Synergistic Effects of Arachidonic Acid Elicitation and Pulsed LED Lighting on Growth and Steviol Glycoside Production in Stevia rebaudiana under Vertical Hydroponics

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Research Article

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Abstract

Background

Vertical farming is emerging as a sustainable solution to meet the rising global demand for high-quality medicinal crops by enabling resource-efficient, year-round production independent of climate. Stevia rebaudiana, valued worldwide for its zero-calorie steviol glycosides, requires robust seedling establishment and optimized secondary metabolites in vertical hydroponic systems. This study investigated the interactive effects of exogenous arachidonic acid (ARA) and three lighting regimens (ambient, continuous LED, and pulsed LED) on the growth, physiology, and phytochemical profile of Stevia rebaudiana in a vertical hydroponic system. A two-factor experiment was conducted using foliar ARA applications (0, 25, and 40 μM L^{-1}) and three light regimes on tissue-cultured seedlings.

Results

Synergistic interactions were observed between 40 μM L^{-1} ARA and pulsed LED light (PSL), significantly enhancing plant height (+ 19%), stem diameter (+ 43%), leaf area (+ 29%), and aerial fresh weight (+ 28%) compared to the control. This combination also improved chlorophyll a (+ 15%), net photosynthetic rate (+ 33%), proline content (+ 32%), and antioxidant enzyme activities (SOD + 54.9%, POD + 50%). Stevioside content reached its highest level (5.80% of leaf dry matter, + 81%) under 40 μM L^{-1} ARA + PSL, while rebaudioside A was maximized with 25 μM L^{-1} ARA under ambient light. Additionally, pulsed LED lighting significantly increased total flavonoid content (+ 38%) and overall antioxidant capacity (+ 30%).

Conclusion

These findings demonstrate that integrating ARA elicitation with pulsed LED illumination is an effective and sustainable approach to simultaneously optimizing biomass yield and the nutritional-pharmaceutical quality of Stevia rebaudiana in vertical farming systems. The synergy between ARA and pulsed light offers a promising strategy for enhancing both growth and bioactive compound production under controlled environments.

1. Introduction

As the global population approaches 9.8 billion by 2050, vertical farming represents a transformative breakthrough in agriculture. It enables consistent year-round production, delivers exceptional resource efficiency, and completely removes farming from the limitations of geography and climate [1]. In these controlled environment systems, the initial stages of plant development, particularly root regeneration and the establishment of high-quality seedlings, are critical determinants of final biomass and metabolic yield. For medicinal species like *Stevia rebaudiana*, which is highly valued for its non-caloric steviol glycosides, ensuring robust physiological establishment in vertical modules is essential for maximizing both economic returns and therapeutic efficacy [2].

Light serves as the primary environmental signal regulating plant morphogenesis, carbon assimilation, and the synthesis of secondary metabolites [1]. In vertical hydroponics, where natural sunlight is often insufficient or unevenly distributed, supplemental lighting is mandatory. Recent physiological reports indicate that supplemental light, particularly when delivered through pulsed light protocols, can significantly enhance photosynthetic quantum yield and prevent photo-inhibition [3, 4]. Pulsed LED lighting, when applied at high frequencies with appropriate duty cycles, delivers multiple strong benefits in vertical hydroponic systems. High-frequency pulsed light significantly enhances plant growth and biomass, often increasing shoot fresh weight and leaf area by 20–32% compared to continuous light at the same average intensity, as plants effectively utilize higher peak photon fluxes during the on periods [5]. Experimental results on pulsed light showed that applying this treatment with long dark periods can reduce energy input by 30% to more than 50% without negatively affecting the observable traits of cabbage, microgreens, and beetroot seedlings. The results of this study on vegetable seedlings demonstrated that implementing short-interval pulsed light strategies significantly reduced the energy requirement for crop growth and improved certain vegetative traits [6].

One of the most compelling advantages is substantial energy savings: lower duty cycles can reduce electricity consumption by 30–50% or more without major yield penalties, markedly improving energy use efficiency in energy-intensive vertical farms [7]. Overall, pulsed lighting enables high-peak intensity delivery with lower average power input, mimicking natural light fluctuations and supporting sustainable, year-round production decoupled from high continuous energy demands. These positive outcomes are highly dependent on frequency and duty cycle, with high frequencies (typically > 1 kHz) and balanced duty cycles producing the strongest results, while very low frequencies (< 2.5 Hz) may reduce performance [8].

Pulsed lighting strategies modulate the kinetics of the photosynthetic apparatus, potentially allowing for better energy dissipation and improved shoot branching compared to continuous illumination. This precise light management is vital for Stevia, as its secondary metabolism is highly sensitive to spectral quality and irradiance patterns, influencing the ratio of stevioside to rebaudioside A [3]. Some studies demonstrated that lettuce grown under multispectral pulsed LED irradiation showed superior growth performance, with significant enhancements in fresh weight (~ 32%), dry weight, and leaf area, alongside improved photosynthetic efficiency and antioxidative properties compared to continuous light. Similarly, high-frequency pulsed lighting (e.g., 2_20 kHz at 50% duty ratio) positively affected lettuce leaf growth and maintained or slightly increased net photosynthetic rates relative to continuous illumination, supporting its use in controlled plant factories for energy-efficient production. In potato plantlets, optimized pulsed LED frequencies and duty ratios promoted optimal in vitro growth. Pulsed LED light also increased phytochemical levels, such as total phenolics, anthocyanins, and antioxidant activity in basil microgreens, with effects varying by wavelength and frequency. These findings highlight pulsed light's potential as a sustainable horticultural tool for boosting yield and quality in crops like lettuce and herbs [8, 9].

Beyond environmental optimization, elicitors have emerged as a potent strategy to enhance plant physiological resilience, secondary metabolite production, and stress tolerance [10]. Arachidonic acid, a long-chain polyunsaturated fatty acid (PUFA) not synthesized de novo by most higher plants, functions as a powerful exogenous signaling molecule and MAMP [11]. When applied exogenously, ARA mimics biotic stress signals from oomycete pathogens such as Phytophthora spp. It rapidly triggers an oxidative burst and accumulation of reactive oxygen species (ROS) [12]. These ROS act as secondary messengers, activating defense genes, promoting phytoalexin biosynthesis, reinforcing the cell wall, and inducing systemic resistance. Although ARA is not a direct substrate of the classical octadecanoid pathway (C18-based jasmonate biosynthesis), it strongly interacts with oxylipin signaling cascades and potentiates jasmonate-mediated responses [11].

Research indicates that arachidonic acid can stimulate root development and modify root architecture in certain species, potentially improving nutrient uptake and plant stability under controlled conditions. Furthermore, in medicinal plants such as sweet basil, arachidonic acid has been shown to upregulate genes involved in the phenylpropanoid pathway (and to a lesser extent, terpenoid pathways), thereby increasing the concentration of bioactive secondary metabolites [13, 14].

Under arachidonic acid (ARA) treatment in maize, the increased resistance to white leaf spot was accompanied by significantly enhanced activity of key defense-related enzymes, including peroxidase (POD), phenylalanine ammonia-lyase (PAL), chitinase (CHI), and laccase (LAC) [13]. Arachidonic acid (ARA) effectively stimulates the production of key defense-related enzymes, enhances the biosynthesis of antimicrobial and antioxidant secondary metabolites such as polyphenols and lignin, and upregulates the accumulation of pathogenesis-related (PR) proteins in treated plants [11, 15].

ARA modulates jasmonic acid (JA) and salicylic acid (SA) pathways, upregulating stress-responsive genes. It also triggers reactive oxygen species (ROS) production and increases the activities of enzymes such as peroxidase, lipoxygenase, and phenylalanine ammonia-lyase [11].

Despite the individual benefits of lighting and elicitation, the synergistic interaction between supplemental pulsed light and arachidonic acid in vertical hydroponics remains poorly understood. Given the increasing demand for high-quality, standardized Stevia products, there is a fundamental need to investigate how these factors influence seedling establishment and biomass partitioning. Therefore, this study was undertaken to investigate the interactive effects of exogenous arachidonic acid and pulsed LED lighting and to test the following hypotheses: H1: The combined application of exogenous arachidonic acid and pulsed LED light will provide significant synergy for plant growth, biomass accumulation, and overall physiological quality of Stevia grown in vertical hydroponic systems. H2: Novel light treatments, including pulsed LED and supplemental light, significantly increase the efficiency of key metabolite synthesis, improve shoot development, and increase energy utilization efficiency compared to natural greenhouse light. H3: Exogenous arachidonic acid significantly promotes root formation and establishment in vertical hydroponic systems, increases nutrient uptake, and drives the biosynthesis of bioactive secondary metabolites, especially steviol glycosides in Stevia. H4: Integrating pulsed LED light with arachidonic acid or continuous light supplemented with arachidonic acid significantly improves the morphological and biochemical quality of Stevia seedlings in vertical hydroponic modular systems.

2. Materials and Methods

2.1 Experimental Plants and Materials

Stevia (*Stevia rebaudiana* Bertoni) seedlings, obtained from the tissue culture laboratory of the Technology College at the 3–4 true-leaf stage, served as uniform, disease-free micropropagated seedlings, providing genetically consistent starting material that minimized the experimental variability typically associated with seed-derived seedlings.

