

Article

From Photosynthesis to Polyphenols: Caffeine Enhances Fruit Quality, Ripening, and Secondary Metabolism in Olive (*Olea europaea* L.) cv. 'Leccino'

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Highlights

- Low-dose caffeine enhanced photosynthetic performance in 'Leccino' olive trees.
- Caffeine accelerated fruit ripening and increased oil accumulation.
- Caffeine strongly enhanced key polyphenols and antioxidant capacity in olive fruits.
- Caffeine remained confined to roots, supporting its potential use as an olive biostimulant.

Abstract

Despite growing interest in caffeine as a potential plant biostimulant, its effects on perennial crops such as olive remain poorly understood. This study investigated the effects of low-dose caffeine application on leaf gas exchange and photosynthetic activity, fruit ripening, and fruit quality in olive trees (*Olea europaea* L. cv. 'Leccino'). Five-year-old potted plants were irrigated for 52 days with either water (control) or water supplemented with ^{13}C -caffeine. Caffeine-treated plants exhibited enhanced photosynthetic performance, characterized by consistently higher stomatal conductance (+44%), net photosynthetic rate (+21%), and transpiration (95%) throughout the experimental period. Fruit ripening progressed more rapidly in treated plants, as reflected by a higher pigmentation index (+8.8%) and an increase in oil accumulation (+14%), while fruit size and water content remained unchanged. Polyphenolic profiling revealed a marked enrichment in several key compounds, including oleuropein, verbascoside, hydroxytyrosol, and ligstroside, indicating a broad stimulation of the phenylpropanoid and secoiridoid pathways. Caffeine was detected exclusively in roots, confirming minimal translocation to aerial tissues. Hormonal analysis showed elevated abscisic acid levels in treated fruits (+44.2%), consistent with the observed advancement in ripening. Although these results demonstrate the potential of caffeine as a biostimulant in olive, the use of potted plants under controlled greenhouse conditions represents a limitation, and further field trials are required to confirm these effects under commercial growing conditions.



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1. Introduction

Caffeine is a naturally occurring purine alkaloid that acts as a potent biochemical stimulant in plants. It is most commonly found in species such as *Coffea*, *Camellia*, and

Theobroma, where it plays multiple ecological and physiological roles. Unlike its well-known stimulatory effects in animals, caffeine in plants functions primarily as a defensive and regulatory compound. It can influence plant metabolism, growth, and interactions with the surrounding environment by affecting cellular signaling pathways and enzyme activity [1,2].

One of the key stimulatory roles of caffeine in plants is its involvement in chemical defense and allelopathy. Caffeine can inhibit the germination and growth of competing plant species, thereby reducing competition for resources such as light, water, and nutrients [3]. Despite its promising physiological and protective effects on plants, the potential use of caffeine as an active ingredient in biostimulant formulations remains poorly explored. Previous studies [4–9] have primarily focused on annual crops, plants exposed to stress conditions, or in vitro systems, whereas the long-term effects of low-dose caffeine on perennial fruit crops, particularly on photosynthetic performance, fruit ripening, and secondary metabolism, remain largely unknown.

In fact, beyond its allelopathic function, caffeine has been shown to influence photosynthesis positively, particularly under stress conditions. One of the primary mechanisms is the protection and enhancement of photosynthetic pigments such as chlorophyll a and b, which are crucial for light absorption. Foliar application of caffeine in spinach (*Spinacia oleracea*) exposed to cadmium stress resulted in increased chlorophyll content, improved stomatal conductance, and higher photosynthetic rate, suggesting that caffeine helps maintain chloroplast integrity and gas exchange under toxic conditions [4]. Similarly, caffeine seed priming in wheat (*Triticum aestivum*) under water-deficit stress improved seedling vigor and germination by increasing the activity of germination-related enzymes like glucosidase, amylase, and protease, which indirectly supports photosynthetic capacity by promoting healthy early development [5]. In legumes such as lentil (*Lens culinaris*), caffeine treatments enhanced vegetative growth and yield while modulating metabolic and hormonal pathways, which likely contributed to improved photosynthetic performance and stress resilience [6,7]. A recent tissue culture study showed that low concentrations of pure caffeine can stimulate callus formation and direct organogenesis in *Ornithogalum dubium* without added hormones. Because callus induction and organogenesis are typically driven by hormones such as auxins and cytokinins in tissue culture, this suggests that caffeine can act in a hormone-like manner, possibly due to its purine-derived structure and its ability to affect cellular signaling [8].

In a recent report by Muratova and collaborators [9], caffeine was used in micropropagation experiments with *Rubus* sp., showing that caffeine was able to increase rhizogenesis rates. This effect was more pronounced when the medium contained auxins.

Collectively, these studies indicate that caffeine can act as a natural photosynthetic stimulant, supporting pigment maintenance, gas exchange, and stress tolerance, particularly under abiotic stress conditions. Through these mechanisms, caffeine serves as a natural stimulant in plants, enhancing growth, reproductive performance, and resilience to environmental stressors, highlighting its potential use as a biostimulant in agriculture. However, there are relatively few studies investigating the effects of caffeine on perennial plants.

The olive tree (*Olea europaea* L.) is a keystone crop of the Mediterranean region, where fruit quality is strongly linked to its polyphenolic profile including oleuropein, hydroxytyrosol, tyrosol, verbascoside, and ligstroside. Recent reviews emphasize the systemic roles of these compounds in antioxidant defense, inflammation modulation, and even neuroprotection, as well as advances in green extraction technologies and valorization of olive by-products. Hydroxytyrosol, in particular, remains a key quality marker, with growing interest in its stability and bioactivity [10,11].

Recently caffeine treatments on almond trees consistently increased kernel weight and nut set, with no adverse effects on nutrient uptake, nutrient distribution, or leaf chlorophyll levels [12]. These findings support the potential agronomic value of caffeine in perennial production. Based on these insights, we hypothesize that low-dose caffeine, consistent with concentrations proposed for biostimulant use, may enhance photosynthetic performance and accelerate fruit ripening, thereby influencing the polyphenolic profile and antioxidant capacity of olive trees.

