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LEDs in Plant Tissue Culture: Boosting Micropropagation of *Castanea sativa* Cultivars

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Abstract

Plant diseases and environmental stresses significantly threaten global biodiversity and agroforestry ecosystems, making traditional management practices insufficient to meet the growing demand for sustainable, high-quality plant production. In this context, the micropropagation emerges as a promising technique for producing disease-free plants; however, its application in chestnuts remains challenging due to rooting recalcitrance and genotype dependency. Recent advances in light-emitting diode (LED) technology offer a potential solution to overcome these obstacles, as specific light wavelengths can be used to optimize the micropropagation process and improve overall efficiency. This study investigates the effects of LED treatments in the *C. sativa* (cv. 'Marrone') in vitro propagation, from multiplication to rooting and acclimatization phases. Four LED treatments were tested, and results were compared to fluorescent light (used as reference). Thirty days after the beginning of the experiment, morphometric and histological analyses were carried out. Two LEDs, Red:Blue:Far-red and Red:Blue:Green:Far-red, significantly improved the multiplication index (calculated as the number of new proliferated shoots from one explant), achieving rates (from 3.9 to 4.3 shoots/explant) that were twice as many as those observed under the reference lamp (2.0 shoots/explant). A rooting induction protocol was optimized and the application of LEDs resulted in 100% rooting success compared to 70% of the reference light. Similarly, LED treatments statistically affected leaf anatomical, morphological, and physiological traits, improving survival rates during the acclimatization phase. This represents a useful protocol for improving *C. sativa* in vitro propagation; it can be easily adopted and applied by nurseries for large-scale production.

Keywords In vitro culture · Chestnut · Light treatment · Multiplication · Rooting · Acclimatization

Introduction

Climate change has significantly intensified environmental stresses and increased plant disease outbreaks, threatening biodiversity and causing significant economic losses (Singh et al. 2023). European chestnut (*Castanea sativa* Mill.), a species valued worldwide for its nuts production (Marino et al. 2024; Bianco et al. 2024), has been severely affected by *Phytophthora* spp. (Gago et al. 2022; Pavese et al. 2024) and *Cryphonectria parasitica* (Pavese et al. 2021) pathogens. Current management strategies, including the removal of infected plant tissues and the use of tolerant hybrids (*C. crenata* × *C. sativa)*, have been partially effective in controlling pathogen spread. However, hybrids often produce nuts of lower quality, particularly in terms of flavor, compared to *C. sativa* (Marino et al. 2024).

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To mitigate economic losses and ensure the sustainability of the chestnut supply chain, efficient propagation systems are necessary to guarantee the availability of high-quality *C. sativa* plants for nurseries. However, chestnut is considered a challenging species to propagate, both through seed and vegetative methods (Corredoira et al. 2017). Traditional grafting techniques (e.g., tongue, cleft, and chip budding) are widely employed in nurseries for vegetative propagation (Uyanık et al. 2022). Despite their widespread use, these techniques are often time-consuming, seasonally limited, dependent on the compatibility between the rootstock and scion, and subjected to the not-uniform and high heterozygosity of the seedling-derived rootstocks (Vieitez et al. 1987; Kunc et al. 2024). As an alternative, micropropagation represents a promising technique for large-scale production of disease-free plants. It serves as an effective tool for plant multiplication and germplasm conservation, while meeting high-quality market standards (Cruz-Cruz et al. 2013; Merkle et al. 2020; Murthy et al. 2023). Nevertheless, its application in woody species remains challenging due to genotype-dependent responses to the in vitro environment. This necessitates the optimization of several parameters, such as basal media composition, phytohormone combinations (Martínez et al. 2017), and growth conditions, to achieve high multiplication rates and high-quality plant materials. *Castanea sativa* micropropagation faces several obstacles, including low multiplication, rooting, and acclimatization rates. These challenges highlight the necessity for an optimized in vitro propagation method that nurseries can easily apply. Currently, different in vitro and ex vitro rooting protocols have been established for chestnut species; Fernandes et al. (2020a) developed a promising protocol involving pre-rooting in a charcoal-supplemented medium, followed by 2 g/L of indol-3-butyric acid (IBA) treatment in paper pots. Other studies have reported the temporary immersion system (TIS) technique (Castro-Camba et al. 2023) as beneficial to enhance biomass and rooting rates by providing continuous nutrient access (Liu et al. 2022; Nongdam et al. 2023). Lastly, Pavese et al. (2022) achieved 35% in vitro rooting in *Castanea sativa*, using a double-layer rooting induction medium enriched with 25 mg/L IBA. However, these protocols lack efficiency and need further implementation to achieve scalability and cost-effectiveness for large-scale production.

Recently, light-emitting diode (LED) technology has been successfully applied during micropropagation in both horticultural (Naznin et al. 2019) and ornamental (Shang et al. 2023) species, improving plant health and sustaining large-scale production. Therefore, modifying in vitro protocols by introducing LEDs could represent an alternative approach to potentially reduce costs and increase plant production (Nacheva et al. 2021). Compared to the commonly used fluorescent lamps, LEDs demonstrated to significantly reduce energy costs and allow for precise modulation of light intensity and emission spectrum (Dutta Gupta and Jatothu 2013; Paradiso and Proietti 2022; Sarropoulou et al. 2023). Wavelengths from 400 to 700 nm were reported to significantly influence plant photosynthesis, and in particular, Red (620–700 nm) and Blue spectra (420–500 nm) represent the two absorption peaks of chlorophyll enhancing the multiplication phase in cherry (Sarropoulou et al. 2023) and blueberry (Hung et al. 2016). In addition, their combination (*i.e.*, Red:Blue spectra) has been demonstrated to improve internode elongation and biomass in some woody species such as pear (Lupo et al. 2022) and olive (Díaz-Rueda et al. 2021). Furthermore, LEDs have the potential to enhance root development, with certain light spectrum bands, such as Red light, already showing significant effects on woody plants (Lotfi et al. 2019; Nacheva et al. 2023; Bertazza et al. 1995). The Far-red and Green components of light play an important role in shaping the shoot morphological traits of woody plants; however, information regarding their role in root development during in vitro propagation remains limited (Li et al. 2017; Li et al. 2020a, b; Wang et al. 2024; Tarakanov 2021).

LEDs were also applied to *C. sativa* seedlings, enhancing rooting and plant growth (Smirnakou and Radoglou 2016). Based on these promising results, the use of LEDs could represent an alternative for enhancing in vitro propagation, contributing to the development of a reliable and large-scale propagation protocol in woody species to meet the nurseries' demand. This study conducted in 2024 focused on boosting *C. sativa* in vitro propagation by evaluating the effect of LED spectra in multiplication, rooting, and acclimatization phases.

