horticulturae

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Comparative Metabolic Fingerprinting of Olive (*Olea europaea* L.) Cultivars Under Boron Foliar Fertilisation

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Abstract

Olive (*Olea europaea* L.) cultivars often exhibit genotype-specific responses to micronutrient management. In this study, we investigated the metabolic leaf fingerprinting of three cultivars 'Rošinjola', 'Leccino', and 'Istarska bjelica' at two sampling periods (SP-I = 64 days after treatment (DAT) and SP-II = 118 DAT), following boron foliar fertilisation (+B = 41.62 mM B; −B = 0 mM B) applied 50 days after anthesis. To our knowledge, this is the first study to provide such a detailed evaluation of boron-induced shifts in phenolic metabolism in olive leaves. At harvest (SP-II), all three cultivars showed higher concentrations of total identified phenolic compounds in +B plants compared with the −B controls. Notably, the concentration of verbascoside at harvest was higher in +B plants of 'Istarska bjelica' and 'Leccino', but not in 'Rošinjola'. Oleuropein content increased in +B plants at harvest to a level higher than 4870 mg/100 g DW, irrespective of cultivar. Conversely, apigenin-7-glucoside declined from SP-I to SP-II in 'Leccino' regardless of treatment, whereas in 'Istarska bjelica', this decrease occurred only in control plants, with boron preventing the seasonal decline. These findings confirm the prolonged effect of boron foliar fertilisation on phenolic metabolism in olive leaves and highlight cultivar-specific differences in metabolic responses. Further research is needed to clarify how these metabolic shifts relate to primary plant metabolism and how they influence olive oil quality traits among cultivars grown under Croatian conditions.

Keywords: *Olea europaea* L.; plant metabolism; olive leaves; LC-MS/MS; phenolic compounds; oleuropein

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Academic Editor: Anoop Kumar Srivastava

Received: 11 November 2025
Revised: 8 December 2025
Accepted: 12 December 2025
Published: 15 December 2025

Citation: Žurga, P.; Pasković, M.P.; Major, N.; Goreta Ban, S.; Marcelić, Š.; Pongrac, P.; Bajoub, A.; Babojelić, M.S.; Petek, M.; Pasković, I. Comparative Metabolic Fingerprinting of Olive (*Olea europaea* L.) Cultivars Under Boron Foliar Fertilisation. *Horticulturae* **2025**, *11*, 1521. https://doi.org/10.3390/horticulturae11121521

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1. Introduction

Olive (*Olea europaea* L.) is a crop of paramount agricultural, economic and cultural importance in the Mediterranean region and beyond. It is cultivated mainly for its fruits, which are used both as table olives and for the production of olive oil, a cornerstone of healthy diets due to its favourable lipid profile and bioactive minor compounds, such as phenolic compounds.

The Istria region, encompassing both Croatian and Slovenian sides, hosts different autochthonous and allochthonous olive cultivars, such as 'Istarska bjelica', 'Rošinjola', and 'Leccino', the latter being the most widespread and economically significant introduced cultivar in newly planted olive orchards in Croatia [1,2]. 'Istarska bjelica' and 'Leccino' are vigorous cultivars characterised by dense canopies. They are partially self-fertile and consistently yield abundantly. 'Istarska bjelica' tends to grow upright, whereas 'Leccino' displays a distinctive drooping habit. 'Rošinjola', on the other hand, is moderately vigorous with a spreading, dense canopy, featuring short elliptic leaves and long inflorescences with small flowers. All three cultivars produce medium-sized, ovoid fruits. Cold tolerance is well documented for 'Istarska bjelica' and 'Leccino', but the agronomic traits of 'Rošinjola' are only partially understood [1,3].

Beyond the fruit and oil, olive leaves represent an abundant by-product in olive orchards. Leaves are recognised as a rich source of nutraceutical compounds, particularly phenolics such as oleuropein, apigenin, luteoline, hydroxytyrosol and verbascoside, which have demonstrated potent antioxidant, anti-inflammatory, antimicrobial, hepatoprotective, antidiabetic and cardiometabolic effects [1,2,4]. Olive leaf extracts have been shown to lower LDL cholesterol, triglycerides and markers of inflammation in humans, highlighting their promise as nutraceuticals [5].

Given this potential, the research focus has recently shifted to pre-harvest agronomic practices, which could modulate the phenolic profiles of olive leaves. Among such practices, foliar fertilisation offers a promising route because it can directly influence leaf nutrient status and its secondary metabolism, potentially enhancing the content or altering the composition of beneficial phenolics.

Boron (B) is a micronutrient with several roles in plant physiology, including cell wall structure, sugar transport, reproductive development and membrane function [6,7]. Boron foliar fertilisation in olives has been shown to enhance in vitro pollen germination and the fruit set, but this increase seems to be year and/or cultivar-dependent [8,9]. It is known that B can affect the phenylpropanoid metabolic pathway, one of the main pathways for phenol synthesis in plants [10]. Indeed, recent research has confirmed its role in increasing total phenol concentration and the sensorial and organoleptic characteristics of extra virgin olive oil [11]. Furthermore, it has been demonstrated that foliar boron application on olive plantlets significantly increased oleuropein levels in both young and old leaves after 90 days [12]. An increase in total phenols in olives using two different boron foliar formulations has also been observed [13]. However, a quite opposite effect has also been noted: boron foliar application increased boron concentration in leaves, buds, and fruits, while total phenol concentrations in leaves and buds decreased with higher boron levels, indicating a complex relationship between boron and phenolic accumulation [14].

