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Article

Use of Light Emitting Diodes on the In Vitro Rooting of Apple Tree Rootstocks

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Abstract: This study presents a novel investigation into the use of Light Emitting Diodes (LEDs) for the in vitro rooting of 'Marubakaido' and 'M9' apple tree rootstocks, marking the first time this approach has been reported in the literature. The research compares four different light sources: blue LED (450 nm), red LED (660 nm), a combination of red and blue LEDs, and traditional fluorescent lamps as a control. Mini-cuttings were inoculated in Murashige & Skoog (MS) medium with reduced nutrient concentrations and supplemented with indoleacetic acid (IAA) and sucrose. The explants were incubated under controlled conditions for 30 days, allowing for a thorough evaluation of the effects of different light sources on various growth metrics. The study found that blue LEDs significantly increased dry mass accumulation in 'Marubakaido' seedlings compared to both red LEDs and fluorescent lamps, demonstrating the effectiveness of LEDs in promoting plant growth. Utilizing LEDs not only enhances seedling development but also offers economic benefits over fluorescent lamps. LEDs are known for their high luminous efficiency, low energy consumption, and long lifespan, which can lead to reduced operational costs in plant production systems. This research not only contributes to the understanding of light effects on plant tissue culture but also suggests that the combination of blue and red LEDs can serve as a viable alternative to fluorescent lamps, potentially transforming practices in the field of horticulture and plant propagation

Keywords: *Malus domestica* Borkh; Marubakaido; M9; plants tissue culture; wavelength; LEDs

1. Introduction

Apples are the most widely grown fruit in temperate climate regions, leading in both cultivated area and consumption volume.[1] Brazil is a major player in the global apple industry, producing approximately 1.38 million tons of apples annually, with 6.9% of this production being exported.[2,3] Apple cultivation ranks among the country's top six fruit crops, predominantly grown in the southern regions, including the municipalities of Vacaria in Rio Grande do Sul, Fraiburgo and São Joaquim in Santa Catarina, and Palmas in Paraná.[4,5]

In the realm of apple cultivation, rootstocks play a crucial role. They are employed for various purposes such as reducing plant vigor, providing resistance to pests and diseases, adapting to different soil conditions, inducing early fruiting, and enhancing orchard productivity. In recent decades, advancements in clonal rootstocks developed through genetic improvement have revolutionized the apple production chain. [6–9]

In Brazil, the predominant rootstocks are the dwarf M.9, the semi-vigorous M.7 and Marubakaido with M.9 interstock, and the vigorous Marubakaido.[10] The 'M9' rootstock is renowned for its dwarfing characteristics and resistance to crown rot.[10,11] It promotes early fruiting and enhances fruit productivity and quality.[12] Meanwhile, the 'Marubakaido' rootstock is recognized for its vigor and resistance to crown rot and woolly aphid.[13]

Traditionally, apple rootstocks are propagated by stool layering.[14,15] This method is slow, yields low output, is labor-intensive, and requires extensive physical space.[14–16] Additionally, it may inadvertently propagate materials with phytosanitary issues[17,18]

In vitro vegetative propagation has been researched to produce vegetative material for apples,[16,19] aiming at mass multiplication of cultivars and production of pathogen-free plants.[15] Also known as micropropagation, this technique involves the true-to-type reproduction of genetically valuable plants through the cultivation of plant segments in an artificial medium under aseptic conditions.[20–23]

High mortality rates of seedlings during the acclimatization phase are common in in vitro propagation systems.[20–23] Therefore, strategies that modify the environment, especially during the final stages of micropropagation, are necessary to make the seedlings more robust and improve their survival in subsequent stages.[24–26] To enhance the efficiency of in vitro propagation techniques, factors such as temperature, humidity, ventilation, and light should be optimized.[27] Among these, light is the most important factor, capable of significantly regulating plant growth and development.[28]

In vitro plant growth rooms are typically equipped with artificial light sources, mainly fluorescent lamps.[29] However, fluorescent lamps have undesirable characteristics such as high energy consumption and heat generation, along with varied wavelength peaks, some of which are not necessary for seedling development.[28]

Light-emitting diodes (LEDs) were introduced in 1960 for plant production in closed systems. From 1960 to 2024, significant advancements were made in the architecture, construction, and enhancement of these artificial light sources. In 1961, the first infrared LEDs were patented. LEDs are known for their high efficiency, high luminous intensity, low-intensity discharge of far-red and red light, and broad wavelength spectrum, which includes ultraviolet (250–380 nm), visible light (380–760 nm), and infrared light (760–1000 nm).[30]

Light Emitting Diodes (LEDs) are being evaluated and have shown satisfactory results in plant tissue culture.[28] LEDs provide specific peaks within the range most favorable to plants,[31] and allow for the precise selection of desired wavelengths.[32] They also offer other advantages, such as high luminous efficiency with minimal heating, long lifespan, absence of heavy metals, and low energy consumption.[28]

Several studies have been conducted to investigate the effects of monochromatic lights, either alone or in combination (two or more colors), on the growth and morphogenesis of a wide variety of in vitro-cultivated seedling species.[28] According to these studies, various characteristics are affected, such as vegetative seedling growth,[33] the formation of photosynthetic pigments,[27,34] and stomatal development, among others.[35,36]

This study aimed to evaluate the effect of using LEDs as light sources on the in vitro rooting of apple rootstocks of the Marubakaido and M9 cultivars.

2. Materials and Methods

Minicuttings approximately 15 mm in length from in vitro pre-established seedlings of the Marubakaido and M9 apple rootstock cultivars were used as explants. Both were obtained from in vitro culture stocks spanning five generations of clonal rootstock cultivations originating from a living collection.

The experiments were conducted in a completely randomized design with four treatments. The treatments consisted of four lighting sources: blue LED (450 nm), red LED (660 nm), red + blue LED (10 diodes of 660 nm and 4 diodes of 450 nm), and fluorescent lamp (control). For the Marubakaido cultivar seedlings, variables such as height, number of leaves and roots, fresh and dry mass of the

aerial part and roots, and chlorophyll and carotenoid content were evaluated. Five replications of eight seedlings each were used. For the M9 cultivar seedlings, stomatal density and size were evaluated using ten replications.

