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The Effect of Far-Red Light and Nutrient Level on the Growth and Secondary Metabolites of the In Vitro Culture of *Prunella vulgaris*

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Abstract: *Prunella vulgaris*, a medicinal plant with antioxidant capacity, was investigated for its response to varying intensities of far-red light and nutrient levels. Plantlets were cultured for 30 d under low far-red light (LFR) or high far-red light (HFR) conditions and different nutrient levels (full, half, and quarter). HFR reduced leaf and branch number, dry weight, and accumulation of chlorophylls (Chl) and carotenoids (Car), while increasing plant height. Lower nutrient levels increased plant height and leaf number, but decreased branch number, Chl, and Car. HFR significantly increased total phenolic content (TPC), rutin, and rosmarinic acid levels, while total flavonoid content decreased. As nutrient levels decreased, TPC and rosmarinic acid declined. HFR induced significant DPPH scavenging activity, while reducing power increased with higher far-red light and nutrient levels. The ferrous ion chelating effect under LFR reduced with lower nutrient levels. There were strong correlations among TPC, rosmarinic acid, DPPH scavenging activity, and reducing power. In conclusion, HFR inhibited plantlet growth but enhanced secondary metabolite accumulation and antioxidant capacity. Different nutrient levels stimulated diverse growth responses, while elevated nutrient levels promoted secondary metabolite production. This study demonstrated the responses of growth, secondary metabolite accumulation, and antioxidant activity in the in vitro cultured *P. vulgaris* to supplemental far-red light and various nutrient levels.

Keywords: *Prunella vulgaris*; far-red light; nutrient levels; plantlet growth; photosynthetic pigments; secondary metabolites; antioxidant activity

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1. Introduction

Prunella vulgaris, a perennial plant of the Lamiaceae family, is widely distributed in Asia, Europe, and North America [1], and is used as a medicinal plant in Asia and Europe [2]. Multiple pharmacological and bioactive properties are found in *P. vulgaris*, such as antioxidant, free radical scavenging, anti-inflammatory, anti-tumor, antibacterial, and antiviral effects. Hence, *P. vulgaris* has great potential for the development of pharmaceutical and nutraceutical products [3]. Flavonoids and phenolic compounds such as rutin, caffeic acid, and rosmarinic acid have been found in *P. vulgaris*. Rosmarinic acid, a major phenolic compound in *P. vulgaris*, is more abundant in leaves than other plant organs [4,5]. The biosynthesis of rosmarinic acid in *P. vulgaris* is regulated by enzymes involved in the phenylpropanoid pathway and tyrosine-derived pathway [6,7]. Far-red light [8] and fertilization [9,10] mediates the growth, physiology, and biosynthesis of secondary metabolites in plants. Herbal yield and biosynthesis of secondary metabolites often vary with environmental and nutrient factors. Light and fertilization are crucial to stabilize the production of high-quality plant raw materials.

Light, including photoperiod, intensity, and spectrum composition, is an important environmental factor regulating photosynthesis, development, and growth in plants [11].

Diverse artificial lighting systems have been applied in modern agriculture over the past few decades, making agricultural production more resilient in the face of seasonal variations and climate change [12,13]. Therefore, optimizing the lighting environment is an important aspect of current agricultural technological advancements. Red and blue light serve as the primary energy sources for photosynthesis and play important roles in plant growth [14]. Far-red light regulates seed germination, morphogenesis, flowering, and biosynthesis of secondary metabolites through phytochromes (Phy) having reversible red-absorbing (Pr) and far-red-absorbing (Pfr) forms [15]. An in vitro study investigated the response of *P. vulgaris* to various monochromatic lights, excluding far-red light [16].

High far-red light or a low red/far-red ratio induces shade avoidance syndromes, which encompass morphological adaptations triggered by shade, including elongated internodes and petioles, leaf hyponasty, and premature flowering tendencies [17], while inhibiting photosynthetic physiology [18]. Nevertheless, several studies show that supplemental far-red light promotes plant growth [19–21]. Furthermore, red and far-red light regulate the biosynthesis of phenolic compounds, such as caffeic acid and rosmarinic acid, and influence antioxidant capacity [8,22,23]. Light-emitting diodes (LEDs) are narrow-band light sources that provide specific wavelengths suitable for use in research on plant processes such as photosynthesis, morphological differentiation, and development [12]. The plant production environment can be optimized via control of the spectral composition [13].

Macronutrients, like nitrogen (N), potassium (K), and phosphorus (P), play a vital role in the growth, differentiation, and physiological metabolism of plants. They also influence the accumulation of secondary metabolites, directly impacting crop yield, quality, and chemical composition [24–29]. At lower N levels, a higher content of flavonoids and phenolic compounds, as well as a better antioxidant capacity, is observed. The content of secondary metabolites is correlated with the activity of phenylalanine ammonia lyase (PAL), which is the key enzyme in the phenylpropanoid pathway [30–34]. Our previous study also demonstrated that N application promoted photosynthetic physiology, whereas N over-fertilization did not increase yield but reduced the antioxidant activity of plant extracts [28]. Appropriate fertilization, including N, P, and K, increases the yield and content of rosmarinic acid in *P. vulgaris* [9] and promotes chlorophyll (Chl) and photosynthetic physiology [10]. Therefore, we were curious about the responses of growth and biosynthesis of secondary metabolites in *P. vulgaris* to different nutrient levels under different far-red light intensities.