Although foliar boron fertilisation has been linked to enhancements of the main commercially valuable traits in olive trees [9,15], its specific impact on the secondary metabolites in olive leaves remains insufficiently explored. Overall, to our knowledge, previous research on this subject has been primarily focused on total phenolics or a limited selection of olive leaf compounds, often within a single cultivar or at a single growth stage. Given the agronomic and physiological importance of phenolic compounds, especially in plant defence, stress resilience, and potential health benefits, a more comprehensive investigation

is necessary. This study was conducted to evaluate the effect of boron foliar fertilisation on a broader olive leaf phenolic profile, including over thirty individual phenolic compounds across three cultivars and two harvest dates. By accounting for both genetic and temporal variability, the results aim to enhance understanding of boron's role in influencing secondary metabolism in olives, with possible implications for nutrient management and the bioactive value of leaves.

Hence, we hypothesised that foliar boron fertilisation would (i) alter the phenolic composition of olive leaves and (ii) trigger cultivar-specific metabolic responses.

2. Materials and Methods

2.1. Olive Leaf Sampling

In the olive orchard collection at the Institute of Agriculture and Tourism in Poreč, Croatia, the trial was set up as a Randomised Complete Block Design (RCBD) with three olive cultivars ('Leccino', 'Istarska bjelica', and 'Rošinjola'), two foliar treatments (−B or +B), and two sampling periods (SP-I and SP-II), with eight replicates. Thus, 32 samples per cultivar were collected during the trial, with each sample representing a single tree. Treatments were applied 50 days after anthesis (12 July 2023), while sampling was conducted 64 (SP-I) and 118 (SP-II) days after treatment (i.e., 114 (14 September 2023), and 168 (7 November 2023) days after anthesis). This timing allowed the evaluation of both short-term and long-term treatment effects on plant physiological and biochemical responses. Consistent with our previous trial [13], the treatments were as follows: (i) Control '0 mM B' (−B) treatment, treated with water and surfactant only (1 mL of Tensiofill® (Loreo, Italy; glycol + dimethylpolysiloxane emulsion) per 1 L of water), and (ii) a '41.62 mM B' (+B) treatment, consisting of water, surfactant, and boron. The '41.62 mM B' (+B) treatment was prepared by diluting boron ethanolamine (FertiGlobal, Larderello, Italy, FOLIAREL® FLUSSIG, containing 150 g B L^{-1}) in water at a rate of 3 mL L^{-1}, resulting in the expected boron concentration of approx. 450 mg B L^{-1} in the final solution. Each tree was treated with approximately 5 L of prepared +B or −B solution until runoff. Each block consisted of six 5-year-old olive trees, two per cultivar. From July to November, mean air temperatures in the region decreased markedly, from 26.5 °C in July to 12.8 °C in November. Over the same period, monthly precipitation fluctuated strongly, ranging from 43.8 to 219.8 mm, highlighting a shift from hot, drier midsummer conditions to cooler, substantially wetter late-autumn weather (https://meteo.hr/, accessed on 1 December 2025). At the sampling time, only fully developed olive leaves from current-season shoots were collected evenly around the tree. In the laboratory, leaves were carefully washed first with tap water, then with a 1% acetic acid solution in deionised water, and finally rinsed twice with deionised water. Afterwards, they were air-dried to constant weight and ground to a fine powder using a Retsch ZM 200 mill (Retsch GmbH, Haan, Germany) before analysis [2].

2.2. Determination of the Leaf Extract Phenolic Profiles

The identification and quantification of polyphenolic compounds in olive leaf lyophilised extracts were performed using an LC-MS/MS system, following the method of Major et al. [16], with slight modifications. The system comprised Nexera HPLC 40XS and a triple quadrupole mass spectrometer (LCMS-8045), all supplied by Shimadzu (Kyoto, Japan). Separation was carried out using a C18 core–shell column (2.1 mm × 150 mm, 2.7 µm; Agilent, Palo Alto, CA, USA). The analyses were performed at 37 °C with an injection volume of 1 µL. Chromatographic separation was achieved using a linear gradient elution with mobile phase A (water containing 0.1% acetic acid) and mobile phase B (methanol containing 0.1% acetic acid) at a flow rate of 0.35 mL/min. The gradient programme was

as follows: 0–0.75 min, 98% A; 0.75–15 min, 98% A to 50% A; 15–15.1 min, 50% A to 0% A; 15.1–20 min, 0% A; 20–20.1 min, 0% A to 98% A; and 20.1–25 min, 98% A. Targeted metabolites were identified and quantified by comparing retention times, precursor/product ion transitions, and peak areas with those of corresponding analytical standards.