Vertical cultivation was performed using uniform cylindrical gutters, each containing 48 planting holes. Each experimental unit consisted of three cylindrical vertical columns constructed from 12 stacked four-hole modules with standard dimensions of 2.8 m in height and 40 cm in width (Rahat_Gigas Sepahan Co., Isfahan, Iran). A modified Cooper's nutrient solution was continuously supplied via a recirculating hydroponic system in two separate stock solutions: Stock A (containing calcium nitrate and micronutrients) and Stock B (containing potassium sulfate and ammonium phosphate), maintained at an electrical conductivity (EC) of 1.7 dS/m and pH 5.8.

2.2 Experimental Design

This experiment was conducted as a two-factor full factorial design in a commercial greenhouse in Kerman, Iran. The study was initiated on April 5, 2025, following the transplanting of uniform, healthy Stevia seedlings at the 3–4 true leaf stage into a vertical hydroponic system. Each experimental unit consisted of a single cylindrical gutter containing 48 seedlings.

2.3. Experimental treatments

Treatments comprised different arachidonic acid concentrations (ARA) (0, 25 and 40 μM L^{-1}) and light regimes (LRs) (ambient/pulsed/continuous), arranged in a completely randomized design (CRD) with nine treatments.

2.3.1 Elicitor treatments

Arachidonic acid (ARA) was used as an exogenous elicitor and applied at three concentrations: 0 μM (control), 25 μM, and 40 μM. The stock solution was prepared by dissolving ARA in a minimal volume of ethanol (final ethanol concentration < 0.1% v/v) and diluting with distilled water containing 0.02% (v/v) Tween-20 as a surfactant to improve leaf wetting and uptake. Foliar sprays were applied once, 3 days before transplanting, using a hand-held sprayer. Control plants received the same solvent solution without AA. Spray application on stevia seedlings was performed in the early morning to maximize stomatal opening and minimize rapid drying.

2.3.2 Light treatments (LRs)

Ambient Light (Control): Plants were grown exclusively under natural solar radiation in the greenhouse, without supplemental lighting. The average Photosynthetic Photon Flux Density (PPFD) at the plant canopy level was adjusted to 520 µmol m^{-2} s^{-1}. A shade net with 60% was used between 12:00 and 24:00 hours, daily.

Continuous Supplemental Light (CSL)

In this light regimen, plants received continuous supplemental lighting using HPS lamps supplemented with monochromatic LEDs designed for horticultural applications, emitting across a photosynthetically active radiation (PAR) range of 400–700 nm. The average Photosynthetic Photon Flux Density (PPFD) at the plant canopy level was adjusted to 400 µmol m^{-2} s^{-1}

Pulsed Supplemental Light (PSL)

Pulsed light treatments were generated by integrating the LED fixtures with a high-precision digital Pulse Width Modulation (PWM) controller. The pulsing regime was set at a frequency of 100 Hz with a 50% duty cycle (5 ms on/ 5 ms off), and monochromatic LEDs were applied. This regime was selected based on previous studies showing improved energy efficiency and maintained or enhanced plant growth under similar conditions in lettuce and other horticultural crops [5, 7]. At the same time, the total daily light integral (DLI) was maintained equivalent to that of the continuous light control. The average Photosynthetic Photon Flux Density (PPFD) at the plant canopy level was adjusted to 400 µmol m^{-2} s^{-1}, based on measurements and calibration with an Apogee SQ-520 PAR meter. This PWM-based pulsed lighting system was employed to examine the effects of intermittent light delivery on the physiological synchrony between light–dark cycles and the photosynthetic induction response of Stevia in a vertical hydroponic setup.

To prevent light contamination between treatments, individual experimental plots were physically separated using light-proof black curtains. These curtains were installed between the continuous supplemental light (CSL) and pulsed supplemental light (PSL) treatment groups, as well as around each replicate unit, to maintain independent and stable light regimes. Supplemental lighting for both CSL and PSL treatments was provided for 14 hours daily, from 06:00 am to 20:00, ensuring a uniform total daily light integral (DLI) across all supplemental treatments and regularly monitored using a quantum sensor. This setup effectively eliminated stray light interference and ensured that each treatment received only its designated light conditions. A schematic representation of the experimental layout and curtain placement is presented in Fig. 1. Two treatments, ARA 0 µM L^{-1} and ambient light, as well as their combined effect, were considered as controls in this experiment.

During the entire experimental period, uniform environmental conditions were maintained across all treatments. Natural light, irrigation frequency and volume, greenhouse temperature, and pest and disease prevention measures were kept consistent to eliminate confounding factors other than the applied treatments. The photoperiod was maintained at 14 h d^{-1}. Ambient light was 520 PPFD at midday by use of shade. The temperature was maintained at 27°C ± 1°C when the lights were on and 20°C ± 1°C when the lights were off, with a humidity level of 60% ± 2%.

2.4 Vegetative traits and biomass

2.4.1 Vegetative Indicators

plant height (PH) was measured from the substrate surface to the apex of the main shoot; stem diameter (SD) was determined at 2 cm above the substrate level using a digital vernier caliper; and leaf area (LA) per plant was quantified using a portable leaf area meter (LI-COR, LI-3000C, USA). The number of shoots per plant was recorded after 6 weeks of transplanting by counting all shoots longer than 5 cm that emerged from each nodal zone.

Fresh weight (FW) of both the aerial parts and roots was determined at 48 days after transplanting (DAT) on six plants per treatment using a precision electronic balance with an accuracy of ± 0.01 g. For dry biomass assessment, samples of aerial parts and roots (three plants per treatment) were oven-dried at 54°C for 48 h in a drying oven (Memmert UF1060, Schwabach, Germany). Dry matter content of the shoots and roots, as well as tissue water content (%), were subsequently calculated.

2.4. 2 Pigment Content (Chlorophyll a, b, and Carotenoids)

At 48 DAT, pigment contents were determined in six plants per treatment. A 500 mg fresh weight leaf sample was ground in liquid nitrogen and extracted with 1.5 mL of 80% (v/v) acetone at 4°C in the dark for 24 h. After centrifugation at 4500 × g for 10 min, the supernatant was used for spectrophotometric analysis using a UV-1800 spectrophotometer (Shimadzu, Japan). Absorbance was recorded at 663, 646, and 470 nm. Chlorophyll a, chlorophyll b, and total carotenoid contents were calculated according to Lichtenthaler and Wellburn [16] and expressed as µg g^{-1} fresh weight.

2.4.4. RWC and Gas exchange measurements

Photosynthetic gas exchange parameters were measured on three randomly selected plants per treatment at 48 DAT. Net photosynthetic rate (Pn), transpiration rate (E), intercellular CO_2 concentration (Ci), and stomatal conductance (gs) were determined using a portable photosynthesis system (KR8700, Korea Tech Inc., Korea) [28]. Measurements were performed on the youngest fully expanded leaf between 10:00 and 13:00 a.m. under consistent environmental conditions to ensure data reliability and comparability across treatments.

Relative water content (RWC) in Stevia leaves is commonly determined using the method of Barrs and Weatherley [17]. Fresh leaf samples are weighed immediately to obtain fresh weight (FW), then immersed in distilled water for 4–6 hours at room temperature to achieve turgid weight (TW). The samples are

then oven-dried at 70–80°C until constant weight to record dry weight (DW). RWC is calculated using the formula: RWC (%) = [(FW – DW) / (TW – DW)] × 100.

2.4.5 Proline measurement

Proline content was determined according to the acid-ninhydrin method described by Bates et al. [18]. Briefly, 0.5 g of fresh Stevia leaves was homogenized in 5 mL of 3% (w/v) sulfosalicylic acid. The homogenate was extracted in a boiling water bath for 10 min, then diluted to a final volume of 10 mL with distilled water and centrifuged. A 2 mL aliquot of the supernatant was reacted with 2 mL distilled water, 2 mL glacial acetic acid, and 4 mL acid-ninhydrin reagent. The mixture was incubated in a boiling water bath for 1 h. After cooling to room temperature, 4 mL of toluene was added and vigorously mixed to extract the chromophore. The absorbance of the toluene phase was measured at 520 nm using a spectrophotometer. Proline concentration was calculated using the following formula and expressed as μg g^{-1} fresh weight:

Proline content (μg g^{-1} FW) = (C × V) / W

where C is the proline concentration (μg mL^{-1}) determined from the standard curve, V is the total volume of the extract (10 mL), and W is the fresh weight of the leaf sample (g).

2.4. 6 Antioxidant enzyme activities

Frozen leaf samples (200 mg) were homogenized in 3 mL of 25 mM sodium phosphate buffer (pH 6.8) and centrifuged at 15,000 × g for 15 min at 4°C. The resulting supernatant was used for the enzyme assay. Catalase activity was determined in a 3 mL reaction mixture containing 10 mM H_2O_2, 25 mM sodium phosphate buffer (pH 6.8), and an appropriate dilution of the enzyme extract. The decrease in absorbance due to H_2O_2 decomposition was recorded at 240 nm for 1 min using a Shimadzu UV-1800 spectrophotometer. CAT activity was expressed as U mg protein min^{-1}. Total soluble protein content was determined using bovine serum albumin (BSA) as the standard [34].