It was hypothesized that an augmentation of far-red light could lead to greater accumulations of secondary metabolites in *P. vulgaris*, while appropriate nutrient levels were expected to enhance both growth and functionality. Several studies have suggested that in vitro culture can be considered as a tool for the production of *P. vulgaris* raw material [16,35,36]. This study reported an in vitro culture trial of *P. vulgaris*. In vitro cultured plantlets were grown under three levels of nutrient media and two levels of supplemental far-red light intensity. Subsequently, we investigated the growth, photosynthetic pigments, secondary metabolites of plantlets, and antioxidant activity of extracts. Furthermore, correlation analyses were conducted to explore relationships between secondary metabolites and antioxidant activity. The objective of this study was to evaluate the growth, biosynthesis of secondary metabolites, and antioxidant activity of *P. vulgaris* cultured under three nutrient levels and two intensities of supplemental far-red light.

2. Materials and Methods

2.1. Plant Culture and Nutrient Levels

P. vulgaris seeds were collected from the Educational Herb Garden of the Ministry of Health and Welfare (25°00′23.0″ N, 121°45′22.0″ E) and incubated in half-strength MS medium for 14 d in a growth chamber with white LED lighting set at 25 °C/20 °C (day/night) under a 12 h photoperiod. Seedlings were then transferred to culture vials with full strength MS medium and cultured under the same conditions for further explant collection. The nutrient levels in MS media were adjusted to full- (FN), half- (HN), and

quarter-optima (QN) of nitrogen sources, while K concentrations were simultaneously altered as potassium nitrate was one of the nitrogen sources (Table 1). Two shoot tips of *P. vulgaris*, each with two fully expanded leaves, were placed in each culture bottle. *P. vulgaris* explants were then incubated in a culture room under two far-red light conditions at an average temperature of 25 °C for 30 d. A total of 90 culture bottles were prepared for each nutrient level and then divided into two lighting conditions.

Table 1. Concentration of nitrogen sources in full (FN), half (HN), and quarter nutrient (QN) MS medium, respectively.

Nutrient Level	KNO_3 (mg L^{-1})	NH_4NO_3 (mg L^{-1})
QN	475	412.5
HN	950	825
FN	1900	1650

The preparation of MS medium followed Paud and Wai [37]. Each nutrient level of MS medium contained 30 g sucrose and 6 g agar per liter, and the pH of MS medium was adjusted to 5.6. Each 550 mL glass bottle contained 100 mL of MS medium. All culture bottles were plugged with one-hole white rubber stoppers blocked by non-absorbent cotton and covered by aluminum foil. The prepared nutrient media in the culture vials were sterilized by autoclaving at 15 psi and 121 °C for 20 min. Before inoculation, the hood environment was sterilized by exposure to laminar flow UV for 30 min.

2.2. Far-Red Light Treatments

The experiment utilized light-emitting diodes (LEDs) as light sources. Two lighting conditions were applied in this study: low far-red light group (LFR) and high far-red light group (HFR). Red and blue LEDs were set with light intensities of 85 μmol m^{-2} s^{-1} and 30 μmol m^{-2} s^{-1}, respectively. Far-red LEDs (740 nm) were set to light intensities of 70 μmol m^{-2} s^{-1} for LFR and 340 μmol m^{-2} s^{-1} for HFR. Their spectral composition and intensities were measured using a HR-550 spectrometer (Taiwan HiPoint Corporation, Kaohsiung, Taiwan) and are shown in Figure 1. All treatments were conducted in 45-replicates in each nutrient level.

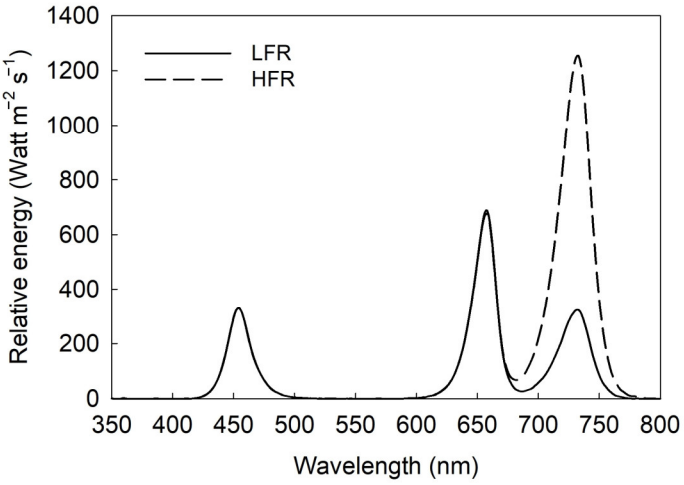

Figure 1. The spectral distribution of the low far-red light (LFR) and the high far-red light (HFR) conditions.

2.3. Plant Growth Parameters

P. vulgaris plantlets were removed from the culture bottles and plant height, leaf number, and branch number recorded for each. The dry weight of each plantlet was

measured after lyophilization. Subsequently, the lyophilized plantlets of each treatment were randomly divided into three groups as replicates and ground for further analysis.

2.4. Photosynthetic Pigments

The photosynthetic pigment content of the *P. vulgaris* plantlets was determined following Yang et al. [38], with modifications. Samples weighing 0.01 g were extracted with 5.5 mL 80% acetone. Mixtures were centrifuged at 4 °C and 4500 rpm for 5 min, and then the supernatant was collected. Absorbance values at 663.6 nm, 646.6 nm, and 440.5 nm were measured using a Hitachi U-3310 spectrophotometer to determine the content of Chl *a*, Chl *b*, and carotenoids (Car). Total Chl content was the sum of Chl *a* and Chl *b*, and Chl *a*/*b* was the ratio of Chl *a* to Chl *b* in each sample.

2.5. Total Phenolic and Flavonoid Content

The total phenolic content (TPC) was determined according to Kujala et al. [39]. Absorbance was measured at 750 nm using a PowerWave X spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA). A standard curve was constructed using gallic acid as the standard, and TPC was expressed as mg gallic acid equivalent (GAE) g^{-1} DW.

The total flavonoid content (TFC) was determined following Bao et al. [40]. Absorbance was measured at 415 nm using a PowerWave X spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA). A standard curve was constructed using rutin as the standard, and TFC of the sample expressed as mg rutin equivalent (RE) g^{-1} DW.