Using the olive cultivar ‘Leccino’ as a model system, this study investigated the effects of low-dose caffeine supplied through the root medium by irrigation, with particular attention to its potential effects on above-ground organs. Specifically, we addressed two key questions: (i) whether root-applied caffeine improves leaf gas exchange and photosynthetic activity and (ii) how it influences fruit polyphenol accumulation and hormone dynamics under controlled greenhouse conditions.

2. Material and Methods

2.1. Plant Material and Treatments

Sixteen 5-year-old olive trees (*Olea europaea* L., cv. ‘Leccino’), similar in canopy size and crop load, were bought in a commercial certified nursery and grown in 9.5 L pots in peat-based substrate (Potgrond H 90 Klasmann) at pH = 6.23–6.56. Freshwater (pH = 7.46–8) was used for irrigation and sampled periodically during the experiment to determine the caffeine baseline concentration.

A completely randomized experimental design was applied with eight plants ($n = 8$) per treatment: (a) control ($0 \mu\text{g L}^{-1}$ caffeine) plants were irrigated with freshwater; (b) caffeine plants were irrigated with freshwater supplemented with caffeine (trimethyl- ^{13}C) (0.014 mg L^{-1}) (from Sigma Aldrich). The concentration of 0.014 mg L^{-1} caffeine was selected as a low-dose treatment within the range of environmentally relevant concentrations reported in water systems [13]. This concentration was chosen to evaluate a potential biostimulant effect while minimizing the risk of phytotoxic responses that may occur at higher doses.

The experiment was carried out in a greenhouse located in San Giuliano Terme (N.43.76926550560972. W.10.407108271387008. Pisa, Italy) under natural light conditions and treated from the end of pit hardening (90 days after flowering (DAF)) until veraison (142 DAF). Caffeine (trimethyl- ^{13}C) was used to discriminate our treatment from eventual plant endogenous caffeine. Plants were irrigated each day with 1 L of solution per plant for 52 days. Caffeine-treated plants received a solution containing 0.014 mg L^{-1} caffeine. Considering the volume supplied at each application and the total number of caffeine applications during the experimental period, each plant received a cumulative caffeine dose of 0.728 mg for 52 days.

2.2. Physiological Measurements

During the experiment, a fully expanded leaf from two shoots with olives of each plant was selected, and fluorescence and gas exchange were measured. Measurements of Chlorophyll *a* fluorescence and gas exchange were performed on 1 leaf per shoot selected, and the values were averaged to obtain one mean value for each plant. Eight plants per treatment were considered as independent biological replicates ($n = 8$). A portable pulse-modulated chlorophyll fluorometer (Hansatech FM2, Hansatech, Inc., UK) was used to detect the efficiency of the Chlorophyll *a* fluorescence of photosystem II (PSII). Measures were taken on dark-adapted leaves (1 h) exposed to a saturating light pulse ($8000 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$; 700 ms). PSII efficiency was determined by the ratio of F_v/F_m , in which F_v is the difference between the maximum fluorescence (F_m) and the

minimum fluorescence yield measured in the dark-adapted state (F_0). Instantaneous gas exchanges and net photosynthesis were recorded between 10:00 and 12:00 am with a portable photosynthesis system (CIRAS-2, PP Systems International, MA, USA) equipped with a leaf chamber. Photosynthetic rate (P_n , $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), stomatal conductance (g_s , $\text{mmol m}^{-2} \text{ s}^{-1}$), and transpiration (E , $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$) were measured at a flow rate of 100 mL min^{-1} , ambient temperature, $424 \pm 8 \text{ ppm CO}_2$, and a photosynthetic flux density of $1000 \mu\text{mol m}^{-2} \text{ s}^{-1}$. Intrinsic water use efficiency (iWUE) and water use efficiency (WUE) were derived from leaf gas-exchange measurements. WUE was calculated as $\text{WUE} = P_n/E$, where P_n is the net photosynthetic rate and E is the transpiration rate, whereas iWUE was calculated as $\text{iWUE} = P_n/g_s$, where g_s is the stomatal conductance. Leaf greenness, correlated with chlorophyll amount, was measured using a SPAD meter (SPAD502 Plus Chlorophyll Meter, Spectrum, Plainfield, IL, USA) on ten consecutive fully expanded leaves near an infructescence, and SPAD-recorded values were averaged, obtaining one single measure for each plant.

2.3. Pigmentation Index, Olive Humidity, and Oil Content

At harvest, all the fruits of each plant were collected, and the pigmentation index was calculated. This method involves the manual separation of the olives, the cutting of the pulp to examine it, and identifying the six groups, according to the following characteristics: group 0, green skin; group 1, $<50\%$ black skin with white flesh; group 2, $\geq 50\%$ black skin with white flesh; group 3, 100% black skin with white flesh; group 4, 100% black skin with $<50\%$ purple flesh; group 5, 100% black skin and $\geq 50\%$ purple flesh ($0 \leq \text{PI} \leq 5$). The pigmentation index (Pi) was calculated with $\text{Pi} = \sum (i * n_i)/N$, where i represents the number of the group, n_i is the number of the fruit group, and N is the total number of the analyzed fruits. The fresh weight of fifteen fruits per plant was determined, and the obtained average was used as one single measure for each plant. For all the olives, collected mesocarps (including also epicarps) were separated from woody pits. Part of the mesocarps was stored at -80°C , while the rest was dried. Mesocarp humidity was calculated when constant dry weight was reached, and mesocarp oil content (% FW) was determined by Soxhlet extraction with petroleum ether (VWR Chemicals, Fontenay-sous-Bois, France) for 8 h.

2.4. Caffeine Determination in Olive Plants

At harvest, fresh roots, leaves, and olives were collected for quantification of caffeine- ^{13}C . From each plant, two aliquots of 0.5 g of fresh material were extracted with 3 mL of 0.1% CH_2O_2 , and 3 μL of 15% HCl was added to each plant organ sample. The extracts were centrifuged at $14,000 \text{ g}$ for 15 min and then filtered through a $0.45 \mu\text{m}$ syringe filter. An analytical standard for caffeine (trimethyl- ^{13}C) was used for analysis. The matrix effect and recovery of caffeine (trimethyl- ^{13}C) were also evaluated. An Agilent PhenylHexyl $2 \times 100 \text{ mm } 2.7 \mu\text{m}$ particle size column (Agilent, Santa Clara, CA, USA) was employed for caffeine chromatographic separation. An elution gradient was performed using acetonitrile containing 0.1% v/v formic acid and MilliQ water with 0.1% v/v formic acid. MS-MS detection was performed in the positive ion mode. The analysis was conducted on a Sciex 5500 QTrap+ mass spectrometer (AB Sciex LLC, Framingham, MA, USA), equipped with a Turbo V ion-spray source, and connected to a custom-made ExionLC AC System (Shimadzu Corporation, Kyoto, Japan). Three instrumental replicates of each sample extract were analyzed, and the average values were used for statistical analysis.

