
Unlocking the Potential of In Vitro Photoautotrophy for *Eryngium foetidum*: Biomass, Morphophysiology, and Acclimatization

Darlyara Reis Silva , Marion Nayon Braga Soares , Maria Cristina Rocha Silva , Mariana Costa Lima , [Vitória Karla de Oliveira Silva-Moraes](#) , Givago Lopes Alves , Anyela Marcela Ríos-Ríos , Aldilene da Silva Lima , Marcos Vinícius Marques Pinheiro , [Thais Roseli Corrêa](#) , [Diego Silva Batista](#) , Fábio Afonso Mazzei Moura de Assis Figueiredo , [Fabrício de Oliveira Reis](#) , [Tiago Massi Ferraz](#) , [Sérgio Heitor Sousa Felipe](#) *

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Article

Unlocking the Potential of In vitro Photoautotrophy for *Eryngium foetidum*: Biomass, Morphophysiology, and Acclimatization

Darlyara Reis Silva ¹, Marion Nayon Braga Soares ¹, Maria Cristina Rocha Silva ¹, Mariana Costa Lima ¹, Vitória Karla de Oliveira Silva-Moraes ², Givago Lopes Alves ², Anyela Marcela Ríos-Ríos ³, Aldilene da Silva Lima ², Marcos Vinícius Marques Pinheiro ⁴, Thais Roseli Corrêa ^{2,5}, Diego Silva Batista ^{5,6}, Fábio Afonso Mazzei Moura de Assis Figueiredo ⁵, Fabrício de Oliveira Reis ², Tiago Massi Ferraz ^{2,5} and Sérgio Heitor Sousa Felipe ^{5,*}

¹ Centro de Ciências Agrárias, Universidade Estadual do Maranhão, São Luís, MA, 65055-310, Brazil; darlyarareissilva.125@gmail.com (D.R.S.), mnbs2018sh@gmail.com (M.N.B.S.), maria.r.silva1508@gmail.com (M.C.R.S.), and lcmarianalima@gmail.com (M.C.L.)

² Programa de Pós-Graduação em Agroecologia, Universidade Estadual do Maranhão, São Luís, MA, 65055-310, Brazil; vitoriakarlaos@gmail.com (V.K.O.S.), engivago@gmail.com (G.L.A.), aldilene29@gmail.com (A.S.L.), thaisroselicorrea@hotmail.com (T.R.C.), fareoli@gmail.com (FOR), and ferraztm@gmail.com (TMF)

³ Programa de Pós-Graduação em Biodiversidade e Biotecnologia da Amazônia Legal – BIONORTE, Universidade Estadual do Maranhão, São Luís, MA, 65055-310, Brazil; anyelamrios@gmail.com (A.M.R.-R.)

⁴ Universidade Regional Integrada do Alto Uruguai e das Missões, Campus Frederico Westphalen (URI-FW), Frederico Westphalen, RS, 98400-000, Brazil; macvini@gmail.com (M.V.M.P.)

⁵ Programa de Pós-Graduação em Agricultura e Ambiente, Universidade Estadual do Maranhão, São Luís, MA, 65055-310, Brazil; figueiredo.uema@gmail.com (F.A.M.M.A.F.) and sergio.h.s.felipe@gmail.com (S.H.S.F.)

⁶ Departamento de Agricultura, Universidade Federal da Paraíba, Campus III, PB, 58220-000, Brazil; diegoesperanca@gmail.com (D.S.B.)

* Correspondence: sergio.h.s.felipe@gmail.com

Abstract: *Eryngium foetidum* L., a biennial herb with diverse applications in food and traditional medicine, holds economic and pharmacological significance. Given its growing commercial interest, implementing biotechnological approaches like plant tissue culture is vital for sustainable propagation and metabolite production. In this study, we investigated the *in vitro* photoautotrophic potential of *E. foetidum*, examining growth, chlorophyll *a* fluorescence, photosynthetic pigments, and anatomical features under different sucrose concentrations and gas exchange rates. Acclimatization and survival rates of plants after *ex vitro* transfer were also assessed. *E. foetidum* exhibited robust growth in both photoautotrophic and photomixotrophic conditions, with natural ventilation significantly enhancing plant development. Chlorophyll *a* fluorescence and photosynthetic performance were influenced by sucrose and gas exchange, highlighting the importance of these factors in plant micropropagation. Moreover, the species demonstrated remarkable plasticity during acclimatization, with high survival rates and rapid inflorescence emission. The research provides valuable insights into optimizing *in vitro* cultivation conditions for *E. foetidum*, emphasizing the potential for large-scale clonal propagation and exploring secondary metabolites. The observed phenotypic plasticity underscores the adaptability of the species to diverse environments. These biotechnological strategies open avenues for future studies, including the application of elicitors for enhanced secondary metabolite production.

Keywords: culantro; gas exchange rate; natural ventilation; plant tissue culture; sucrose supplementation; wild coriander

1. Introduction

Eryngium foetidum L. (Apiaceae family), commonly known as culantro or wild coriander, is a biennial herb with an erect growth habit, featuring a rosette of long spatulate spiny-toothed leaves and reaching a height of 10–50 cm. It is commonly found in tropical and subtropical regions [1,2]. This species serves a dual purpose, functioning both as a food source (unconventional seasoning vegetable) and a traditional medicine in Latin America, Central and Pacific Islands, Africa, and Asia [3]. The leaves of *E. foetidum* are notably rich in phosphorus, potassium, and dietary micronutrients, making them suitable for potential use in food fortification [4–6]. In the realm of medicine, *E. foetidum* is recognized for its high bioactive and antioxidant value. It is utilized in the treatment of diseases related to the gastrointestinal tract and exhibits antibacterial, analgesic, anti-inflammatory, anthelmintic, anticonvulsant, and anticancer properties [7–9].