Peroxidase (POD) activity was determined spectrophotometrically by monitoring the oxidation of guaiacol in the presence of H_2O_2. The reaction mixture (1 mL) contained 20 mM guaiacol, 10 mM H_2O_2, and 0.1 M sodium phosphate buffer (pH 6.0). The increase in absorbance due to tetraguaiacol formation was recorded at 470 nm. One unit of peroxidase activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol of product per minute. The molar extinction coefficient of guaiacol used for calculation was 26.6 mM^{-1} cm^{-1} [19].

Superoxide dismutase (SOD) activity was determined using the xanthine oxidase inhibition assay (EC 1.1.3.22), which is one of the most widely used methods for SOD measurement [8, 28]. The assay was performed according to Sigma-Aldrich's manufacturer protocol. Absorbance changes were monitored at 550 nm for 1 min, with readings taken at 10-second intervals using a Shimadzu UV-160A UV-Vis spectrophotometer (Shimadzu, Japan. The resulting changes in absorbance were converted into enzyme activity units [20].

2.4.7 Stevioside and Rebaudioside A determination

Leaves from 45-day-old plants were used for RP-HPLC analysis. For the preparation of Stevia leaf extract, 0.5 g of dried leaves was extracted three successive times with 5 mL of distilled water each in a boiling water bath at 100°C for 30 minutes. After each extraction, the mixtures were cooled to room temperature and centrifuged at 2500 × g for 10 minutes at 10°C. The supernatants were collected and combined in a 25 mL volumetric flask, brought to final volume with distilled water, and filtered through a 0.45 μm membrane filter before HPLC analysis.

Stevioside (Cat. No. S3572) and Rebaudioside A (Cat. No. 01432) reference standards were procured from Sigma-Aldrich. The glycoside standards were lyophilized under vacuum (133 × 10^{-3} Bar) at -40°C using a Labconco freeze-dryer, then dissolved in HPLC-grade water, filtered through 0.45 μm membranes, and stored at − 20°C until use. Quantitative analysis of these steviol glycosides was performed by high-performance liquid chromatography (HPLC) according to JECFA (2010) [21] using an Agilent 100 HPLC system equipped with a UV-Vis detector. Chromatographic separation was carried out on a Luna C18(2) column (250 mm × 4.6 mm i.d., 5 μm particle size; Phenomenex, CA, USA) at ambient temperature. The mobile phase consisted of a 32:68 (v/v) mixture of acetonitrile and 10 mmol/L sodium phosphate buffer (pH 2.6), delivered at a flow rate of 1.0 mL/min. The injection volume was 20 μL, and detection was performed at 210 nm. Data acquisition and processing were carried out using Clarity software 8.5.14.

2.4.8 Determination of total phenolic and flavonoid content

The total phenolic content in the leaf extract of S. rebaudiana was determined spectrophotometrically using the Folin-Ciocalteu method, which is based on the colorimetric oxidation-reduction reaction of phenolic compounds [17]. This assay was used to quantify the phenolic levels in the samples. A standard calibration curve was first prepared using gallic acid as the reference standard. The measurement was performed according to the procedure described by Ghanta et al. [21]. In brief, 2 ml of 2% Na_2CO_3 solution was added to 10 μl of the plant extract, and the mixture was incubated at 25°C for 2 minutes. Then, 100 μl of 50% Folin-Ciocalteu's phenol reagent was added to the mixture and vortexed thoroughly. The final mixture was allowed to stand in the dark for 30 minutes at room temperature. Absorbance was recorded at 760 nm, and the results were expressed as milligrams of gallic acid equivalents per gram of dry weight of the plant material.

Total flavonoid content in Stevia leaves is commonly determined using the Aluminum Chloride ($AlCl_3$) colorimetric method. Briefly, 1 g of dried leaf powder is extracted with 20–50 mL of 80% methanol or ethanol. Then, 0.5–1 mL of extract is mixed with 10% $AlCl_3$, 1 M potassium acetate, and methanol, brought to 5 mL with distilled water, incubated for 30 min, and the absorbance is measured at 415 nm. Results are expressed as mg Quercetin Equivalents per gram dry weight (mg QE/g DW) [22].

2.4.9 Data Processing and Statistical Analysis

This study employed a two-factor completely randomized design, with arachidonic acid (ARA) concentration and light regimes (LRs) as the two independent factors. All statistical analyses were conducted in triplicate (n = 3) using SAS software version 9.2. The effects of ARA, LRs, and their interactions on the measured parameters were examined by two-way analysis of variance (ANOVA). When significant differences were detected, Duncan's multiple range test was applied for mean separation. A probability level of $p < 0.05$ was considered statistically significant for all analyses. The data were reported as mean ± SD.

3. Results

3.1. Vegetative Traits and Biomass

Significant ARA × LRs interactions were observed for key vegetative parameters. Combined application of 40 μM L^{-1} ARA and PSL significantly promoted plant height, reaching 43.44 cm (19% higher than the control; $F = 15.24$, $p \leq 0.01$; Fig. 2a, Table 1). Considering the non-significant interaction effects of ARA × LRs, the main effect of ARA with 40 μM L^{-1} concentration significantly increased stem diameter to 0.43 cm, a 18.0% improvement compared with the control value of 0.35 cm ($F = 49.91$, $p \leq 0.05$; Fig. 3a, Table 1). The main effect of LRs was significant and increased stem diameter to 0.47 cm, a 47.0% improvement in PSL treatment compared with the control value of 0.27 cm ($F = 49.91$, $p \leq 0.05$; Fig. 3b, Table 1). This combined application of 40 μM L^{-1} ARA and PSL also enhanced leaf area to 2400 cm^2, which was 29.0% greater than the control (1700 cm^2) ($F = 1162609$, $p \leq 0.01$; Fig. 2b, Table 1).

This combined treatment increased the number of shoots to 4.7, which was 57.0% greater than the control (2) ($F = 2.83$, $p \leq 0.01$; Fig. 2c, Table 1).

Significant ARA × LRs interaction was also detected for shoot fresh weight. The combination of 40 μM L^{-1} ARA and PSL maximized shoot fresh weight at 159.88 g, representing and 28.0% increase over the lowest value (115.66%) ($F = 21.10$, $p \leq 0.01$; Fig. 2d, Table 1).

For shoot dry weight, no significant interaction occurred ($F = 4.67$, $p > 0.01$), but both factors showed significant main effects. Treatment with 40 μM L^{-1} ARA raised shoot dry weight to 25.47g (7.22% above the control), and PSL increased it to 26.40 g (11.0% above the control) (Figs. 3c, d).

No significant interaction was observed for root fresh weight ($F = 6.60$, $p > 0.01$). However, only the ARA exhibited significant main effects. Application of 40 μM L^{-1} ARA increased root fresh weight to 16.42 g (9.0% higher than the control), For root dry weight, there was no significant interaction between the factors ($F = 14.51$, $p > 0.01$), only the LRs showed a significant main effect, and PSL raised it to 17.64 g (18.0% above the control) (Figs. 3e, f).

Table 1
Pearson correlation coefficients among morphological, physiological, and phytochemical traits of *Stevia rebaudiana* under ar