2.5. Polyphenol and Hormones Analyses in Olives

Phenols and hormones were quantified in mesocarps of olive at harvest. For each treatment, eight trees were considered independent biological replicates ($n = 8$). For each

plant, two replicates were extracted and analyzed for each parameter, and the resulting values were averaged to obtain a single value for each biological replicate. According to Machado et al. [14], 5 g of mesocarp was dissolved in 50 mL of MeOH: H₂O (80/20, *v/v*) and shaken (90 min, RT) for polyphenol extraction. Samples were then filtered and washed twice with hexane (3:1). A Sciex 5500 QTrap+ mass spectrometer (AB Sciex LLC, Framingham, MA, USA), equipped with a Turbo V ion-spray source and coupled to an ExionLC AC System custom made by Shimadzu (Shimadzu Corporation, Kyoto, Japan), was used to study 23 known phenols (tyrosol, hydroxytyrosol, protocatechuic acid, 4-coumaric acid, vanillic acid, caffeic acid, t-ferulic acid, naringenin, apigenin, luteolin, quercetin, chlorogenic acid, rosmarinic acid, piceid, phloridzin, kaempferol-7-O-glucoside, cichoric acid, cynarin, ligstroside, oleuropein, eriodictiol, rutin, verbascoside). Chromatographic separation was performed with a Phenomenex Kinetex[®] Biphenyl 100 × 2.1 mm, 2.6 µm particle size column (Phenomenex, Torrance, CA, USA). An elution gradient was performed with acetonitrile containing 0.1% formic acid and water with 0.1% formic acid. The gradient program was as follows: 0–10.0 min, 5–95% A; 10.0–12.0 min, 95% A; 12.0–12.1 min, 95–5% A; and 12.1–16.0 min, 5% A. The flow rate of the mobile phase was 0.3 mL/min.

Hormones (abscisic acid, jasmonic acid, and gibberellic acid) present in the olive mesocarp (0.5 g) were extracted using 5 mL of methanol, water, and formic acid (75:20:5, *v:v:v*). The mixture was stirred overnight in a dark cold room (4 °C); then, the samples were sonicated for 10 min in an ice bath and centrifuged for 15 min at 4000 rpm (5810 R Eppendorf). The supernatant was filtered through a 0.45 µm filter and used for chromatography on a Phenomenex Kinetex F5 100 × 2.1 mm, 2.6 µm particle size column (Phenomenex, Torrance, CA, USA). The elution was performed in gradient mode as reported above. MS/MS experiments were carried out using electrospray ionization, in negative ion mode for acidic components and in positive ion mode for basic analytes, using nitrogen as the collision gas. The compound parameters declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP) were adjusted for the specific SRM for each component. The MRM transitions and corresponding compound parameters are listed in Table 1. Data collection and processing utilized Analyst 1.7.3 software and OS 1.7 software from AB Sciex. The information-dependent acquisition (IDA) criteria were used for qualitative validation. This process involved switching from selected reaction monitoring (SRM) to enhanced product ions (EPI) to obtain the MS/MS spectrum using a collision energy (CE) of 35 eV with a CE spread of 15 eV. All data recorded were normalized considering the matrix effect. For quantitative analysis, calibration curves were constructed using a standard mix containing all the analytes at concentrations in the range of 0.25–512 ng/mL.

Table 1. MRM acquisition parameters of caffeine, selected polyphenols and hormones. Compound-dependent parameters for scheduled MRM scan survey, per metabolite: retention time (RT) in minutes, precursor mass (Q1), product mass (Q3), declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP) in volts.

Name	RT (min)	Q1	Q3	DP (V)	CE (eV)	CXP (V)
Tyrosol	2.43	137.0	106.0	−78.0	−22.0	−13.0
Hydroxytyrosol	1.92	153.0	123.0	−100.0	−20.0	−17.0
Protocatechuic acid	1.92	153.0	109.0	−86.0	−21.0	−7.0
4-Coumaric acid	3.35	163.0	119.0	−65.0	−18.0	−11.0
Vanillic acid	2.86	166.9	108.0	−62.0	−26.0	−13.0
Caffeic acid	2.80	178.9	135.0	−86.0	−23.0	−11.0
t-Ferulic acid	3.65	193.0	134.0	−62.0	−20.0	−8.0

Table 1. Cont.

Name	RT (min)	Q1	Q3	DP (V)	CE (eV)	CXP (V)
Naringenin	5.13	270.9	150.9	−120.0	−25.0	−10.5
Apigenin	5.02	268.9	117.0	−120.0	−49.0	−14.0
Luteolin	4.60	284.9	133.0	−130.0	−44.6	−17.4
Quercetin	4.64	301.0	150.9	−113.0	−38.0	−8.0
Chlorogenic acid	2.56	353.0	151.0	−61.0	−24.0	−9.0
Rosmarinic acid	3.97	359.0	161.0	−70.0	−22.5	−10.0
Piceid	3.54	389.1	227.0	−125.0	−32.0	−11.0
Phloridzin	4.01	435.1	272.9	−135.0	−23.0	−5.0
Kaempferol-7-O-glucoside	3.85	447.1	284.9	−158.0	−38.0	−5.0
Cichoric acid	3.75	473.1	149.0	−76.0	−30.0	−8.0
Cynarin	3.08	515.1	191.0	−120.0	−41.0	−7.0
Ligstroside	4.42	523.1	291.0	−117.0	−32.0	−11.8
Oleuropein	4.09	539.1	275.0	−137.0	−32.0	−12.0
Eriodictiol	4.6	287.1	135.0	−121.0	−34.0	−10.0
Rutin	3.29	609.2	299.9	−154.0	−48.0	−11.0
Verbascoside	3.48	623.1	160.9	−82.0	−43.0	−7.0
Caffeine- ¹³ C	2.13	198.1	140.1	−119	−28	−15
Jasmonic acid	20	209.1	58.9	−138	−17	−9
Absciscic acid	20	263.2	153.1	−77	−15	−7
Gibberellic acid	20	345.1	143	−70	−36	−7