2.3. Statistical Analysis

A three-way analysis of variance (three-way ANOVA) was conducted to examine the effects of the main factors (treatment, cultivar, and sampling period) and their interactions. When applicable, Tukey's post hoc test was used, and differences between means (n = 8) were regarded as significant at $p < 0.05$. These analyses were conducted with TIBCO Statistica 14.1.0.8 software (TIBCO StatSoft®, Palo Alto, CA, USA).

3. Results

3.1. Flavonoids

3.1.1. Flavones

Among all detected flavones (8 in total), the most abundant were apigenin-7-glucoside, luteolin-7-glucoside, luteolin-4-rutinoside, and luteolin-7-rutinoside (Table 1). The interaction between cultivar (Cv), treatment (T), and sampling period (SP), Cv × T × SP, was significant for apigenin-7-glucoside ($p < 0.001$). It underwent a significant reduction between SPs for control plants in 'Istarska bjelica' and 'Leccino', but not in 'Rošinjola'. The same level of reduction was achieved with the treatment in the first SP in both cultivars, and it remained unchanged throughout the experiment. There was no observed effect of boron on apigenin-7-glucoside in 'Rošinjola' across two SPs, regardless of the treatment (Table 1).

There were no significant Cv × T × SP interactions for major monitored luteolin derivatives ($p = 0.121$ for luteolin-7-glucoside; $p = 0.254$ for luteolin-4-rutinoside; $p = 0.070$ for luteolin-7-rutinoside). However, when observing Cv × T interactions, treatment with boron caused a significant increase in all three monitored major luteolin derivatives in 'Istarska bjelica' and 'Leccino', except for luteolin-7-rutinoside in 'Leccino', which showed a slight increase, but without statistical significance. Luteolin derivatives in 'Rošinjola' cultivar remained unaffected with boron treatment (Table 1). Significant T × SP interactions were observed: boron treatment increased the concentration of luteolin derivatives at the second SP, thereby either increasing concentrations that were otherwise stable over time or stabilising concentrations that tended to decrease over time (Table 1).

Table 1. Effect of foliar treatment, sampling periods, and their interactions on the concentration of flavonoids in the leaves of three olive cultivars.