The pharmacological importance attributed to *E. foetidum* is particularly notable due to the various classes of compounds produced in its secondary metabolism, including aldehydes, carotenoids, phenols, and anthraquinones [7]. Furthermore, it is important to note that essential oils are found in the leaves, including molecules such as (2*E*)-2-dodecenal, *trans*-2-tetradecenal, 1-dodecanal, 1-decanal, tetradecanal, γ -terpinene, mesitylene, ρ -cymene, α -pinene, 1-undecanal, carotol, and 1-tetradecene [10]. Collectively, this phytochemical profile adds economic value to international trade and in the cosmetic, pharmaceutical, and food industries [7–11].

Due to the increasing commercial interest in *E. foetidum*, it becomes essential to implement biotechnological approaches, such as plant tissue culture, for the rational propagation of the species. This is crucial to mitigate the risk of genetic erosion resulting from the disorderly exploitation of the population [12]. Furthermore, apart from being a biotechnological strategy, plant tissue culture offers the potential for controlled production of metabolites of interest, in contrast to field conditions. It also enables the exploration of complex biosynthetic pathways that have not been achievable artificially until now [13,14].

Plant tissue culture serves as a platform for plant cloning under aseptic conditions and environmental control in the laboratory [15]. Traditionally, *in vitro* multiplication relies on three nutritional types of cultures: heterotrophic, where there are no photosynthetically active organs, and the carbon source is solely the carbohydrate supplied in the medium; photomixotrophic, where photosynthetically active organs use the carbohydrate in the medium, along with the consumption of CO₂ in the flask headspace; and photoautotrophic (or sugar-free medium), where photosynthetically active organs rely on CO₂ in the flask headspace as the carbon source [16,17]. The latter two cultivation systems, photoautotrophic and photomixotrophic, promote *in vitro* plant growth compared to the first (heterotrophic), which is more suitable for callus cultivation [16].

While photoautotrophic cultivation requires the use of gas-permeable films to enhance gas exchange between the flask headspace and the external environment, these films can also be employed in photomixotrophic (sugar-provided medium) cultivation to improve the ventilation rate of the culture flask [17–19]. Investigating the impact of natural ventilation on both photoautotrophy and photomixotrophy in the *in vitro* cultivation of *E. foetidum* plants will offer valuable insights into the morphophysiological characteristics influencing the growth and development of plants in these systems. This research can contribute new knowledge for implementing strategies applicable to clonal propagation on a large scale and in the *in vitro* germplasm banks.

Considering the information presented above, our objective was to assess the *in vitro* photoautotrophic potential of *E. foetidum*. In this study, we initially characterized the growth and development of *E. foetidum* plants under *in vitro* photomixotrophic conditions (with- and without-natural ventilation) and photoautotrophic systems. Subsequently, we examined the acclimatization of these plants. Our research provides novel insights into how *E. foetidum* responds to photomixotrophy and photoautotrophy, demonstrating its adaptability to different carbon sources.

2. Materials and Methods

2.1. Plant material and experimental design

The *Eryngium foetidum* plants utilized in the experiments were sourced from the Germplasm Bank at the Tissue Culture Laboratory (LCT), State University of Maranhão, MA, Brazil (2°34'00" S and 44°12'00" W). These plants are regularly maintained in 350-mL transparent glass flasks, each containing 60 mL of Murashige and Skoog (MS) medium with vitamins [20] (PhytoTechnology®, Lenexa, KS, USA), 3% sucrose (w/v; Dinâmica® Química Contemporânea Ltda, São Paulo, SP, Brazil), and solidified with 5.5 g L⁻¹ agar (Dinâmica® Química Contemporânea Ltda, São Paulo, SP, Brazil). The maintenance conditions include a temperature of 25 ±2 °C, an irradiance of 85 μmol m⁻² s⁻¹ (provided by four tubular white LED lamps, T8, 9 W, Avant, São Paulo, SP, Brazil), and a 16-hour photoperiod.

Explants (approximately 2.0–2.5 cm) were inoculated into 350-mL transparent glass flasks containing 60 mL of MS culture medium [20] (PhytoTechnology®, Lenexa, KS, USA), following the conditions employed *in vitro* cultivation in the Germplasm Bank, with sucrose concentrations of 0 and 30 g L⁻¹. Two explants were inoculated per flask. We employed two flask-sealing systems, resulting in different gas exchange rates [21]: polypropylene lids without membranes (14 μL L⁻¹ s⁻¹ CO₂ exchange rate) and polypropylene lids with two 10 mm holes covered by microporous tape membranes, as proposed by Saldanha et al. [18] (25 μL L⁻¹ s⁻¹ CO₂ exchange rate) (Figure 1A).

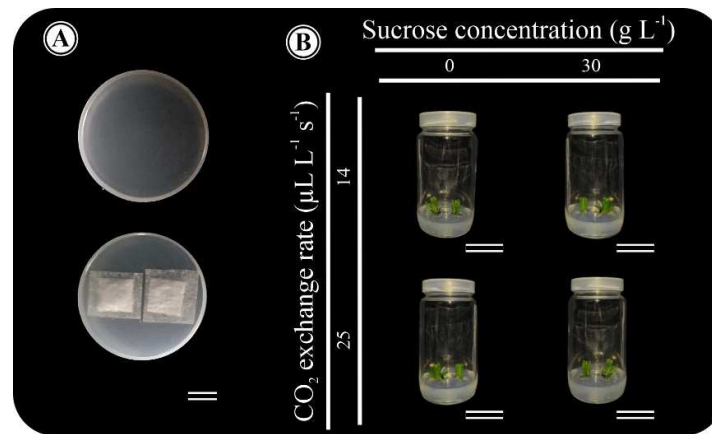


Figure 1. Schematic representation of the experimental design. (A) Polypropylene lids without a membrane (14 μL L⁻¹ s⁻¹ CO₂ exchange rate) and with two membranes (25 μL L⁻¹ s⁻¹ CO₂ exchange rate). (B) Explants of *Eryngium foetidum* at the beginning of *in vitro* culture. Further details can be found in the Materials and Methods section. Scale bars: 1A = 1 cm and 1B = 5 cm.