The culture medium used was MS[37] with half the concentration of macronutrients and micronutrients, and iron chelate (FeEDTA) and ethylenediaminetetraacetic acid (EDTA), supplemented with 1.0 mg/L IAA and 30.0 g/L sucrose.

Stock solutions were prepared according to Murashige & Skoog[37] (**Error! Reference source not found.**) using analytically pure reagents and pipetted into a flask containing deionized water (30% of the total volume of the medium to be prepared). The volumes used for the preparation of one liter of culture medium were: 25.0 mL of macronutrient solution, 2.5 mL of micronutrient solution, 5.0 mL of FeEDTA, 5.0 mL of vitamins and amino acids, and 10.0 mL of IAA. Sucrose (30.0 g) was then added. The pH of the chemical preparation was adjusted to 5.8, and 6.0 g of agar and deionized water were added to reach the pre-established total volume, followed by boiling in a microwave oven.

Table 1. MS medium composition.

Stok solution	Components	Concentration in the stock solution (g L ⁻¹)	Final concentration in culture medium (g L ⁻¹)
Macronutrients			
A	NH ₄ NO ₃	33	1.65
B	KNO ₃	38	1.9
C	CaCl ₂ .2H ₂ O	8,8	0.44
D	MgSO ₄ .7H ₂ O	7.4	0.37
E	KH ₂ PO ₄	3.4	0.17
Micronutrients ⁴			
	MnSO ₄ .H ₂ O	3.38	0.0169
	H ₃ BO ₃	1.24	0.0062
	ZnSO ₄ .7H ₂ O	1.72	0.0086
F	KI	0.166	0.00083
	Na ₂ MoO ₄ .2H ₂ O	0.05	0.00025
	CuSO ₄ .5H ₂ O	0.005	0.000025
	CoCl ₂ .6H ₂ O	0.005	0.000025
FeEDTA			
G	Na ₂ EDTA.2H ₂ O	3,7	0.0373
	FeSO ₄ .7H ₂ O	2,78	0.0278
Vitamins and amino acids			
	Thiamine.HCl	0.04	0.0002
H	Nicotinic acid	0.2	0.001
	Pyridoxine.HCl	0.2	0.001
	Glycine	0.8	0.004
-	Myo-inositol	-	0.1
	Sucrose	-	30

The culture medium was distributed in 6.0 mL aliquots into 50 cm³ test tubes. The tubes were sealed with 7.0 x 7.0 cm aluminum foil and sterilized by autoclaving at 121°C and 1.05 kg/cm² pressure for 15 minutes.

After disinfecting the Laminar Flow Hood with 70% ethanol and sterilizing it with UV light (100 to 280 nm) for 20 minutes, the explant inoculation process began. Using tweezers and scissors, the mini-cuttings were isolated and inoculated into the culture medium vertically in the tubes, ensuring each tube contained one explant.

In the growth room, the shelves were equipped with two tubular lamps each. The first shelf was equipped with blue LED lamps (Tecnal®, Tec-Lamp, 14 diodes of 450 nm, 28 W, 99.6 ± 20.4 μmol m⁻² s⁻¹, 900 mm), the second with red LED lamps (Tecnal®, Tec-Lamp, 14 diodes of 660 nm, 28 W, 82.2

$\pm 13.0 \mu\text{mol m}^{-2} \text{s}^{-1}$, 900 mm), the third with red and blue LED lamps (Tecnal®, Tec-Lamp, 10 diodes of 660 nm and 4 diodes of 450 nm, 28 W, $81.8 \pm 14.3 \mu\text{mol m}^{-2} \text{s}^{-1}$, 900 mm), and the last with fluorescent lamps (Osram®, T8 FO 32W/640, $24.4 \pm 4.4 \mu\text{mol m}^{-2} \text{s}^{-1}$, 1200 mm). Light-blocking curtains (Blackout®) were installed at the ends to prevent interference from other light sources.

The tubes containing the inoculated explants were transferred to the Growth Room, where they remained for 30 days under controlled environmental conditions: a temperature of $25 \pm 2^\circ\text{C}$, a 16-hour photoperiod, and different lighting conditions. The environmental factors were monitored throughout the rooting period of the seedlings.

After removing the plant material from the test tubes, evaluations were conducted for height, number of leaves and roots, and fresh and dry mass of shoots and roots. Height was measured using a caliper, recording the distance between the collar region and the insertion of the last leaf. The number of leaves and roots was then counted. The shoots and roots were separated and weighed to obtain the fresh mass, then placed separately in paper bags and transferred to an oven for drying at 60°C . After 96 hours, the dry mass was measured.

Samples (approximately 150 mg of fresh mass) were randomly collected and ground in a mortar with a pestle. The macerate was filtered, and ethanol (NEON®, 95%) was added to make up 50 mL in a volumetric flask, previously wrapped with aluminum foil. The extract was analyzed using a UV-VIS spectrophotometer (Varian Cary 50) with readings at 664 nm for chlorophyll a, 648 nm for chlorophyll b, and 470 nm for carotenoids. The absorbance values obtained were substituted into LICHTENTHALER's equations (1987), and the final values were expressed in milligrams per gram of fresh mass (mg/g).

To evaluate the density (number of stomata per mm^2) and average area (μm^2) of stomata, the technique of impression with clear nail polish, adapted from the universal instant adhesive impression technique (Superbonder®). The leaves of the seedlings were placed on a small layer of clear nail polish on a glass slide, with the abaxial surface in contact with the substance. After complete drying, the leaves were removed and discarded.

The slides were focused at 40x magnification on an optical microscope (AAKER®) and the images were captured using a scientific camera (Opticam®) with a resolution of 14.0 MP. The stomatal density and average area were obtained by counting the number of stomata and measuring the area of each stoma in a 0.0536 mm^2 image using ToupView 3.7 software.