2.6. Caffeic Acid, Rutin, and Rosmarinic Acid

We extracted 0.02 g samples with 2 mL 50% EtOH solution. Each mixture was centrifuged at 4 °C and 3500 rpm for 15 min. Then, 1.4 mL of the supernatant was filtered through a 0.22 µm Millex-GV PVDF membrane filter for HPLC analysis. The HPLC method followed Hong Kong Chinese Materia Medica Standards [41], with modifications. Separation was performed using a LC-40D XR HPLC pump system (Shimadzu, Kyoto, Japan) equipped with a DGU-405 degassing unit (Shimadzu, Kyoto, Japan), and a Cosmosil 5C18AR-II column (4.6 × 250 mm, Nacalai Tesque, Kyoto, Japan). Detection was performed utilizing a SPD-M20A photodiode array detector (Shimadzu, Kyoto, Japan). Mobile phase A was 0.1% (*v*/*v*) formic acid, and mobile phase B was acetonitrile. The gradient elution of mobile phase B was as follows: 10% to 13% from 0–10 min, 13% to 14.5% from 10–14 min, held at 14.5% from 14–15 min, 14.5% to 19% from 15–18 min, maintained at 19% from 18–25 min, 19% to 20% from 25–27 min, 20% to 25% from 27–35 min, decreased from 25% to 10% from 35–40 min, and maintained at 10% from 40–55 min. The flow rate was 1.0 mL per min, and sample injection volume was 10 µL. The column temperature was set at 35 °C. Caffeic acid, rutin, and rosmarinic acid in the sample solution were analyzed at 295 nm, 353 nm, and 330 nm, respectively. Concentrations were calculated using the standard calibration curves of each chemical standard, and the results expressed as mg g^{-1} DW.

2.7. Antioxidant Activity

Samples were extracted using MeOH and subsequently centrifuged at 3200 rpm for 10 min. The supernatant was collected and then subjected to gradient dilution for antioxidant activity assays. DPPH radical scavenging activity was determined following the method described by Braca et al. [42]. Reducing power was assessed as per the procedure outlined by Ferreira et al. [43]. The ferrous ion chelating capacity was evaluated according to the method by Dinis et al. [44]. The antioxidant capacity of each individual sample was determined using MeOH as the blank. Butylated hydroxytoluene (BHT) served as the standard for both DPPH radical scavenging activity and reducing power, while ethylene diamine tetraacetic acid (EDTA) was used as the standard for ferrous ion chelating capacity. Absorbance measurements were taken using an EPOCH2 microplate reader (Bio-Tek Instruments, Winooski, VT, USA). Finally, the half maximal inhibitory concentration (IC_{50}) was calculated based on the concentration gradient of the sample solutions. The

concentration gradient ranged from 1.25 to 4.00 mg mL^{-1} for DPPH radical scavenging activity, from 0.5 to 4.0 mg mL^{-1} for reducing power, and from 3 to 15 mg mL^{-1} for the ferrous ion chelating capacity.

2.8. Statistical Analysis

Statistical analysis was applied to all experimental data using a two-way analysis of variance (ANOVA), followed by a least significant difference (LSD) test at a significance level of $p < 0.05$. Correlation analysis between the IC_{50} values of antioxidant capacities and secondary metabolites was performed using Pearson's correlation coefficient. All statistical procedures were executed using the GLM procedure within SAS 9.2 software (SAS Institute, Cary, USA).

3. Results

3.1. Growth of In Vitro Cultured Plantlets

According to our growth analysis ANOVA, the light factor showed significance in all variables, while the nutrient factor was insignificant in dry weight alone. Furthermore, the interaction effects of all variables were also insignificant (Table 2). The plant height of plantlets under HFR, ranging from 5.0 to 7.7 cm, was taller than that under LFR, which ranged from 4.8 to 5.8 cm. Among all nutrition levels, the average plant height of FN-treated plantlets was lower than that of HN and QN-treated ones (Table 2). The leaf number of plantlets under LFR, ranging from 9.0 to 10.0, was higher than that under HFR, which ranged from 7.5 to 8.9. Overall, QN-treated plantlets showed a higher leaf number than FN-treated ones (Table 2). The branch number of plantlets under LFR, ranging from 0.6 to 1.2, was higher than that under HFR, which ranged from 0.2 to 0.6. On average, the QN-treated plantlets showed a lower branch number compared to the other nutrition levels (Table 2). The dry weight of LFR plantlets, ranging from 58 to 65 mg per plantlet, was higher than HFR plantlets, ranging from 47 to 53 mg per plantlet. The difference among all nutrition levels was not significant (Table 2).

Table 2. Plant height, leaf number, branch number, and dry weight of *Prunella vulgaris* plantlet cultured on full (FN), half (HN), and quarter nutrient (QN) of MS medium under low far-red light (LFR) and the high far-red light (HFR) conditions, respectively.

Light	Nutrient Level	Plant Height (cm)	Leaf Number	Branch Number	Dry Weight (mg per Plantlet)
LFR	QN	5.3 ± 3.3 b	10.0 ± 1.8 a	0.6 ± 1.1 bc	61 ± 20 ab
	HN	5.8 ± 4.1 b	9.6 ± 2.0 ab	0.8 ± 1.4 b	58 ± 24 ab
	FN	4.8 ± 3.5 b	9.0 ± 2.5 b	1.2 ± 1.3 a	65 ± 27 a
HFR	QN	7.7 ± 3.3 a	8.9 ± 1.7 b	0.2 ± 0.6 c	47 ± 20 b
	HN	6.0 ± 3.3 b	7.7 ± 2.6 c	0.6 ± 1.0 bc	48 ± 28 b
	FN	5.0 ± 3.2 b	7.5 ± 2.3 c	0.5 ± 0.9 bc	53 ± 66 ab
ANOVA					
Light		*	***	**	**
Nutrient		**	***	**	ns
L × N		ns	ns	ns	ns

Values are presented as means ± SD ($n = 45$). Different letters indicate significant differences within each column, determined by ANOVA followed by LSD test ($p < 0.05$). * $p < 0.05$ level; ** $p < 0.01$ level; *** $p < 0.001$; ns—no significant difference.