The flasks were maintained for 45 days in a growth room under an irradiance of 85 μmol m⁻² s⁻¹, a 16-hour photoperiod, and a temperature of 25±2 °C. The design was completely randomized in a 2 × 2 factorial [sucrose concentrations (0 and 30 g L⁻¹) and gas exchange rate (14 and 25 μL L⁻¹ s⁻¹ CO₂)], consisting of twelve replications for each treatment (Figure 1B). The experiments were repeated twice.

2.2. Chlorophyll *a* fluorescence

Chlorophyll fluorescence measurements were conducted on the third fully expanded leaf from the apex. The following parameters: initial fluorescence (F₀), maximum fluorescence (F_m), variable fluorescence (F_v), PSII maximum quantum yield (F_v/F_m), total number of active reaction centers per absorption (RC/ABS), maximum primary efficiency of PSII (F_v/F₀), and performance index (PI), were assessed using a portable non-modulated fluorimeter (Pocket PEA, Hansatech Instrument Ltd., King's Lynn, Norfolk, UK). The instrument was calibrated to the leaf under dark conditions for 30 min using a leaf-clip system (Hansatech Instrument Ltd., King's Lynn, Norfolk, UK). This calibration

ensured the complete opening of reaction centers with minimal heat loss and the complete oxidation of QA [22].

2.3. Determination of photosynthetic pigments

Pigments were extracted from leaf discs (5 mm in diameter), which were obtained from the third fully expanded leaf from the apex. The leaf discs were immersed in 5 mL of dimethyl sulfoxide (DMSO; Isofar® Ltda, Duque de Caxias, RJ, Brazil) and left in the dark for 48 h [23]. Absorbance measurements of the samples at 665 nm, 649 nm, and 480 nm were taken using a UV-visible spectrophotometer (mono-beam) (model UV-M51; BEL Engineering Company, Monza, Italy) in a 10-mm quartz cuvette. The calculation of chlorophyll *a*, *b*, and carotenoids followed the method outlined by Wellburn [24].

2.4. Quantification of photosynthetic performance

Gas exchange and quantification of the *in vitro* photosynthetic rate were conducted following the procedures outlined by Castro et al. [25]. A CO₂ gas analyzer (Model SBA-5; PP Systems International, Amesbury, MA, USA) and LoggerLite 1.9.3 software (Vernier Software & Technology Caliper, Beaverton, OR, USA) were used for these measurements. Gas exchange was determined by calculating the difference between the reference CO₂ and the CO₂ levels of plants exposed to atmospheric air. This calculation was based on the leaf area per plant (cm²).

2.5. Growth analyzes

To analyze the growth and development of plants grown *in vitro* for 45 d, various parameters were assessed, including shoot length (cm), root length (cm), base diameter of the rosette (mm), leaf area (cm²), number of leaves, shoot dry mass (g), root dry mass (g), and shoot-to-root dry mass ratio. The leaf area was determined following the method outlined by Silva et al. [26]. To determine the dry mass, the samples were subjected to 60 °C in an oven with forced air circulation (AmbiKontrol, Arujá, SP, Brazil) until a constant weight was reached.

2.6. Leaf anatomical analysis

For an anatomical structural characterization of plants grown *in vitro* for 45 days, samples of the third pair of expanded leaves of the median region were fixed in FAA solution (formalin: acetic acid: 50 % ethyl alcohol in 1:1:18 ratio) [27]. After fixation, the samples were dehydrated in increasing ethanolic series (10, 20, 30, 40, 50, 60, 70, 80, 85, 90, and 95%) and included in acrylic resin (Historesin; Leica Instruments, Jena, Germany). Cross-sections of 6- μ m thickness were obtained on a semi-automated rotary microtome (model MRP2015, Lupetec Tecnologia Aplicada, São Carlos, SP, Brazil) and stained with toluidine blue (pH 3.2) [28]. Slides were mounted and images were captured using a light microscope (model B20T; Biotika, Colombo, PR, Brazil) with the U-photo system, coupled to a digital photographic camera (model CMOS-5.0; Biotika, Colombo, PR, Brazil) and computer with Capture V2.1 software.

2.7. Ex vitro acclimatization

The culture medium was removed from the root systems of 45-day-old *E. foetidum* plants grown in cultivation systems by gently rinsing them under running water. The plants of each *in vitro* treatment were then transplanted into 350-mL polystyrene pots (model KP-350 TR; Cristalcopo Descartaveis S/A, Içara, SC, Brazil) containing commercial substrate (Carolina soil®, Santa Cruz do Sul, RS, Brazil). The plants were grown in a greenhouse with an average temperature of 28.5 \pm 2°C; Humidity of 80 \pm 5%; and natural light conditions with an intensity of ~1050 μ mol photons m⁻² s⁻¹.

2.8. Statistical analyzes

The data were statistically analyzed by two-way analysis of variance (ANOVA), with the means compared by Tukey's test. All statistical analyses were performed using the software Genes [29].

3. Results

3.1. Gas exchange rates improve chlorophyll *a* fluorescence and photosynthetic rate in *E. foetidum* grown *in vitro*

Our results indicate that *E. foetidum* plants exhibit phenotypic traits indicative of robust growth and development under photoautotrophic conditions (0 g L⁻¹ sucrose and 25 μL L⁻¹ s⁻¹ CO₂) and photomixotrophic conditions (30 g L⁻¹ sucrose) both without (14 μL L⁻¹ s⁻¹ CO₂; lower gas exchange rate) and with (25 μL L⁻¹ s⁻¹ CO₂; higher gas exchange rate) natural ventilation. However, plants under 0 g L⁻¹ sucrose and 14 μL L⁻¹ s⁻¹ CO₂ were negatively impacted, likely due to the limiting condition of carbon availability — considered as the control treatment—(Figure 2).