Materials and Methods

Light Design and Plant Material

Four different LED types were selected to evaluate their effect on *C. sativa* plant growth: Hot White (HW), Red:Blue (RB), Red:Blue:Far-red (RBFr), and Red:Blue:Green:Far-red (RBGFr). These combinations were chosen based on existing literature demonstrating the effectiveness of Red:Blue wavelengths in enhancing both multiplication and rooting phases in several plant species (Gupta and Jatothu 2013), while the addition of Green and Far-red light aimed to investigate their potential influence in shoot morphology and root induction (Tan et al. 2022; Wu et al. 2024; Li et al. 2021). The objective was to select a single LED spectrum capable of supporting all stages of in vitro propagation, from multiplication to acclimatization thus offering a scalable and practical solution for nursery applications. LED lamps

were designed to emit the desired light spectra (Prisma Srl, Turin, Italy). The HW spectrum featured broad continuous emission across the photosynthetically active radiation range (400–700 nm), while the other spectra combined LEDs with specific monochromatic emissions: Blue (peak at 450 nm), Red (peak at 660 nm), Green (peak at 530 nm), and Far-red (peak at 730 nm). Spectral characteristics and color ratios were calculated according to the methodology described by Kim et al. (2004) and are presented in Fig. 1 and Table 1. Fluorescent Light (FL), being the most popular one used in tissue culture, was used as a reference (L58 CoolWhite, Osram, Germany). Cultures were maintained under a photoperiod of 16 h light and 8 h darkness. The photosynthetic photon flux density (PPFD) was checked with a photosynthetically active radiation meter (Model: PG200N; UPRtek, Seoul, South Korea) and per all treatments equaled 60 ± 2 µmol/sm^2.

Seventy-five in vitro shoots of *C. sativa* (cv. 'Marrone'), coming from the establishment done by Pavese and co-authors (2022) and uniform in length (3 cm), were used as starting material for the experiment. In vitro establishment was carried out using nodal explants, surface sterilized, and cultured on MS3B media (MS medium with half-strength NH_4NO_3 and KNO_3) (Duchefa Biochemie, Haarlem, The Netherlands) supplied with 0.5 mg/L benzylaminopurine (BAP) and 100 mg/L ascorbic acid to reduce polyphenol exudation.

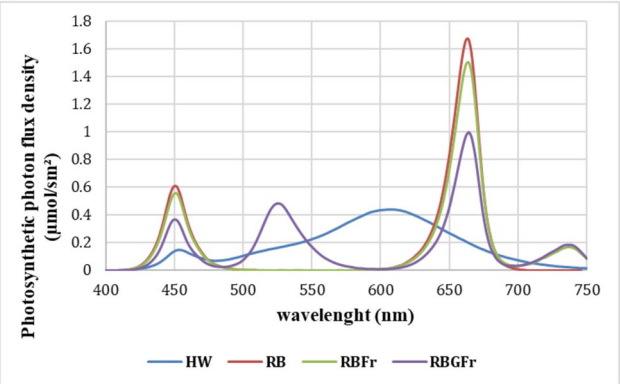

Fig. 1 Spectral distribution of relative photosynthetic photon flux density for the four LEDs used for experimental purposes. Abbreviations used are as follows: HW—Hot White; RB—Red:Blue; RBFr—Red:Blue:Far-red; RBGFr—Red:Blue:Green:Far-red (Color figure online)

Multiplication Phase

The selected plant material had undergone multiple subcultures in a full-strength MS3B multiplication medium (Duchefa Biochemie, Haarlem, The Netherlands) supplemented with 30 g/L sucrose, 8 g/L agar, 100 mg/L ascorbic acid, 0.1 mg/L benzylaminopurine (BAP) and 0.05 mg/L indole-3-butyric acid (IBA), adjusting the pH to 5.6 (Pavese et al. 2022). Shoots were maintained at 16/8 h light/dark photoperiod, 25 °C temperature, and 70% relative humidity for 30 days.

To evaluate the effect of light treatments on *C. sativa* shoot development, shoot growth (mm) and number (No.) of new leaves were measured after 30 days in multiplication phase (T30). Shoot growth measurements were carried out using a digital caliper (Digimatic Caliper, Mitutoyo, Japan) and, for each explant, the newly developed shoots were counted to assess the multiplication index.

The fresh (FW) and dry weights (DW) were also determined. To this extent, five shoots per treatment were collected at T30, and their weight was calculated through an analytical balance (Model: W3200-3200-E; Merck Group, Darmstadt, Germany) before (FW) and after (DW) drying them in an oven at 100 ± 2 °C for 24 h, until a constant mass was achieved. Furthermore, before drying the shoots, three leaves per shoot (per treatment) were scanned at 600-dpi resolution with a scanner (Model: CanoScan LiDE 300; Canon Ltd., London, UK). As previously done by other authors while dealing with image analysis (Mozzanini et al. 2023a; 2023b), pictures were used to measure the leaf area for each treatment through ImageJ software (https://imagej.net/ij/index.html; version: 1.54 g). Specific Leaf Area (SLA; cm^2/g) was calculated as the ratio between leaf area (cm^2) and leaf dry weight (g).

Leaf Anatomy and Stomata Structure Analysis

The anatomical structure of leaves and stomata morphology exposed to light treatments (T30) were analyzed. First, for leaf anatomy structure analysis, five expanded leaves, for each treatment, were collected from the apex at the first internode and fixed in 70% alcohol with 3% acetic acid-alcohol (Merck, Darmstadt, Germany) for 48 h, and then embedded in isoparaffin. Microtome sections of leaves were taken perpendicularly to the leaf axis at 12 µm thickness with a rotatory microtome (Leica Microsystems, Wetzlar, Germany). Sections were hydrated and stained with a mixture of 0.1% safranin O (Carlo Erba, Milan, Italy) and 0.1% fast green FCF (0.1%)

Table 1 Ratios of different light color bands within the spectra used for experimental purposes

Hot White	Red:Blue	Red:Blue:Far-red	Red:Blue:Green:Far-red	Fluorescent
R:B:G	R:B:G	R:B:G	R:B:G	R:B:G
5:1:2.5	3:1:0	3:1:0 + 10% Fr	3:1:2 + 10% Fr	2.3:1:2.3

(Serva, Heidelberg, Germany) for 1 h at room temperature (Moreno-Sanz et al. 2020). After three washes in distilled water, the sections were dehydrated and mounted with isoparaffin (Girelli Alcool, Milan, Italy), and images were captured using the B-190 Series OPTIKA microscope (OPTIKA, Bergamo, Italy). Pictures were then analyzed via ImageJ software (https://imagej.net/ij/index.html; version: 1.54 g).

Second, for the stomatal density analysis, other five leaves (for each treatment) were sampled as above mentioned and analyzed through the nail polish method (Meeus et al. 2020). The epidermal imprints of the abaxial side were performed using transparent nail polish, and after 30 min, the thin film was peeled off and mounted on a microscope slide. Images were taken using the microscope B-190 Series OPTIKA microscope (Italy), and ImageJ software (https://imagej.net/ij/index.html; version: 1.54 g) was used to evaluate the stomatal density (No. of stomata/mm^2), length (µm), and width (µm).