3.2. Photosynthetic Pigments of Plantlets

The ANOVA results for photosynthetic pigments indicated that light factor, nutrient factor, and their interaction effect were all significant for Chl, Chl *a*/*b*, and Car (Table 3). Chl was significantly highest in the LFR + FN treatment among all groups ($p < 0.05$), with a value of 10.59 mg g^{-1} DW (Table 3). Under HFR lighting, Chl increased with higher nutrient levels, but was still lower compared to LFR (Table 3). Under LFR lighting, there was no significant difference in Chl *a*/*b* among different nutrient levels. However, under

HFR lighting, Chl *a*/*b* of the FN group was significantly higher ($p < 0.05$), followed by QN and HN (Table 3). Car exhibited a trend similar to Chl. The LFR + FN treatment significantly had the highest total carotenoid content, at 4.71 mg g^{-1} DW, followed by LFR + QN, and LFR + HN, ranging from 4.24 to 4.39 mg g^{-1} DW. Car in the high far-red lighting group was significantly lower than low far-red lighting, and Car increased as nutrient levels increased, showing significant differences among different nutrient levels (Table 3).

Table 3. Total chlorophylls content (Chl), Chl *a*/*b* ratio, and total carotenoids content (Car) in *P. vulgaris* plantlet cultured on full (FN), half (HN), and quarter nutrient (QN) of MS medium under low far-red light (LFR) and the high far-red light (HFR) conditions, respectively.

Light	Nutrient Level	Chl (mg g^{-1}DW)	Chl *a*/*b*	Car (mg g^{-1}DW)
LFR	QN	9.67 ± 0.48 b	3.22 ± 0.08 a	4.39 ± 0.20 b
	HN	9.36 ± 0.34 b	3.13 ± 0.05 ab	4.24 ± 0.14 b
	FN	10.59 ± 0.18 a	3.17 ± 0.05 a	4.71 ± 0.08 a
HFR	QN	4.17 ± 0.08 e	2.81 ± 0.09 c	2.03 ± 0.04 e
	HN	4.86 ± 0.23 d	2.86 ± 0.06 c	2.32 ± 0.09 d
	FN	6.08 ± 0.13 c	3.04 ± 0.04 b	2.90 ± 0.06 c
ANOVA				
Light		***	***	***
Nutrient		***	*	***
L × N		*	*	**

Values are presented as means ± SD ($n = 3$). Different letters indicate significant differences within each column, determined by ANOVA followed by LSD test ($p < 0.05$). * $p < 0.05$ level; ** $p < 0.01$ level; *** $p < 0.001$.

3.3. Total Phenolic and Flavonoid Content in Plantlets

Light and nutrient factors showed significant effects on TPC, while the interaction effect was not significant (Figure 2A). The TPC of plantlets under HFR, ranging from 38.25 to 44.73 mg GAE g^{-1} DW, was higher than that under LFR, ranging from 35.90 to 39.34 mg GAE g^{-1} DW. The TPC of FN-treated plantlets under both HFR and LFR were 44.73 and 39.34 mg GAE g^{-1} DW, respectively, and these values were significantly higher than the QN-treated ones (Figure 2A). ANOVA results of TFC showed that only the light factor was significant, while the interaction effect between light and nutrient was insignificant (Figure 2B). The TFC of plantlets under LFR, ranging from 94.60 to 105.57 mg RE g^{-1} DW, was higher than that under HFR, ranging from 84.00 to 93.91 mg RE g^{-1} DW.

3.4. Caffeic Acid, Rutin, and Rosmarinic Acid Content in Plantlets

Light, nutrient factors and their interactions with caffeic acid were all insignificant (Figure 3A). Caffeic acid content ranged from 0.14 to 0.21 mg g^{-1} DW, with no significant differences among all groups (Figure 3A). Rutin content displayed significance only with the light factor, and there were no significant interaction effects between light and nutrient factors (Figure 3B). Rutin content in plantlets under HFR, ranging from 0.17 to 0.18 mg g^{-1} DW, was higher than that under LFR, ranging from 0.15 to 0.17 mg g^{-1} DW (Figure 3B). Light and nutrient factors both showed significant effects on rosmarinic acid, and their interaction effect was insignificant (Figure 3C). Under LFR lighting, the rosmarinic acid content of QN, HN, and FN-treated plantlets was 8.99, 12.23, and 16.60 mg g^{-1} DW, respectively. Under HFR, the rosmarinic acid content of QN, HN, and FN-treated plantlets was 12.44, 16.79, and 20.43 mg g^{-1} DW, respectively. At each nutrient level, the rosmarinic acid content in plantlets under HFR was significantly higher than that under LFR, and the rosmarinic acid content significantly increased with elevated nutrition levels (Figure 3C).