x1	x1	x2	x3	x4	x5	x6	x7	x8	x9	x10	x11	X12	x13	x14	x15
	1														
x2	-0.52^{ns}	1													
x3	-0.56^{ns}	0.97**	1												
x4	0.36^{ns}	0.42^{ns}	0.35^{ns}	1											
x5	-0.28^{ns}	0.70*	0.63^{ns}	0.23^{ns}	1										
x6	-0.31^{ns}	0.80**	0.72*	0.38^{ns}	0.94**	1									
x7	-0.27^{ns}	0.88**	0.83**	0.52^{ns}	0.89**	0.95**	1								
x8	-0.15^{ns}	0.58^{ns}	0.61^{ns}	0.17^{ns}	0.76*	0.64^{ns}	0.68*	1							
x9	-0.50^{ns}	0.45^{ns}	0.46^{ns}	-0.19^{ns}	0.65^{ns}	0.53^{ns}	0.53^{ns}	0.29^{ns}	1						
x10	-0.37^{ns}	0.81**	0.72*	0.36^{ns}	0.74*	0.71*	0.76*	0.51^{ns}	0.63^{ns}	1					
x11	-0.73*	0.85**	0.79**	0.04^{ns}	0.73*	0.80*	0.76*	0.37^{ns}	0.67*	0.72*	1				
x12	0.24^{ns}	0.36^{ns}	0.44^{ns}	0.70*	0.04^{ns}	0.14^{ns}	0.34^{ns}	0.41^{ns}	-0.30^{ns}	0.15^{ns}	-0.14^{ns}	1			
x13	-0.65^{ns}	0.55^{ns}	0.67*	0.08^{ns}	0.33^{ns}	0.27^{ns}	0.41^{ns}	0.46^{ns}	0.49^{ns}	0.50^{ns}	0.42^{ns}	0.33^{ns}	1		
x14	0.07^{ns}	-0.61^{ns}	-0.50^{ns}	-0.47^{ns}	-0.83*	-0.80*	-0.81*	-0.46^{ns}	-0.58^{ns}	-0.75*	-0.61^{ns}	0.02^{ns}	-0.21^{ns}	1	
x15	-0.37^{ns}	0.83**	0.81**	0.49^{ns}	0.85**	0.86**	0.93**	0.62^{ns}	0.65^{ns}	0.83*	0.72*	0.31^{ns}	0.64^{ns}	-0.81*	1
x16	-0.26^{ns}	0.75*	0.68**	0.40^{ns}	0.96**	0.94**	0.94**	0.676*	0.62^{ns}	0.77*	0.73*	0.12^{ns}	0.40^{ns}	-0.91**	0.93**
x17	-0.50^{ns}	0.92**	0.89**	0.41^{ns}	0.80**	0.84*	0.91**	0.53^{ns}	0.68*	0.87**	0.86*	0.20^{ns}	0.60^{ns}	-0.80*	0.95**
x18	-0.55^{ns}	0.35^{ns}	0.48^{ns}	-0.17^{ns}	0.24^{ns}	0.09^{ns}	0.21^{ns}	0.48^{ns}	0.40^{ns}	0.30^{ns}	0.29^{ns}	0.07^{ns}	0.82*	-0.19^{ns}	0.39^{ns}
X19	0.19^{ns}	-0.51^{ns}	-0.49^{ns}	-0.12^{ns}	-0.92**	-0.82*	-0.79*	-0.66*	-0.75*	-0.61^{ns}	-0.58^{ns}	-0.02^{ns}	-0.32^{ns}	0.71^{ns}	-0.78*
x20	-0.18^{ns}	0.47^{ns}	0.43^{ns}	0.01^{ns}	0.82*	0.65^{ns}	0.61^{ns}	0.91**	0.40^{ns}	0.57^{ns}	0.41^{ns}	0.12^{ns}	0.34^{ns}	-0.51^{ns}	0.58^{ns}
x21	-0.29^{ns}	0.73*	0.70**	0.46^{ns}	0.85**	0.85*	0.91**	0.57^{ns}	0.70*	0.77*	0.67^{ns}	0.26^{ns}	0.56^{ns}	-0.79*	0.97**
x22	-0.29^{ns}	0.88**	0.87**	0.61^{ns}	0.77*	0.88**	0.96**	0.59^{ns}	0.42^{ns}	0.67^{ns}	0.72*	0.44^{ns}	0.48^{ns}	-0.74*	0.91**
x23	-0.56^{ns}	0.89**	0.89**	0.32^{ns}	0.80*	0.82*	0.89**	0.58^{ns}	0.74*	0.82*	0.84**	0.21^{ns}	0.70*	-0.73*	0.95**
x24	-0.60^{ns}	0.80**	0.72*	0.01^{ns}	0.80*	0.79*	0.75*	0.46^{ns}	0.76*	0.86*	0.93**	-0.16^{ns}	0.38^{ns}	-0.67*	0.73*

Significance levels are denoted as: * ($P < 0.05$), ** ($P < 0.01$), and ns (non-significant, $P > 0.05$).

1: plant height (PH), 2: stem diameter (SD), 3: number of shoots, 4: shoot fresh weight, 5: shoot dry weight, 6: root fresh weight, 7: root dry weight, 8: leaf area, 9: relative water content (RWC), 10: Chlorophyll a (Chl a), 11: Chlorophyll b (Chl b), 12: carotenide, 13: superoxide dismutase (SOD), 14: catalase activity (CAT), 15: peroxidase activity (POD), 16: proline, 17: net photosynthesis rate (P_n), 18: transpiration rate (E), 19: stomatal conductance (g_s), 20: intercellular CO_2 (C_i), 21: stevioside (SVglys), 22: Rebaudioside A, 23: Total phenolic content, 24: Total flavonoide content.

3.2. Pigment Content (Chlorophyll a, b, and Carotenoids)

No significant interactive effects were observed for chlorophyll a. The treatment 40 µM L^{-1} ARA increased chlorophyll a to 1456.88 µg g^{-1} fresh weight, representing a 15.0% improvement over the control (1242.33 µg g^{-1}).

The treatment of PSL enhanced chlorophyll a to 1460.78 µg g^{-1} fresh weight, representing a 10.0% improvement over the control (1310.34 µg g^{-1}) ($F = 0.73$, $p \leq 0.05$; Fig. 3g, h, Table 1).

Chlorophyll b concentration was significantly enhanced by the combination of 25 µM L^{-1} ARA and PSL, with the maximum value reaching 584.0 µg g^{-1} fresh weight (27.0% higher than the control) ($F = 283.78$, $p \leq 0.01$; Fig. 4a, Table 1). A strong and positive correlation was obtained between the net photosynthesis rate and chlorophyll a ($r > 0.87$, $p < 0.01$) and especially between the net photosynthesis rate and chlorophyll b ($r > 0.86$, $p < 0.05$) according to the results in

Table 1. Meanwhile, total carotenoids reached a maximum of 388.33 µg g^{-1} fresh weight (6.0% higher than the control of 363.11 µg g^{-1}) at 40 µM L^{-1} ARA and PSL. For carotenoids, no significant interaction or main effects were observed ($F = 1.51$, $p > 0.05$).

3.3. RWC and Gas exchange measurements

The combined treatment of 40 mg L^{-1} ARA and PSL maintains the RWC on 84.33%, a 7.0% increase compared with the control value of 78.33 ($F = 7.1$, $p \leq 0.01$; Fig. 4b, Table 1).

Significant ARA × LRs interactions were observed for gas exchange parameters. Combined application of 40 µM L^{-1} ARA and PSL significantly promoted P_n, reaching 16.92 mol m^{-2} s^{-1} (33% higher than the control; $F = 111.86$, $p \leq 0.01$; Fig. 4c, Table 1).

The combined treatment of 25 mg L^{-1} ARA and CSL increased E to 4.40 $mmol^{-1}$ H_2O m^{-2} s^{-1}, a 25.0% increase compared with the control value of 3.30 $mmol^{-1}$ H_2O m^{-2} s^{-1} ($F = 9.61$, $p \leq 0.01$; Fig. 4d, Table 1).

This application also enhanced g_s to 0.33 mmol m^{-2} s^{-1}, which was 48.0% greater than the control (0.17 mmol m^{-2} s^{-1}) ($F = 11.72$, $p \leq 0.05$; Fig. 4e, Table 1).

The combination of 40 µM L^{-1} ARA and PSL maximized C_i at 306.33 µmol mol^{-1}, representing and 22.0% increase over the lowest value (238.66%) ($F = 47.49$, $p \leq 0.01$; Fig. 4f, Table 1).

3.4. Biochemical Defenses

The interaction between the two factors was also clearly reflected in the biochemical responses. Combined application of 40 µM L^{-1} ARA and PSL significantly enhanced proline content, reaching 24.69 mol m^{-2} s^{-1} (32.0% higher than the control; $F = 358.53$, $p \leq 0.01$; Fig. 5a, Table 1).

The combination of 0 mg L^{-1} ARA and ambient light (AL) had the lowest value in catalase (CAT) activity, decreasing 9.U mg protein min^{-1} (a 44.0% decrease compared to the control value of 17.49; $F = 1296.94$, $p \leq 0.01$; Fig. 5b, Table 1). Meanwhile, the treatment combination 40 µM L^{-1} ARA and PSL elevated superoxide dismutase (SOD) activity to 0.236 U mg protein min^{-1}, representing a 54.9% rise compared to the control (0.143 U mg protein min^{-1}; $F = 6.07$, $p \leq 0.01$; Fig. 5c, Table 1). This treatment combination also elevated Peroxidase (POD) activity to 22.95 U mg protein min^{-1}, representing a 50.0% rise compared to the control (11.50 U mg protein min^{-1}; $F = 273.98$, $p \leq 0.001$; Fig. 5d, Table 1). Peroxidase (POD) activity increased by 91.7% with 300 mg L^{-1} L-Arg and by 61.5% with 2 g L^{-1} SW (Figs. 4c, d; Table 1).

3.5. Stevioside and Rebaudioside A

The interaction between the two factors was also reflected in these parameters. Combined application of 40 µM L^{-1} ARA and PSL significantly enhanced stevioside content, reaching 5.80 percent of leaf dry matter (81.0% higher than the control; $F = 30.93$, $p \leq 0.01$; Fig. 5e, Table 1). The combination of 25 mg L^{-1} ARA and ambient light (AL) had the maximum value in rebaudioside A, increasing 1.50 (a 57.0% increase compared to the control value of 0.60; $F = 3.67$, $p \leq 0.01$; Fig. 5f .(A very strong and positive correlation was obtained between peroxidase activity and stevioside content ($r > 0.97$, $p < 0.01$) and between peroxidase activity and rebaudioside A content ($r > 0.91$, $p < 0.01$, Table 1(. A very strong and positive correlation was obtained between root dry weight and stevioside content ($r > 0.96$, $p < 0.01$) and between root dry weight and rebaudioside A content ($r > 0.88$, $p < 0.01$). Also, a strong correlation was obtained between root fresh weight and stevioside content ($r > 0.82$, $p < 0.05$) and between root fresh weight and rebaudioside A content ($r > 0.89$, $p < 0.01$, Table 1(. Results from another study on stevia showed that stronger root systems supported greater shoot/leaf biomass (fresh and dry weight), which directly increased total steviol glycoside yield. This study attributed better root development to improved plant growth and accumulation of related secondary metabolites [23].