2.1. Statistical Analysis

The data obtained from the four independent treatments were assessed for normality and homogeneity using the Shapiro-Wilk test and Bartlett's test, respectively. When the data were both homogeneous and normally distributed, the four treatments were compared using a one-way ANOVA (a parametric test). If the data were not normally distributed, the treatments were compared using the Kruskal-Wallis test (a nonparametric test).[38] Hypothesis testing (one-way ANOVA and Kruskal-Wallis) was carried out at a 95% confidence level ($p\text{-value} < 0.05$). The Tukey test was used as the post hoc test following the one-way ANOVA, while the Dwass-Steel-Critchlow-Fligner pairwise comparisons were used with the Kruskal-Wallis test. All post hoc tests were conducted with a 95% confidence interval. These tests were performed using JAMOVI (version 2.3.28).[39] Boxplots with violin plots, created using JASP (version 0.18.3.0), were used to represent the data from the four independent treatments.[40–42] Principal component analysis (PCA) was conducted using JAMOVI's MEDA plugin.[43]

3. Results and Discussion

The vegetative growth parameters measured for Marubakaido apple rootstock seedlings which were grown using LEDs and a florescent lamp, were provided in **Error! Reference source not found.. Error! Reference source not found.** shows the Seedlings of the Marubakaido apple rootstock cultivar obtained using LEDs and a fluorescent lamp (control).

Table 2. Vegetative growth of seedlings of 'Marubakaido' apple tree rootstocks rooted in vitro under different sources of light.

Tratamentos	Height (cm)	Number of leaves	Number of roots	Fresh mass (mg)		Dry mass (mg)	
				Aerial parts	roots	Aerial parts	Roots
Blue LED	1.54 ^{ns}	12.50 ^{ns}	7.45 ^{ns}	118 a	127 ^{ns}	26 a	15 ^{ns}
Red LED	1.76	11.30	6.85	84 bc	100	18 bc	15
Red + Blue LED	1.39	12.05	4.80	100 ab	104	23 ab	15
Control	1.63	10.55	7.50	64 c	131	13 c	15
RSD (%)	17.8	14.2	26.5	18.9	17.7	18.7	16.2

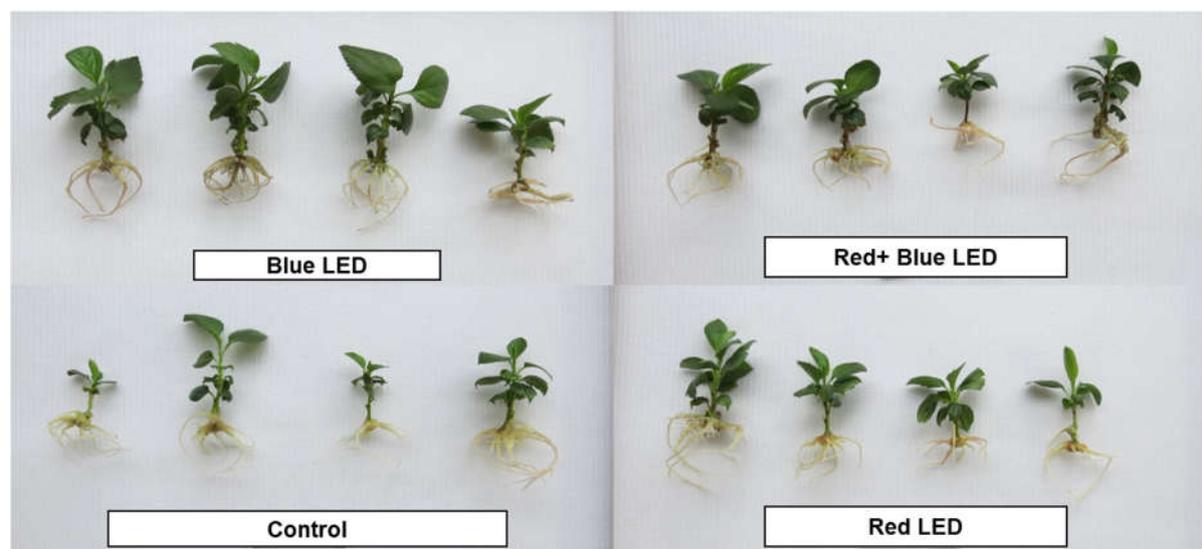


Figure 1. Seedlings of the Marubakaido apple rootstock cultivar obtained after 30 days of in vitro rooting under different light source.

3.1. Height

There were no differences between the four treatments for the height of Marubakaido apple rootstock seedlings. The four treatments provided equivalent heights (**Error! Reference source not found.**). The Shapiro-Wilk test showed that the data was not normally distributed (p -value < 0.001). Then, the Kruskal-Wallis test showed that heights obtained using the four treatments were equivalent (p -value = 0.304).

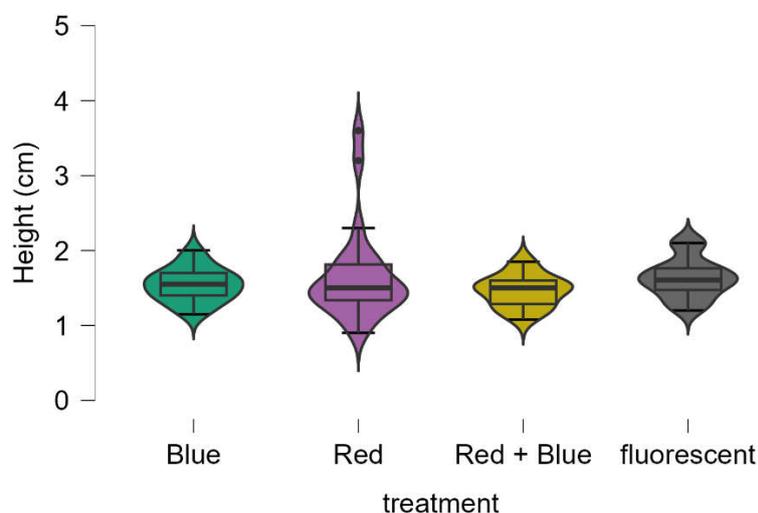


Figure 2. Boxplot of height of Marubakaido apple rootstock seedlings obtained using four independent treatments.

3.2. Dry and Fresh Mass of the Aerial Parts

The treatments with blue LED and the combination of red + blue LED resulted in a significant increase in both fresh and dry mass compared to the treatment with fluorescent lamps (**Error! Reference source not found.**).

Means followed by the same letter in the column do not differ statistically according to Tukey's test at 5%. ns: not significant according to Tukey's test at 5%.