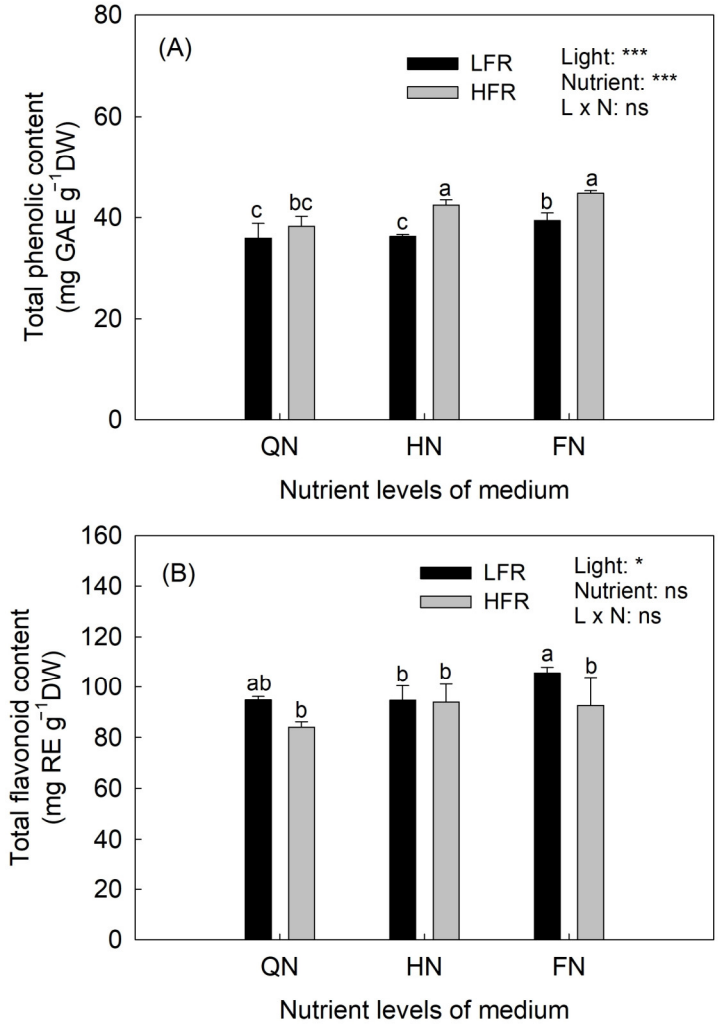

Figure 2. Total phenolic (**A**) and total flavonoid content (**B**) in *P. vulgaris* cultured on full (FN), half (HN), and quarter nutrient (QN) of MS medium under low far-red light (LFR) and the high far-red light (HFR) conditions, respectively. Vertical bars depict means with standard deviation (n = 3). Different lowercase letters indicate significant differences, as determined by ANOVA followed by the LSD test ($p < 0.05$). * $p < 0.05$ level; *** $p < 0.001$; ns—no significant difference; GAE—gallic acid equivalent; RE—rutin equivalent.

3.5. Antioxidant Activity of Methanolic Plantlet Extract

Five different sampling concentrations were measured for DPPH radical scavenging activity, reducing power, and ferrous ion chelating effect, and their IC_{50} values for each treatment were calculated. Only the light factor was significant according to the ANOVA results for DPPH radical scavenging activity, and there was no interaction between light and nutrient factors (Table 4). The IC_{50} values of HFR plantlet extract for DPPH radical scavenging activity, ranging from 1.95 to 2.22 mg mL^{-1}, were lower than those of LFR, ranging from 2.34 to 2.60 mg mL^{-1}. The ANOVA results for reducing power indicated that light factor, nutrient factor, and their interaction effect were all significant (Table 4). On average, the IC_{50} values of reducing power were lower under high far-red light. There were significant differences in nutrient levels within individual lighting conditions ($p < 0.05$), with low and high far-red lighting both showing the lowest IC_{50} values in the FN treatment (Table 4). Overall, the HFR + FN treatment showed the lowest IC_{50} value of 2.33 mg mL^{-1}. The IC_{50} values for ferrous ion chelating effect showed that only the nutrient factor reached statistical significance, and there was no interaction between light and nutrient levels (Table 4). The IC_{50} values of FN plantlet extracts for ferrous ion chelating effect under LFR

and HFR are 4.82 and 4.96 mg mL^{-1}, respectively. Among the three nutrition levels, the average IC_{50} values of extracts from FN-treated plantlets were lower.