3.6. Total phenolic and flavonoid content

In terms of phenolic content, significant interaction was observed for this parameter ($F = 13.69$, $p > 0.01$, Table 1). However, the combined application of 40 µM L^{-1} ARA and PSL significantly enhanced this parameter to 17.46 mg GAE g^{-1} FW (a 46.0% rise over the 9.47 mg GAE g^{-1} FW in the control plants. The treatment combination of 40 µM L^{-1} ARA and PSL elevated total flavonoid content to 1.35 mg QE g^{-1} DW, representing a 38.0% rise compared to the control (0.83; $F = 15.86$, $p \leq 0.01$; Fig. 5g, h, Table 1).

A very strong and positive correlation was obtained between peroxidase activity and total phenol content)$r > 0.95$, $p < 0.01$, Table 1). The tight association suggests coordinated regulation between enzymatic activity and phenolic metabolism, possibly linked to antioxidant defense or mild stress response mechanisms to physicochemical elicitors (40 µM L^{-1} ARA and PSL) in this experiment.

Also, a very strong and positive correlation was obtained between the amount of chlorophyll b and the total flavonoid content ($r > 0.93$, $p < 0.0$, $p < 0.01$, Table 1). This suggests a close relationship between photosynthetic pigment accumulation and flavonoid biosynthesis. The strong association may indicate that flavonoids play a protective role in stabilizing or supporting the photosynthetic apparatus, possibly through antioxidant activity or regulation under varying light conditions.

These results suggest that peroxidase may play a significant role in the biosynthesis or accumulation of steviol glycosides in Stevia. The strong associations indicate possible involvement of peroxidase in oxidative reactions or stress-related pathways that regulate the production of these important sweetening compounds. In another experiment in stevia, in response to salt stress through the use of chitosan as an elicitor, the amount of rebudioside A synthesis in the leaves of this plant increased, while increasing the activity of the peroxidase enzyme [24].

Table 1 presents the Pearson correlation coefficients among various morphological, physiological, and phytochemical traits of Stevia in response to arachidonic acid elicitation under different light regimes. The analysis revealed several significant positive and negative correlations, indicating strong interrelationships between plant growth parameters, physiological responses, and secondary metabolite accumulation.

4. Discussion

4.1. Interactive Effects of ARA and Pulsed Light on Stevia Vegetative Growth

The present study demonstrated a significant synergistic interaction between arachidonic acid (ARA) elicitation and pulsed supplemental LED lighting (PSL) on the vegetative growth of Stevia under vertical hydroponic cultivation. The combination of 40 μM L^{-1} ARA and PSL resulted in the highest plant height (43.44 cm, + 19%), stem diameter (0.48 mm, + 43%), leaf area (2400 cm^2, + 29%), and aerial fresh weight (159.88 g, + 28%) compared to the control.

The synergistic effect can be attributed to the complementary roles of both factors. These findings are supported by researchers, who demonstrated enhanced biomass accumulation and photosynthetic performance in leafy crops under pulsed LED regimes. Arachidonic acid, as an effective elicitor, activates defense signalling pathways that enhance plant growth and metabolic activity at low concentrations [11, 12]. A similar study found that pulsed light increased fresh biomass in baby leaf lettuce by up to 32%, dry biomass by 36%, and leaf area by 48% in lettuce compared to continuous lighting [7, 25].

The integration of these two stimuli likely optimized carbon assimilation and resource allocation toward vegetative growth, explaining the pronounced interaction observed in morphological parameters.

For shoot and root dry weights, although no significant interaction was recorded, both ARA and PSL exerted significant main effects, increasing dry matter accumulation. These findings are consistent with previous reports showing that pulsed light enhances dry biomass production in leafy crops [8]. While the ARA application promotes overall plant vigor through improved physiological performance. These effects are consistent with recent findings in which ARA treatment improved physiological traits and plant performance under both normal and stress conditions in maize [13]. In controlled environment systems such as vertical farming, the initial stages of plant development, particularly root regeneration and the establishment of high-quality seedlings, are critical determinants of final biomass and metabolic yield. Supporting this principle, our results demonstrated that both ARA and PSL significantly enhanced early root development. Application of 40 μM L^{-1} ARA increased root fresh weight by 9.0% (16.42 g) and root dry weight by 7.0% (0.80 g) compared to the control, while PSL showed even stronger effects, improving root fresh weight by 18.0% (17.64 g) and root dry weight by 9.0% (0.82 g). Therefore, despite the lack of significant interaction between the two elicitors, each alone naturally induces structure and increases in stevia seedlings grown in vertical hydroponics, demonstrating the promising potential of each.

4.2. RWC, Pigment Accumulation, and Gas Exchange Parameters

The combined application of 40 mg L^{-1} arachidonic acid (ARA) and pulsed supplementary lighting (PSL) resulted in the highest relative water content (RWC) in hydroponically grown Stevia leaves. PSL supplies energy efficiently with recovery phases, while ARA enhances membrane protection and stress signaling, making the combination highly effective for superior leaf water status in Stevia [5, 11].

The combined application of arachidonic acid (ARA) and pulsed supplemental LED lighting (PSL) significantly enhanced photosynthetic pigment contents and gas exchange parameters in *Stevia rebaudiana*. The highest chlorophyll a concentration (1456.88 μg g^{-1} FW, + 15%) was recorded in plants treated with 40 μM L^{-1} ARA + PSL, while chlorophyll b reached its maximum (584.0 μg g^{-1} FW, + 27%) under 25 mg L^{-1} ARA + PSL. Total carotenoids also increased slightly (6%) under the optimal combination. These increases in chlorophyll content are consistent with previous findings showing that ARA acts as an effective elicitor that stimulates chlorophyll biosynthesis and delays chlorophyll degradation. It seems that ARA triggers the production of reactive oxygen species (ROS) and activates key signaling molecules such as jasmonic acid (JA), salicylic acid (SA), and nitric oxide (NO). These signals upregulate transcription factors involved in stress responses and secondary metabolism, indirectly promoting the expression of genes related to chlorophyll biosynthesis [13].

Pulsed LED lighting enhances chlorophyll accumulation by optimizing light absorption and minimizing photo-oxidative stress. The intermittent light–dark cycles allow better recovery of the photosynthetic apparatus and upregulation of chlorophyll biosynthesis pathways while reducing ROS-induced pigment degradation [25]. This mechanism has been well documented in lettuce and basil, where pulsed light significantly increased chlorophyll a and b contents compared to continuous illumination [25]. While direct studies on Stevia are limited, the mechanism is transferable to C3 plants like Stevia, especially under vertical hydroponic systems.

Pulsed light allows brief dark intervals between light pulses. These intervals enable the photosynthetic electron transport chain to reset, reducing the over-reduction of photosystem II (PSII). This leads to better utilization of light energy and upregulation of genes involved in chlorophyll biosynthesis (such as those encoding 5-aminolevulinic acid synthesis and Mg-chelatase). As a result, plants produce and accumulate more chlorophyll a and b [9, 26].

A significant positive correlation was observed between the elevated chlorophyll contents and improved gas exchange parameters. The combination of 40 μM L^{-1} ARA and PSL resulted in the highest net photosynthetic rate (Pn) of 16.92 μmol m^{-2} s^{-1} (+ 33% over control), along with substantial increases in stomatal conductance (gs), transpiration rate (E), and intercellular CO_2 concentration (Ci). This strong relationship indicates that the increase in chlorophyll content enhanced light-harvesting capacity, which, together with improved stomatal behavior under pulsed light, led to higher photosynthetic efficiency. These results align with previous studies demonstrating that higher chlorophyll levels are directly associated with increased Pn in various crops [2, 5]. Furthermore, pulsed light regimes have been shown to enhance photosynthetic induction and reduce energy loss, resulting in superior gas exchange performance compared to continuous lighting [25].

4.3. Biochemical Responses and Enzyme Activities

The interaction between arachidonic acid (ARA) and pulsed LED lighting (PSL) significantly affected the biochemical responses of *Stevia rebaudiana*. The combination of 40 μM L^{-1} ARA and PSL increased proline content by 32% and boosted SOD and POD activities by 54.9% and 50%, respectively, indicating stronger antioxidant defense. In contrast, catalase (CAT) activity was lowest in control plants under ambient light.

This elevation in proline suggests an enhanced osmoprotective and antioxidant capacity under the combined treatment. Proline acts as a compatible solute that stabilizes cellular structures, scavenges reactive oxygen species (ROS), and maintains redox balance [27, 28].

Furthermore, the same treatment combination markedly enhanced the activity of key antioxidant enzymes. These increases indicate a more efficient enzymatic ROS-scavenging system, which helps protect cells from oxidative damage. Interestingly, catalase (CAT) activity was lowest in the control treatment (ambient light + 0 mg L^{-1} ARA), suggesting that the synergistic application of ARA and pulsed light helped maintain higher antioxidant defense capacity.

In baby leaf lettuce, pulsed LED light (especially at 1 kHz frequency) and significantly increased total phenolic content and antioxidant power compared to continuous light [8, 25].

4.4. Antioxidant Capacity and Secondary Metabolites Accumulation

A significant interaction between arachidonic acid (ARA) and pulsed supplemental LED lighting (PSL) markedly influenced the accumulation of SVglys and phenolic compounds in *Stevia rebaudiana*. The controlled light cycling enhances carbon availability and energy efficiency, which supports the energy-demanding biosynthesis of SVglys and flavonoids. Moreover, pulsed light and supplement frequency are known to stimulate the phenylpropanoid pathway by upregulating phenylalanine ammonia-lyase (PAL) activity, leading to higher flavonoid accumulation and overall antioxidant capacity [8, 29].