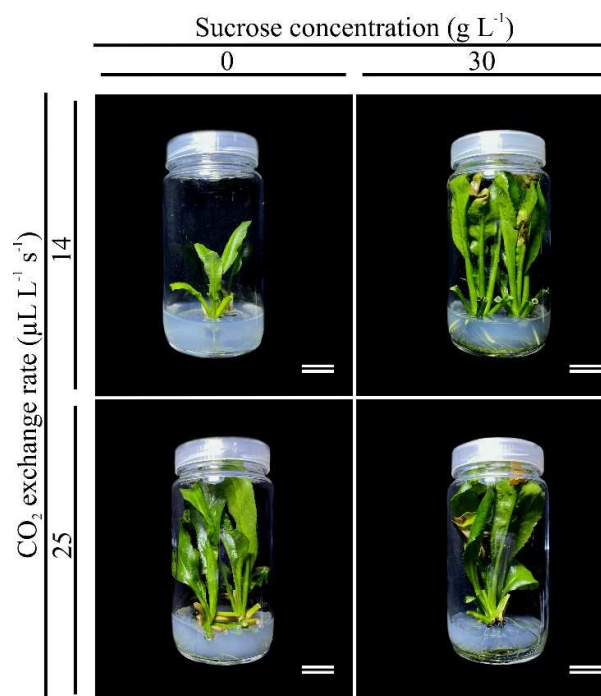


Figure 2. Forty-five-day-old *Eryngium foetidum* plants under different sucrose concentrations and gas exchange rates *in vitro*. Scale bars: 2 cm.

E. foetidum plants grown under 0 g L⁻¹ sucrose and 14 μL L⁻¹ s⁻¹ CO₂ displayed a significantly higher initial fluorescence compared to the other treatments (45% higher) (Figure 3A). In parallel, there were no significant differences in the maximum fluorescence for all treatments (ranged from 2112 to 2250) (Figure 3B). Plants under a higher gas exchange rate (25 μL L⁻¹ s⁻¹ CO₂), regardless of sucrose concentration, displayed better results for variable fluorescence, total number of active reaction centers per absorption, maximum primary efficiency of PSII, and performance index (Figures 3C; 3E–G). Lastly, only plants under the limiting condition of *in vitro* cultivation (0 g L⁻¹ sucrose and 14 μL L⁻¹ s⁻¹ CO₂) exhibited an average of 0.61 for PS II maximum quantum yield (Figure 3C), which indicates that they are facing stress.

The photosynthetic rate showed that *E. foetidum* plants in the absence of sucrose exhibited better performance compared to treatments with the presence of this carbohydrate in the culture medium (3–8 times in sucrose-free with 14 and 25 μL L⁻¹ s⁻¹ CO₂) (Figure 3H). Notably, plants under cultivation with sucrose and low gas exchange (30 g L⁻¹ sucrose and 25 μL L⁻¹ s⁻¹ CO₂) did not show photosynthetic carbon assimilation when their gas exchange was analyzed (Figure 3H). Collectively,

the data indicate that sucrose negatively affects photosynthetic performance, but is mitigated when using a higher gas exchange rate ($25 \mu\text{L L}^{-1} \text{s}^{-1} \text{CO}_2$).