The fresh mass of the aerial parts obtained using LED treatments provided larger fresh mass than the control (fluorescent lamp). Between the LED treatments the blue LED provided larger fresh mass of the aerial parts than the other two LED treatments (**Error! Reference source not found.**). The Shapiro-Wik test showed that the data was normally distributed (p-value = 0.600), the Bartlett's test showed that variances were equivalent (p-value = 0.904), the one-way ANOVA showed that the four treatments provided different fresh mass of the aerial parts (p-value < 0.001). The Tukey test showed that red LED and red + blue LED treatments provided equivalent dry masses of the aerial parts (p-value = 0.383).

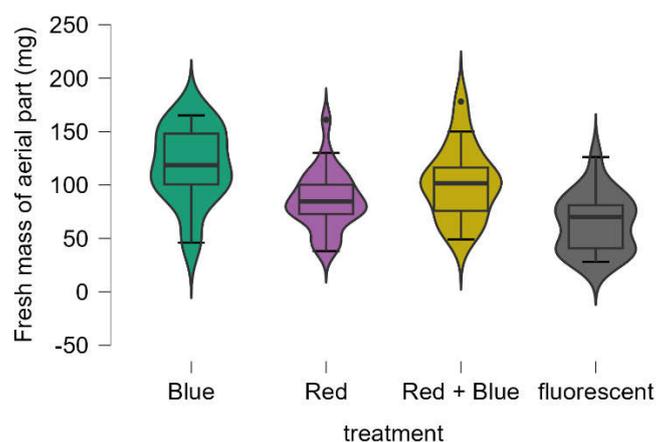


Figure 3. Boxplot of fresh mass of aerial part of Marubakaido apple rootstock seedlings obtained using four independent treatments.

The dry mass of the aerial parts obtained using LED treatments provided larger masses than the control (**Error! Reference source not found.**). The Shapiro-Wik test showed that the data was normally distributed (p-value = 0.615), the Bartlett's test showed that variances were equivalent (p-value = 0.432), the one-way ANOVA showed that the four treatments provided different dry mass of the aerial parts (p-value < 0.001). The Tukey test showed that red LED treatment was equivalent to the red + blue LED treatment (p-value = 0.255). All treatments carried out using LED provided larger dry masses than the control.

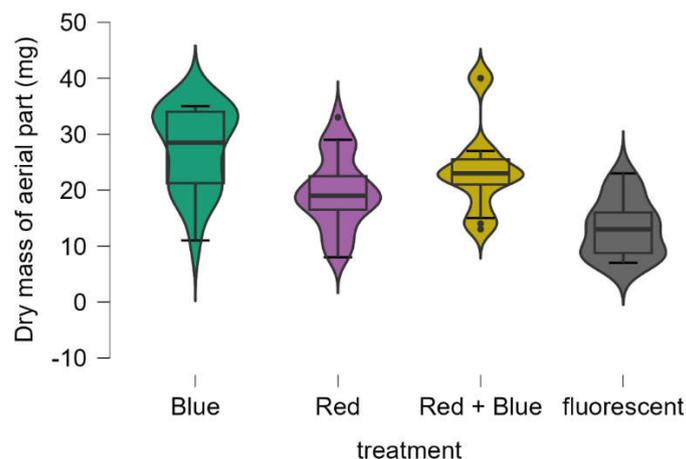


Figure 4. Boxplots of dry mass of aerial part (mg) of Marubakaido apple rootstock seedlings obtained using four independent treatments.

Table 3. Tukey Post hoc test for the dry mass of aerial part (mg) obtained for the four treatments.

		Blue	Red	Red + Blue	Red + Blue	fluorescent
Blue	p-value	—	0.002	0.750	0.568	< .001
Red	p-value		—	0.877	0.402	0.018
Red + Blue	p-value			—	1.000	0.065
Red + Blue	p-value				—	< .001
fluorescent	p-value					—

Note. * $p < .05$, ** $p < .01$, *** $p < .001$.

The light emission peaks in the blue and red wavelengths coincide with the maximum absorption of chlorophylls, thereby allowing photosynthesis to occur with maximum efficiency.[44,45] This effect may be related to the greater accumulation of dry mass in the seedlings exposed to blue LED and red + blue LED compared to those exposed to fluorescent lamps.

The results of the current experiment indicate that blue light (450 nm wavelength) positively influences the development of the aerial parts of Marubakaido apple rootstock seedlings. Beyond its role in photosynthesis, light is essential for regulating growth and morphogenesis processes. Plant responses to blue light are attributed to the presence of pigments such as phytochromes, cryptochromes, and phototropins. When stimulated, these pigments control the expression of certain genes, stomatal opening, flowering, and other processes.[46] Blue light-induced stomatal opening enhances gas exchange during photosynthesis, directly impacting crop productivity.[45]

A similar effect was found by Shin et al.[46] when cultivating orchids in vitro. They observed that seedlings grown under a combination of red and blue LEDs exhibited higher fresh and dry leaf mass compared to those grown under fluorescent lamps. In studies on *Gossypium hirsutum* L., Li et al.[47] found that the red and blue light combination also resulted in greater fresh and dry seedling mass than fluorescent lamps. The increase in fresh and dry mass can help seedlings survive the acclimatization phase,[48] which is the most critical stage in micropropagation systems due to the high mortality rate of seedlings.

When used alone, red LED light (660 nm) produced results inferior to blue light but comparable to fluorescent lamps and the red + blue LED combination in terms of fresh and dry mass of the aerial part (Table 2). This indicates that red LEDs at 660 nm can also be used for in vitro cultivation of Marubakaido apple rootstock.

Similarly, Lin et al.[34] revealed that fluorescent lamps and red LEDs were less efficient than blue LEDs, leading to lower shoot formation and dry mass in *Dendrobium officinale* explants cultivated in vitro. Liu et al.[49] observed that *Platycodon gradiflorum* seedlings showed greater dry mass increase when grown under blue LEDs compared to red LEDs. Red light may induce starch accumulation in chloroplasts, which can inhibit photosynthesis in plants.[36] According to SÆBØ et al.[50] one response induced by exposure to red light is the reduction of photoassimilate translocation from leaves to other parts of the plant. This can lead to starch accumulation in chloroplasts and indirectly reduce the photosynthetic rate, potentially explaining the lower dry mass production in seedlings exposed to red light compared to blue light.