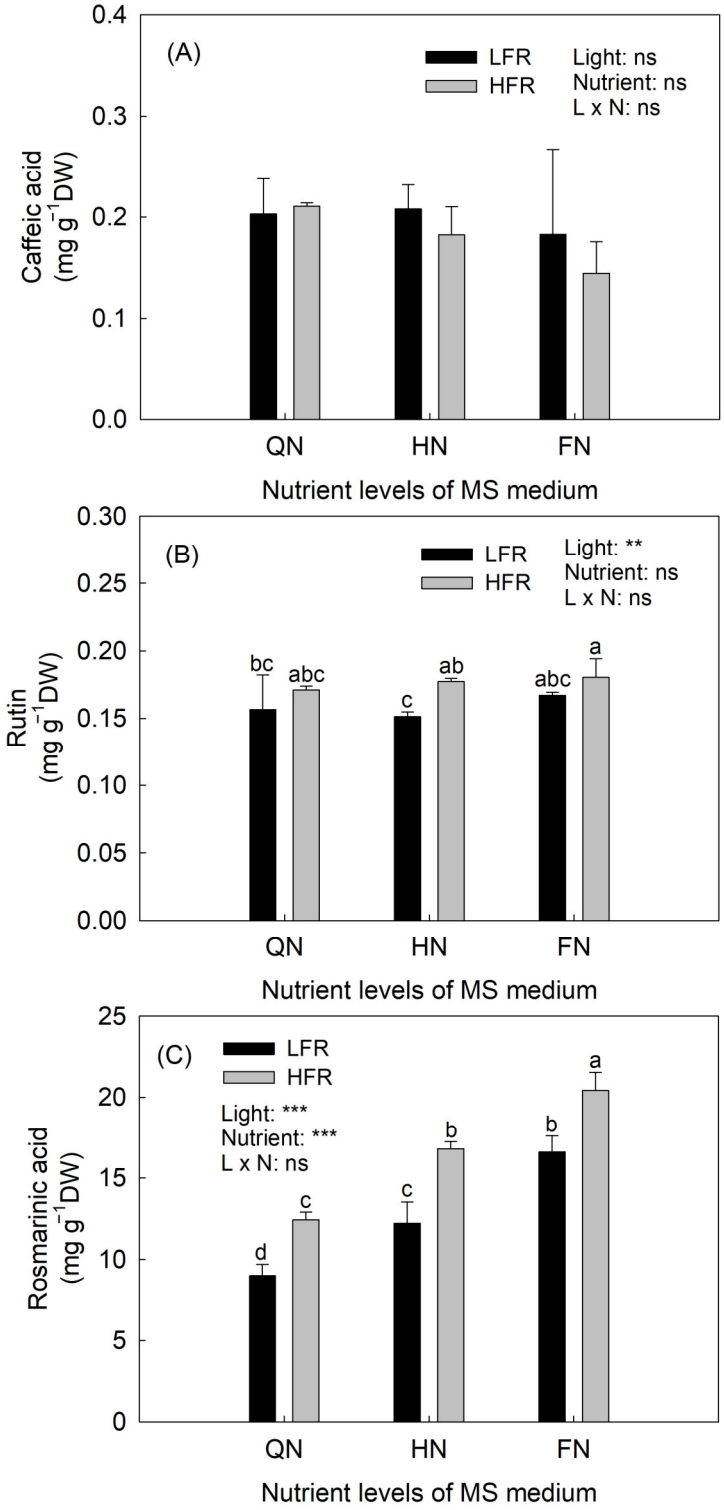

Figure 3. Caffeic acid (**A**), rutin content (**B**), and rosmarinic acid content (**C**) in *P. vulgaris* cultured on full (FN), half (HN), and quarter nutrient (QN) of MS medium under low far-red light (LFR) and the high far-red light (HFR) conditions, respectively. Vertical bars depict means with standard deviation ($n = 3$). Different lowercase letters indicate significant differences, as determined by ANOVA followed by the LSD test ($p < 0.05$). ** $p < 0.01$ level; *** $p < 0.001$; ns—no significant difference.

Table 4. The IC_{50} value for DPPH scavenge activity, reducing power, and Fe^{2+} chelating effect of extract from *P. vulgaris* plantlet cultured on full (FN), half (HN), and quarter nutrient (QN) of MS medium under low far-red light (LFR) and the high far-red light (HFR) conditions, respectively.

Light	Nutrient Level	DPPH Scavenge (mg mL^{-1})	Reducing Power Rate (mg mL^{-1})	Fe^{2+}-Chelating Effect (mg mL^{-1})
LFR	QN	2.60 ± 0.18 a	3.22 ± 0.02 a	5.67 ± 0.04 a
	HN	2.46 ± 0.37 a	2.86 ± 0.09 b	5.48 ± 0.48 ab
	FN	2.34 ± 0.32 ab	2.61 ± 0.12 c	4.82 ± 0.41 c
HFR	QN	2.22 ± 0.14 ab	2.78 ± 0.07 b	5.01 ± 0.17 bc
	HN	2.03 ± 0.12 b	2.53 ± 0.04 c	5.42 ± 0.54 abc
	FN	1.95 ± 0.11 b	2.33 ± 0.05 d	4.96 ± 0.16 bc
Standard		0.53 ± 0.09	0.31 ± 0.02	0.02 ± 0.00
ANOVA				
Light		**	***	ns
Nutrient		ns	***	*
L × N		ns	*	ns

Values are presented as means ± SD (n = 3). Different letters indicate significant differences within each column, determined by ANOVA followed by LSD test ($p < 0.05$). * $p < 0.05$ level; ** $p < 0.01$ level; *** $p < 0.001$; ns—no significant difference.

3.6. Correlation of Secondary Metabolites and Antioxidant Activity

Correlation analyses between antioxidant capacity and secondary metabolites (Table 5) showed significant correlations between caffeic acid, rutin, and rosmarinic acid content with TPC. Caffeic acid had a negative correlation with TPC. Among them, the correlation coefficient between TPC and rosmarinic acid was 0.85 ($p < 0.001$) (Table 5). DPPH scavenging activity showed significant correlations with TPC, rutin, and rosmarinic acid, with stronger correlations with TPC and rosmarinic acid (Table 5). With the exception of TFC, reducing power exhibited correlations with secondary metabolites, having the highest correlation coefficient with rosmarinic acid (Table 5). Conversely, ferrous ion chelating ability did not show significant correlations with any secondary metabolite (Table 5).

Table 5. The correlation analysis between total phenolic content (TPC), total flavonoid content (TFC), caffeic acid, rutin, rosmarinic acid (RA) and IC_{50} values for DPPH scavenge activity, reducing power and Fe^{2+} chelating effect.

	TPC	TFC	Caffeic Acid	Rutin	RA	DPPH Scavenging	Reducing Power	Fe^{2+}-Chelating
TPC	--							
TFC	0.02 ns	--						
Caffeic acid	−0.65 **	−0.39 ns	--					
Rutin	0.52 *	−0.28 ns	−0.11 ns	--				
RA	0.85 ***	0.18 ns	−0.48 *	0.55 *	--			
DPPH scavenging	−0.74 ***	0.21 ns	0.23 ns	−0.48 *	−0.72 ***	--		
Reducing power	−0.85 ***	−0.08 ns	0.51 *	−0.56 *	−0.95 ***	0.71 **	--	
Fe^{2+}-chelating	−0.25 ns	−0.10 ns	0.00 ns	−0.33 ns	−0.43 ns	0.29 ns	0.43 ns	--

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns—no significant difference.

