| | 0.3 | 8.00 ± 3.74[ab] | 1.20 ± 0.37[a] | 0.11 ± 0.04[a] |
| | 0.5 | 4.00 ± 2.45[b] | 0.80 ± 0.58[a] | 0.07 ± 0.04[a] |
| | 0.7 | 2.00 ± 2.00[b] | 0.20 ± 0.20[a] | 0.04 ± 0.04[a] |

Values are presented as mean ± SE of three biological replicates. Means followed by different lowercase letters within the same auxin treatment are significantly different according to Duncan's Multiple Range Test ($P < 0.05$).

Plantlets regenerated on the optimal rooting medium developed healthy shoots and roots and were suitable for subsequent acclimatization (Figure 2D).

## 4. Discussion

The present study established an efficient *in vitro* regeneration system for *L. rugosa* through callus-mediated indirect organogenesis. To the best of our knowledge, this is the first report describing a complete regeneration protocol based on indirect shoot organogenesis in this medicinal species. The protocol includes sequential procedures for explant disinfection, callus induction, shoot regeneration, and root formation, thereby providing a reproducible platform for future micropropagation and conservation studies.

Successful establishment of aseptic cultures is a prerequisite for any plant tissue culture system because contamination and tissue damage directly influence subsequent morphogenic responses. In the present study, explant survival was significantly affected by the duration of $HgCl_2$ treatment. Sterilization with 0.1% $HgCl_2$ for 2 min produced the highest survival rate while maintaining acceptable contamination levels, whereas prolonged exposure markedly reduced explant viability. These observations indicate that excessive exposure to mercuric chloride causes phytotoxic effects that outweigh its disinfecting capacity. Similar responses have been reported in several medicinal plant species, in which optimization of sterilization duration was essential to achieve a balance between effective microbial elimination and preservation of explant viability.

Callus induction was strongly influenced by both explant type and plant growth regulator composition. Petiole explants exhibited the highest callus induction frequency, followed by leaf explants, whereas nodal stem segment explants showed a comparatively lower response. Differences in morphogenic competence among explant types are commonly associated with variations in endogenous hormonal balance, physiological status, and the abundance of actively dividing parenchymatous cells. The superior response of petiole tissues observed in the present study therefore suggests that this explant possesses a greater capacity for cellular dedifferentiation under *in vitro* conditions.

Among the tested growth regulators, TDZ was considerably more effective than 2,4-D in promoting callus formation. In particular, 0.1 mg $L^{-1}$ TDZ produced the highest callus induction frequencies and generated compact, cream- to light-green calli with vigorous growth. In contrast, calli induced on 2,4-D-containing media were generally smaller and exhibited weaker development. TDZ has been recognized as one of the most potent cytokinin-like compounds for inducing morphogenic responses because it promotes cell division while maintaining the regenerative competence of cultured tissues (Thomas and Philip, 2005; Dogan, 2020). Similar responses have been reported in *L. aromatica* (Dogan, 2020), where TDZ effectively stimulated callus formation and shoot regeneration. Comparable results have also been described in other aquatic medicinal plants (Karataş and Aasim, 2014). The decline in callus induction observed at higher TDZ concentrations further indicates that excessive cytokinin activity may inhibit cellular dedifferentiation and reduce morphogenic potential, highlighting the importance of optimizing growth regulator concentrations for each species (Thomas and Philip, 2005; Yorgançilar, 2011).

Shoot regeneration in *L. rugosa* occurred through indirect organogenesis, with green nodular structures developing on the callus surface before differentiating into multiple shoots. The highest regeneration efficiency was achieved on MS medium supplemented with 0.3 mg $L^{-1}$ TDZ and 0.1 mg $L^{-1}$ IAA, whereas replacement of IAA with IBA substantially reduced shoot regeneration. These findings demonstrate that the interaction between cytokinin and auxin plays a decisive role in regulating shoot organogenesis. The superior performance of the TDZ–IAA combination may be attributed to a more favorable hormonal balance

that supports the initiation and development of shoot meristems. Similar cytokinin–auxin interactions have been reported in regeneration systems of several medicinal species, where low concentrations of auxin combined with TDZ significantly enhanced shoot proliferation and regeneration efficiency.