3.3. Roots Dry and Fresh Mass

The roots dry mass obtained using LED treatments were equivalent to those obtained using the control (**Error! Reference source not found.**). The Shapiro-Wik test showed that the data was not normally distributed (p -value = 0.001), the Bartlett's test showed that variances were not equivalent (p -value = 0.01), the Kruskal-Wallis test showed that the four treatments provided equivalent results (p -value < 0.014). The p -value provided by the Kruskal-Wallis test was smaller than 0.05, but the Dwass-Steel-Critchlow-Fligner pairwise comparisons (**Error! Reference source not found.**) confirmed that results obtained using LEDs were equivalent from those obtained using the control.

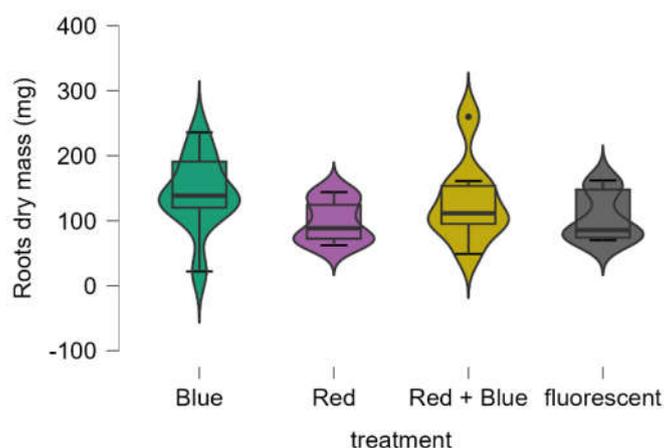


Figure 5. Boxplot of roots dry mass (mg) of Marubakaido apple rootstock seedlings obtained using four independent treatments.

Table 4. Pairwise comparisons of Roots dry masses (mg) obtained using the four treatments.

		W	p
Blue LED	Control	-3.37	0.080
Blue LED	Red + Blue LED	-1.62	0.660
Blue LED	Red LED	-3.81	0.036
Control	Red + Blue LED	2.16	0.419
Control	Red LED	-1.20	0.831
Red + Blue LED	Red LED	-2.66	0.237

The roots fresh mass obtained using LED treatments were equivalent to those obtained using the control (**Error! Reference source not found.**). The Shapiro-Wik test showed that the data was normally distributed (p -value = 0.501), the Bartlett's test showed that variances were equivalent (p -

value = 0.629), the one-way ANOVA showed that the four treatments provided equivalent results (p-value < 0.835).

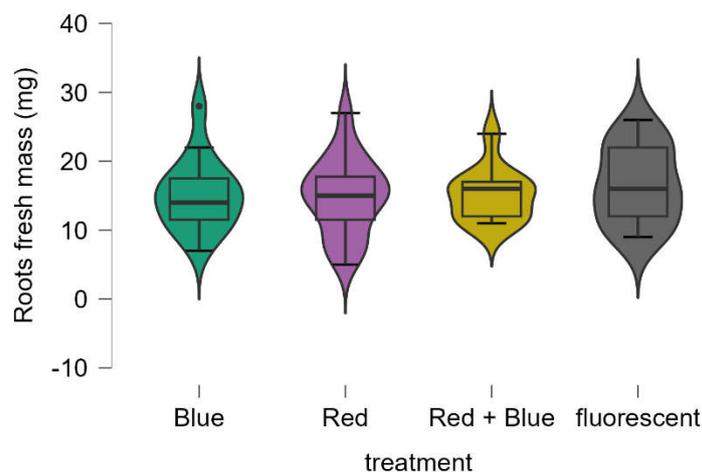


Figure 6. Boxplot of roots fresh mass (mg) of Marubakaido apple rootstock seedlings obtained using four independent treatments.

3.4. Number of Leaves and Roots

The number of leaves obtained using LED treatments were equivalent to those obtained using the control (**Error! Reference source not found.**). The Shapiro-Wik test showed that the data was not normally distributed (p-value = 0.001), the Bartlett's test showed that variances were not equivalent (p-value = 0.012), the Kruskal-Wallis test showed that the four treatments provided equivalent results (p-value < 0.213).

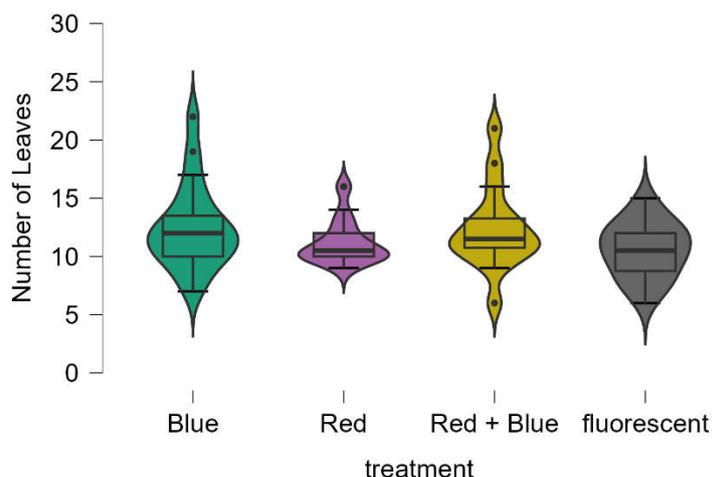


Figure 7. Boxplots of number of leaves in Marubakaido apple rootstock seedlings obtained using four independent treatments.

The number of roots obtained using LED treatments were equivalent to those obtained using the control (**Error! Reference source not found.**). The Shapiro-Wik test showed that the data was normally distributed (p-value = 0.134), the Bartlett's test confirmed data homogeneity (p-value = 0.999), the one-way ANOVA test showed that the four treatments provided equivalent results (p-value < 0.127).

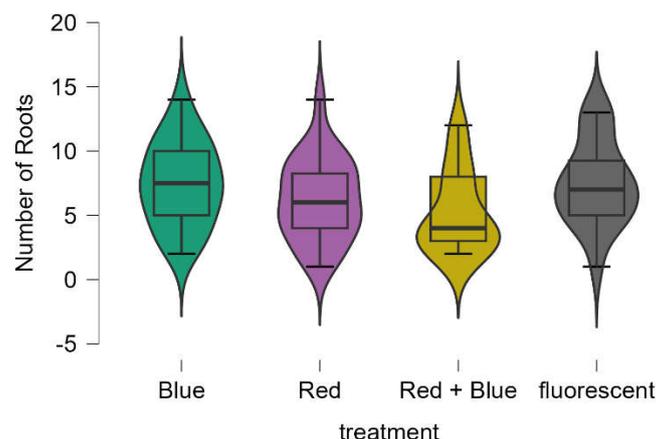


Figure 8. Boxplot of the number of roots fresh mass (mg) of Marubakaido apple rootstock seedlings obtained using four independent treatments.