	Flavonoids (mg/100 g DW)									
	Flavones						Flavonols			Dihydroflavonols
CULTIVAR (Cv.)	**Apigenin**	**Apigenin-7-glucoside (Cosmosiin)**	**Luteolin-4-rutinoside**	**Luteolin-7-glucoside**	**Luteolin-7-rutinoside**	**Quercetin**	**Quercetin-3-glucoside (Isorquercitroside)**	**Quercetin-3-rhamnoside (Quercitrin)**	**Quercetin-3-rutinoside (Rutin)**	**Dihydroquercetin (Taxifolin)**
Istarska bjelica (IB)	0.77 ± 0.06	125.82 ± 10.23	99.12 ± 5.55	299.24 ± 15.47	123.09 ± 4.57	40.19 ± 3.62	17.86 ± 1.09	43.69 ± 2.3	145.32 ± 9.25	47.92 ± 3.44
Leccino (L)	0.75 ± 0.07	134.23 ± 13.57	166.32 ± 7.73	405.68 ± 19.77	130.29 ± 3.74	438.89 ± 43.1	25.82 ± 1.31	12.67 ± 0.53	47.75 ± 2	77.12 ± 3.09
Rošinjola (R)	1.16 ± 0.07	74.23 ± 3.21	75.5 ± 2.53	359.64 ± 13.68	84.36 ± 2.93	195.31 ± 16.2	13.1 ± 0.91	12.74 ± 0.58	53.36 ± 4.4	79.52 ± 3.28
TREATMENT (T)										
Control (C)	1.06 ± 0.07	139.64 ± 10.91	102.14 ± 5.66	315.2 ± 16.27	105.85 ± 3.79	177.1 ± 33.86	17.1 ± 1.28	20.15 ± 2.09	75.38 ± 7.85	64.48 ± 3.83
Boron (B)	0.73 ± 0.04	83.21 ± 2.92	125.15 ± 8.25	394.5 ± 10.43	119.31 ± 4.49	272.49 ± 28.99	20.75 ± 1.02	25.91 ± 2.64	88.92 ± 8.33	71.89 ± 2.76
SAMPLING PERIOD (SP)										
I	0.84 ± 0.05	136.07 ± 11.04	104.4 ± 5.88	388.42 ± 9.25	108.72 ± 4.05	290.84 ± 34.91	21.42 ± 1.19	25.61 ± 2.55	89.6 ± 8.92	81.95 ± 2.88 a
II	0.96 ± 0.07	86.78 ± 3.72	122.88 ± 8.21	321.28 ± 17.52	116.44 ± 4.41	158.75 ± 26.07	16.43 ± 1.06	20.45 ± 2.21	74.69 ± 7.14	54.43 ± 2.56 b
Cv. × T										
IB × C	0.94 ± 0.11	159.56 ± 16.38	86.35 ± 7.74 d	241.53 ± 21.59 c	108.65 ± 6.7 b	26.43 ± 3.92	14.82 ± 1.78 c	36.39 ± 3.54	126.47 ± 15.4	38.84 ± 4.21 b
IB × B	0.61 ± 0.04	92.08 ± 3.55	111.88 ± 6.76 c	356.94 ± 8.91 b	137.53 ± 3.69 a	53.95 ± 3.7	20.89 ± 0.69 b	50.99 ± 1.47	164.17 ± 8.3	57.01 ± 4.48 b
L × C	0.95 ± 0.13	182.92 ± 20.31	142.84 ± 7.51 b	358.07 ± 30.21 b	123.08 ± 5.36 ab	360.76 ± 80	23.59 ± 2.27 ab	11.57 ± 0.89	43.23 ± 2.81	78.14 ± 4.69 a
L × B	0.56 ± 0.04	85.53 ± 5.74	189.79 ± 10.84 a	453.28 ± 20.04 a	137.49 ± 4.72 a	517.02 ± 21.52	28.06 ± 1.15 a	13.76 ± 0.48	52.27 ± 2.45	76.1 ± 4.15 a
R × C	1.3 ± 0.1	76.44 ± 4.62	77.22 ± 3.99 d	346 ± 23.73 b	85.81 ± 3.78 c	144.11 ± 22.93	12.91 ± 1.64 c	12.5 ± 1.12	56.42 ± 8.2	76.46 ± 5.68 a
R × B	1.02 ± 0.08	72.01 ± 4.54	73.78 ± 3.19 d	373.28 ± 13.65 b	82.92 ± 4.58 c	246.51 ± 14.46	13.29 ± 0.85 c	12.97 ± 0.37	50.3 ± 3.38	82.57 ± 3.31 a
Cv. × SP										
IB × I	0.66 ± 0.05	154.6 ± 17.37	96.53 ± 8.13 c	329.99 ± 8.72	129.27 ± 4.39 ab	47.62 ± 3.96	20.73 ± 0.98 b	49.31 ± 2.04	164.16 ± 12.39	60.36 ± 4.12
IB × II	0.89 ± 0.11	97.04 ± 4.54	101.7 ± 7.77 c	268.49 ± 28.06	116.91 ± 7.87 b	32.76 ± 5.59	14.98 ± 1.68 c	38.07 ± 3.67	126.48 ± 12.35	35.49 ± 3.35
L × I	0.6 ± 0.04	173.05 ± 22.44	146.23 ± 7.42 b	457.52 ± 8.24	122.09 ± 2.93 b	607.24 ± 16.84	30.44 ± 0.86 a	14.44 ± 0.35	52.44 ± 1.96	92.07 ± 2
L × II	0.9 ± 0.13	95.4 ± 7.54	186.41 ± 11.76 a	353.84 ± 34.5	138.48 ± 6.35 a	270.54 ± 60.11	21.21 ± 1.88 b	10.9 ± 0.8	43.07 ± 3.13	62.17 ± 2.37
R × I	1.25 ± 0.08	80.56 ± 4.56	70.45 ± 2.45 d	377.77 ± 10.34	74.81 ± 3.06 d	217.67 ± 13.45	13.11 ± 1.27 c	13.1 ± 0.77	52.2 ± 5.96	93.41 ± 3.21
R × II	1.07 ± 0.1	67.89 ± 4.07	80.54 ± 4.13 cd	341.52 ± 24.97	93.92 ± 3.73 c	172.95 ± 28.93	13.1 ± 1.34 c	12.38 ± 0.89	54.53 ± 6.65	65.62 ± 2.89
T × SP										
C × I	0.84 ± 0.08	180.48 ± 17.65	101.68 ± 6.91 b	381.99 ± 14.23 a	108.85 ± 5.11 b	289.57 ± 52.96	21.8 ± 1.83 a	25.38 ± 3.54	92.14 ± 13.47	80.54 ± 4.55
C × II	1.28 ± 0.09	98.8 ± 5.49	102.6 ± 9.11 b	248.42 ± 22.17 b	102.84 ± 5.64 b	64.63 ± 27.93	12.41 ± 1.17 b	14.93 ± 1.69	58.61 ± 6.77	48.42 ± 4.11
B × I	0.83 ± 0.07	91.66 ± 3.86	107.13 ± 9.64 b	394.86 ± 11.98 a	108.59 ± 6.39 b	292.11 ± 46.64	21.05 ± 1.56 a	25.85 ± 3.74	87.06 ± 11.98	83.36 ± 3.61
B × II	0.63 ± 0.04	74.75 ± 3.7	143.17 ± 12.53 a	394.15 ± 17.34 a	130.04 ± 5.61 a	252.87 ± 35.02	20.45 ± 1.35 a	25.97 ± 3.81	90.77 ± 11.84	60.43 ± 2.6
Cv. × T × SP										
IB × C × I	0.67 ± 0.06 c	215.09 ± 14.94 a	96.98 ± 13.71	317.82 ± 14.92	126.73 ± 7.78	40.59 ± 2.79 e	20.56 ± 1.89	47.79 ± 3.71 a	166.04 ± 21.84 a	53.84 ± 2.67
IB × C × II	1.21 ± 0.16 ab	104.03 ± 6.76 bc	75.73 ± 6.07	165.25 ± 10.58	90.58 ± 6.2	12.27 ± 0.85 e	9.08 ± 0.8	24.98 ± 1.65 b	86.9 ± 9.6 b	23.83 ± 2.14
IB × B × I	0.64 ± 0.07 c	94.12 ± 4.91 bc	96.09 ± 9.76	342.16 ± 7.79	131.82 ± 4.49	54.65 ± 6.72 e	20.9 ± 0.75	50.82 ± 1.83 a	162.29 ± 13.4 a	66.88 ± 7.31
IB × B × II	0.58 ± 0.04 c	90.05 ± 5.36 bc	127.68 ± 5.39	371.73 ± 14.74	143.24 ± 5.38	53.25 ± 3.65 e	20.89 ± 1.23	51.16 ± 2.43 a	166.06 ± 10.7 a	47.14 ± 2.19