Rooting Phase

After the multiplication phase (T30), shoots were subjected to rooting. The rooting phase was performed according to a modified version of the double-layer rooting technique described by Pavese et al (2022). First, shoots were exposed to extended treatment with 25 mg/L IBA for seven days (Vieitez et al. 2007) and then were transferred to a hormone-free expression medium supplemented with 0.05% activated charcoal. After six weeks in culture, corresponding to 72 days from the beginning of the experiment (T72), rooting percentage (%) and number of roots/explant were recorded, and mean root length (mm) was measured using a digital caliper (Digimatic Caliper, Mitutoyo, Japan). Rooting percentage was calculated as the number of explants with at least one visible root divided by the total number of cultured shoots, multiplied by 100.

Acclimatization

After the rooting phase (T72)**,** shoots were collected and subjected to acclimatization. Briefly, roots were cleaned to remove the adhering agarized medium and transferred to jars containing a sterile peat:perlite: vermiculite (1:1:1) mixture (Fernandes et al. 2020a). After sealing the jars with a plastic film layer, to maintain high humidity, they were kept in the growth chamber for twenty days (T92) at 25 ± 2 °C, 16/8 h light/dark photoperiod. To maintain consistency throughout the experimental process, each explant was acclimatized under the same light treatments (Table 1) used during the in vitro micropropagation phases (*i.e.*, multiplication and rooting phases). After ten days of acclimatization (T82), the plastic film was gradually perforated to allow plant adaptation to the external environment. Plants were then maintained for ten more days (T92) and then transplanted in pots (7 cm diameter, 0.27 L volume) containing peat:perlite:vermiculite (1:1:1) mixture. At this stage, the acclimatization survival rate was registered, and plants in pots were placed in a greenhouse at 25 ± 2 °C and 80% relative air humidity.

Maximum Quantum Efficiency and Spectral Reflectance Signature Measurements

To assess the plants' health status following the acclimatization phase (T92), the maximum quantum efficiency and spectral reflectance signatures were analyzed. To this extent, the second fully expanded leaf from the apex of transplanted plants was used. In all cases, three biological replicates were performed.

To analyze the maximum quantum efficiency, after 30 min of dark acclimation of the selected leaf, the chlorophyll fluorescence parameters were obtained through a continuous excitation chlorophyll fluorometer (Handy PEA +, Hansatech, UK) to measure the ground (F_0) and maximum (Fm) chlorophyll fluorescence. The maximum quantum efficiency (Fv/Fm) of the Photosystem II (PSII) photochemistry was then calculated using the following equation:

$$Fv/Fm = (Fm - Fo)/Fm$$

The spectral reflectance signatures were then measured using a spectrometer (Model: RS-5400, Spectral Evolution, Canada). The following spectral bands were considered:

Wavelength Band 1 (B1): 430–450 nm
Wavelength Band 2 (B2): 450–550 nm
Wavelength Band 3 (B3): 520–600 nm
Wavelength Band 4 (B4): 645–680 nm
Wavelength Band 5 (B5): 690–720 nm
Wavelength Band 6 (B6): 730–750 nm
Wavelength Band 7 (B7): 770–800 nm

The indices analyzed included.

- Anthocyanin reflectance Index (ARI)

$$ARI = \left(R_{B5} - R_{B3}\right) / \left(R_{B5} + R_{B3}\right)$$

- Chlorophyll index (CI)

$$CI = \left(R_{B7} / R_{B3}\right) - 1$$

- Carotenoid reflectance index (CRI)

$$CRI = \left(R_{B5} / R_{B2}\right)$$

- Plant senescence reflectance index (PSRI)

$$PSRI = \left(R_{B4} - R_{B2}\right) / R_{B6}$$

- Green normalized difference vegetation index (GNDVI)

$$GNDVI = \left(R_{B7} - R_{B3}\right) / \left(R_{B7} + R_{B3}\right)$$

- Normalized difference vegetation index (NDVI)

$$NDVI = \left(R_{B7} - R_{B5}\right) / \left(R_{B7} + R_{B5}\right)$$

- Normalized pigment chlorophyll index (NPCI)

$$NPCI = \left(R_{B4} - R_{B1}\right) / \left(R_{B4} + R_{B1}\right)$$

Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistic (Version 29) predictive analytical software for Windows. A one-way ANOVA was used to establish the effects on the dependent variable evaluated. Means were compared using a Duncan *post-hoc* test for multiple comparisons ($p < 0.05$).

Results

Multiplication Phase

Castanea sativa shoot multiplication (T30) was strictly influenced by the different light treatments tested (Table 2). Regarding the shoot growth and the number of new leaves, no significant differences were observed among the five light treatments. Nevertheless, RB LED, with respect to the other treatments, resulted in the least shoot elongation and the lower number of newly developed leaves. The multiplication index showed a statistically significant difference among light treatments with a wide range of new shoots/explants from FL to RBGFr LEDs. The RBFr (Fig. 2d) and RBGFr (Fig. 2e) did not significantly differ from each other but outperformed the other treatments in multiplication index (FL (Fig. 2b), RB (Fig. 2c), and HW (Fig. 2a)). Furthermore, RBGFr and RBFr treatments showed a significant increment in biomass with values equal to 964 ± 34.0 mg and 914 ± 23.9 mg, respectively, compared to the other light treatments. Moreover, a statistical reduction of the SLA parameter was obtained with RBGFr and RBFr, compared to the light treatments HW and FL.

Leaf Anatomy and Stomata Structure Analysis

The anatomy of *C. sativa* leaves showed marked responses to different light treatments, particularly in the morphology of the leaf lamina and its components (mesophyll, palisade parenchyma, spongy parenchyma, and adaxial

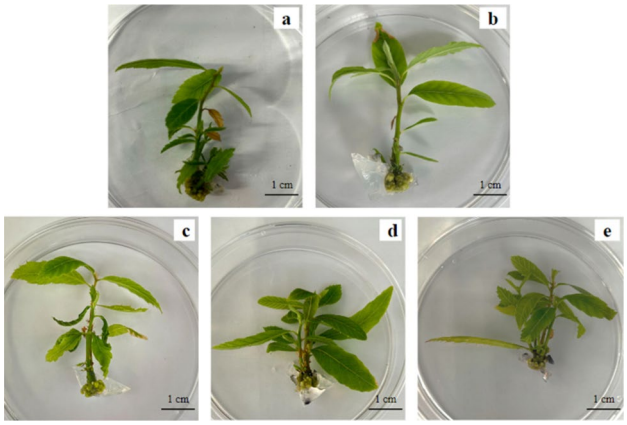

Fig. 2 *Castanea sativa* in vitro shoots grown under different LED lights and fluorescent lamps (T30). **a** Hot White (HW); **b** Fluorescent (FL); **c** Red:Blue (RB); **d** Red:Blue:Far-red (RBFr); **e** Red:Blue:Green:Far-red (RBGFr)

Table 2 Growth and morphological parameters of in vitro chestnut grown under different light treatments (T30)