Plant height, number of leaves, number of roots, and fresh and dry root mass were not affected by the different light sources (**Error! Reference source not found.**). According to Moon et al.[27] light quality can influence plant morphology. For example, plant height can be promoted or inhibited depending on the different interactions between blue and red-light receptors and phytochromes.[51]

A similar result was obtained by Li et al.[47] when they cultivated *Brassica napus* L. in vitro. The researchers observed that the stem length of seedlings treated with LEDs was comparable to those treated with fluorescent lamps.

The wavelength of light can affect the rooting of in vitro seedlings, varying according to the cultivated species.[27] A study by Chée[52] showed that blue LEDs had more promising effects on the rooting of grapevine seedlings compared to red LEDs. On the other hand, Moon et al.[27] observed that the number of roots in *Tripterospermum japonicum* was induced by fluorescent lamps and the red + blue LED combination but inhibited by isolated red and blue LEDs. Shin et al.[46] reported that the fresh and dry root masses of in vitro orchids increased when grown under the red + blue LED combination. However, Jao et al.[53] found that *Zantedeschia jucunda* seedlings subjected to fluorescent lamps had greater dry root mass formation than those treated with LEDs.

3.5. Principal Component Analysis (PCA)

Finally, an analysis of vegetative growth was conducted using PCA,[54,55] The score plot would be used to observe correlations and trends of the data.[56–59] The score plot (**Error! Reference source not found.**) shows that number of roots, root fresh mass, and height were correlated variables samples places in the top of the right side had larger values of those parameters than samples placed in the left side. It also shows that samples placed on the right side had larger number of leaves, fresh mass of the aerial parts, dry mass of aerial parts, and roots dry mass than samples placed in the left side.

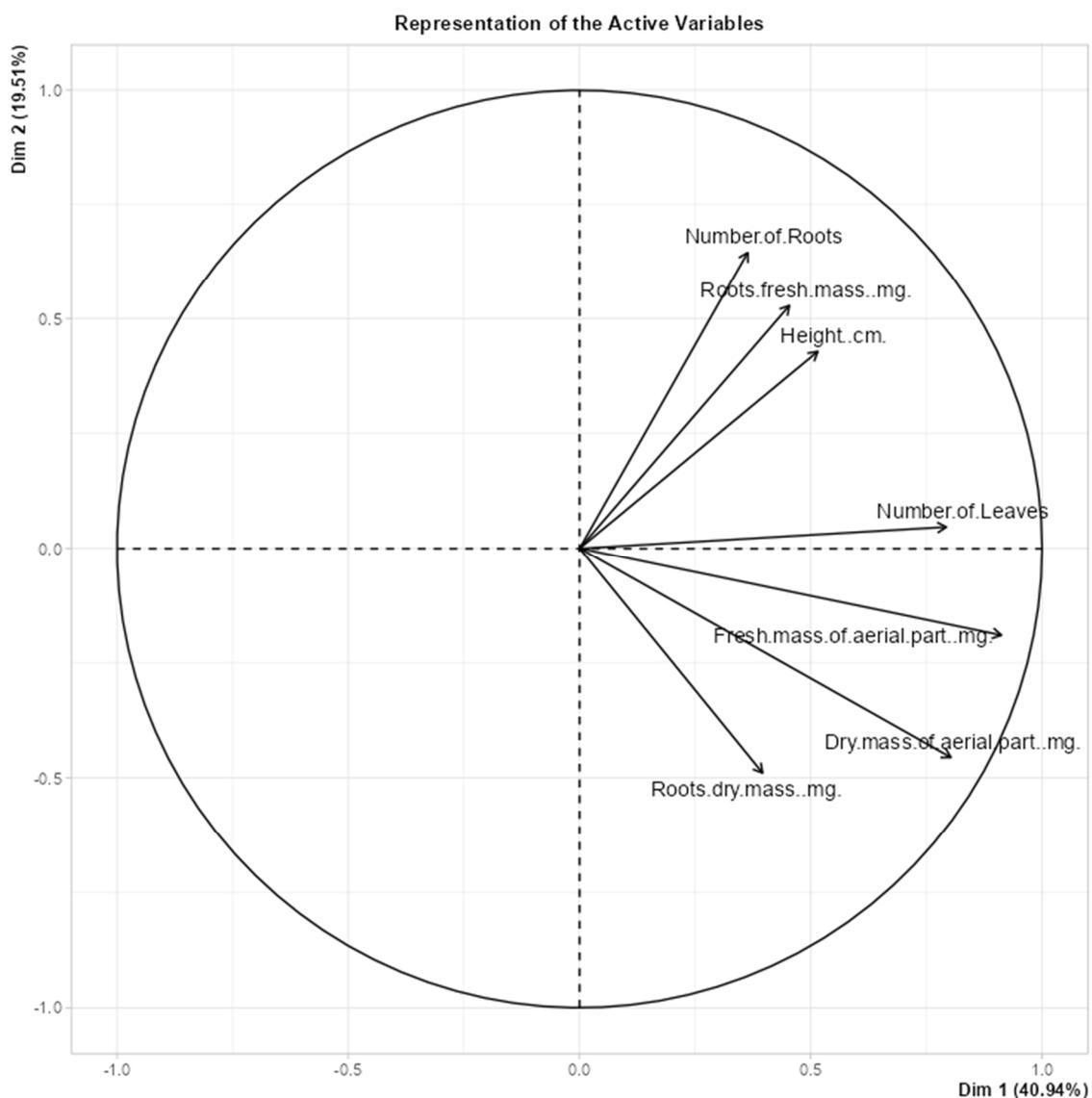


Figure 9. Loading plot of the vegetative growth of seedlings of 'Marubakaido' apple tree rootstocks rooted in vitro under different sources of light.

