INFLUENCE OF LIGHT AND CYTOKININ CONCENTRATION ON THE *IN VITRO* MORPHOGENESIS OF *HELICHRYSUM ARENARIUM* LEAF SEGMENTS

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Abstract

Although *Helichrysum arenarium* (L.) Moench micropropagation is documented, efficient regeneration from somatic leaf tissue remains uncharacterized. This preliminary study evaluated the combined effects of thidiazuron (TDZ) within a concentration range of - 0.00 µM to –22.70 µM and 2 lighting regimes (standard 16/8-h photoperiod vs. a dark-to-light transition) on leaf explants over an 8-week culture period. Explants exhibited high viability (77.1%–100.0%) and robust callus formation (>91%) across all treatments. Shoot organogenesis was strictly dependent on exogenous TDZ concentration. The optimal treatment combination was 0.91 µM TDZ under standard photoperiodic conditions, yielding the highest absolute multiplication rate of 4.19 ± 0.95 shoots per explant. Further increasing in TDZ concentrations caused a decline in shoot numbers, decreasing to a minimal 0.17 ± 0.09 at 22.70 µM. Organogenic efficiency decreased under dark-to-light transition, which restricted the average shoot number to 2.48 ± 0.44. The current study represents the first successful shoot regeneration from leaf tissue, which can contribute to develop an efficient micropropagation methodology for the conservation of this endangered medicinal species.

Introduction

In vitro regeneration from non-meristematic somatic tissues represents a cornerstone of plant biotechnology, offering a reliable pathway for germplasm conservation and clonal propagation. For endangered or over-exploited botanical resources, establishing high-frequency organogenic protocols from somatic explants like leaf laminas is crucial to safeguard wild populations while generating uniform experimental material (Stanilova et al., 2022). *Helichrysum arenarium* (L.) Moench (Asteraceae) is a protected, ecologically vulnerable perennial plant highly valued for its specialized secondary metabolites utilized in modern phytotherapy (Czinner et al., 2000; Pljevljakušić et al., 2018). Due to the rapid decline of its natural sand-grassland habitats across Europe and its poor natural seed-regeneration traits under changing climatic conditions, developing robust *in vitro* protocols has become a priority for both conservationists and agricultural biotechnologists (Sawilska, 2008; Van Rossum et al., 2024).

While previous micropropagation efforts in the genus *Helichrysum* have primarily relied on pre-existing meristems, such as nodal segments or apical buds, the morphogenic potential of differentiated leaf tissues remains largely uncharacterized (Figas et al., 2016; Gülsen, 2025). Utilizing foliar explants for direct or indirect organogenesis needs a precise protocol for adventitious shoot induction (Teixeira da Silva and Dobránszki, 2014). This process heavily depends on the type and concentration of exogenous plant growth regulators, as well as their interaction with ambient physical factors during the early phases of culture initiation.

Thidiazuron (TDZ) has gained widespread recognition as an exceptionally potent agent for inducing shoot organogenesis in recalcitrant plant species. Unlike conventional adenine-type cytokinins, this phenylurea derivative exhibits unique stability and high biological activity, often triggering rapid cell division and meristemoid formation at nanomolar or low micromolar

concentrations. However, the application of TDZ is frequently a double-edged sword; supra-optimal levels can trigger hyper-induction of some metabolic pathways, leading to structural abnormalities or severe growth inhibition. To modulate these powerful physiological responses, environmental factors, specifically light quality and photoperiodic regimes, are applied to regulate early signal transduction and balance endogenous hormonal fluxes during tissue differentiation (Teixeira da Silva and Dobránszki, 2014).

Because the initial exposure to light or darkness can profoundly alter tissue browning, callus formation, and subsequent shoot induction, understanding the precise interplay between TDZ concentrations and early photomorphogenic regimens is a mandatory prerequisite for successful micropropagation (Teixeira da Silva and Dobránszki, 2014). Consequently, this study was designed to explore the basic organogenic potential of *H. arenarium* leaf tissues, serving as a critical foundation for the future development of a precise micropropagation protocol.