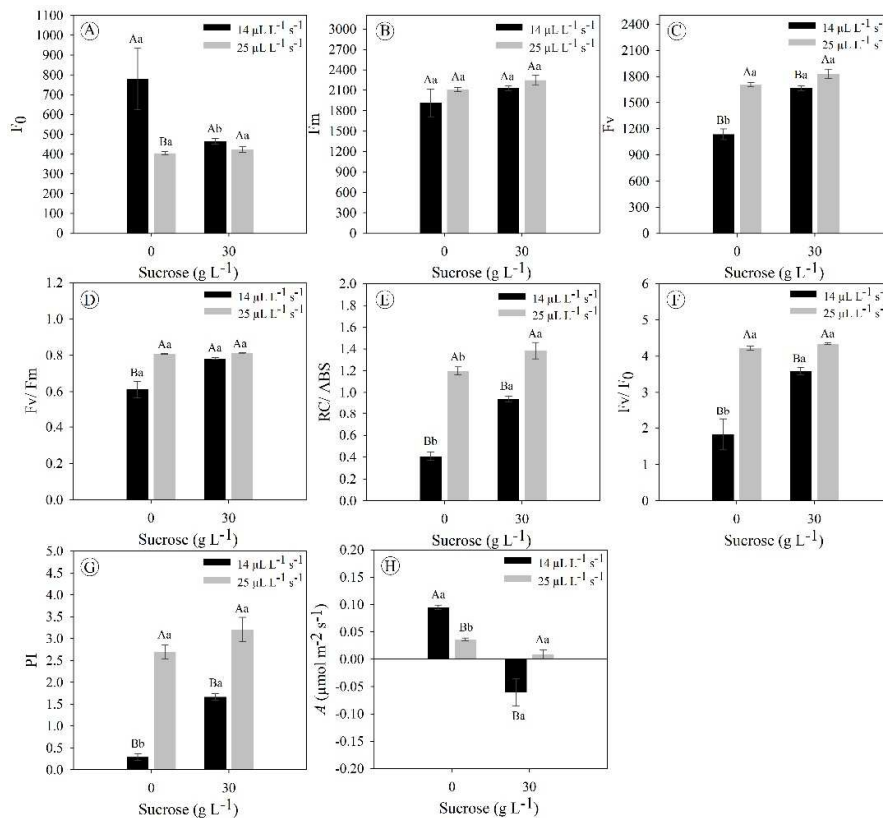


Figure 3. Chlorophyll fluorescence parameters and photosynthetic rate in *Eryngium foetidum* after 45 days of *in vitro* cultivation under different sucrose concentrations (0 and 30 g L⁻¹) and gas exchange levels (14 and 25 μL L⁻¹ s⁻¹ CO₂ exchange rates). F₀: initial fluorescence; F_m: maximum fluorescence; F_v: variable fluorescence; F_v/F_m: PS II maximum quantum yield; RC/ABS: total number of active reaction centers per absorption; F_v/F₀: maximum primary efficiency of PSII; PI: performance index; and A: photosynthetic rate. Uppercase letters compare the means of plants under different CO₂ exchange rates at the same sucrose concentration, while lowercase letters compare plants under the same CO₂ exchange rate at different sucrose concentrations (Tukey's test; $P \leq 0.05$). The bars represent the standard error (n = 5).

3.2. Sucrose concentrations and gas exchange rates do not modulate pigment concentration in *E. foetidum* grown *in vitro*

E. foetidum plants grown under photoautotrophic (0 g L⁻¹ sucrose and 25 μL L⁻¹ s⁻¹ CO₂), photomixotrophic (0 g L⁻¹ sucrose and 14 μL L⁻¹ s⁻¹ CO₂) and photomixotrophic with ventilation natural (0 g L⁻¹ sucrose and 14 μL L⁻¹ s⁻¹ CO₂) did not differ statistically from each other for the variables chlorophyll *a* (ranged from 37 to 49 μg cm⁻²), total chlorophyll (ranged from 45 to 57 μg cm⁻²), carotenoids (ranged from 10 to 12 μg cm⁻²) and total chlorophyll/carotenoid ratio (~5 μg cm⁻²); but these three cultivation systems differed significantly for cultivation under 0 g L⁻¹ sucrose and 14 μL L⁻¹ s⁻¹ CO₂ (Figure 4A,D-F). Chlorophyll *b* concentrations and the chlorophyll *a/b* ratio did not show significant differences between all treatments, with averages of 8 and 6 μg cm⁻², respectively (Figure 4B,C).

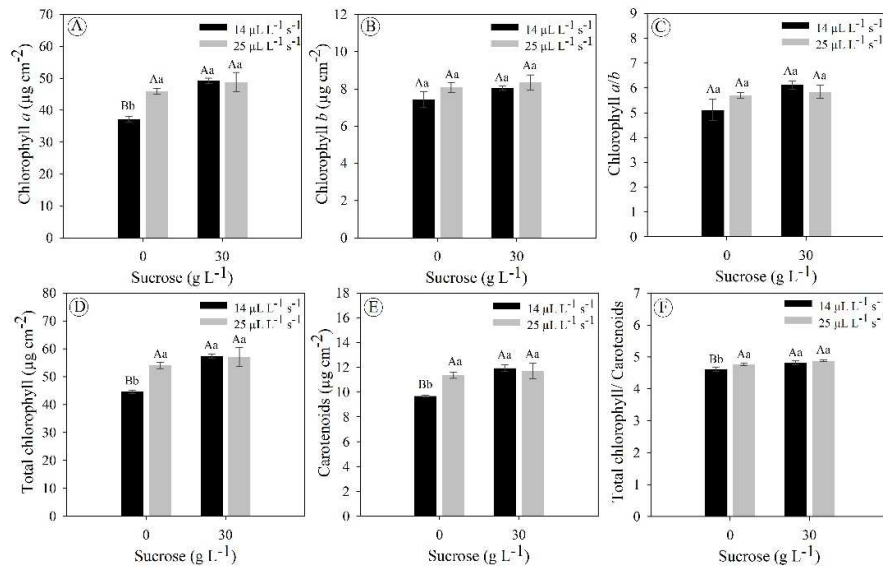


Figure 4. Concentration of photosynthetic pigments in *Eryngium foetidum* after 45 days of *in vitro* cultivation under different sucrose concentrations (0 and 30 g L⁻¹) and gas exchange levels (14 and 25 μL L⁻¹ s⁻¹ CO₂ exchange rates). Uppercase letters compare the means of plants under different CO₂ exchange rates at the same sucrose concentration, while lowercase letters compare plants under the same CO₂ exchange rate at different sucrose concentrations (Tukey's test; $P \leq 0.05$). The bars represent the standard error (n = 5).

3.3. Sucrose concentrations and gas exchange rates affect the *in vitro* growth and development of *E. foetidum*

E. foetidum plants grown under a higher gas exchange rate (25 μL L⁻¹ s⁻¹ CO₂) displayed an increase in shoot size of 74% and 11% under concentrations of 0 and 30 g L⁻¹ sucrose, respectively (Figure 5A). Furthermore, in this same condition of greater gas exchange, there was a significant increase in root length by 382% and 39% at concentrations of 0 and 30 g L⁻¹ sucrose, respectively (Figure 5B). Plants under a lower gas exchange rate (14 μL L⁻¹ s⁻¹ CO₂) displayed larger rosette base diameter compared to the treatment with a higher gas exchange rate (25 μL L⁻¹ s⁻¹ CO₂) under the concentration of 30 g L⁻¹ sucrose (10% larger diameter); while at the concentration of 0 g L⁻¹ sucrose, the opposite occurred, an expected result since this treatment (0 g L⁻¹ and 14 μL L⁻¹ s⁻¹ CO₂) is an extremely limiting cultivation condition for growth and plant development (Figure 5C).