Although phenylalanine ammonia lyase (PAL) activity was not directly measured in the present study, the observed increases in total phenolic content, flavonoid accumulation, and antioxidant capacity (ABTS and FRAP) under pulsed LED light indicate stimulation of the phenylpropanoid pathway. This interpretation is supported by previous studies showing that pulsed light regimes increase PAL gene expression and enzyme activity, the rate-limiting step in the phenylpropanoid pathway, leading to increased biosynthesis of phenolic compounds and flavonoids in leafy vegetables such as lettuce [8]. Pulsed light may act as a mild abiotic stimulus and stimulate defense-related signaling cascades that contribute to the production of secondary metabolites. Similar light-mediated responses have been reported in Stevia, where specific LED spectra (blue, red-blue or complementary UV compounds) increased the accumulation of flavonoids and other phenolics under controlled conditions [30]. These findings are consistent with the results of the current experiment and suggest that the use of pulsed LED light at optimal frequencies can effectively enhance the nutritional quality and bioactivity of products without the need for additional chemicals. A similar study demonstrated that application of supplementary red and blue light effectively alleviated these negative effects by enhancing plant growth, photosynthetic performance, antioxidant capacity, and nutrient accumulation the application of supplementary red and blue light effectively alleviated these negative effects by enhancing plant growth, photosynthetic performance, and antioxidant capacity [31].

At the higher concentration (40 μM L^{-1}), ARA appears to shift metabolic flux more strongly toward SVglys biosynthesis, especially when combined with pulsed light, which provides improved carbon availability and energy efficiency. In contrast, the lower concentration (25 μM L^{-1}) under ambient light favored rebaudioside A accumulation. This suggests a dose-dependent fine-tuning effect, where milder elicitation may favor the hydroxylation and glycosylation steps leading to rebaudioside A. This elicitor-induced accumulation of SVglys is a classic defense response, as these compounds serve as protective secondary metabolites. The synergistic interaction with pulsed LED lighting further amplifies this response by optimizing photosynthetic performance and providing the necessary carbon skeletons and reducing power for secondary metabolite biosynthesis.

The differential response between SVglys (favored by ARA + PSL) and rebaudioside A (favored by ARA + ambient light) suggests that the optimal light regime for each glycoside may vary due to differences in their biosynthetic regulation and sensitivity to oxidative signals.

The superior accumulation of rebaudioside at a low arachidonic acid concentration (25 μM) combined with ambient light, in contrast to enhanced stevioside under pulsed light and higher ARA levels, can be attributed to differential activation of the steviol glycoside biosynthetic branches. Low-dose ARA likely induces a moderate oxidative signal that, under stable ambient light, optimizes energy availability for additional glucosylation steps required for rebaudiosides [32]. In contrast, pulsed light known to improve photosynthetic efficiency and create rhythmic signaling, synergizes with stronger ARA-elicited ROS to favor earlier pathway intermediates or specific UGT isoforms leading to stevioside.

This light-elicitor interaction aligns with previous findings in *Stevia rebaudiana*, where elicitors such as methyl jasmonate, chitosan, or salinity interact with light spectra to shift SG profiles [33]. Similar concentration- and light-dependent divergences have been reported with arachidonic acid in other systems, where low doses preferentially enhance complex secondary metabolites under continuous conditions, while higher doses plus pulsed/rhythmic stress favor core defense compounds.

Overall, the synergistic application of ARA and pulsed LED lighting represents an effective strategy to enhance both the yield and the quality of bioactive compounds in Stevia through the coordinated activation of defense signaling and the optimization of photosynthetic metabolism.

5. Conclusion

This study demonstrates that the combined application of arachidonic acid (ARA) and pulsed supplemental LED lighting (PSL) synergistically enhanced both vegetative growth and nutritional quality of Stevia under vertical hydroponic cultivation. The interaction significantly improved plant height, stem diameter, leaf area, biomass, photosynthetic pigments, gas exchange, antioxidant capacity, and the accumulation of steviol glycosides, phenolics, and flavonoids.

The present study provides robust empirical support for all four hypotheses through significant main effects and synergistic interactions between exogenous arachidonic acid (ARA) and pulsed supplemental LED lighting (PSL) in vertical hydroponic cultivation of Stevia. In strong support of H1 and H4, the combined application of 40 μM L^{-1} ARA and PSL produced the most pronounced synergistic improvements, yielding the highest plant height (43.44 cm, + 19%), stem diameter (0.48 mm, + 43%), leaf area (2400 cm^2, + 29%), and aerial fresh weight (159.88 g, + 28%) compared to controls. This synergy arises from complementary mechanisms in which ARA activates defense signaling pathways (via jasmonic acid, salicylic acid, and nitric oxide), enhancing metabolic activity, while pulsed light optimizes carbon assimilation through intermittent dark periods that allow photosynthetic recovery, reduce photo-oxidative stress, and improve energy utilization efficiency.

These combined stimuli resulted in superior vegetative growth, biomass partitioning, and overall physiological quality of seedlings in vertical hydroponic systems.

Supporting H2, PSL significantly outperformed ambient greenhouse light by enhancing shoot development, dry matter accumulation, photosynthetic pigments (chlorophyll a + 15%, chlorophyll b + 27%), and gas exchange parameters, achieving the highest net photosynthetic rate (16.92 μmol m^{-2} s^{-1}, + 33%) under the combined treatment. Pulsed regimes upregulated chlorophyll biosynthesis genes, prevented over-reduction of photosystem II, and improved quantum yield, thereby increasing metabolite synthesis efficiency and energy utilization compared to natural light.

In alignment with H3, ARA alone exerted strong positive main effects by promoting early root formation (root fresh weight + 9%, dry weight + 7%), boosting proline content, antioxidant enzyme activities (SOD + 54.9%, POD + 50%), and shifting metabolic flux toward secondary metabolite biosynthesis. Higher ARA concentrations (40 μM) strongly favored total steviol glycosides (SVglys) accumulation, particularly when paired with pulsed light, while lower doses (25 μM) under ambient light preferentially enhanced rebaudioside A, confirming ARA's role in improving nutrient uptake, root establishment, and bioactive compound production through controlled ROS signaling and phenylpropanoid pathway stimulation.

Overall, the integration of pulsed LED with ARA (H4) created an optimized physiological state in which enhanced photosynthesis supplied precursors and energy that ARA's elicitation efficiently channeled into both growth and defense metabolites. This approach not only improved the morphological and biochemical quality of Stevia seedlings but also enabled fine-tuning of SVglys profiles (stevioside vs. rebaudioside A) according to light regime and elicitor dose, offering a promising strategy for tailoring industrial traits in vertical hydroponic systems.

Abbreviations

LRs
Lighting Regimes
CAT
Catalase
Chl
Chlorophyll (Chl a, Chl b)
Ci
Intercellular CO2 Concentration
EC
Electrical Conductivity
E
Transpiration Rate
FW
Fresh Weight
GAE
Gallic Acid Equivalent
Gs
Stomatal Conductance
HPLC
High-Performance Liquid Chromatography
Pn
Net CO_2 Assimilation Rate
Reb A
Rebaudioside A
ROS
Reactive Oxygen Species
RWC
Relative Water Content
SD
Standard Deviation
SOD
Superoxide Dismutase
SVglys

Steviol Glycosides
ARA
Arachidonic Acid
AL
Ambient Light
CSL
Continuous Supplemental Lighting
PSL
Pulsed Supplemental Lighting
CRD
Completely Randomized Design
CSL
Continuous Supplemental Light
PAR
Photosynthetically Active Radiation
PWM
Pulse Width Modulation
DLI
Daily Light Integral
PPFD
Photosynthetic Photon Flux Density
PH
Plant Height
LA
Leaf Area
FW
Fresh Weight
DAT
Days After Transplanting
POD
Peroxidase
PAL
Phenylalanine Ammonia-Lyase
H1, H2, H3, H4
Hypotheses 1 to 4
ANOVA
Analysis of Variance
PUFA
Polyunsaturated Fatty Acid

Declarations

Ethics approval and consent to participate

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Consent for publication

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Author Contribution

NZ: designed the research, Writing – review & editing, Methodology, AM: Methodology, Data curation, SS: Methodology, review, MD: Formal analysis

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Data Availability

All data supporting the findings of this study are available within the paper and its Supplementary Information.