Root induction represented the final stage of plant regeneration and was markedly affected by auxin type. IBA consistently outperformed IAA with respect to rooting percentage, root number, and root development. Although the highest rooting frequency was obtained at 0.3 mg $L^{-1}$ IBA, the medium containing 0.1 mg $L^{-1}$ IBA produced a well-developed and vigorous root system suitable for acclimatization. The superiority of IBA over IAA has been widely documented because IBA is generally more stable during in vitro culture and promotes the initiation of adventitious roots more effectively than naturally occurring auxins. Similar rooting responses have been reported in aquatic and medicinal plants, including those described by Dogan et al. (2016), Mosoh et al. (2024), and Fatima et al. (2024), supporting the suitability of IBA as the preferred auxin for root induction in *L. rugosa*.

Beyond its practical value for plant propagation, the regeneration protocol established in this study provides an important experimental platform for future biotechnological applications. An efficient callus-mediated regeneration system is a prerequisite for genetic transformation, genome editing, in vitro selection, and the enhancement of secondary metabolite production. In addition, the protocol may facilitate ex situ germplasm conservation and reduce harvesting pressure on natural populations of *L. rugosa*. Although these applications were not investigated in the present study, the regeneration system described here provides a foundation for future research aimed at the sustainable utilization and conservation of this medicinal species.

## 5. Conclusion

The present study established an efficient regeneration protocol for *L. rugosa* through callus-mediated indirect organogenesis. Among the tested explants, petiole tissues exhibited the highest callus induction response, particularly on MS medium supplemented with 0.1 mg $L^{-1}$ TDZ. The highest shoot regeneration frequency and shoot multiplication were achieved on MS medium containing 0.3 mg $L^{-1}$ TDZ in combination with 0.1 mg $L^{-1}$ IAA, whereas 0.1–0.3 mg $L^{-1}$ IBA proved to be the most suitable treatment for root induction and complete plantlet development.

To the best of our knowledge, this is the first report describing an efficient callus-mediated regeneration system for *L. rugosa***.** The regeneration protocol established in this study provides a reliable foundation for future research on large-scale micropropagation, germplasm conservation, genetic transformation, genome editing, and the production of valuable secondary metabolites. Although these applications were not evaluated in the present study, the protocol represents an important step toward the sustainable utilization and conservation of this medicinal species.

## Acknowledgements

This research was funded from University of Education, Hue University, Grant No. T.26.TN.106.10 and Hue University under the Core Research Program, Grant No.NCTB.DHH.2024.10.

## Data Availability Statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

## References

CHUNG, H.H. and OUYANG, H.Y., 2021. Use of thidiazuron for high-frequency callus induction and organogenesis of wild strawberry (*Fragaria vesca*). *Plants*, vol. 10, no. 1, pp. 67. https://doi.org/10.3390/plants10010067. PMid:33396927.

DOGAN, M., 2020. Effect of salt stress on in vitro organogenesis from nodal explants of two medicinal aquatic plants. *In Vitro Cellular & Developmental Biology. Plant : Journal of the Tissue Culture Association*, vol. 56, no. 2, pp. 242-251. http://doi.org/10.1007/s11627-020-10045-0.

DOGAN, M., KARATAŞ, M. and AASIM, M., 2016. In vitro shoot regeneration of *Limnophila aromatica* from nodal and internodal explants. *Fresenius Environmental Bulletin*, vol. 25, no. 11, pp. 4777-4782.

FAISAL, M., QAHTAN, A.A. and ALATAR, A.A., 2023. Thidiazuron induced in vitro plant regeneration, phenolic contents, antioxidant potential, GC-MS profiles and nuclear genome stability of *Plectranthus amboinicus* (Lour.) Spreng. *Horticulturae*, vol. 9, no. 2, pp. 277. https://doi.org/10.3390/horticulturae9020277.