Table 1. *Cont.*

	Flavonoids (mg/100 g DW)									
	Flavones					Flavonols				Dihydroflavonols
CULTIVAR (Cv.)	**Apigenin**	**Apigenin-7-glucoside (Cosmosiin)**	**Luteolin-4-rutinoside**	**Luteolin-7-glucoside**	**Luteolin-7-rutinoside**	**Quercetin**	**Quercetin-3-glucoside (Isorquercitroside)**	**Quercetin-3-rhamnoside (Quercitrin)**	**Quercetin-3-rutinoside (Rutin)**	**Dihydroquercetin (Taxifolin)**
L × C × I	0.55 ± 0.03 c	245.85 ± 24.38 a	132.74 ± 5.77	451.23 ± 14.11	118.56 ± 2.45	631.84 ± 24.5 a	31.2 ± 1.27	14.7 ± 0.34 c	51.69 ± 1.87 bc	93.21 ± 2.65
L × C × II	1.35 ± 0.14 a	119.99 ± 6.49 b	152.94 ± 13.39	264.92 ± 35.1	127.6 ± 10.56	89.67 ± 76.35 de	15.97 ± 1.96	8.45 ± 0.7 c	34.78 ± 3.13 c	63.08 ± 4.75
L × B × I	0.66 ± 0.06 c	100.25 ± 7.09 bc	159.72 ± 12.29	463.8 ± 8.97	125.63 ± 5.23	582.64 ± 21.04 ab	29.68 ± 1.19	14.18 ± 0.62 c	53.18 ± 3.59 bc	90.94 ± 3.13
L × B × II	0.46 ± 0.02 c	70.82 ± 5.38 c	219.87 ± 9.69	442.76 ± 40.12	149.36 ± 5.27	451.41 ± 17.64 b	26.45 ± 1.88	13.34 ± 0.73 c	51.36 ± 3.54 bc	61.26 ± 1.09
R × C × I	1.31 ± 0.11 a	80.49 ± 6.72 bc	75.31 ± 2.94	376.92 ± 17.27	81.28 ± 4.81	196.28 ± 14.26 cd	13.65 ± 2.38	13.66 ± 1.49 c	58.68 ± 11.64 bc	94.57 ± 6.06
R × C × II	1.29 ± 0.18 ab	72.4 ± 6.46 bc	79.12 ± 7.65	315.09 ± 42.91	90.34 ± 5.67	91.94 ± 35.68 de	12.17 ± 2.38	11.35 ± 1.67 c	54.16 ± 12.29 bc	58.36 ± 2.79
R × B × I	1.19 ± 0.13 ab	80.62 ± 6.63 bc	65.59 ± 3.23	378.62 ± 12.62	68.34 ± 2.27	239.05 ± 21.01 c	12.57 ± 1.06	12.54 ± 0.46 c	45.72 ± 2.09 bc	92.26 ± 2.67
R × B × II	0.84 ± 0.03 bc	63.39 ± 4.83 c	81.96 ± 3.74	367.95 ± 25.12	97.5 ± 4.89	253.96 ± 20.95 c	14.02 ± 1.34	13.41 ± 0.56 c	54.89 ± 6.21 bc	72.88 ± 3.62
CULTIVAR (Cv.)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
TREATMENT (T)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.011	0.001
SAMPLING PERIOD (SP)	0.033	<0.001	<0.001	<0.001	0.014	<0.001	<0.001	<0.001	0.005	<0.001
Cv. × T	0.746	<0.001	**<0.001**	**0.031**	**<0.001**	0.007	**0.031**	<0.001	0.004	**0.001**
Cv. × SP	0.001	<0.001	**0.011**	0.144	**<0.001**	<0.001	**<0.001**	<0.001	0.008	0.626
T × SP	<0.001	<0.001	**0.001**	**<0.001**	**<0.001**	<0.001	**<0.001**	<0.001	0.001	0.034
Cv. × T × SP	**0.046**	**<0.001**	0.254	0.121	0.07	**<0.001**	0.071	**<0.001**	**0.011**	0.292

Results are expressed as means ± standard errors (n = 8). Different letters for each variable indicate significant differences between mean values for treatment, sampling period, cultivar, and their interaction ($p \leq 0.05$) using three-way ANOVA and Tukey's post hoc test ($p \leq 0.05$). The absence of letters indicates either non-significant differences or the presence of a higher-order interaction. DW—dry weight.