Interestingly, plants grown in a photoautotrophic system (0 g L⁻¹ sucrose and 25 μL L⁻¹ s⁻¹ CO₂) displayed as good an increase in leaf area as the photomixotrophic with natural ventilation (30 g L⁻¹ sucrose and 25 μL L⁻¹ s⁻¹ CO₂; it ranged from 43 to 59 cm²), both treatments being significantly superior concerning the system with the lowest gas exchange rate (14 μL L⁻¹ s⁻¹ CO₂) (Figure 5D). However, these differences in leaf area did not follow for the variable number of leaves, in which the plants under 0 g L⁻¹ sucrose and 14 μL L⁻¹ s⁻¹ CO₂ and the plants under 30 g L⁻¹ sucrose and 14 or 25 μL L⁻¹ s⁻¹ CO₂ displayed number of leaves with close values (~5 leaves) (Figure 5E).

Plants grown under a higher concentration of sucrose displayed greater accumulation of dry mass in the shoot and root, independent of the gas exchange rate (Figures 5F and 5G). Concerning the shoot-to-root dry mass ratio, it was observed that in plants under 0 g L⁻¹ sucrose, there was a greater carbon allocation in the shoot, while plants under 30 g L⁻¹ sucrose displayed a more balanced carbon allocation between the shoot and root (Figure 5H).

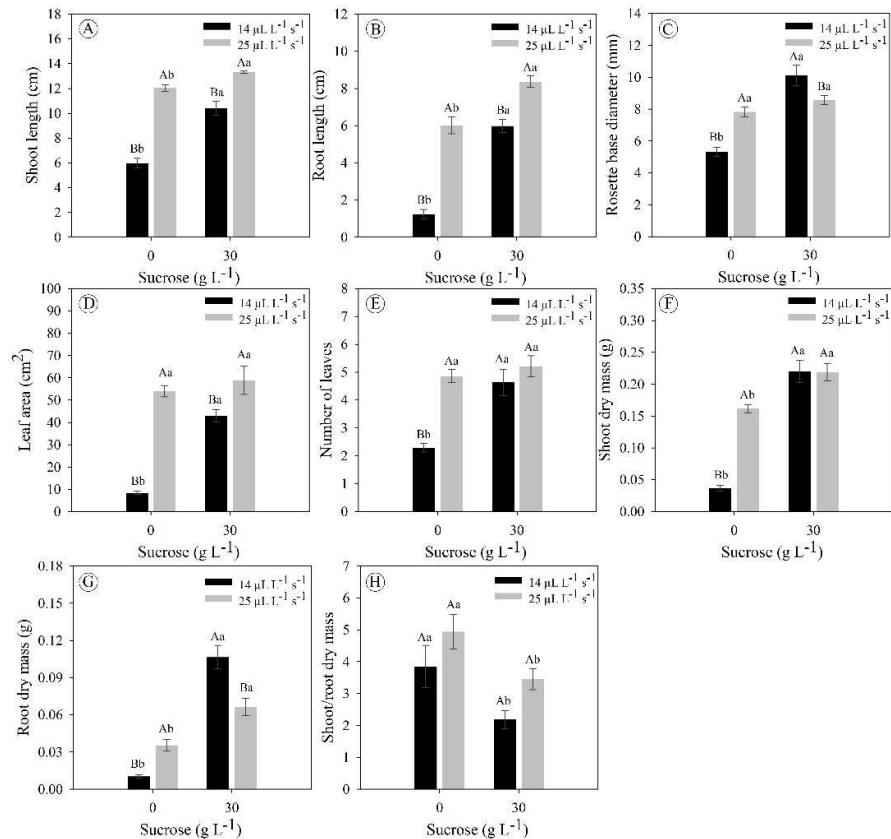


Figure 5. Growth parameters of *Eryngium foetidum* after 45 days of *in vitro* cultivation under different sucrose concentrations (0 and 30 g L⁻¹) and gas exchange levels (14 and 25 μL L⁻¹ s⁻¹ CO₂ exchange rate). Uppercase letters compare the means of plants under different CO₂ exchange rates at the same sucrose concentration, while lowercase letters compare plants under the same CO₂ exchange rate at different sucrose concentrations (Tukey's test; $P \leq 0.05$). The bars represent the standard error (n = 7).

3.4. Sucrose concentrations and gas exchange rates affect leaf anatomy

E. foetidum plants displayed the same leaf histological organization in all *in vitro* cultivation conditions accessed. The anatomy of the midrib consists of a uniseriate and amphistomatic epidermis, subepidermal collenchyma on the adaxial and abaxial surfaces; secretory canal and idioblasts distributed throughout the fundamental parenchyma; vascular bundles and fundamental parenchyma distributed throughout the central cavity (Figure 6). However, natural ventilation affected the histological differentiation of leaves compared to treatments with less gas exchange, leading to the formation of thicker and larger leaves (Figure 6).