This preliminary investigation had two primary objectives: first, to determine the survival limits and optimize shoot induction from leaf explants across a wide range of TDZ concentrations; and second, to evaluate whether a temporary dark incubation at the onset of the regeneration could reduce TDZ toxicity and further maximize shoot yield compared to a standard photoperiod.

Materials and Methods

Helichrysum arenarium L. seeds were provided by the Botanical Garden of the University of Nyíregyháza. These seeds originated from a natural population in Brandenburg, Germany (51°51′ N, 13°43′ E) and were obtained via the Index Seminum exchange program of the Botanical Garden Klagenfurt, Austria (accession No. 174; DE-0-HAL-141102_2). The donor plants were cultivated at the University of Nyíregyháza under controlled horticultural conditions with drip irrigation, following standard protocols (Sawilska, 2006). After harvesting in the summer of 2023, aseptic cultures were established in 2024 at the Centre for Agricultural Genomics and Biotechnology, University of Debrecen.

Seed surface sterilization was performed according to Cseh et al. (2023). Disinfected seeds were germinated on full-strength Murashige and Skoog (MS; 1962) medium supplemented with 3% sucrose and 6.5 g l^{-1} plant agar (Merck - Sigma-Aldrich, Budapest, Hungary). The medium pH was adjusted to 5.8 prior to autoclaving at 121 °C (1.2 bar). Germination was carried out at 23 ± 2 °C with a light intensity of 80–106 µmol s^{-1} m^{-2}, inoculating five seeds per 390 ml jar containing 70 ml of medium.

An 85% germination rate was achieved within seven days. After three weeks of culture, shoots were excised and subcultured onto the identical hormone-free MS medium. Jars were sealed with ventilated steel caps equipped with 0.2-micron HEPA sponge plugs. *In vitro* stock cultures were maintained at 23 ± 2 °C under a 16/8-hour photoperiod provided by a 1:1 mixture of daylight and warm white fluorescent lamps. This three-week subculture cycle was repeated to generate adequate plant material for the experiments.

For the regeneration studies, three young, fully expanded leaves located directly below the shoot apex were harvested from each donor plant. Leaf explants were prepared by removing the petiole, basal segment, and leaf apex, discarding approximately 5 mm from both ends. The remaining lamina was sectioned transversely into two equal segments (approx. 5 mm in width). To prevent tissue browning during excision, a sterile antioxidant solution containing 0.1 g l^{-1} ascorbic acid and 0.15 g l^{-1} citric acid was applied (Teixeira da Silva and Dobránszki, 2014). Explants were inoculated adaxial side down into 92 mm Petri dishes containing 25 ml of regeneration medium, with six explants allocated per dish.

The combined effects of thidiazuron (TDZ) at various concentrations and the 2 lighting regimes on leaf explant regeneration were evaluated. The regeneration medium consisted of full-strength MS medium supplemented with 3% sucrose, 6.5 g l^{-1} plant agar, and 0.57 µM indole-3-acetic acid (IAA). TDZ was supplemented at concentrations of 0.00, 0.91, 2.27, 5.68, 11.35, and 22.70 µM. The pH was adjusted to 5.8 before autoclaving (121 °C, 1.2 bar).

Following inoculation, the explants were subjected to two distinct lighting treatments for an 8-week culture period:

1) Cultures were maintained continuously at 23 ± 2 °C under a 16/8 h (light/dark) photoperiod (80–106 µmol m^{-2} s^{-1} light intensity, 1:1 daylight/warm white fluorescent mix) (Standard lighting condition).

2) Cultures were initially kept in complete darkness for 1 week. Subsequently, they were transferred to low light intensity (10–30 µmol m^{-2} s^{-1}) for 1 week, followed by intermediate light intensity (50–70 µmol m^{-2} s^{-1}) for another week, before being placed under standard lighting conditions (80–106 µmol m^{-2} s^{-1}) for the final 5 weeks of the culture period (Dark-to-Light transition).