4. Discussion

P. vulgaris is widely utilized in traditional medicine in Europe and Asia [1], with its major compounds being rosmarinic acid [4,5]. The effect of nutrient levels on yield and

rosmarinic acid content in *P. vulgaris* has been previously reported [9]. Additionally, photosynthesis and chlorophyll fluorescence also respond to nutrient levels [10]. Conversely, the response of *P. vulgaris* to far-red light is little investigated. In this study, nutrient levels and far-red light intensity were well-controlled through an in vitro culture trial for researching the response of *P. vulgaris* to these two environmental factors.

In natural environments, a higher proportion of far-red light typically occurs in shaded conditions where light intensity is reduced, limiting the plant's ability to undergo sufficient photosynthesis [45]. However, far-red light drives the photosynthetic system when it interacts with the photosynthetically active radiation spectrum [46]. Furthermore, far-red light contributes 10–25% of plant photosynthesis in shaded environments [47]. Previous studies showed that far-red light promoted plant height [8,19,22], while a low red/far-red light ratio reduced branch number [48]. In the present study, inhibition of branch number at all nutrient levels and promotion of QN plant height under HFR (Table 2) indicated the response of shade avoidance [17]. Supplemental far-red light did not necessarily reduce leaf number in *Forsythia saxatilis* plantlets [49]; however, a suppressed leaf number was observed in *P. vulgaris* under high intensity of far-red light in this study (Table 2). In vitro studies displayed that far-red light did not effectively increase dry weight compared to red or blue light [50,51]. Additionally, supplemental far-red light had a negative effect on dry weight [52]. In this study, a reduced dry weight occurred under HFR (Table 2). High far-red light or a low red/far-red ratio may decrease Chl content and photosynthetic capacity [18]. A lower Chl in plantlets was also observed under HFR in this study (Table 3). Therefore, we speculated that the presence of high far-red light severely inhibits photosynthesis, leading to a decrease in dry weight (Table 2)

N, P, and K are macronutrients that impact the development and biosynthesis of secondary metabolites in plants [25,26]. In vitro studies revealed that plant height, leaf number, and branch number were inhibited with a reduced N source (KNO_3 and/or NH_4NO_3) [53,54]. In this study, lower nutrient levels resulted in a lower branch number, but they led to a greater plant height and a higher leaf number (Table 2). The study conducted by Chen et al. [9] indicated that proper fertilization promoted the yield of *P. vulgaris*. Combatt Caballero et al. [29] revealed that the yield of *Stevia rebaudiana* is positively correlated with N, while K and P showed no correlation with yield. According to the in vitro study conducted by Georgiev et al. [55], the dry weight of plantlets was influenced by the composition of sucrose and macro nutrients, including KNO_3, NH_4NO_3, and KH_2PO_4 in MS media. The insignificant difference in dry weight among all nutrient levels (Table 2) might suggest that none of the compositions of MS media in this study (Table 1) were optimal for increasing production yield in *P. vulgaris*.

Lower red/far-red ratios or reduced expressions of phytochrome lead to decreases in chlorophyll in various plants [18]. Different intensities of far-red light or plant species might lead to these diverse responses in biosynthesis of photosynthetic pigments [13,19,49]. The present study observed reduced Chl and Car content, as well as a significant decrease in Chl a/b under HFR conditions (Table 3). These results were similar to those of the in vitro study conducted by Klimek-Szczykutowicz et al. [52]. Phytochrome inhibits the expression of one kind of protochlorophyllide oxidoreductase (POR) involved in the chlorophyll biosynthesis pathway via PENTA 1, resulting in hindered Chl synthesis [15]. The phytoene synthase (PSY) involved in the biosynthetic pathway of Car is repressed by the transcription factor Phytochrome Interacting Factor 1 (PIF1) in darkness or under low R/Fr (red to far-red light) conditions, while it is activated by the transcription factor Long Hypocotyl 5 (HY5), which is stabilized by light or high R/Fr conditions [56]. Therefore, we speculated that the HFR treatment in this study led to an inhibition of POR and PSY activity, resulting in lower accumulations of Chl and Car (Table 3).

Several plant species have increased Chl with increases in nitrogen [27,32]. Furthermore, Chl in *Amaranthus viridis* increased with nitrogen levels, while the Chl a/b ratio remained stable across all nitrogen levels [28]. A similar trend was observed in this study, where the Chl a/b ratio of the FN-treated plantlets under HFR was significantly higher than

in the HN and QN-treated ones (Table 3). Nevertheless, the content of photosynthetic pigments is enhanced through appropriate fertilization, as opposed to over-fertilization [10,34]. Increasing N application induces the gene expression of enzymes involved in chlorophyll biosynthesis such as glutamyl-tRNA reductase and POR [57,58]. Conversely, N deficiency inhibits chlorophyll biosynthesis while inducing the chlorophyll degradative pathway [59]. Under the combined effect of far-red light and nutrient level, the accumulations of Chl and Car decreased significantly with reduced nutrient levels under HFR. In contrast, the Chl and Car of FN plantlets accumulated significantly in low far-red light (Table 3).

Red light and far-red light regulate the biosynthetic pathways of secondary metabolites through phytochrome transformation between Pr and Pfr [15]. Far-red light stimulates the biosynthesis of caffeic acid and rosmarinic acid [22] and leads to an accumulation of total phenolic in plants [23]. In vitro studies have demonstrated that blue, red, and far-red light stimulated the accumulation of phenolic acids and flavonoids [50,51]. However, the effect of supplemental far-red light on the content of total phenolic and flavonoids was not consistent across different spectrum compositions [49]. This study showed that *P. vulgaris* plantlets exhibited higher TPC and rosmarinic acid under HFR (Figures 2A and 3B,C), while there was an insignificant dynamic in caffeic acid (Figure 3A), similar to the findings of Schwend et al. [8]. Caffeic acid and rosmarinic acid are derived compounds from the phenylpropanoid pathway [60]. Far-red light is known to induce PAL activity [61] and catalyze 3- and 3′-hydroxylation through cytochrome P450-monooxygenase [7,62]. These mechanisms should contribute to an increase in TPC and rosmarinic acid. Additionally, a lower TFC but higher rutin content in *P. vulgaris* plantlets was observed under HFR in this study (Figures 2B and 3B). The genes involved in the flavonoid biosynthetic pathway, including chalcone synthase (CHS), chalcone isomerase, flavanone 3-hydroxylase, and flavanone 3′-hydroxylase (F3′H), are regulated by HY5 [15]. Thus, under HFR, a lower R/FR condition, the biosynthesis of flavonoids might be inhibited, resulting in a reduction in TFC. As speculated by Zhang et al. [63], F3′H expression is upregulated via a MYB transcription factor under red light, leading to an increase in rutin content. The far-red light LEDs used in this study cover a portion of the red-light spectrum (Figure 1), suggesting that HFR lighting may provide additional red light and potentially lead to an increase in rutin.