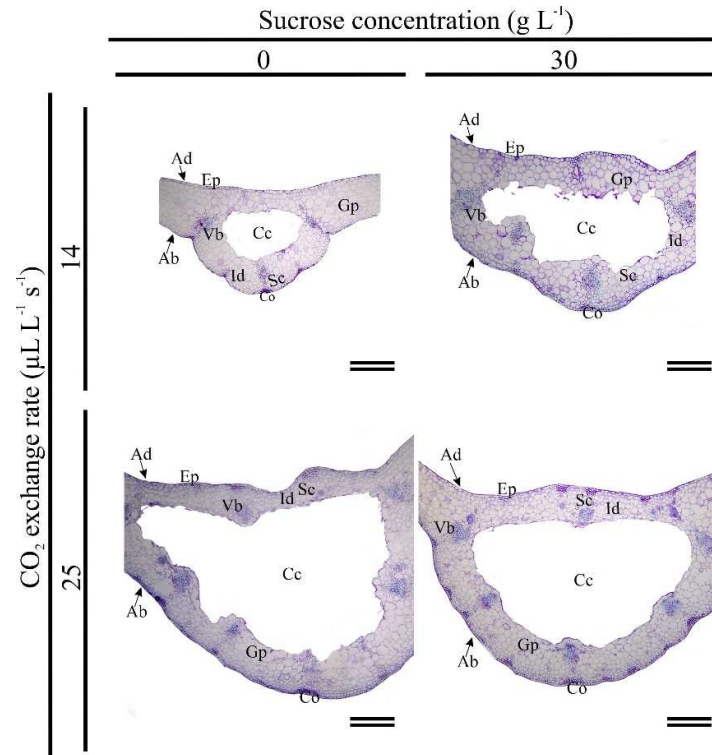


Figure 6. Leaves of *Eryngium foetidum* after 45 days of *in vitro* cultivation under different sucrose concentrations (0 and 30 g L⁻¹) and gas exchange levels (14 and 25 μL L⁻¹ s⁻¹ CO₂ exchange rate). Ab: abaxial epidermis; Ad: adaxial epidermis; Cc: central cavity; Co: collenchyma; Ep: epidermis; Gp: ground parenchyma; Id: idioblast; Sc: secretory canal; and Vb: vascular bundle. Scale bars: 300 μm.

3.5. *Ex vitro* acclimatization of *E. foetidum* grown under different sucrose concentrations and gas exchange rates

Plants showed interesting plasticity to face the acclimatization phase (Figure 7A). *E. foetidum* plants grown under photoautotrophic (0 g L⁻¹ sucrose and 25 μL L⁻¹ s⁻¹ CO₂), photomixotrophic (30 g L⁻¹ sucrose) without- (14 μL L⁻¹ s⁻¹ CO₂) and with- (25 μL L⁻¹ s⁻¹ CO₂) natural ventilation displayed 100% survival, while only the limiting (0 g L⁻¹ sucrose and 14 μL L⁻¹ s⁻¹ CO₂) displayed a low survival (~33%) (Figure 7B). An interesting fact was the emission of inflorescence in plants grown in these photoautotrophy and photomixotrophy treatments (Figure 7C).

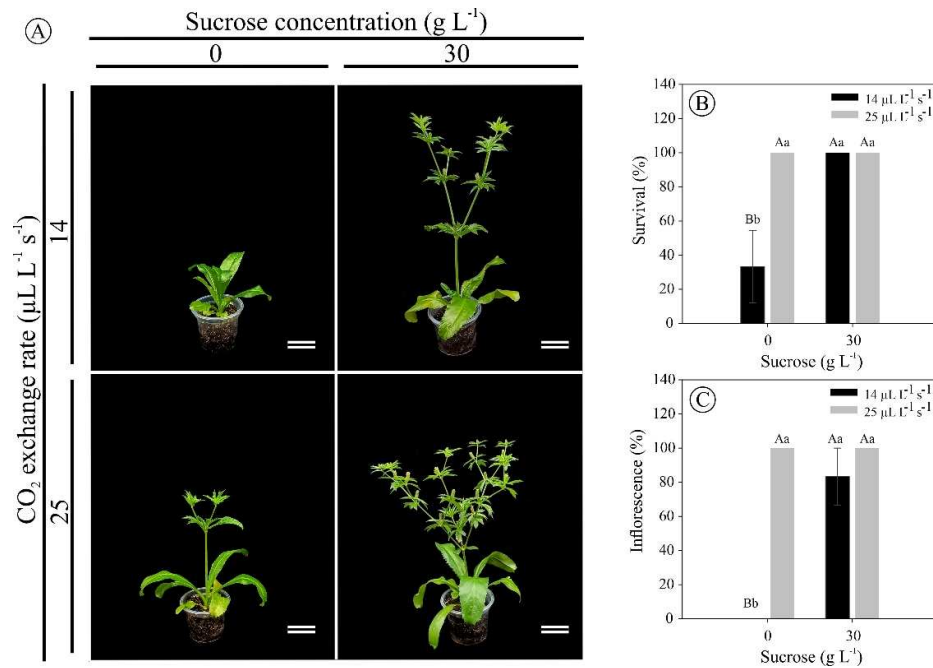


Figure 7. Acclimatization of *Eryngium foetidum* plants after 45 days of *in vitro* cultivation under different sucrose concentrations (0 and 30 g L⁻¹) and gas exchange levels (14 and 25 µL L⁻¹ s⁻¹ CO₂ exchange rate). (A) Sixty-five-day-old *Eryngium foetidum* plants during acclimatization; (B) Survival after 60 days of *ex vitro* cultivation; and (C) Inflorescence emission after 60 days of *ex vitro* cultivation. Uppercase letters compare the means of plants under different CO₂ exchange rates at the same sucrose concentration, while lowercase letters compare plants under the same CO₂ exchange rate at different sucrose concentrations (Tukey's test; $P \leq 0.05$). Scale bars: 7A = 5 cm and 7B–C = the bars represent the standard error (n = 6).

4. Discussion

Plants coordinate their growth and development with carbon availability and source *in vitro* [30–32]. Understanding how sucrose levels (carbon source provided in the cultivation medium) and natural ventilation (carbon source provided in the flask headspace) can affect plant micropropagation offers new perspectives for producing plants with improved morphophysiological characteristics [19]. Our results here demonstrate that *E. foetidum* plants exhibit *in vitro* photoautotrophic potential. However, the addition of sucrose in the photomixotrophic cultivation system increased the accumulation of plant dry mass.