Light treatment/parameter	HW	FL	RB	RBFr	RBGFr	Sign[a]
Shoot growth (mm)	6.0 ± 2.0	11.7 ± 2.8	4.5 ± 1.1	9.1 ± 1.9	10.1 ± 1.5	N.S
No. of new leaves	1.9 ± 0.4	2.6 ± 0.8	0.5 ± 0.3	1.6 ± 0.5	1.4 ± 0.4	N.S
Multiplication index	2.4 ± 0.2^{a}	2.0 ± 0.2^{a}	2.2 ± 0.4^{a}	3.9 ± 0.4^{b}	4.3 ± 0.5^{b}	***
Plant FW (mg)	352 ± 41.2^{a}	346.6 ± 31.8^{a}	453.7 ± 32.1^{a}	914 ± 23.9^{b}	964 ± 34.0^{b}	***
Plant DW (mg)	44.2 ± 5.2^{a}	38.3 ± 3.5^{a}	41.1 ± 2.9^{a}	74.4 ± 1.9^{b}	78.4 ± 2.7^{b}	***
SLA (cm^2/g)	185.4 ± 39.2^{c}	169.5 ± 21.3^{bc}	116.4 ± 6.7^{ab}	102.55 ± 13.6^{a}	87.5 ± 9.7^{a}	***

HW Hot White, *FL* Fluorescent, *RB* Red:Blue, *RBFr* Red:Blue:Far-red, *RBGFr* Red:Blue:Green:Far-red

Shoot growth (mm), number of new leaves, multiplication index, plant fresh (FW) and dry (DW) weight, and SLA (Specific Leaf Area) parameter (mean ± standard error of the mean) are reported. Different letters indicate significant differences per considered parameter ($p < 0.05$; Duncan *post-hoc* test)

[a]Statistical significance levels: N.S. $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

and abaxial epidermis) (Table 3). Significant values were noted in leaf thickness: leaves grown under FL (Fig. 3b) and RBFr (Fig. 3d) showed the highest values for overall leaf thickness (84.58 ± 3.55 µm and 84.15 ± 0.49 µm, respectively), followed by HW (Fig. 3a), RB (Fig. 3c) and RBGFr (Fig. 3e). A similar trend was observed for mesophyll thickness; FL (Fig. 3b) and RBFr (Fig. 3d) drove to thicker mesophyll layers with values equal to 61.20 ± 2.77 µm and 59.08 ± 0.47 µm with respect to HW (Fig. 3a), RBGFr (Fig. 3e), and RB (Fig. 3c).

Regarding the palisade parenchyma, it was reported a remarkable variability in cell morphology and organization among the light treatments. RB (Fig. 3c) showed the highest palisade parenchyma thickness (18.58 ± 1.02 µm) with densely packed cells. HW (Fig. 3a) presented thick palisade parenchyma constituted by a single layer of closely packed cylindrical cells. FL-treated leaves (Fig. 3b) exhibited single-layered palisade parenchyma with small, nearly cylindrical cells and visually exhibited more intercellular spaces. In contrast, leaves grown under RBFr (Fig. 3d) and RBGFr (Fig. 3e) treatments resulted in the thinnest palisade layers, with cells appearing in both cases thickened and tightly packed.

Regarding the spongy parenchyma architecture, RBFr (Fig. 3d) led to a thick and compact spongy tissue (46.68 ± 0.70 µm) with few intercellular spaces, followed

Table 3 Leaf anatomical parameters of the in vitro chestnut grown under different light treatments (T30)

Light treatment/thickness (µm)	HW	FL	RB	RBFr	RBGFr	Sign[a]
Leaf lamina	79.80 ± 2.91^{b}	84.58 ± 3.55^{b}	70.25 ± 1.50^{a}	84.15 ± 0.49^{b}	69.10 ± 2.26^{a}	***
Mesophyll	55.17 ± 2.16^{b}	61.20 ± 2.77^{c}	47.27 ± 1.40^{a}	59.08 ± 0.47^{bc}	48.99 ± 1.30^{a}	***
Palisade parenchyma	17.91 ± 0.85^{b}	16.76 ± 1.25^{b}	18.58 ± 1.02^{b}	12.40 ± 0.31^{a}	12.81 ± 0.85^{a}	***
Spongy parenchyma	37.26 ± 1.79^{b}	44.43 ± 3.04^{c}	28.69 ± 0.99^{a}	46.68 ± 0.70^{c}	36.18 ± 1.49^{b}	***
Adaxial epidermis	13.25 ± 0.56^{bc}	12.16 ± 0.54^{ab}	12.56 ± 0.60^{abc}	14.27 ± 0.61^{c}	11.15 ± 0.67^{a}	*
Abaxial epidermis	11.38 ± 0.67^{b}	11.23 ± 0.76^{b}	10.42 ± 0.48^{ab}	10.80 ± 0.34^{b}	8.96 ± 0.64^{a}	*

HW Hot White, *FL* Fluorescent, *RB* Red:Blue, *RBFr* Red:Blue:Far-red, *RBGFr* Red:Blue:Green:Far-red

Leaf lamina (µm), mesophyll (µm), palisade parenchyma (µm), spongy parenchyma (µm), adaxial epidermis (µm), and abaxial epidermis (µm) (mean ± standard error of the mean) are reported. Different letters indicate significant differences per parameter tested ($p < 0.05$; Duncan *post-hoc* test)

[a]Statistical significance levels: N.S. $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