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Figures

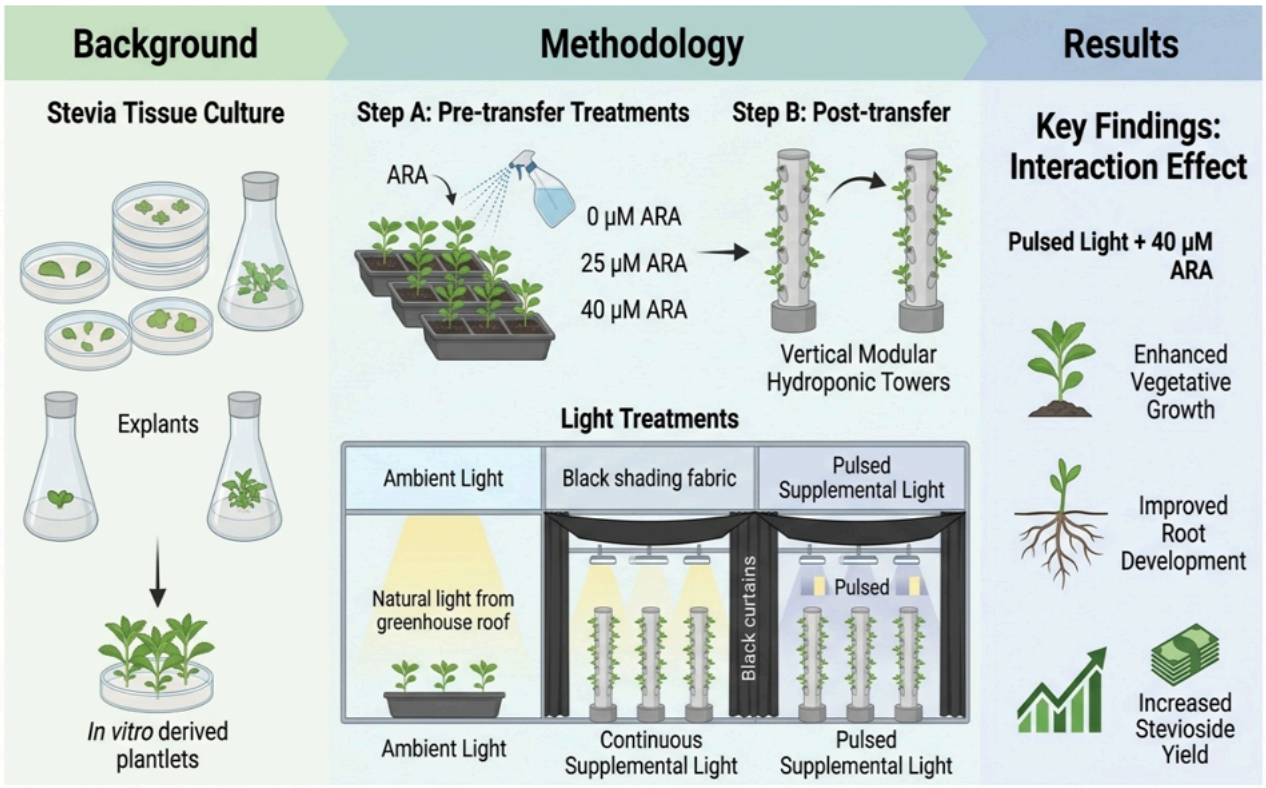

Figure 1

Graphical overview of the experimental design illustrating the factorial combinations of arachidonic acid (ARA) elicitor treatments (0, 25, and 40 µM) and light regimes (LRs) (ambient, continuous supplemental light (CSL), and pulsed supplemental light (PSL) in a vertical hydroponic system.

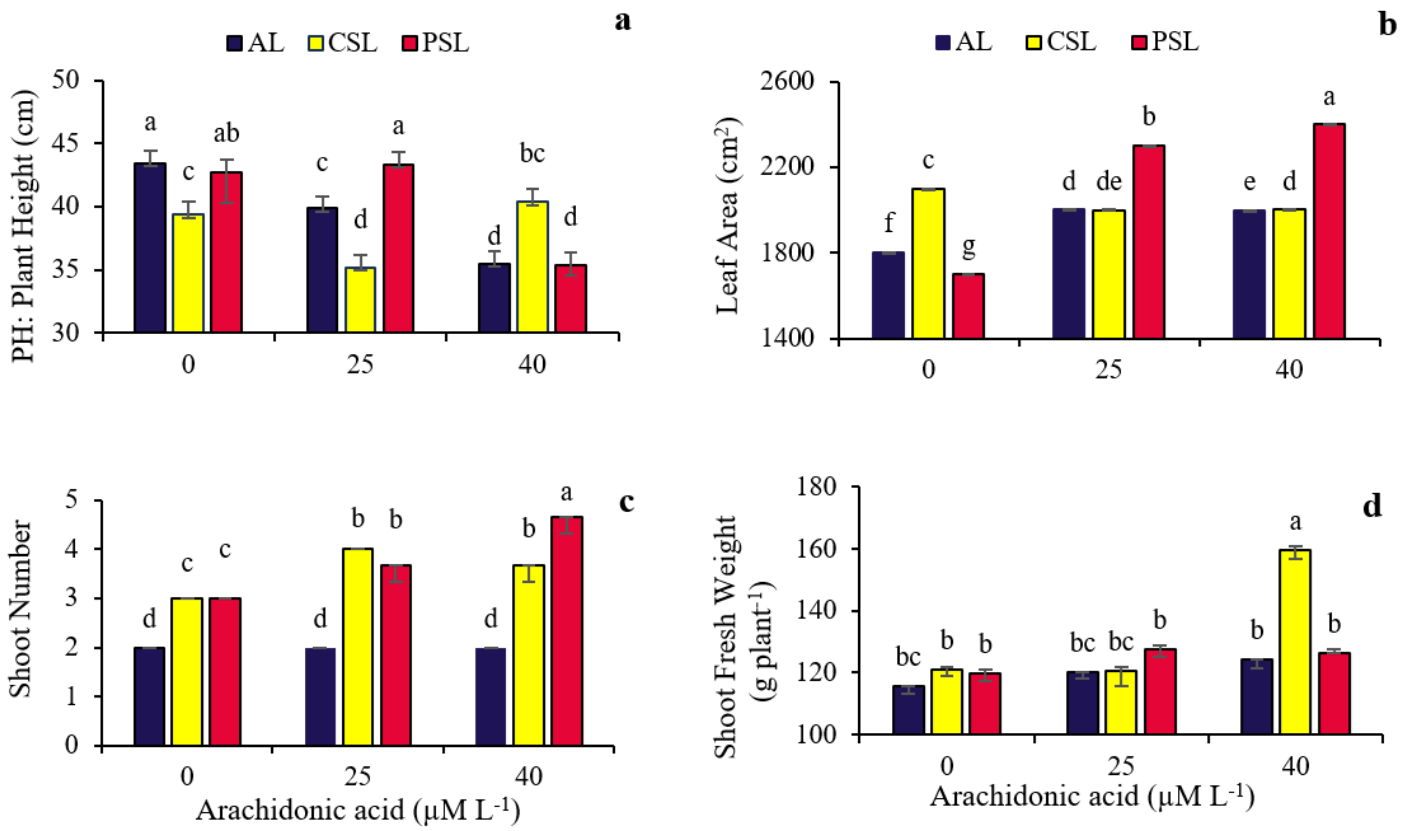

Figure 2

Interaction effects of ARA and LRs on PH: Plant height (a), on Leaf area (b), on number of shoots (c), and shoot fresh weight (d), of stevia in hydroponic conditions. Values are means of three replicates ± standard error (SE). Different letters above each bar indicate significant differences according to Duncan's Multiple Range Test ($p \leq 0.05$).

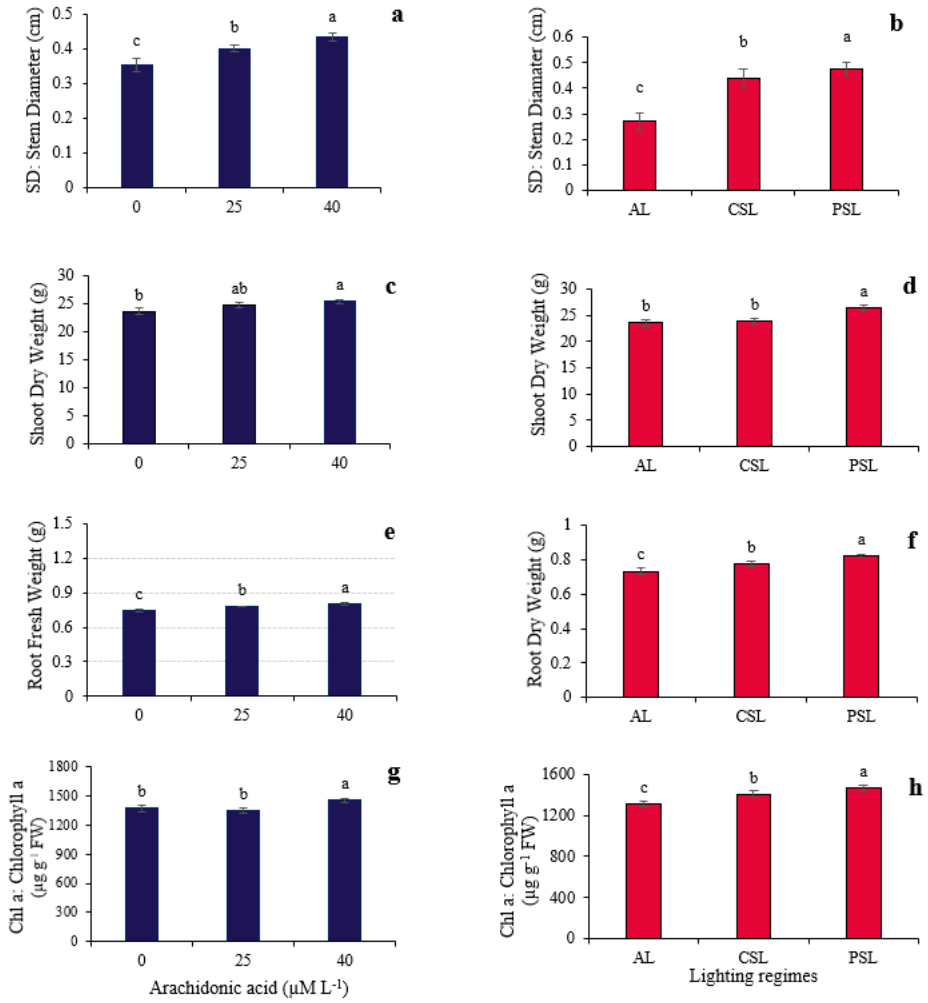

Figure 3

Main effect of ARA on stem diameter (SD)(a), main effect of LRs on stem diameter (SD)(b), main effect of ARA on shoot dry weight (c), main effect of LRs on shoot dry weight (d), main effect of ARA on root fresh weight (e), main effect of LRs on root dry weight (f), main effect of ARA on chlorophyll a (Chl a)(g), and main effect of LRs on chlorophyll a (Chl a)(h) of stevia in hydroponic conditions. Values are means of three replicates ± standard error (SE). Different letters above each bar indicate significant differences according to Duncan's Multiple Range Test ($p \leq 0.05$). (AL: Ambient lighting, CSL: Continuous supplemental lighting, PSL: Pulsed supplemental lighting).