The loading plot (**Error! Reference source not found.**) shows that Marubakaido apple rootstock samples that had larger vegetative growth were placed in the right side, while samples that had smaller vegetative growth were placed in the left side. Most of the samples placed on the right side were growth under blue LED, while most of the samples placed at the left side were controls. It showed that LED lights were more efficient than control.

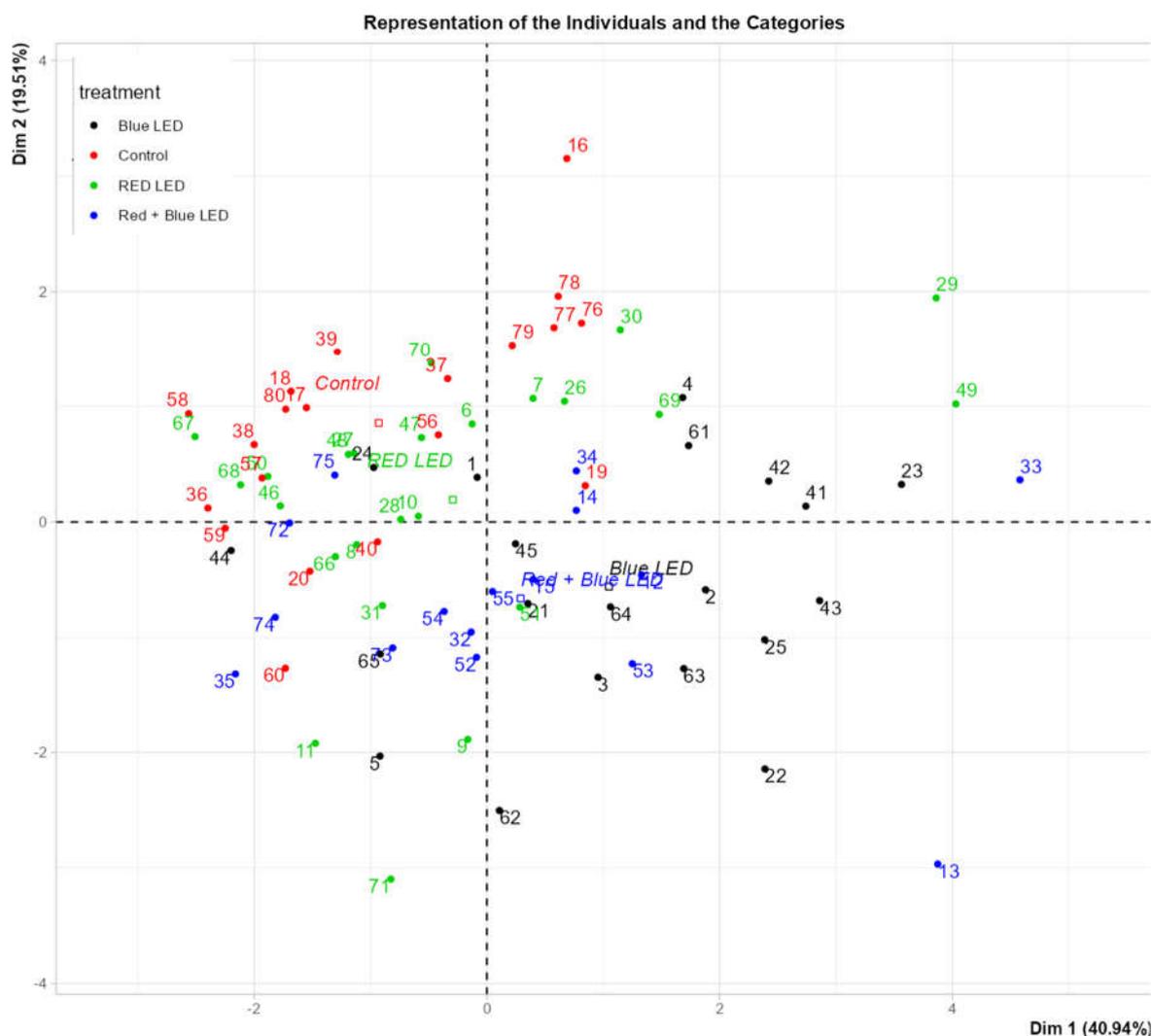


Figure 10. Score plot of the vegetative growth of seedlings of 'Marubakaido' apple tree rootstocks rooted in vitro under different sources of light.

3.6. Chlorophyll a, b, Total (a + b) and Carotenoids Content

Treatment with fluorescent lamps resulted in higher concentrations of chlorophylls a, b, total chlorophyll (a+b), and carotenoids in leaf samples compared to LED treatments. The different LED wavelengths did not show significant differences among themselves for pigment formation (**Error! Reference source not found.**). Jao et al.[53] cultivated *Zantedeschia jucunda* in vitro and reported that fluorescent lamps yielded more promising results for chlorophyll formation than LED treatments. Another study by Moon et al.[27] showed that chlorophyll content in *Tripterosperrum japonicum* was higher when seedlings were treated with fluorescent lamps and red + blue LED combinations but inhibited under isolated red and blue LEDs. However, Shin et al.[46] observed that in vitro-cultivated *Doritaenopsis* plants under the red + blue LED combination had higher chlorophyll and carotenoid content than those grown under fluorescent lamps. These studies indicate that the synthesis of chlorophylls and carotenoids in plants exposed to different light sources may vary depending on the species.

Table 5. Chlorophyll a, b, total (a + b) and carotenoids content in leaf samples of 'Marubakaido' apple tree rootstock seedlings rooted in vitro under different light sources.

Treatments	Chlorophyll a (mg g ⁻¹)	Chlorophyll b (mg g ⁻¹)	Total Chlorophyll (a+b) (mg g ⁻¹)	Carotenoids (mg g ⁻¹)
Blue LED	2.37 ^b	0.54 ^b	2.9 ^b	0.74 ^b
RED LED	2.49 ^b	0.63 ^b	3.12 ^b	0.78 ^b
Red+ Blue LED vermelho + azul	2.59 ^b	0.62 ^b	3.21 ^b	0.79 ^b
Control	3.17 ^a	0.83 ^a	3.99 ^a	0.94 ^a
RSD (%)	7.7	8.1	7.7	6.9

Means followed by the same letter in the column do not differ statistically according to Tukey's test at 5%.