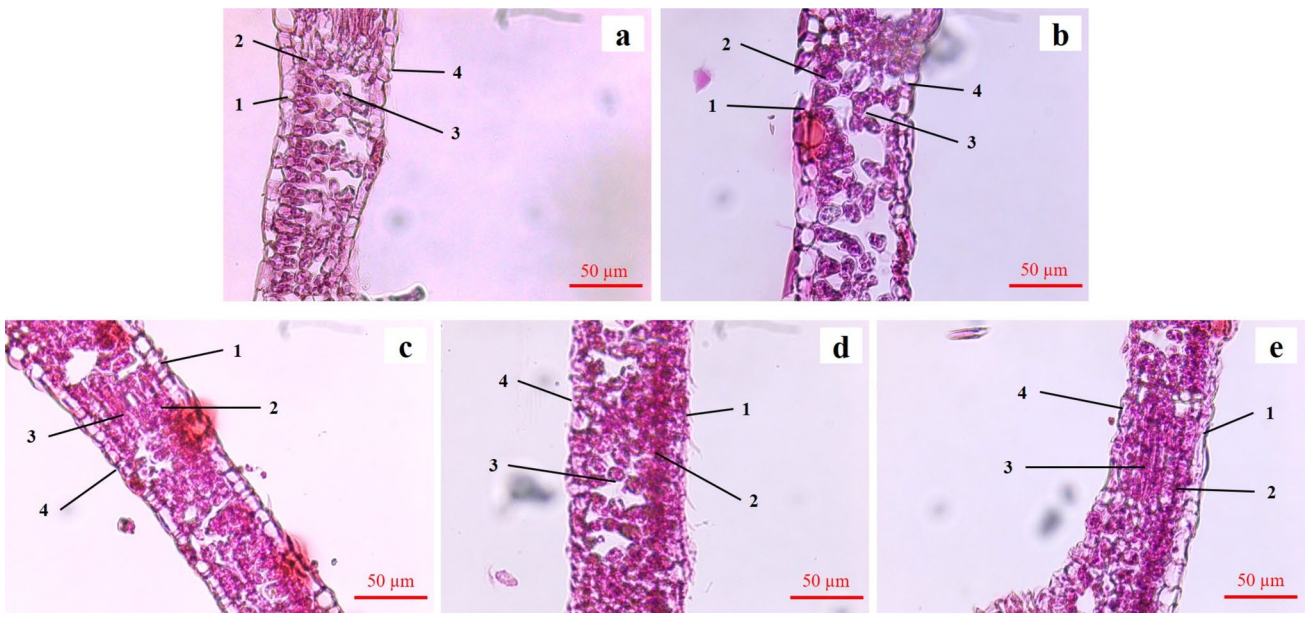

Fig. 3 Leaf anatomy of chestnut grown in vitro under different light treatments (T30). **a** Hot White (HW); **b** Fluorescent (FL); **c** Red:Blue (RB); **d** Red:Blue:Far-red (RBFr); **e** Red:Blue:Green:Far-red (RBGFr). The numbers used are as follows: (1) Adaxial epidermis. (2) Palisade parenchyma. (3) Spongy parenchyma. (4) Abaxial epidermis. The scale bar is 50 µm (40×)

by FL (Fig. 3b), which presented conspicuous intercellular spaces between the cells. HW (Fig. 3a) presented spongy parenchyma with global cells characterized by the presence of intercellular spaces, while RBGFr (Fig. 3e) developed a denser spongy parenchyma composed of round cells with few intercellular spaces. RB (Fig. 3c) drove to the thinnest spongy parenchyma (28.69 ± 0.99 µm), with round cells.

Epidermal thickness showed a similar trend among the different light treatments. RBFr drove to obtain the thickest adaxial epidermis, (14.27 ± 0.61 µm), while the HW and FL induced the thickest abaxial epidermis (11.38 ± 0.67 µm and 11.23 ± 0.76 µm, respectively). In contrast, RBGFr treatment resulted in the thinnest epidermis on both surfaces.

The stomata size was also significantly affected by the light treatments and showed an elliptical/round morphology (Table 4). Significant statistical differences were observed regarding the stomatal length: RBFr (Fig. 4d) produced the maximum stomatal length (32.49 ± 0.64 µm), followed by RBGFr (Fig. 4e), RB (Fig. 4c), while the shorter stomata were observed under FL (Fig. 4b) and HW (Fig. 4a).

Stomatal width showed a similar pattern. In fact, the widest stomata were detected under RBGFr (Fig. 4e), followed by RBFr (Fig. 4d), RB (Fig. 4c), and HW (Fig. 4a), whereas FL (Fig. 4b) showed the narrowest values. The stomatal frequency parameter did not show statistically significant differences among light treatments.

Rooting Phase

The effect of different light treatments on *C. sativa* rooting (T72) was evaluated through rooting percentage, number of roots/explant, and root length (Table 5). All the assessed

Table 4 Leaf stomatal parameters of the in vitro chestnut plants grown under different light treatments (T30)

Light treatment/parameter	HW	FL	RB	RBFr	RBGFr	Sign.[a]
Stomata length (µm)	26.16 ± 0.62[b]	21.25 ± 0.38[a]	30.17 ± 0.85[c]	32.49 ± 0.64[d]	30.25 ± 0.86[c]	***
Stomata width (µm)	20.72 ± 0.50[b]	15.75 ± 0.39[a]	22.03 ± 0.58[bc]	23.08 ± 0.33[c]	24.57 ± 0.59[d]	***
Stomata frequency (No./mm^2)	218.12 ± 5.1	233.34 ± 5.1	152.18 ± 15.2	221.1 ± 29.16	180.21 ± 32.38	N.S

HW Hot White, *FL* Fluorescent, *RB* Red:Blue, *RBFr* Red:Blue:Far-red, *RBGFr* Red:Blue:Green:Far-red

Stomata frequency, length (µm), and width (µm) (mean ± standard error of the mean) are reported. Different letters indicate significant differences per considered parameter ($p < 0.05$; Duncan *post-hoc* test)

[a]Statistical significance levels: N.S. $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

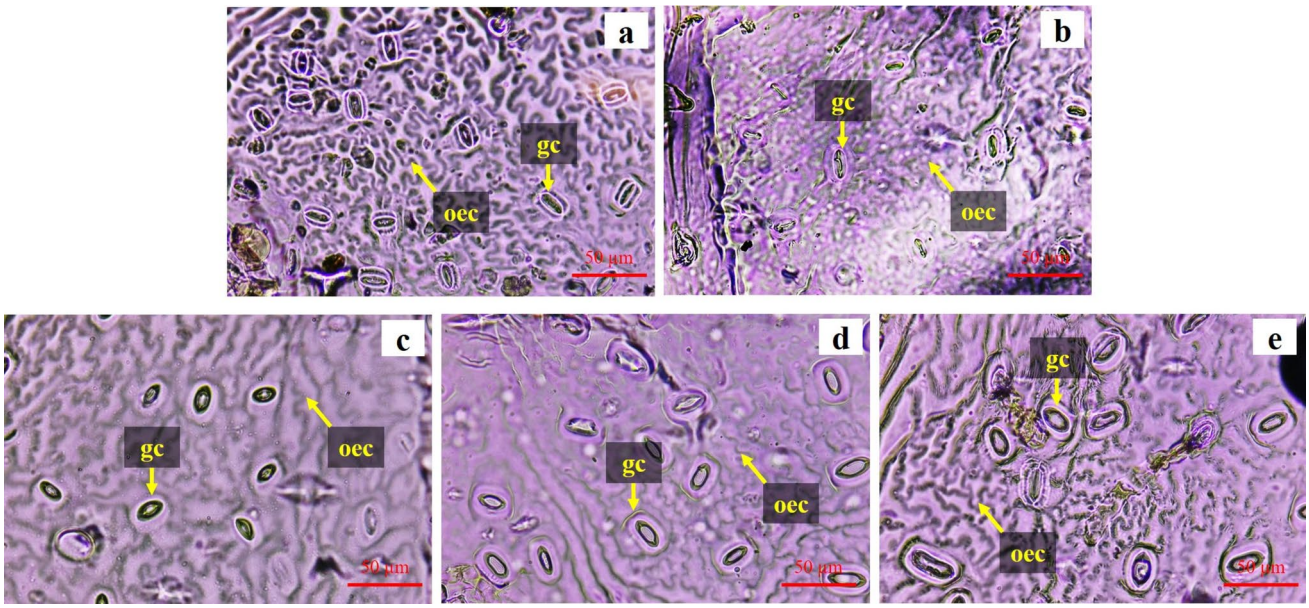

Fig. 4 Leaf epidermis and stomata of the in vitro chestnut plants grown under different light treatments. (T30) **a** Hot White (HW); **b** Fluorescent (FL); **c** Red:Blue (RB); **d** Red:Blue:Far-red (RBFr); **e** Red:Blue:Green:Far-red (RBGFr). In detail, "oec" stands for ordinary epidermal cells, whereas "gc" for guard cells. The scale bar is 50 µm (40×)