3.1.2. Flavonols and Dihydroflavonols

Quercetin was the most abundant among the detected flavonols and dihydroflavonols (11 in total), followed by quercetin-3-rutinoside (rutin), dihydroquercetin, quercetin-3-rhamnoside (quercitrin) and quercetin-3-glucoside (Table 1). There were statistically significant Cv × T × SP interactions for quercetin, quercitrin and rutin ($p < 0.001$; <0.001; 0.011, respectively), but not for quercetin-3-glucoside ($p = 0.071$) and dihydroquercetin ($p = 0.292$). In the control plants, quercetin, quercitrin, and rutin showed a significant decrease between sampling periods in at least one of the two cultivars, 'Istarska bjelica' and 'Leccino' (Table 1). At SP-II, boron treatment significantly increased quercetin concentrations in both 'Leccino' and 'Rošinjola', while no significant increase was observed in 'Istarska bjelica'. In contrast, boron increased the concentrations of both quercetin-3-glucoside and quercetin-3-rhamnoside only in 'Istarska bjelica', with no increases detected in 'Leccino' or 'Rošinjola' (Table 1). Quercetin-3-glucoside showed significantly increased concentration following treatment (Cv × T) but only in 'Istarska bjelica'; however, its concentration decreased between SPs (Cv × SP) in 'Istarska bjelica' and 'Leccino' and was stabilised following treatment (T × SP) approximately at the level of the control at the first SP. Dihydroquercetin showed a significant decrease over time, which was not improved with boron treatment (T × SP) (Table 1).

3.2. Phenolic Acids

3.2.1. Hydroxybenzoic Acids

The highest concentration of all detected hydroxybenzoic acids (6 in total) was measured for vanillic-4-glucoside, followed by much less abundant vanillic acid, 4-hydroxybenzoic acid, and 3,4-dihydroxybenzoic acid (protocatechuic acid) (Table 2). No significant Cv × T × SP interactions were observed in this group. There was no significant effect of boron treatment on vanillic-4-glucoside. Its concentration was significantly higher in 'Istarska bjelica' than in 'Leccino' and 'Rošinjola', and it increased over time regardless of the cultivar and treatment. However, neither of the interactions (Cv × T, Cv × SP, T × SP, and Cv × T × SP) appeared significant for vanillic-4-glucoside (Table 2). Temporal decreases were compound- and cultivar-dependent: protocatechuic acid decreased in all cultivars, 4-hydroxybenzoic acid in 'Istarska bjelica' and 'Rošinjola', and vanillic acid only in 'Istarska bjelica' (Table 2). The differences in their concentrations among cultivars remained significant in Cv × T interactions for protocatechuic acid and vanillic acid, despite treatments mainly not affecting the concentrations, except vanillic acid in 'Rošinjola' (Table 2).

3.2.2. Hydroxycinnamic Acids

Among hydroxycinnamic acids and their derivatives (7 in total), verbascoside was by far the most abundant, followed by chlorogenic acid (Table 2). In both cases, the Cv × T × SP interactions were statistically significant ($p = 0.001$; <0.001, respectively). While the concentration of chlorogenic acid was much lower in 'Istarska bjelica' and 'Rošinjola' and remained unchanged regardless of treatment and SPs, 'Leccino' cultivar showed a significant reduction in its concentration between SPs for control plants, which was notably altered with boron treatment regardless of sampling period (Table 2). Verbascoside concentrations showed numerical variations across sampling periods in all cultivars under control conditions, but none of these changes were statistically significant. At SP-II, boron treatment significantly increased verbascoside levels in both 'Istarska bjelica' and 'Leccino' compared to the control, while no significant change was observed in 'Rošinjola' (Table 2).

Table 2. Effect of foliar treatment, sampling periods, and their interactions on the concentration of phenolic acids in the leaves of three olive cultivars.