Firstly, it is worth highlighting that the *E. foetidum* plants in limiting cultivation (14 µL L⁻¹ s⁻¹ CO₂ and 0 g L⁻¹ sucrose) were drastically affected in their physiological entirety, reducing growth and affecting plant development. This response was expected, since there is a strong limitation in the supply of carbon to plants, both in the cultivation medium due to only organic components making carbon available in small quantities (e.g., vitamins and myoinositol), and in the headspace of the flask due to the use of rigid lids making it difficult for CO₂ to diffusion [16,19]. Although this cultivation condition is an obstacle, the explants did not die, which may indicate the high phenotypic plasticity of *E. foetidum* to face adverse environments. In contrast, the other three *in vitro* culture systems exemplified by photoautotrophic and photomixotrophic with- and without- natural ventilation provided better responses at the plant morphophysiological level.

In the photomixotrophic system with a lower gas exchange rate (or without natural ventilation), the microenvironment of the flask is the factor with the greatest influence on the growth and development of plants, since carbon is made available through the supply of sucrose in the medium cultivation [33,34]. Undeniably, when using sealing the bottles (rigid lids or without membranes) provides high humidity inside them, greater accumulation of the phytohormone ethylene, reduced

gas exchange, and low CO₂ concentration [36–39]. These factors lead to the emergence of plants with morphophysiological disorders such as leaf formation displaying mesophyll with large intercellular spaces, poorly developed vascular system, lack of epicuticular wax formation, and non-functional stomata [16,18,19,40]. Still, in this system, the accumulation of ethylene is a factor that can strongly affect the growth and development of plants [36].

Ethylene affects several processes in plant growth and development, in which its synthesis increases as the concentration of its precursor increases, suggesting that 1-aminocyclopropane-1-carboxylic acid can act as a signaling molecule in plants [41]. Depending on the plant species and type of flask sealing, there may be a high accumulation of ethylene in the flask headspace, causing a lower photosynthetic rate due to inducing leaf senescence pathways and, consequently, lower plant growth [36,42,43]. In the current study, although some symptoms of leaf chlorosis were seen in plants under systems with a lower gas exchange rate (data not shown), there was no leaf abscission; thus, we believe that ethylene may indeed harm plants, but not to the level of being decisive for lower photosynthetic performance. Probably, the sucrose supplied in the cultivation medium is the main inhibitor of photosynthesis in these systems without natural ventilation [19,44].

Sucrose supplementation in the culture medium affects carbon assimilation by photosynthesis done activity reduction of the ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) enzyme [44] since that active carboxylation sites of the Rubisco are blocked with phosphorylated sugars [45]. To face these morphophysiological disorders, there is the photoautotrophic system, which improves the environment in the flask headspace in line with the elimination of the carbon source (carbohydrate; e.g., sucrose) [16,40,42]. Despite this, we cannot consider the sucrose supplemented in the cultivation medium to be a deleterious factor in its entirety. Here, sucrose supplemented in the *in vitro* culture medium increased the absolute values of plant dry weight.

Previous studies with *Solanum tuberosum* plants grown under 3% sucrose estimated that CO₂ assimilation contributed about 40% of the dry mass, while the other 60% is from sucrose absorption of culture medium [33]. This may be the explanation for the greater biomass of *E. foetidum* plants in a photomixotrophic with- and without- natural ventilation compared to photoautotrophic, as, often, these plants grown in a photoautotrophic system may not be achieving maximum photosynthesis performance due to extrinsic factors such as quantity (irradiance) and quality (spectral) of light, in addition to the low concentration of CO₂ in the headspace of the bottle. Based on this, CO₂ enrichment – or forced ventilation – has been applied to plant tissue culture, see [30–32,46].

Considering the chlorophyll *a* fluorescence and photosynthesis of *E. foetidum* plants, it appears that the response of CO₂ assimilation by photosynthesis was not likely associated with photochemical limitations between cultivation systems. Instead, it suggests potential obstacles in terms of biochemical reactions to CO₂ fixation. The utilization of natural ventilation in the *in vitro* cultivation of *E. foetidum* plants (both photoautotrophic and photomixotrophic with natural ventilation) significantly improved fluorescence aspects in correlation with the photosynthetic rate, compared to photomixotrophic cultivation without natural ventilation.

Photoautotrophic or photomixotrophic with natural ventilation systems also provided greater development of the leaf area of *E. foetidum*, in addition to altering the cellular differentiation of the leaves. These results corroborate findings observed in other species, for example, *Vernonia condensata* [19], *Pfaffia glomerata* [40], *Aechmea blanchetiana* [47], *Lippia dulcis* [48], and *Brassavola tuberculata* [49], in which natural ventilation led to the formation of thicker and larger leaves. These response patterns are important in photosynthetic performance; however, even better, natural ventilation also leads to the formation of functional stomata even in the *in vitro* cultivation, capable of better controlling gas exchange and evapotranspiration during the acclimatization phase [16,42].

E. foetidum plants grown *in vitro* displayed an excellent survival percentage, indicating that not only the photoautotrophic system but also photomixotrophy without and with natural ventilation are effective in the micropropagation of the species. An interesting fact was the rapid emission of inflorescences in plants. We do not know for sure whether the cultivation conditions caused an effective stress capable of leading to this or whether it could be related to the vegetative propagation

that favored the rapid emission of inflorescences in the photoautotrophic and photomixotrophic treatments with- and without- natural ventilation.

The pioneering results of this research, based on biotechnological strategies, open avenues for future studies. These studies could explore the application of elicitors to develop methods promoting the exploration of secondary metabolites in the medicinal context of the species. The spectacular phenotypic plasticity observed in the *in vitro* cultivation of the species, as demonstrated here, emphasizes the potential for further advancements in this field.

5. Conclusions

Eryngium foetidum plants exhibit *in vitro* photoautotrophic potential. Simultaneously, the supplementation of sucrose, along with natural ventilation, resulted in the highest increase in plant biomass. The findings of this research could be applied to the mass propagation of this species, aligning with the exploration of secondary metabolites, particularly those of interest in the phytochemical industry, such as essential oils.

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