Table 5 Rooting parameters of the in vitro chestnut plants grown under different light treatments (T72)

Light treatment/parameter	HW	FL	RB	RBFr	RBGFr	Sign[a]
Rooting percentage	60%[a]	70%[ab]	60%[a]	100%[b]	100%[b]	*
No. of roots/explant	1.2 ± 0.4[a]	1.3 ± 0.4[a]	1.4 ± 0.5[a]	2.6 ± 0.3[b]	3.2 ± 0.7[b]	*
Mean roots length (mm)	29.4 ± 9.1[a]	71.0 ± 10.9[a]	47.3 ± 15.6[ab]	102.6 ± 11.4[c]	74.4 ± 11.9[bc]	**

HW Hot White, *FL* Fluorescent, *RB* Red:Blue, *RBFr* Red:Blue:Far-red, *RBGFr* Red:Blue:Green:Far-red

Rooting percentage, roots number/explant, and roots length (mean ± standard error of the mean) are reported. Different letters indicate significant differences per parameter tested ($p < 0.05$; Duncan *post-hoc* test)

[a]Statistical significance levels: N.S. $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

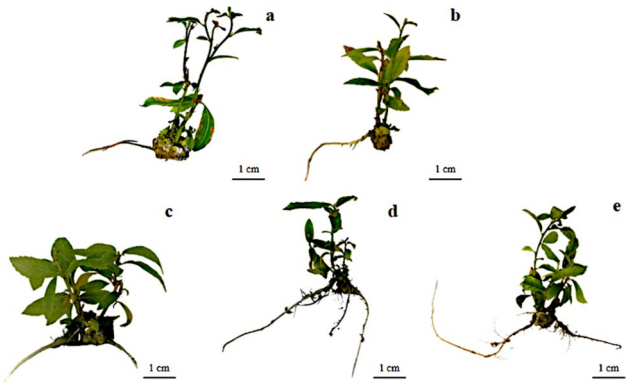

Fig. 5 Rooting of in vitro chestnut plants grown under different light treatments (T72). **a** Hot White (HW); **b** Fluorescent (FL); **c** Red:Blue (RB); **d** Red:Blue:Far-red (RBFr); **e** Red:Blue:Green:Far-red (RBGFr)

Fig. 6 Chestnut plants acclimatized and grown under different light treatments (T92) before transplanting. **a** Hot White (HW); **b** Fluorescent (FL); **c** Red:Blue (RB); **d** Red:Blue:Far-red (RBFr); **e** Red:Blue:Green:Far-red (RBGFr)

parameters were statistically significant between the light treatments. In detail, the RGBFr and RBFr LEDs promoted rooting formation, achieving a 100% rooting rate compared to the other light treatments. Concerning the number of roots/explant, RBGFr (Fig. 5e) and RBFr (Fig. 5d) were more capable to promote the rooting response (3.2 ± 0.7 and 2.6 ± 0.3, respectively) compared to RB (Fig. 5c), FL (Fig. 5b), and HW (Fig. 5a). Finally, regarding root length, RBFr (Fig. 5d) proved to be the most effective among the treatments tested followed by RBGFr (Fig. 5e). On the other hand, FL (Fig. 5b), RB (Fig. 5c), and HW (Fig. 5a) treatments resulted in a significant reduction in root growth (*i.e.*, characterized by fewer and small roots).

Acclimatization and Assessment of Plant Physiological Status

After the rooting phase, 100% of the RBGFr (Fig. 6e) and RBFr (Fig. 6d) rooted plants were successfully acclimatized. Lower values were observed for FL (Fig. 6b), RB (Fig. 6c), and HW (Fig. 6a) (71.4%, 57.1%, and 16.7%, respectively). The plant health was estimated through physiological parameter evaluation (T92) (Table 6). No statistical differences among light treatments were observed referring to the Photosystem II (PSII) parameters. Despite this, a maximum efficiency value of PSII higher than 0.8 was obtained per treatment. This result confirmed the health status of all the *C. sativa* plants. The Anthocyanin Reflectance Index (ARI) showed higher values in HW, FL, and RB compared to RBFr and RBGFr, indicating a significant effect of light treatments. Chlorophyll Index (CI) showed a similar pattern to the ARI index, recording a higher value in HW, followed by RB, FL, RBGFr, and RBFr. The Chlorophyll Reflectance Index (CRI) remained consistent across the treatments, with no statistical significance. The CI is correlated with the

Table 6 Spectral parameters of leaves measured under different light treatments (T92)

Light treatment/ index	HW	FL	RB	RBFr	RBGFr	Sign[a]
ARI	1.15 ± 0.02^{b}	1.18 ± 0.02^{b}	1.16 ± 0.02^{b}	1.11 ± 0.02^{a}	1.10 ± 0.02^{a}	**
CI	2.05 ± 0.12^{b}	1.84 ± 0.12^{b}	1.91 ± 0.18^{b}	1.14 ± 0.09^{a}	1.18 ± 0.09^{a}	***
CRI	2.65 ± 0.20	2.69 ± 0.08	2.43 ± 0.11	2.57 ± 0.12	2.64 ± 0.11	N.S
PSRI	-0.03 ± 0.002^{b}	-0.04 ± 0.002^{b}	-0.04 ± 0.003^{b}	-0.05 ± 0.002^{a}	-0.05 ± 0.003^{a}	***
GNDVI	0.50 ± 0.02^{b}	0.47 ± 0.02^{b}	0.46 ± 0.03^{b}	0.36 ± 0.02^{a}	0.36 ± 0.02^{a}	***
NDVI	0.44 ± 0.02^{b}	0.40 ± 0.01^{b}	0.40 ± 0.03^{b}	0.31 ± 0.01^{a}	0.32 ± 0.01^{a}	***
NPCI	-0.04 ± 0.02	-0.05 ± 0.01	-0.05 ± 0.01	-0.02 ± 0.01	-0.01 ± 0.02	N.S
Fv/Fm	0.83 ± 0.004	0.83 ± 0.002	0.82 ± 0.003	0.83 ± 0.002	0.83 ± 0.001	N.S

HW Hot White, *FL* Fluorescent, *RB* Red:Blue, *RBFr* Red:Blue:Far-red, *RBGFr* Red:Blue:Green:Far-red

[a]Statistical significance levels: N.S. $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