FATIMA, T., MUJIB, A., BANSAL, Y., DEWIR, Y.H. and MENDLER-DRIENYOVSZKI, N., 2024. Indirect organogenesis of *Calendula officinalis* L. and comparative phytochemical studies of field-grown and in vitro-regenerated tissues. *Agronomy (Basel)*, vol. 14, no. 8, pp. 1743. https://doi.org/10.3390/agronomy14081743.

GU, F.G., SUN, B.Y., YUN, Y.F. and LIAN, Z.Y., 2008. Studies on tissue culture and rapid propagation technique of *Limnophila sessiliflora* BI. *Zhiwu Kexue Xuebao*, vol. 26, no. 6, pp. 639-643.

HOTA, R., KUMAR NANDA, B., BEHERA, B. and KUMAR DALAI, M., 2023. Ethno-botanical and phytopharmacological study of *Limnophila rugosa* Roth. Merr. (Scrophulariaceae): mini review. *Current Traditional Medicine*, vol. 9, no. 5, pp. 137-149. https://doi.org/10.2174/2215083808666220610123934.

KARATAŞ, M. and AASIM, M., 2014. Efficient in vitro regeneration of medicinal aquatic plant water hyssop (*Bacopa monnieri* L. Pennell). *Pakistan Journal of Agricultural Sciences*, vol. 51, no. 3, , pp. 1-6.

KARATAŞ, M. and AASIM, M., 2015. In vitro whole plant regeneration of medicinal aquatic plant *Limnophila aromatica. Fresenius Environmental Bulletin*, vol. 24, no. 10, pp. 2747-2750.

KUMAR, S. and SINGH, R., 2023. In vitro propagation of aquatic plants: a review. *International Journal of Novel Research and Development*, vol. 8, no. 10, pp. 406-418.

LINH, N.T. and THACH, L.N., 2011. Study of the essential oil of *Limnophila rugosa* (Roth.) Merr. in the South of Vietnam. *Journal of Essential Oil-Bearing Plants*, vol. 14, no. 3, pp. 366-372. https://doi.org/10.1080/0972060X.2011.10643947.

LIU, M.C., CHEN, Z.S., CHUNG, L.C., YANG, M.S., HO, S.T. and CHEN, M.T., 1991. Studies on hypotensive constituents of *Limnophila*

*rugosa. Zhongguo Yao Xue Za Zhi (Zhongguo Yao Xue Hui)*, vol. 43, no. 1, pp. 35-40.

MOSOH, D.A., KHANDEL, A.K., VERMA, S.K. and VENDRAME, W.A., 2024. Optimizing callus induction and indirect organogenesis in non-dormant corm explants of *Gloriosa superba* (L.) via media priming. *Frontiers in Horticulture*, vol. 3, pp. 1378098. https://doi.org/10.3389/fhort.2024.1378098.

MURASHIGE, T. and SKOOG, F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum*, vol. 15, no. 3, pp. 473-497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x.

PHONG, H.X., VIET, N.T., QUYEN, N.T.N., VAN THINH, P., TRUNG, N.M. and NGAN, T.T.K., 2022. Phytochemical screening, total phenolic, flavonoid contents, and antioxidant activities of four spices commonly used in Vietnamese traditional medicine. *Materials Today: Proceedings*, vol. 56, pp. A1-A5. https://doi.org/10.1016/j.matpr.2021.12.142.

THOMAS, T.D. and PHILIP, B., 2005. Thidiazuron-induced high-frequency shoot organogenesis from leaf-derived callus of a medicinal climber, *Tylophora indica* (Burm. f.) Merrill. *In Vitro Cellular & Developmental Biology. Plant : Journal of the Tissue Culture Association*, vol. 41, no. 2, pp. 124-128. https://doi.org/10.1079/IVP2004575.

YORGANÇILAR, M., 2011. The effect of thidiazuron (TDZ) on shoot regeneration of the endemic *Astragalus schizopterus. Turkish Journal of Botany*, vol. 35, no. 5, pp. 521-526.

YU, X.J. and CHENG, B.Q., 1986. Studies on the chemical constituents of the essential oil from *Limnophila rugosa. Plant Diversity*, vol. 8, no. 1, pp. 1-3.