	Phenolic Acids (mg/100 g DW)					
		Hydroxybenzoic Acids			Hydroxycinnamic Acids	
CULTIVAR (Cv.)	**3.4-Dihydroxybenzoic Acid (Protocatechuic Acid)**	**4-hydroxybenzoic Acid**	**Vanillic Acid**	**Vanillic-4-glucoside**	**Chlorogenic Acid**	**Verbascoside**
Istarska bjelica (IB)	2.52 ± 0.18	3.92 ± 0.18	3.13 ± 0.13	20.1 ± 1.36 a	2.8 ± 0.05	86.79 ± 15.4
Leccino (L)	4.34 ± 0.19	3.44 ± 0.12	2.42 ± 0.06	14.8 ± 0.49 b	40.75 ± 1.48	141.96 ± 21.11
Rošinjola (R)	2.99 ± 0.14	3.3 ± 0.17	3.72 ± 0.12	14.46 ± 0.42 b	12.95 ± 0.36	48.4 ± 9.27
TREATMENT (T)						
Control (C)	3.33 ± 0.19	3.58 ± 0.14	3.21 ± 0.14	16.14 ± 0.75	18.54 ± 2.49	47.1 ± 8.35
Boron (B)	3.23 ± 0.17	3.53 ± 0.13	2.97 ± 0.09	16.77 ± 0.84	19.13 ± 2.4	137.66 ± 15.62
SAMPLING PERIOD (SP)						
I	4.1 ± 0.14	3.99 ± 0.13	3.13 ± 0.13	14.78 ± 0.65 b	20.19 ± 2.67	63.12 ± 8.01
II	2.46 ± 0.12	3.12 ± 0.11	3.05 ± 0.11	18.12 ± 0.86 a	17.48 ± 2.18	121.64 ± 17.32
Cv. × T						
IB × C	2.4 ± 0.25 d	3.72 ± 0.28	2.99 ± 0.2 bc	19.04 ± 1.94	2.76 ± 0.1	35.9 ± 10.38
IB × B	2.63 ± 0.25 cd	4.12 ± 0.23	3.26 ± 0.17 b	21.16 ± 1.92	2.84 ± 0.05	137.67 ± 22.96
L × C	4.47 ± 0.26 a	3.56 ± 0.18	2.48 ± 0.09 cd	14.41 ± 0.64	40.56 ± 2.54	63.32 ± 17.34
L × B	4.21 ± 0.27 a	3.33 ± 0.17	2.37 ± 0.09 d	15.19 ± 0.74	40.94 ± 1.62	220.6 ± 26.78
R × C	3.11 ± 0.21 b	3.46 ± 0.28	4.17 ± 0.16 a	14.96 ± 0.58	12.3 ± 0.51	42.09 ± 14.79
R × B	2.86 ± 0.19 bc	3.14 ± 0.22	3.28 ± 0.1 b	13.97 ± 0.59	13.61 ± 0.47	54.71 ± 11.44
Cv. × SP						
IB × I	3.33 ± 0.16b	4.51 ± 0.21 a	3.42 ± 0.16 a	17.68 ± 1.63	2.85 ± 0.09	65.32 ± 8.42
IB × II	1.7 ± 0.11 d	3.33 ± 0.22 bc	2.83 ± 0.18 b	22.52 ± 2.04	2.75 ± 0.06	108.25 ± 29.12
L × I	5.33 ± 0.1 a	3.54 ± 0.19 b	2.24 ± 0.07 c	13.58 ± 0.43	44.5 ± 2.05	107.93 ± 15.8
L × II	3.35 ± 0.07 b	3.35 ± 0.17 bc	2.6 ± 0.09 bc	16.03 ± 0.77	36.99 ± 1.73	175.99 ± 37.92
R × I	3.63 ± 0.08 b	3.93 ± 0.23 ab	3.74 ± 0.19 a	13.1 ± 0.48	13.2 ± 0.49	16.1 ± 0.99
R × II	2.34 ± 0.13 c	2.68 ± 0.15 c	3.71 ± 0.15 a	15.83 ± 0.48	12.7 ± 0.53	80.7 ± 14.66
T × SP						
C × I	4.15 ± 0.22	4.1 ± 0.18	3.3 ± 0.21	14.17 ± 0.74	21.27 ± 4.14	55.08 ± 7.37
C × II	2.51 ± 0.19	3.06 ± 0.17	3.12 ± 0.18	18.11 ± 1.2	15.81 ± 2.75	39.12 ± 14.99
B × I	4.05 ± 0.2	3.89 ± 0.19	2.97 ± 0.14	15.4 ± 1.06	19.1 ± 3.46	71.15 ± 14.22
B × II	2.42 ± 0.14	3.17 ± 0.15	2.97 ± 0.12	18.14 ± 1.26	19.16 ± 3.41	204.17 ± 20.24
Cv. × T × SP						
IB × C × I	3.23 ± 0.21	4.52 ± 0.26	3.42 ± 0.28	15.86 ± 2.02	2.93 ± 0.17 e	67.64 ± 13.14 cde
IB × C × II	1.57 ± 0.18	2.92 ± 0.31	2.56 ± 0.2	22.22 ± 3.03	2.6 ± 0.06 e	4.15 ± 1.01 e
IB × B × I	3.44 ± 0.26	4.5 ± 0.35	3.42 ± 0.18	19.5 ± 2.53	2.78 ± 0.06 e	62.99 ± 11.38 cde
IB × B × II	1.83 ± 0.11	3.73 ± 0.26	3.1 ± 0.28	22.83 ± 2.95	2.9 ± 0.08 e	212.35 ± 23.15 b
L × C × I	5.46 ± 0.1	3.5 ± 0.27	2.27 ± 0.08	13.26 ± 0.43	48.09 ± 2.64 a	80.12 ± 6.82 cde
L × C × II	3.48 ± 0.09	3.61 ± 0.25	2.69 ± 0.13	15.56 ± 1.1	33.02 ± 2.12 c	46.52 ± 34.08 de
L × B × I	5.2 ± 0.16	3.58 ± 0.28	2.22 ± 0.12	13.89 ± 0.77	40.92 ± 2.72 b	135.74 ± 28.33 bc
L × B × II	3.22 ± 0.08	3.09 ± 0.19	2.52 ± 0.12	16.49 ± 1.14	40.96 ± 1.96 b	305.45 ± 14.62 a
R × C × I	3.75 ± 0.15	4.27 ± 0.31	4.2 ± 0.25	13.37 ± 0.76	12.8 ± 0.81 d	17.49 ± 1.57 de
R × C × II	2.48 ± 0.21	2.65 ± 0.21	4.13 ± 0.21	16.54 ± 0.37	11.8 ± 0.62 d	66.68 ± 27.61 cde
R × B × I	3.52 ± 0.04	3.59 ± 0.3	3.27 ± 0.19	12.82 ± 0.63	13.6 ± 0.58 d	14.71 ± 1.1 de
R × B × II	2.2 ± 0.16	2.7 ± 0.23	3.29 ± 0.06	15.11 ± 0.85	13.61 ± 0.77 d	94.71 ± 10.11 cd
CULTIVAR (Cv.)	<0.001	0.003	<0.001	**<0.001**	<0.001	<0.001
TREATMENT (T)	0.246	0.752	0.032	0.466	0.474	<0.001
SAMPLING PERIOD (SP)	<0.001	<0.001	0.437	**<0.001**	0.001	<0.001
Cv. × T	**0.024**	0.111	**0.000**	0.344	0.816	<0.001
Cv. × SP	**0.005**	**0.007**	**0.003**	0.471	<0.001	0.566
T × SP	0.972	0.285	0.44	0.49	0.001	<0.001
Cv. × T × SP	0.975	0.103	0.469	0.73	**<0.001**	**0.001**