The anthocyanin reflectance index (ARI), chlorophyll index (CI), chlorophyll reflectance index (CRI), plant senescence reflectance Index (PSRI), green normalized difference vegetation index (GNDVI), normalized difference vegetation index (NDVI), normalized pigment chlorophyll index (NPCI), and maximum quantum efficiency (Fv/Fm) (mean ± standard error of the mean) are reported. Different letters indicate significant differences per parameter tested ($p < 0.05$; Duncan *post-hoc* test)

Green Normalized Difference Vegetation Index (GNDVI) and Normalized Difference Vegetation Index (NDVI), which decreased under RBFr and RBGFr. The CI, NDVI, and GNDVI spectral indices, which positively correlate to chlorophyll content, indirectly indicate higher chlorophyll levels in treatments without Far-red spectrum (*i.e.*, FL, HW, and RB). On the other hand, significant statistical differences were observed analyzing the Plant Senescence Reflectance Index (PSRI). PSRI results were consistent with the other indexes with low values under RBFr and RBGFr followed by FL, RB, and HW treatments. Finally, the Normalized Pigment Chlorophyll Index (NPCI) showed stable values across all treatments, with no significant differences.

Discussion

This study represents the first application of LEDs for the multiplication, rooting, and acclimatization of *C. sativa,* specifically focusing on the cv. 'Marrone.' Light is a key factor in plant morphogenesis, significantly influencing growth by activating light receptors (Sarropoulou et al. 2023). Fluorescent lamps are commonly used in micropropagation; however, they emit a broad spectrum of wavelengths (350–730 nm) (Ramírez-Mosqueda et al. 2017a), making it challenging to identify which wavelengths most effectively enhance plant growth (Kim et al. 2004; Bello-Bello et al. 2017). In contrast, LEDs allow for precise setting of specific wavelengths, better matching plant needs and enhancing their growth (Huo et al. 2024).

An effective in vitro culture protocol should ensure a high multiplication rate, enabling the production of multiple healthy clones from each explant. This capability enhances yield efficiency, helps meet market demand, and enables nurseries to remain competitive in the plant market. Chestnut represents a challenging species to introduce in vitro and manipulate during the micropropagation steps (Fernandes et al. 2020b); therefore, the interest in micropropagation using axillary buds has increased by pushing the development of different protocols for mass propagation (Corredoira et al. 2017; Pavese et al. 2022). Therefore, the use of LEDs in micropropagation can efficiently improve existing in vitro protocols, promoting better shoot proliferation and rooting rates without requiring significant adjustments to the protocol.

In the present study, RBFr and RBGFr LEDs significantly increased the shoot multiplication index, allowing the rapid formation of new shoots/explants. This improvement is linked to the presence of Far-red light, which plays an important role in modulating the activity of phytochrome (PHY) (Zaytseva et al. 2023). In its active form, PHY shifts the endogenous hormonal balance by triggering cytokinin accumulation and reducing auxin biosynthesis (Raspor et al. 2021). Similar findings were observed in pineapple (Cavallaro et al. 2023) and blueberry (Noè et al. 1998), where the Red/Far-red lights enhanced cytokinin synthesis and counteracted apical dominance (Teichmann and Muhr 2015). As reported in other species, such as *Gerbera jamesonii* (Pawłowska et al. 2018) and *Malus domestica* (Muleo and Morini 2008), the combination of specific wavelengths could enhance the shoot proliferation and limit the inhibitory effects of Blue light. Indeed, the addition of Far-red light combined with Blue wavelengths seems to mitigate the Blue light inhibitory effects, promoting the shoot proliferation. This effect suggests a possible cross-talk between the

pathways regulated by PHY and the cryptochrome (CRY) (Cavallaro and Muleo 2022).

Far-red light was also demonstrated to improve in vitro shoot elongation as reported in different woody species, such as blueberry (Wang et al. 2024) and Chinese red pine (Li et al. 2020a, b).

The elongation effect is linked to the Shade Avoidance Syndrome (SAS), a physiological response in plants exposed to low Red/Far-red light ratios. In shaded environments, this response promotes internode and hypocotyl elongation. This growth adaptation helps them reach higher light levels above the surrounding vegetation (Teichmann and Muhr 2015). Indeed, according to Li et al. (2020a, b), Red light and low Red/Far-red addition enhanced shoot elongation in *Pinus tabuliformis*, triggering gibberellin (GA) accumulation, which is fundamental for plant cell elongation and for stimulating the synthesis of auxin inhibitory genes (Li et al. 2017).

In our study on *C. sativa* explants, it has been observed that the reduction of Far-red wavelengths within the Red-Blue (RB) spectrum significantly decreases shoot elongation. The RB spectrum alone appears suboptimal for chestnut propagation due to the inhibitory impact of Blue light on growth, as previously noted in *Gerbera jamesonii* (Pawłowska et al. 2018) and *Picea abies* (OuYang et al. 2015). In Norway spruce, the Blue light negatively affected the shoot development due to a minor concentration of GAs compared to the concentration in the Red light treatment. Gibberellins play a central role in promoting stem elongation; indeed, transcriptome analyses of *Picea abies* grown under Blue light revealed a marked upregulation of the *GIBBERELLIN INSENSITIVE DWARF1* (*GID1), DELLA* and *GRAS* family genes, which are involved in negative GA signaling (OuYang et al. 2015). Consequently, the inclusion of Far-red LED appears essential for mitigating the negative growth effects of Blue light while enhancing the beneficial growth effects of Red light (Li et al. 2017; Nacheva et al. 2021). Additionally, introducing Green wavelengths to the light spectrum has been found to significantly increase the multiplication rate in chestnut; Green light may act synergistically with Far-red light by enhancing canopy penetration and counteracting Blue light-mediated growth inhibition (Tarakanov et al. 2021).

The RBFr and RBGFr light treatments influenced the development of new shoots/explants and enhanced leaf morphological traits. Combined with an adequate photosynthetic rate, these treatments were associated with rapid growth kinetics, as indicated by increased biomass and fresh weight accumulation under LED lighting conditions (Nacheva et al. 2023).

Plants grown under RB light treatments, including the RBFr and RBGFr spectra, showed reduced thickness of the palisade parenchyma, forming a thinner leaf structure with densely packed cells optimized for photosynthesis (Zheng and Van Labeke 2017). The palisade parenchyma plays a crucial role in photosynthesis, allowing light penetration inside the chloroplasts, absorbing the Blue and Red wavelengths, and the Green light to penetrate the other leaf layers (Cioć and Pawłowska 2020). Additionally, exposure to Far-red light (RBFr and RBGFr) altered mesophyll structure in a shade-adapted pattern, increasing the layers of spongy parenchyma and decreasing intercellular spaces (Ivanova and P'yankov 2002). These anatomical adjustments, including increased spongy parenchyma layers and denser mesophyll organization under RBFr and RBGFr treatments, likely enhance internal light scattering and absorption, promoting more efficient light capture and contributing to the improved photosynthetic capacity observed under these spectra (Sarropoulou et al. 2023; Li et al. 2020a, b). The RBFr treatment also led to a thicker adaxial epidermis in *C. sativa* leaves, an advantage during acclimatization as it offers protection against UV radiation and reduces water loss as reported in *Ficus carica* (Chirinéa et al. 2012; Mommer et al. 2005).