2.6. Olive DPPH Assay

The scavenging activity of 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was evaluated according to the protocol of Francini et al. [15]. For each plant, two replicate samples were extracted and analyzed in duplicate, and the resulting values were averaged to obtain a single value for each biological replicate. Methanolic olive extract (200 µL) for polyphenols was mixed with 100 mM Tris-HCl solution pH 7.0 (800 µL). Samples were then supplemented with 250 µM DPPH (1 mL) and kept in the dark (30 min). As a reference control, methanol (80:2, *v:v*) was used, and absorbance was measured at 517 nm using a spectrophotometer (Infinite 200 PRO, Tecan Italia Srl Milan, Italy). The inhibition of DPPH (%) was calculated considering: Inhibition ratio (%) = ((absorbance of control − absorbance of sample)/(absorbance of control)) × 100.

2.7. Statistical Analysis

Statistical analyses were performed in RStudio and Prism-GraphPad 9.0 for Mac (R Project for Statistical Computing, version 1.4.1103). Each dataset was analyzed with the two-tailed *t*-test, ***, *p* < 0.001; **, *p* < 0.01; *, *p* < 0.05. The Shapiro–Wilk test was used to check normality. The graphs were drawn in Prism-GraphPad 9.0 for Mac and RStudio. Metabolomic Data Analysis with MetaboAnalyst 5.0 was also carried out.

3. Results

Caffeine treatment significantly affected leaf gas exchange performance of ‘Leccino’ olive plants throughout the experimental period. Both stomatal conductance (*g_s*) and net

photosynthetic rate (P_n) increased compared to controls throughout the experimental period (Figure 1A,B). Differences in g_s were statistically significant at days 18, 30, and 37, with significant increases of 39%, 36%, and 44%, respectively (Figure 1A). Similarly, P_n rose by 24%, 23%, and 21% on the same dates, reaching values between 8 and 19 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ (Figure 1B). Transpiration rate (E) also showed marked increases (+59%, +35%, and +95% at days 18, 30, and 37) (Figure 1C). PSII efficiency (F_v/F_m) exhibited a positive trend (Figure 1D), with a significant difference at day 18. Although not significant, the trend of the relative amount of chlorophyll expressed as SPAD units is higher in the treated leaves than in the control ones (Figure 1E).

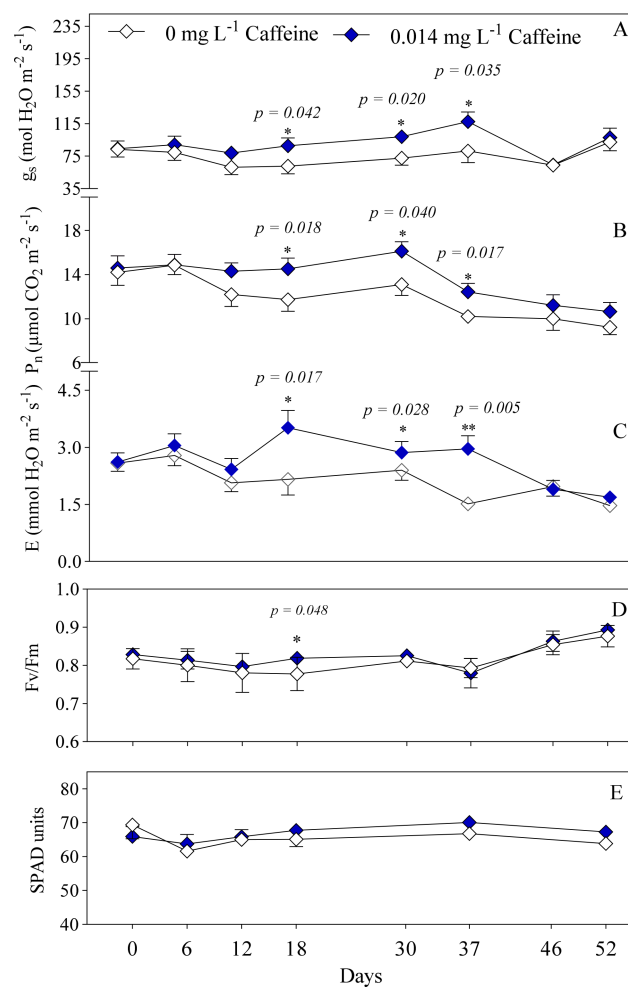


Figure 1. Time course of plant gas exchange measurements and CO₂ assimilation during the experiment. (A) g_s (mmol H₂O m⁻² s⁻¹) = stomatal conductance; (B) P_n ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) = estimated net photosynthetic rate at environmental CO₂ measurements; (C) E (mmol H₂O m⁻² s⁻¹) = transpiration; (D) F_v/F_m ; and (E) SPAD units in *Olea europaea* cv. ‘Leccino’ during 52 days of exposure to tap water (Control) and 0.014 mg L⁻¹ of caffeine (Treated). Values show means (n = 8) ± SE. The results for each day were analyzed by a two-tailed *t*-test. Significantly different data are denoted by *, *p* < 0.05; **, *p* < 0.01.

Intercellular CO₂ concentration (C_i), water use efficiency (WUE), and intrinsic water use efficiency (iWUE) were not significantly affected by caffeine treatment throughout the experimental period (Table 2). Despite the observed increases in photosynthetic rate, stomatal conductance, and transpiration, C_i , WUE, and iWUE remained comparable between control and caffeine-treated plants at all sampling times (Table 2).

Table 2. Effects of caffeine (0.014 mg L^{−1}) on intercellular CO₂ concentration (Ci), water use efficiency (WUE), and intrinsic water use efficiency (iWUE) in comparison to control plants. Values (n = 8) are reported as mean ± SD. (Student's *t*-test, ns = *p* > 0.05.)