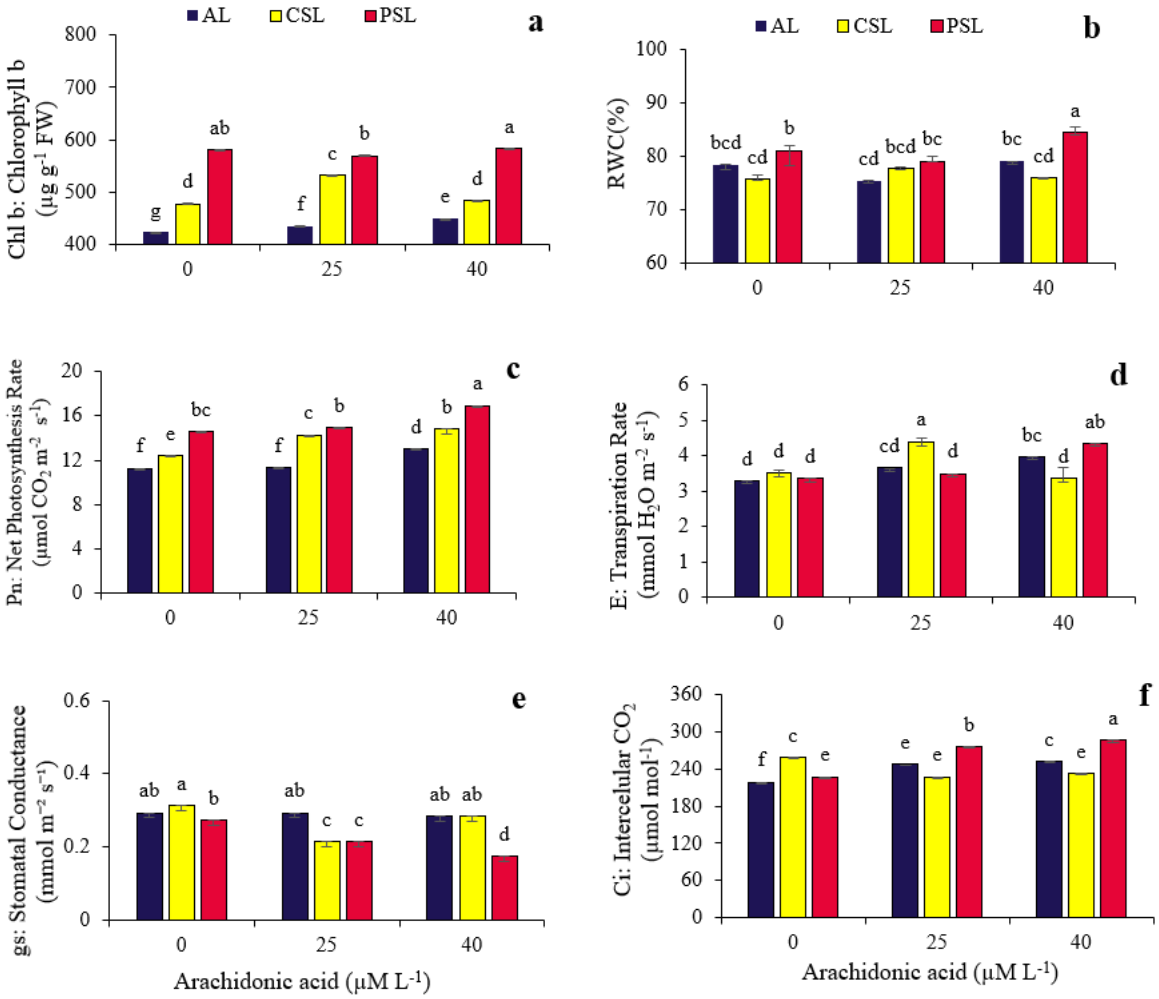

Figure 4

Interaction effects of ARA and LRs on Chl b: Chlorophyll b (a), on relative water content (RWC) (b), on P_n: Net photosynthesis rate (c), on E: Transpiration rate (d), on g_s: Leaf stomatal conductance (e), and C_i: Intercellular CO_2 (f), of stevia in hydroponic conditions. Values are means of three replicates ± standard error (SE). Different letters above each bar indicate significant differences according to Duncan's Multiple Range Test ($p \leq 0.05$).

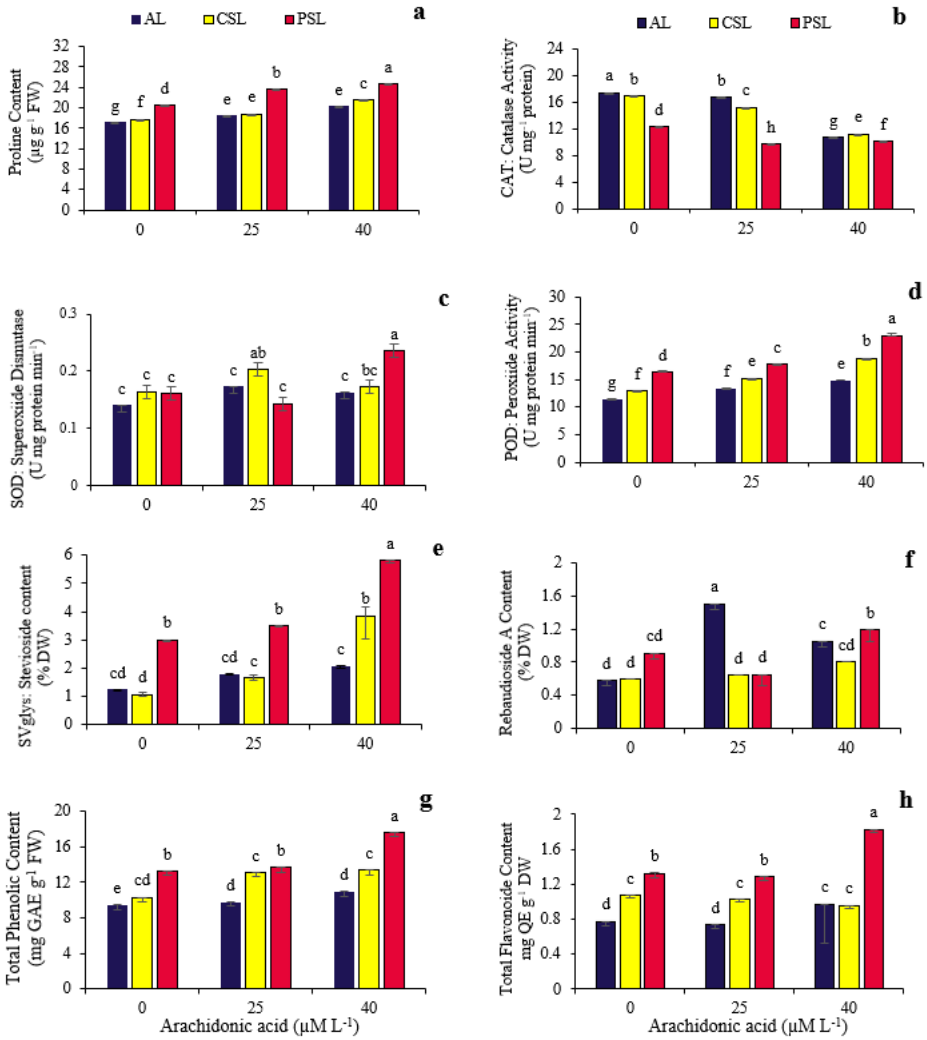

Figure 5

Interaction effects of ARA and LRs on proline content (a), on catalase activity (CAT) (b), on superoxide dismutase activity (SOD) (c), on peroxidase activity (POD) (d), on stevioside content (SVglys) (e), on rebaudioside A content (f), on total phenolic content (g), on total flavonoide content (h) of stevia in hydroponic conditions. Values are means of three replicates ± standard error (SE). Different letters above each bar indicate significant differences according to Duncan's Multiple Range Test ($p \leq 0.05$).

Supplementary Files

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- Steviadatas.xlsx