After the 8-week culture period, the morphogenic and organogenic responses of the leaf explants were comprehensively evaluated. All experiments were established using a completely randomized design. Five independent Petri dish replicates were utilized per treatment combination, and each dish contained six explants, representing a total of 30 individual leaf explants evaluated per treatment. Quantitative data, including explant viability rate (Alive %), callus formation percentage (Callus %), and shoot induction rate (Shoot induction %), were expressed as percentages. The mean number of regenerated shoots per explant (Shoot number) was expressed as mean ± standard error (SE).

The experimental data were subjected to statistical evaluation using IBM SPSS Statistics software (Version 26.0; IBM Corp., Armonk, NY, USA). Main and interaction effects of thidiazuron (TDZ) concentrations and lighting regimes were analyzed using analysis of variance (ANOVA). Significant differences among the treatment means were determined using a post-hoc multiple comparison test (Duncan's Multiple Range Test) at a significance level of $P < 0.05$. Homogeneous statistical groups were assigned lowercase letter codes in descending order.

Results and Discussion

The morphogenic and organogenic responses of *Helichrysum arenarium* L. leaf explants were profoundly influenced by both TDZ concentrations and lighting regimes (Table 1).

Table 1. Morphogenic and organogenic responses of *H. arenarium* L. explants.

	TDZ (µM)	Alive %	Callus %	Shoot induction %	Shoot number
Standard conditions	0.00	77.1	97.2	0.0	0.00 ± 0.00 e
	0.91	82.9	97.2	33.3	4.19 ± 0.95 a
	2.27	100.0	100.0	47.2	1.93 ± 0.62 bcd
	5.68	100.0	100.0	66.7	2.14 ± 0.52 bc
	11.35	88.2	94.4	11.1	3.03 ± 0.61 ab
	22.70	77.1	97.2	61.1	0.17 ± 0.09 e
Dark-to-Light transition	0.00	87.9	91.7	0.0	0.00 ± 0.00 e
	0.91	100.0	100.0	63.9	2.48 ± 0.44 bc
	2.27	100.0	100.0	36.1	1.08 ± 0.19 cde
	5.68	100.0	100.0	25.0	0.51 ± 0.17 de
	11.35	97.2	100.0	5.6	0.50 ± 0.17 de
	22.70	87.9	91.7	69.4	0.09 ± 0.07 e

The explants demonstrated remarkably high organogenic plasticity. *In vitro* survival rates (Alive %) remained high across all treatments, ranging between 77.1% and 100.0%. Notably, complete survival (100%) was achieved at intermediate TDZ concentrations (2.27–5.68 µM) under standard conditions, and spanning from 0.91 to 5.68 µM under the dark-to-light transition. Callus formation (Callus %) was exceptionally vigorous, exceeding 91% in all experimental groups,

including the cytokinin-free control. This high callus induction on cytokinin-free media indicates a strong endogenous hormone content or a highly sensitive wounding response in *H. arenarium* leaf tissues, which aligns with observations in other recalcitrant Asteraceae species (Stanilova et al., 2022).

Shoot organogenesis was critically dependent on exogenous TDZ application, as no viable shoots were induced on the control, cytokinin-free medium. Under standard lighting conditions, the lowest TDZ concentration (0.91 μM) promoted the highest shoot number (4.19 ± 0.95 shoots per explant). While this value was statistically comparable to the 11.35 μM TDZ treatment (3.03 ± 0.61 shoots/explant; significance group ab), it was significantly higher to all other tested concentrations. Increasing the TDZ concentration to 2.27 and 5.68 μM led to a notable decline in shoot numbers (1.93 and 2.14, respectively), despite an increase in the overall shoot induction percentage (up to 66.7%). At the highest tested concentration (22.70 μM), a severe inhibitory effect was observed, with the shoot yield restricted to a mere 0.17 ± 0.09. This peak in shoot production followed by a rapid decline typifies the concentration-dependent effects of TDZ. At elevated levels, this potent regulator often stimulates excessive endogenous ethylene biosynthesis or cytokinin-oxidase activity, thereby suppressing subsequent shoot elongation and development (Nisler, 2018).