Day	Ci			WUE			iWUE		
	Control	Caffeine (0.014 mg L ^{−1})	<i>t</i> -Test	Control	Caffeine (0.014 mg L ^{−1})	<i>t</i> -Test	Control	Caffeine (0.014 mg L ^{−1})	<i>t</i> -Test
0	127 ± 58.8	89 ± 58.59	ns	5.05 ± 1.25	5.82 ± 1.43	ns	0.160 ± 0.043	0.183 ± 0.045	ns
6	104 ± 57.5	108 ± 48.41	ns	5.05 ± 1.13	5.04 ± 0.835	ns	0.181 ± 0.052	0.176 ± 0.036	ns
12	88 ± 35.2	81 ± 38.02	ns	5.14 ± 0.55	5.69 ± 0.911	ns	0.193 ± 0.042	0.198 ± 0.043	ns
18	112 ± 57.1	104 ± 47.13	ns	4.91 ± 1.31	4.84 ± 1.335	ns	0.373 ± 0.577	0.174 ± 0.031	ns
30	102 ± 38.1	130 ± 68.99	ns	5.24 ± 0.89	6.17 ± 2.539	ns	0.175 ± 0.043	0.206 ± 0.109	ns
37	176 ± 65.6	227 ± 51.94	ns	5.79 ± 1.88	4.61 ± 1.725	ns	0.144 ± 0.045	0.112 ± 0.033	ns
46	102 ± 47.4	102 ± 21.15	ns	8.67 ± 8.76	6.30 ± 2.105	ns	0.252 ± 0.230	0.175 ± 0.028	ns
52	222 ± 39.4	218 ± 32.72	ns	5.94 ± 1.17	6.39 ± 0.885	ns	0.105 ± 0.023	0.113 ± 0.023	ns

At the end of the experiment, all olives were harvested from each plant and counted. No significant differences in fruit number were observed between control and caffeine-treated plants, with 90 ± 19 and 94 ± 31 fruits per plant, respectively. The total fruit yield per plant was also determined at harvest and averaged 163 ± 35 g plant^{−1} in control plants and 175 ± 58 g plant^{−1} in caffeine-treated plants, with no significant difference between treatments (*p* = 0.633).

After 52 days of treatment, fruits from caffeine-treated plants exhibited a significantly higher pigmentation index (+8.8%) and oil content (+14%), indicating accelerated ripening and enhanced commercial quality (Figure 2A,B,I), while fruit fresh weight and water content remained unchanged (Figure 2C,D). Hormonal profiling indicated a significant increase in abscisic acid (ABA) (+44.2%), whereas jasmonic acid and gibberellic acid showed no significant variation (Figure 2E,F,G). Moreover, olive treated with caffeine showed a significant improvement in DPPH scavenging capacity (Figure 2H).

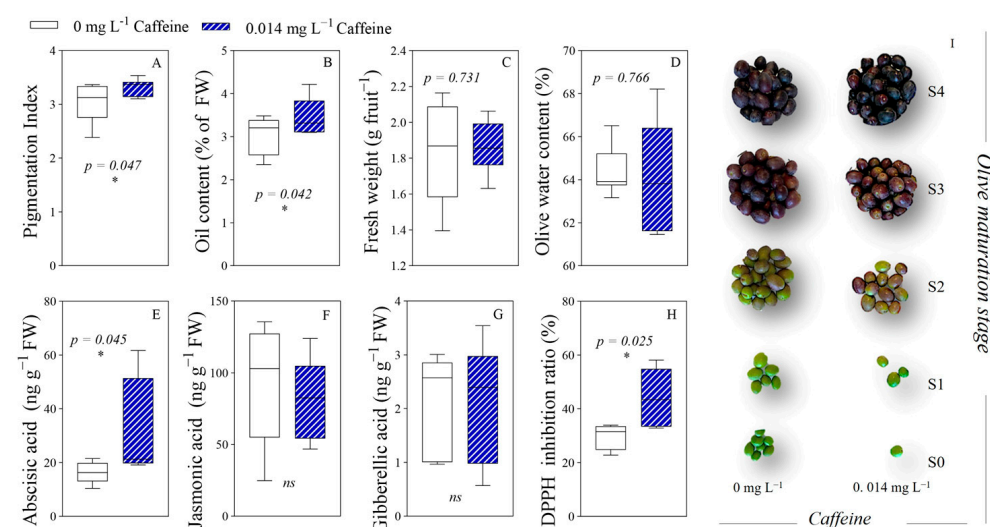


Figure 2. The effects of treatments with tap water (control) and caffeine (0.014 mg L^{−1}) on fruit of 'Leccino' plants after 52 days of the experiment. (A) Pigmentation index, (B) oil content (% of FW), (C) fresh weight (g fruit^{−1}), (D) olive water content (%), (E) abscisic acid concentration (ng g^{−1} FW), (F) jasmonic acid (JA), (G) gibberellic acid (GA3), (H) DPPH assay, and (I) olive distribution among different stages (S0, green skin; S1, <50% black skin with white flesh; S2, ≥50% black skin with white flesh; S3, 100% black skin with white flesh; S4, 100% black skin with <50% purple flesh). Box plots represent data from 8 biological replicates. Significantly different data are denoted by *, *p* < 0.05. ns = not significant.

Caffeine uptake was detected exclusively in roots ($0.07\text{--}0.62\text{ ng g}^{-1}\text{ FW}$), confirming its limited translocation to leaves and fruits. Principal Component Analysis (Figure 3A) of polyphenol fruits of *Olea europaea* cv. ‘Leccino’ plants clearly separated control and caffeine-treated samples, with PC1 and PC2 explaining 53.9% and 17.0% of variance. Among the 24 measured polyphenols in PC1, rutin is responsible for the maximum variation (Figure 3B). Caffeine induced extensive remodeling of the fruit polyphenol profile, with significant increases in several compounds associated with antioxidant activity and nutraceutical value. Among secoiridoids, oleuropein and ligstroside increased 2.7-fold ($p = 0.0003$) and 1.04-fold ($p = 0.025$), respectively (Figure 4). Phenolic alcohols tyrosol and hydroxytyrosol rose by 44.6% and 69.8%. Significant increases were also observed for phenolic acids such as chlorogenic acid (+42.9%), piceid (+62.7%), vanillic acid (+58.8%), 4-coumaric acid (+44.9%), verbascoside (+188%), and caffeic acid (+38.3%). Among flavonoids, rutin and naringenin increased by 87.7% and 15.2%, respectively. Conversely, cynarin, quercetin, and rosmarinic acid decreased by 67.8%, 54.7%, and 78%. Other compounds (e.g., protocatechuic acid, ferulic acid, apigenin, luteolin) showed no significant changes (Figure 4).