Although carotenoids are known for their important role in protecting organisms from light-induced damage,[60,61] both carotenoids and chlorophylls are involved in energy capture by plants.[62–64] Light wavelengths play a crucial role in regulating photosynthesis, with blue and red LEDs being the most used for seedling growth. Their wavelengths, approximately 460 nm and 660 nm, respectively, represent the ranges of highest photosynthetic efficiency.[28]

Fluorescent lamps have wavelength peaks ranging from 350 to 750 nm in the electromagnetic spectrum, emitting light in a broad range of colors, many of which are unnecessary for seedling development.[28] Plants exposed to white light preferentially absorb light in the blue, red, and part of the green spectra.[65,66]

Alvarenga et al.[67] showed that green LEDs induced greater synthesis of chlorophylls a, b, and total (a+b) and carotenoids in *Achillea millefolium* seedlings compared to blue and red LEDs. According to the same authors, the increase in pigment levels in plants when exposed to green light may be associated with stress in response to a lack of photosynthetically active light.

3.7. Density and Average AREA of stomata

Despite having higher chlorophyll and carotenoid content, seedlings treated with fluorescent lamps did not show an increase in the photosynthetic rate, as the dry mass accumulation was lower with fluorescent lamps than with blue LEDs and the red + blue LED combination. This result may be due to the provision of light in the wavelengths of interest for photosynthesis, which is more favorable for seedlings in the case of LEDs.

Optical microscopy images of the abaxial surface of leaf samples showed that the density and average area of stomata were not affected by the different light sources (**Error! Reference source not found.**). A similar result was obtained by Li et al.[47] when they observed the development of *Brassica napus* L. seedlings subjected to blue LEDs and the red + blue LED combination. The researchers reported that the number of stomata in these seedlings did not differ from those treated with fluorescent lamps. **Error! Reference source not found.** shows the stomata of the abaxial face of leaf samples of 'M9' apple tree rootstock obtained using LEDs and a fluorescent lamp.

Table 6. Density and mean area of stomata of the abaxial face of leaf samples of 'M9' apple tree rootstock seedlings rooted in vitro under different light sources.

Treatments	Density (stomata, mm ⁻²)	Area (µm ⁻²)
Blue LED	404.85 ^{ns}	377.8 ^{ns}
Red LED	322.76	453.94
Red+Blue LED	382.46	434.75
Control	317.16	438.27
RSD (%)	20.6	32.4

ns: not significant according to Tukey's test at 5%.

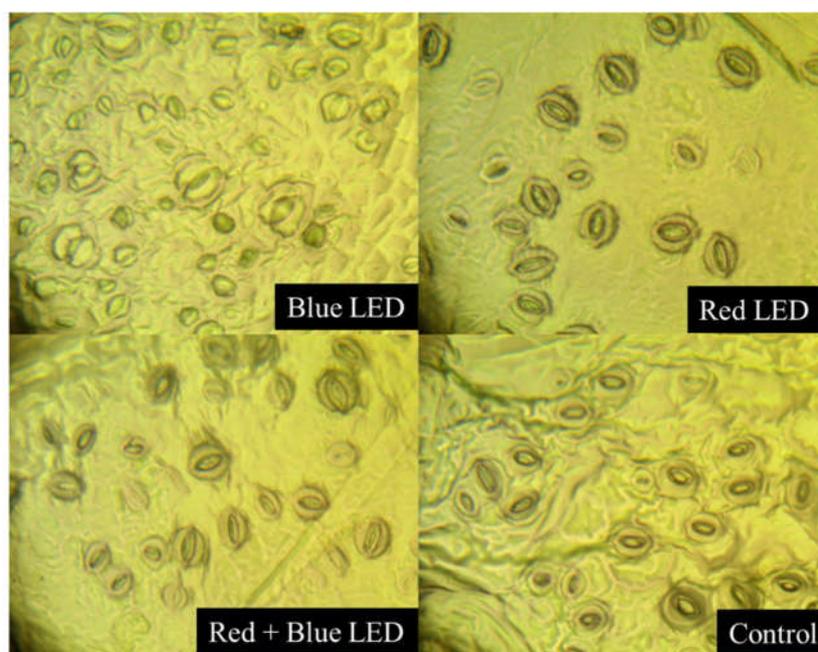


Figure 11. Images obtained by optical microscopy of the abaxial surface of leaf samples from in vitro rooted seedlings of the M9 apple rootstock cultivar using LEDs and a fluorescent lamp.

In contrast, Li et al.[47] found that the stomatal density of *Gossypium hirsutum* L. seedlings was stimulated by red LEDs and inhibited by blue LEDs and the red + blue LED combination. However, the stomata were larger when the plants were exposed to blue LEDs. The researchers concluded that blue LEDs might be beneficial for the stomatal opening of cotton plants.

Therefore, it is likely that the different light sources did not affect stomatal anatomy, as they did not alter the size or density of stomata during the growth and development of the seedlings. However, it is important to consider that exposure to different light sources may have influenced processes affecting the photosynthetic rate, such as stomatal opening and conductance, which could impact dry mass accumulation in the seedlings.

4. Conclusions

Blue LEDs and the red + blue LED combination, which resulted in greater dry mass accumulation in the seedlings, may be a promising alternative to fluorescent lamps for in vitro rooting of Marubakaido apple rootstocks. This would lead to more developed seedlings with a higher likelihood of survival during the acclimatization phase. Although red LEDs inhibit dry mass production compared to blue LEDs, they can still be used, as they produced similar dry mass levels to fluorescent lamps. Additionally, LEDs offer several advantages beyond their specific peaks within the favorable range for plants, such as high luminous efficiency, minimal heating, long lifespan, absence of heavy metals, and low energy consumption, enhancing profitability in in vitro plant propagation.

Fluorescent lamps induce a higher accumulation of chlorophylls and carotenoids compared to LEDs but result in lower dry mass production compared to blue LEDs and the red + blue LED combination in the in vitro rooting of Marubakaido apple rootstock seedlings.

Blue LEDs are more favorable than red LEDs and fluorescent lamps for dry mass accumulation in the in vitro rooting of Marubakaido apple rootstock seedlings.

Blue LEDs, red LEDs, and the red + blue LED combination can be used as alternatives to fluorescent lamps in the in vitro rooting of Marubakaido apple rootstock seedlings.

The different light sources used in the treatments do not affect the density and average area of stomata in M9 apple rootstock seedlings.

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