The initial dark period (Dark-to-Light transition) fundamentally altered the regeneration dynamics. In contrast to the standard photoperiod, the highest shoot induction percentage under dark-to-light conditions was recorded at 0.91 μM TDZ (63.9%), which also yielded the highest shoot number within this lighting regime (2.48 ± 0.44 bc). Interestingly, further increases in TDZ concentration under darkness caused a progressive decline in both shoot induction rates and shoot numbers, reaching near-zero values at 22.70 μM (0.09 ± 0.07 e).

Comparing the two lighting regimes, standard photoperiodic conditions combined with low TDZ levels (0.91 μM) optimize *H. arenarium* micropropagation compared to a dark-induction period. The 0.91 μM TDZ treatment under standard light significantly outperformed its dark-to-light counterpart (2.48 ± 0.44 bc). While darkness is frequently used in plant tissue culture to prevent tissue browning, our results indicate that light is essential during early TDZ exposure to promote effective shoot induction and development in *H. arenarium* leaf explants.

In conclusion, these data indicate that a low concentration of 0.91 μM TDZ under standard 16/8 h photoperiod provides the optimal environmental and hormonal equilibrium for efficient shoot regeneration for normal leaf explants. Since this concentration achieves maximum shoot output without the toxicity associated with higher hormone levels, the beforementioned TDZ concentration combined with standard lighting regimen serves as a starting point for establishing large-scale protocols aimed at species conservation and germplasm preservation.

Conclusions

Our findings demonstrate that *Helichrysum arenarium* L. leaf explants possess high morphogenic ability, as indicated by near-complete viability and robust callus and shoot formation across almost all tested conditions. However, successful shoot organogenesis is heavily restricted by specific hormonal thresholds and environmental conditions (e.g. lighting).

We concluded that the application of low-dose thidiazuron represents the most effective strategy for shoot multiplication, while higher concentrations are inhibitory, likely due to excessive production of endogenous ethylene or triggering high cytokinin-oxidase activity. Furthermore, an initial dark incubation period was proved to be detrimental to regeneration efficiency, indicating that light is an essential “co-factor” for TDZ-mediated signaling and meristemoid differentiation in this species.

Ultimately, these conclusions shift the focus of *H. arenarium* leaf tissue culture from complex dark-induction treatments toward simplified protocols using standard photoperiods together with low-concentration cytokinin.

Summary

This study successfully established a preliminary *in vitro* shoot regeneration protocol for *Helichrysum arenarium* L. using leaf explants. The combined effects of varying TDZ concentrations (0–22.70 µM) and two lighting regimes (Standard photoperiod vs. Dark-to-Light transition) were systematically evaluated over an 8-week culture period.

The experimental data showed that the optimal treatment combination was 0.91 µM TDZ under standard 16/8 h lighting conditions, which produced an average multiplication rate of 4.19 ± 0.95 shoots per explant. While this performance was statistically equivalent to the 11.35 µM treatment under standard light, the low-dose 0.91 µM treatment significantly outperformed all other experimental groups by supposedly avoiding the inhibitory responses associated with high cytokinin concentrations. Increasing the TDZ concentration to 22.70 µM triggered a decline in shoot numbers, dropping to 0.17 ± 0.09 shoots per explant. The dark-to-light transition generally reduced organogenic efficiency, with the average shoot number reaching only 2.48 ± 0.44 at 0.91 µM TDZ.

In summary, this research provides a verified, quantified methodological baseline for early-stage shoot induction, which can be directly integrated into future large-scale micropropagation and genetic conservation programs for this valuable medicinal species.

Keywords: Thidiazuron; Shoot organogenesis; Medicinal plant; *In vitro* culture; Photoperiod; Callogenesis

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