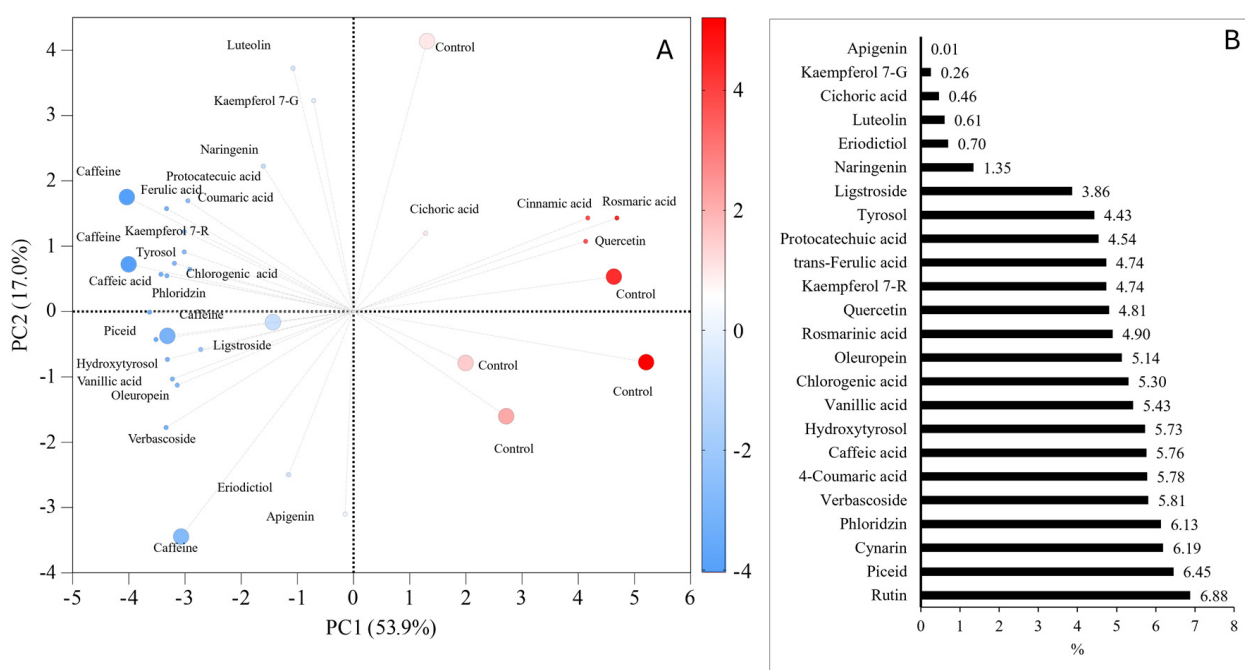


Figure 3. Principal Component (PC) Analysis plot (A) and PC1 loading values (B) of the 24 detected polyphenols in fruits of *Olea europaea* cv. ‘Leccino’ plants after 52 days of exposure to 0 (control) or 0.014 mg L^{-1} of caffeine. The explained variances are shown in brackets. The color gradient from red to blue represents polyphenol concentration, indicating high to low concentration levels, respectively.

The enhancement of DPPH scavenging activity observed in caffeine-treated olives was closely associated with the accumulation of specific phenolic compounds, particularly oleuropein, hydroxytyrosol, and verbascoside (Figure 5). Correlation analyses revealed significant positive relationships between the concentrations of these metabolites and antioxidant capacity, suggesting that they were among the major contributors to the improved radical scavenging activity observed in treated fruits.