The macronutrients N, P, and K influence the synthesis of plant secondary metabolites through potential crosstalk among shared transcription factors that are involved in the pathway [64]. N application can affect the internal carbon/nitrogen ratio of plants and inhibit PAL activity, resulting in a decrease in flavonoids and phenolic compounds content [30,32,34]. Under low N, enzyme activity and the expression of genes related to flavonoid biosynthesis are higher, including CHS, flavonoid-3′,5′-hydroxylase, dihydroflavonol-4-reductase [65], and flavanone-3-hydroxylase [34]. The results of Nguyen and Niemeyer [31] showed that basil had higher phenolics, caffeic acid, and rosmarinic acid under low N applications. Allahdadi and Farzane [33] reported that total flavonoid content in artichoke (*Cynara scolymus*) decreased with increasing N. However, Zhao et al. [66] demonstrated that N increased TPC in *Allium fistulosum*. Chen et al. [9] indicated that an appropriate nutrient level enhanced rosmarinic acid content in *P. vulgaris*. In this study, secondary metabolite results showed that TPC and rosmarinic acid were reduced with decreasing nutrient level (Figures 2A and 3C). As several plant enzymes require K for activation [26], the concentration of K in the MS medium also affected the activity of enzymes involved in the biosynthesis pathway of phenolic compounds, leading to the dynamics of TPC and rosmarinic acid. Moreover, there is a strong correlation between TPC and rosmarinic acid (Table 5), indicating that the variation in rosmarinic acid, which serves as the main phenolic compound in cultured *P. vulgaris* plantlets, is sufficient to reflect the TPC.

P. vulgaris possesses antioxidant properties and the ability for free radical scavenging, and various flavonoids and phenolic compounds isolated from *P. vulgaris* are considered sources of antioxidants [3]. The antioxidant activities of rosmarinic acid, caffeic acid, and rutin, including DPPH scavenging ability, reducing power, and ferrous ion chelating capacity, have been previously demonstrated [67–70]. Furthermore, phenolic compounds

exhibit a strong correlation with the antioxidant activity mentioned above [71]. In this study, we also observed a strong correlation between rosmarinic acid and DPPH scavenging activity, as well as reducing power, similar to the results for TPC (Table 5).

The results of this study revealed that the HFR groups exhibited lower IC_{50} levels for DPPH scavenging activity, indicating that *P. vulgaris* plantlets exposed to high far-red light were more capable of scavenging free radicals. Furthermore, the IC_{50} value for DPPH scavenging also exhibited a slight decline with increasing nutrient levels (insignificant). The IC_{50} values for reducing power in extracts declined with increases in far-red light intensity and nutrient level, indicating that high far-red light and high nutrient level enhanced electron donating ability. The dynamics of IC_{50} for DPPH scavenging and reducing power (Table 4) corresponded to the TPC and rosmarinic acid content in the plantlets (Figures 2A and 3C). The finding agrees with Nguyen and Oh [20] who reported supplementing far-red light enhanced the antioxidant capacity, as well as TPC and rosmarinic acid. Previous studies have indicated that elevated N application reduces total phenolic and rosmarinic acid levels, which in turn leads to worse DPPH scavenging and reducing power [31,33]. However, in this study, lower IC_{50} values for DPPH scavenging and reducing power (Table 4), along with higher TPC and rosmarinic acid content (Figures 2A and 3C), were observed in elevated nutrient levels. Similar results were reported by Zhao et al. [66], which revealed a greater dependence on increased N application for inducing DPPH scavenging ability. Extracts of plantlets under LFR + FN conditions exhibited lower IC_{50} values for the ferrous ion chelating effect, indicating a better ion-chelating ability and reduced formation of ferrozine complex from ferrous ions (Table 4). However, there was no significant correlation found between the ferrous ions chelating capacity and all the analyzed secondary metabolites (Table 5). These results imply that other bioactive compounds, such as triterpenes, sterols, volatile oils, and polysaccharides in *P. vulgaris* [3], might be more effective at chelating ferrous ions compared to the analyzed secondary metabolites.

5. Conclusions

In this study, high far-red light induced shade avoidance responses, hindered plantlet growth, and reduced the content of photosynthetic pigments and TFC, but enhanced TPC, the content of rutin, rosmarinic acid, and antioxidant capacity, such as DPPH scavenging activity and reducing power. Meanwhile, for the light regimes assessed, adequate nutrient levels did not necessarily promote plantlet dry weight but increased branch number, photosynthetic pigment content, and accumulation of TPC and rosmarinic acid, as well as antioxidant capacity, including reducing power and ferrous ion chelating effect. Furthermore, DPPH scavenging activity and reducing power were highly correlated with TPC and rosmarinic acid. An increase in the intensity of far-red light and elevated nutrient levels could enhance the content of bioactive compounds of *P. vulgaris*. However, a high intensity of far-red light might inhibit growth. Additionally, plantlet growth could potentially respond differently to varying nutrient levels.

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