As for stomatal characteristics, the RBFr and RBGFr light treatments produced larger and more evenly distributed stomata, which support improved CO_2 uptake and greater biomass accumulation, enhancing photosynthetic efficiency as observed in *Amelanchier alnifolia* (Bošnjak Mihovilović et al. 2020) and *Quercus ithaburensis* var. *macrolepis* (Smirnakou et al. 2017). These traits positively influence both in vivo and ex vitro plant growth. In contrast, HW and FL lights led to the formation of smaller, densely packed stomata, a characteristic of highly transpiring plants (Shilpha et al. 2024). However, this feature could potentially increase water loss during the acclimatization phase, making this stage more challenging (Bertolino et al. 2019; Willmer and Fricke 1996). A similar trend was observed in other woody species such as *Olea europaea* (Díaz-Rueda et al. 2021), *Juglans regia* (Saeedi et al. 2023), and *Vanilla planifolia* (Ramírez-Mosqueda et al. 2017b).

These light spectra (RB, RBFr, and RBGFr) emerge as the ideal choices for preparing plants for ex vitro adaptation, supporting successful greenhouse acclimatization.

The Specific Leaf Area (SLA), which measures leaf area relative to dry mass, confirmed the effectiveness of RBFr and RBGFr treatments. In our study, under these LEDs spectra, plants exhibited lower SLA values, indicating that although the leaves are thinner, the mesophyll structure is denser. This suggests a more compact leaf morphology, which may enhance the plant's ability to tolerate environmental stress while optimizing its physiological functions.

Rooting is a crucial phase in the micropropagation of woody plant species, particularly in chestnut. However, there is a lack of an effective and rapid rooting system that can accelerate chestnut propagation. Previous studies

demonstrated that rooting could be achieved using an induction medium supplemented with IBA, as also observed in chestnut hybrids (Vieitez et al. 2007; Gonçalves et al. 1998), holm oak (Martínez et al. 2020), and American oaks (Vieitez et al. 2009).

This study presents an optimized rooting system that increased the rooting rate to 70% under reference FL light, compared to the 35% of the protocol developed by Pavese et al. 2022. Further improvements were achieved by exposing *C. sativa* explants to LED treatments, specifically RBFr and RBGFr spectra, resulting in a remarkable 100% rooting success. This highlights the relevant role of Far-red light in promoting root induction and development. The influence of the light spectrum on rooting is supported by several studies in other species: Red light promoted rooting in *Pyrus communis* (Bertazza et al. 1995) and in *Prunus domestica*, reaching the highest rooting percentage (98.67%) (Nacheva et al. 2023).

However, our results in chestnut suggest that a combination of light spectra enhances rooting due to synergistic cooperation among light receptors (Daud et al. 2013; Lotfi et al. 2019). While monochromatic Far-red light inhibits the in vitro root formation (Iacona and Muleo 2010), low-intensity Far-red combined with RB promotes root formation (Kurilčik et al. 2008). In *Chrysanthemum morifolium*, a low Red:Far-red ratio improved the rooting formation and countered the inhibitory effect of auxin transport inhibitors, underscoring the relationship between light quality and hormone homeostasis (Christiaens et al. 2019). Indeed, auxin represents an essential plant hormone for root formation due to the directional auxin flux, regulated by *PIN-FORMED* (*PIN*) family transporters, whose expression and localization are regulated by light signal (Yun et al. 2023). In *Picea abies,* the Red light exposure has a positive effect on rooting development, reducing the cytokinin accumulation and repressing jasmonate biosynthesis (Alallaq et al. 2020).

In our study, spectrometric data indicate that plants exposed to the Far-red light (RBFr and RBGFr) exhibit lower chlorophyll concentration, as reflected by the chlorophyll index (CI) and the normalized difference vegetation index (NDVI), along with reduced carotenoid (CRI) and anthocyanin levels (ARI). Despite the pigment reduction under the Far-red light treatments (RBFr and RBGFr), the Plant Senescence Reflectance Index (PSRI) and the Normalized Pigment Chlorophyll Index (NPCI) (Anderegg et al. 2020) indicated that the acclimated plants maintained a healthy phenotype, with no visible signs of photodamage or photochemical stress, as confirmed by Fv/Fm values. All light treatments showed maximum PSII efficiency values ($Fv/Fm > 0.8$), confirming overall plant health. However, only plants subjected to RBFr and RBGFr achieved 100% survival during acclimatization, whereas FL, RB, and HW treatments showed progressively lower survival rates (71.4%, 57.1%, and 16.7%, respectively). These data suggest that the physiological benefits induced by Far-red light, although not reflected in pigment content, translate into markedly improved ex vitro performance.

These results are consistent with previous studies on hazelnut cultivars, where plants grown under a higher Red and Far-red ratio showed a decrease in total chlorophyll content (Silvestri et al. 2019) suggesting that Far-red may have a stronger impact on pigment reduction than Blue light (Lorrain et al. 2008). Although physiological measurements such as the chlorophyll concentration indices (CI, NDVI, and GNDVI), carotenoid, and anthocyanin levels are higher in FL, HW, and RB light treatments, these parameters did not directly correlate with better acclimatization. On the contrary, Far-red exposure promotes leaf morphology and physiological adaptations, contributing to greater stress resilience and higher survival rates in ex vitro conditions. Previous studies observed that Far-red light can enhance acclimatization success by stimulating leaf area expansion and root development, both of which are critical for water and nutrient uptake in new environments (Wang et al. 2024). While plants exposed to HW and FL light exhibited higher pigment content (Bello-Bello et al. 2016; Chen et al. 2020), chestnut plants developed under these lights had a low survival rate when exposed to ex vitro conditions.

Conclusion and Future Perspectives

This study provides valuable insights into the influence of LED light quality on the growth and development of European chestnut (*Castanea sativa*), a species known for its propagation challenges. Our study demonstrates the effectiveness of LEDs of specific wavelength in each step of the chestnut micropropagation protocol; in particular, the incorporation of Far-red wavelengths can optimize shoot multiplication and achieve a high rooting percentage promoting the acclimatization phase and enhancing plantlet performance after in vitro culture. The protocol established in this study presents an efficient and cost-effective approach for the large-scale propagation of valuable chestnut genotypes, making it well suited for commercial applications.

Further studies will take into consideration the molecular and hormonal pathways involved in the light-driven response to better understand the mechanisms underlying the observed improvements in shoot multiplication, rooting, and acclimatization. This will provide deeper insights into the physiological processes regulated by specific light wavelengths, further refining the micropropagation protocol and enhancing its efficiency for large-scale applications.

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Data Availability Data will be made available on request.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical Approval This article does not contain any studies with human or animal subjects.

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