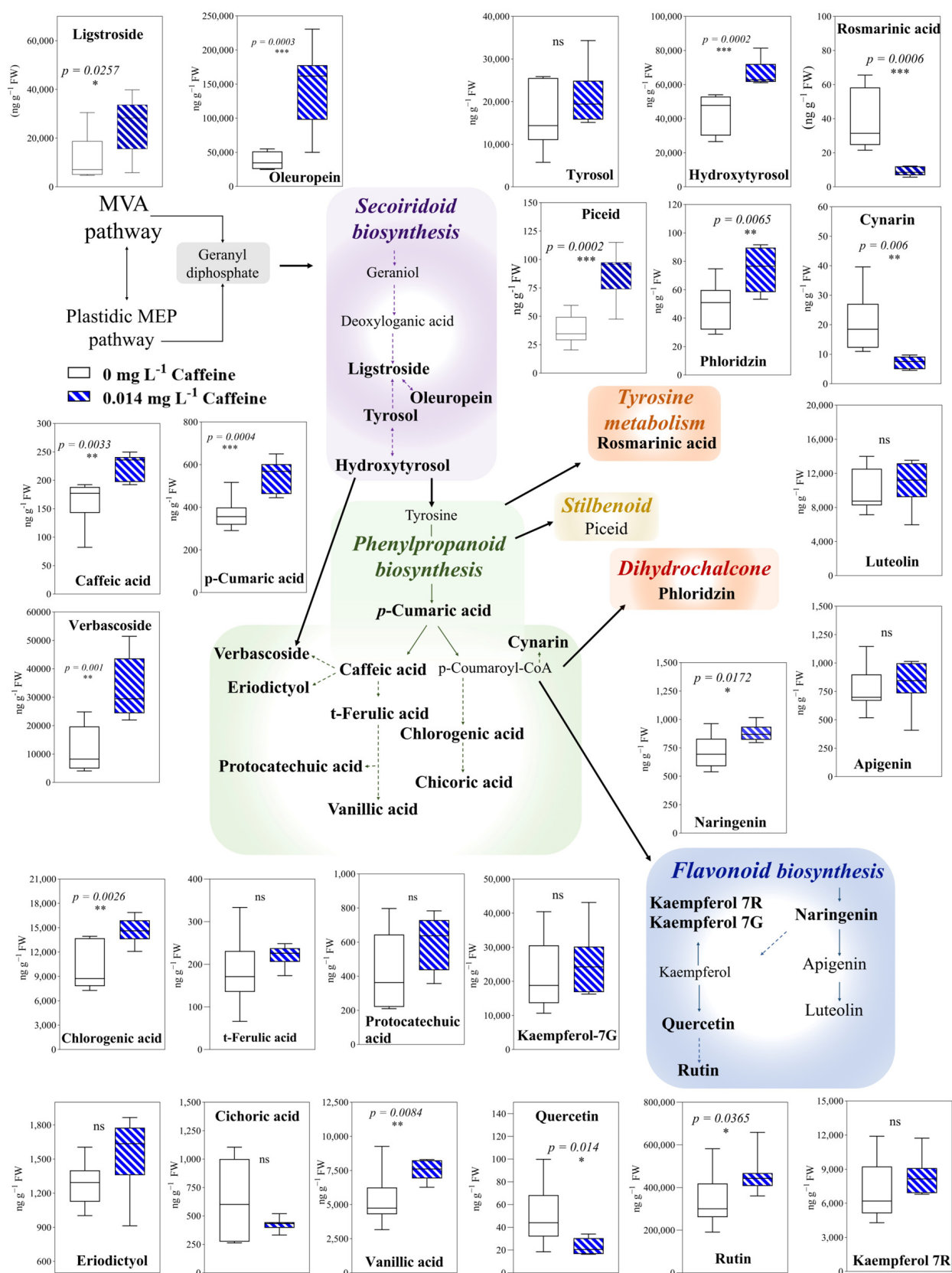


Figure 4. Schematic representation of the putative biosynthetic pathways of the main polyphenol in fruits of *Olea europaea* cv. 'Leccino' plants after 52 days of exposure to 0 (control) or 0.014 mg L⁻¹ of caffeine. Box plots represent the concentration (ng g⁻¹ FW) of specific polyphenols that were analyzed by a two-tailed *t*-test (n = 6). Significantly different data are denoted by *, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.001. ns = not significant.

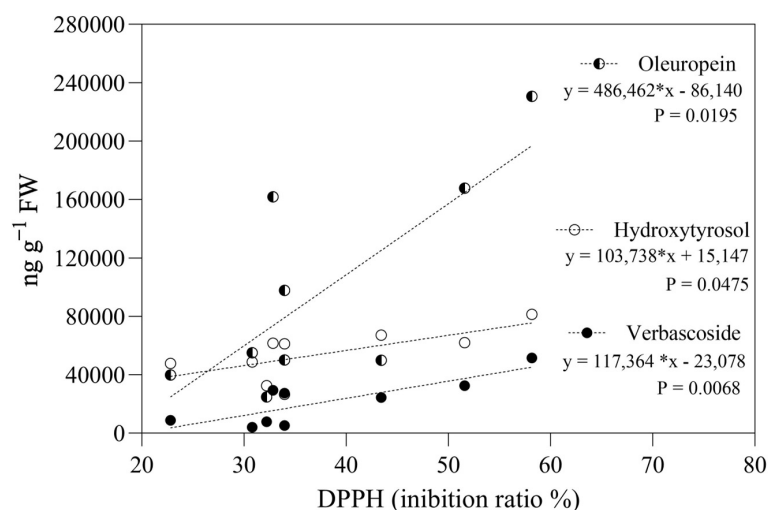


Figure 5. Linear regression between DPPH inhibition ratio and specific polyphenol oleuropein, hydroxytyrosol, and verbascoside concentration (ng g^{-1} FW) in fruits of *Olea europaea* cv. 'Leccino' plants after 52 days of exposure to 0 (Control) or 0.014 mg L^{-1} of caffeine.

4. Discussion

To our knowledge, this is the first study investigating the long-term effects of low-dose ^{13}C -caffeine applied through root irrigation to potted olive trees under controlled greenhouse conditions, with particular emphasis on photosynthetic performance, fruit ripening, and polyphenol accumulation.

Regarding the accumulation of caffeine in olive plants, roots seem to act as a selective barrier limiting translocation to the aerial parts. The retention of caffeine in olive roots may result from species-specific differences in root uptake, compartmentalization, metabolism, or xylem loading, which could restrict its translocation to aerial tissues. This behavior may differ from that reported in *Populus*, where caffeine can be transported to leaves [16,17]. Future studies combining time-course ^{13}C -caffeine tracing with the analysis of caffeine metabolites in different plant organs could help clarify the mechanisms responsible for its root retention and limited translocation in olive.

Although caffeine was not detected in the leaves, root-applied caffeine increased photosynthetic performance. This suggests that the observed response may not require the direct translocation of caffeine to the aerial tissues but could involve an indirect root-to-shoot signaling mechanism. These results are consistent with data from *Spinacia oleracea*, where foliar application of caffeine improved stomatal conductance and photosynthetic rate [4]. Similarly, caffeine application promoted the opening of stomata in *Vicia faba* L. plants [18]. Despite the higher stomatal conductance and net photosynthetic rate observed in treated plants, C_i remained comparable to controls, suggesting that the increased diffusion of CO_2 into the leaf was balanced by a parallel enhancement in carbon fixation. Similarly, neither WUE nor iWUE showed significant changes, indicating that the stimulation of photosynthesis by caffeine was accompanied by a proportional increase in transpiration and stomatal opening. These findings suggest that caffeine enhanced overall gas exchange activity without altering the efficiency of water use or the balance between CO_2 supply and assimilation.

PSII also shows a greater efficiency trend, with differences 18 days after the start of the experiment. All the values obtained from the F_v/F_m are coherent with the optimal values known for the 'Leccino' cultivar, as also reported by Moretti et al. [19] in olive plants.

The effects of caffeine treatments appear to stimulate natural plant processes, similar to those observed with other biostimulants [20]. Caffeine treatment did not significantly affect the number of fruits per plant. However, the positive effects of caffeine observed in

this study are consistent with those reported for other biostimulants applied to olive trees. Seaweed-based biostimulants have been shown to influence fruit maturation, productivity, and oil quality, whereas protein hydrolysates can promote vegetative growth in young olive trees [21,22]. In the present study, low-dose caffeine similarly stimulated photosynthesis, stomatal conductance, and transpiration, while also promoting fruit ripening, oil accumulation, and changes in the polyphenolic profile, suggesting its potential as a biostimulant for olive cultivation.

As a natural alkaloid, caffeine is degraded into secondary metabolites such as uric acid, xanthine, and allantoin, which can facilitate nitrogen assimilation [23]. In the study conducted by Jené et al. [7], the use of caffeine induced a significant increase in the biomass of lentils. Caffeine can also be used as a rhizogenesis stimulator by adding it to the rooting medium for rooting of the genus *Rubus* in vitro plants [9]. In *Solanum lycopersicum* cv. 'Panarea', 56 days of caffeine (1 mg L⁻¹) application induced an increment of the fruit number per plant of 20% compared to the control [24].

In our experimental condition, the treatments significantly influenced the pigmentation index of the cultivar 'Leccino', with an acceleration of the ripening process of the olive fruits followed by an increased oil yield, as also observed in other studies on olive trees after treatments with hormones [25].

Although caffeine-treated olives exhibited a more advanced ripening stage, their polyphenol content remained high. Considering the general decline of several major phenolic compounds during olive fruit maturation [26,27], this finding suggests that caffeine treatment may have contributed to maintaining phenolic levels despite the progression of ripening. This response may be related to a mild stress or elicitation effect induced by caffeine, which could stimulate secondary metabolism and promote phenolic accumulation.

Moreover, we observed an increase in ABA concentrations that is usually associated with the onset of ripening and metabolic changes in the fruit [25].

According to Alagna [26], among the secoiridoids, oleuropein is present at the highest concentration and is notable for its nutritional and antioxidant properties. Among flavonoids, rutin, and among phenolic alcohols, hydroxytyrosol and verbascoside (the major derivative of hydroxycinnamic acid) are most common [27]. Caffeine treatment increased the concentrations of these compounds, as well as ligstroside, chlorogenic acid, vanillic acid, naringenin, 4-coumaric acid, caffeic acid, piceid, and phloridzin. Among the factors influencing polyphenol concentration, the ripening stage of the fruit is particularly important. In this study, plants treated with caffeine exhibited a more advanced ripening stage. This advanced ripening stage is consistent with the higher ABA content observed in caffeine-treated plants, aligning with findings by Baktir et al. [28], who reported increased ABA levels proportional to fruit maturation in olives. Considering the well-established role of ABA in promoting stomatal closure, the increased stomatal conductance observed following caffeine treatment suggests that the root-to-shoot response may involve changes in hormonal signaling; however, because ABA was not quantified in roots, the involvement of root-derived ABA cannot be directly assessed in the present study.

The increased concentration of the main polyphenols in olive is in line with a general increase in DPPH scavenging activity. The increased DPPH scavenging activity reflects enhanced in vitro antioxidant capacity and should not be directly interpreted as evidence of health benefits in humans. However, the relationship between the concentration of phenolic compounds and DPPH radical removal activity in the fruit is well known in the literature [15,29,30], and in 'Leccino' cv., oleuropein, with a higher concentration in treated olives, may have played an important role. Xie et al. [31] observed a dose-dependent behavior of oleuropein in relation to a high antioxidant capacity due to the synergism of its phenolic compounds, compared to the other polyphenols present in olives. In our olive

fruits, correlation analyses indicated that not only oleuropein but also verbascoside and hydroxytyrosol played key roles in improving DPPH scavenging activity. The improvement of olive phenolic compounds is a major objective, as they represent key components of the healthy Mediterranean diet [32].

Under our experimental condition, oleuropein increased by approximately 2.7-fold following caffeine treatment, representing one of the largest metabolic changes detected. The strong correlation between verbascoside concentration and DPPH values also suggests that caffeine stimulated not only the secoiridoid pathway but also other branches of phenylpropanoid metabolism involved in antioxidant defense. Oleuropein, hydroxytyrosol, and verbascoside collectively accounted for the main metabolic shifts induced by caffeine treatment, supporting the hypothesis that caffeine enhances the nutraceutical quality of olive fruits through a coordinated stimulation of secondary metabolism. In addition, the stronger antioxidant activity may be linked to the advancement of ripening observed in treated fruits, as indicated by the higher pigmentation index and ABA concentration [33]. Nevertheless, despite the accelerated ripening stage, caffeine-treated olives accumulated higher levels of several key phenolics, suggesting that caffeine actively promoted the biosynthesis and/or accumulation of antioxidant metabolites beyond the effects expected from ripening alone. The significant correlations between DPPH activity and oleuropein, hydroxytyrosol, and verbascoside highlight these compounds as key biochemical markers of the caffeine-induced improvement in olive fruit quality, supporting the potential of caffeine as a biostimulant capable of enhancing both antioxidant capacity and polyphenol compounds.

5. Conclusions

Although caffeine was detected exclusively in the roots and was not detected in leaves or fruits, root-applied caffeine induced clear physiological and metabolic responses in above-ground organs. This finding suggests that the observed effects may involve indirect root-to-shoot signaling rather than direct caffeine translocation. In fact, long-term exposure to low-dose caffeine positively influenced both leaf gas-exchange activity and fruit quality in *Olea europaea* L. cv. 'Leccino'. Caffeine enhanced photosynthetic activity by stimulating stomatal conductance and carbon assimilation, while maintaining PSII efficiency within optimal physiological ranges. At the fruit level, caffeine application induced accelerated ripening, ABA accumulation, promoted oil deposition, and substantially enhanced the concentration of several health-promoting polyphenols, including oleuropein, hydroxytyrosol, verbascoside, and ligstroside. These changes translated into a greater antioxidant capacity, highlighting the potential of caffeine as an innovative biostimulant for olive cultivation. Future studies should clarify the molecular mechanisms underlying caffeine-induced metabolic responses and assess their effectiveness across a wider range of caffeine concentrations, under field conditions, and in climate-related stress scenarios.

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