Results are expressed as means ± standard errors (n = 8). Different letters for each variable indicate significant differences between mean values for treatment, sampling period, cultivar, and their interaction ($p \leq 0.05$) using three-way ANOVA and Tukey's post hoc test ($p \leq 0.05$). The absence of letters indicates either non-significant differences or the presence of a higher-order interaction. DW—dry weight.

3.3. Secoiridoids

Oleuropein, the most abundant olive phenol, did not reach statistical significance in Cv × T × SP interactions ($p = 0.051$), as well as in the other two studied secoiridoids, oleacein ($p = 0.354$) and oleuropein aglycone ($p = 0.580$) (Table 3). However, T × SP interactions showed that the boron treatment had no effect on oleuropein concentration at SP-I but significantly increased its levels at SP-II. The other two secoiridoids showed different responses at SP-II: in B+ olives, oleacein showed a significant decrease, while oleuropein aglycone levels

were also notably lower, although the reduction was less pronounced. Conversely, the Cv × SP interaction revealed that the average oleuropein aglycone concentration increased only in the 'Rošinjola' cultivar when two SPs are compared (Table 3).

Table 3. Effect of foliar treatment, sampling periods, and their interactions on the concentration of secoiridoids, simple phenols and total phenols (TPC) in the leaves of three olive cultivars.

	Secoiridoids (mg/100 g DW)			Simple Phenols (mg/100 g DW)	LC-MS/MS TPC (mg/100 g DW)
	Dialdehydic Form of Oleuropein Aglycone	Glycosylated Secoiridoid	Aglycone Form of Oleuropein	Phenylethyl Alcohols	
CULTIVAR (Cv.)	**Oleacein**	**Oleuropein**	**Oleuropein Aglycone**	**Hydroxytyrosol**	
Istarska bjelica (IB)	42.68 ± 11.33	4064.22 ± 247.58	16.18 ± 1.82	16.26 ± 0.66	5237.47 ± 286.32
Leccino (L)	35.35 ± 9.62	4399.36 ± 273.63	18.97 ± 2.47	19.86 ± 0.65	6197.79 ± 344.88
Rošinjola (R)	54.6 ± 11.59	4228.69 ± 218.12	42.51 ± 5.25	21.41 ± 0.6	5436.03 ± 238.96
TREATMENT (T)					
Control (C)	75.82 ± 10.66	3438.28 ± 218.28	33.68 ± 4.02	17.97 ± 0.62	4727.13 ± 269.08
Boron (B)	12.61 ± 1.69	5023.24 ± 84.99	18.1 ± 1.86	20.39 ± 0.53	6520.4 ± 118.87
SAMPLING PERIOD (SP)					
I	18.17 ± 2.81	4647.74 ± 107.99	18.59 ± 2.28	18.44 ± 0.5	6122.79 ± 144.43
II	70.25 ± 11.05	3813.78 ± 250.06	33.18 ± 3.84	19.92 ± 0.68	5124.74 ± 298.81
Cv. × T					
IB × C	79.86 ± 18.62	3147.73 ± 335.74	22.85 ± 2.63	14.01 ± 0.44 c	4203.8 ± 395.87
IB × B	5.5 ± 0.44	4980.72 ± 169.24	9.51 ± 0.91	18.51 ± 0.96 b	6271.14 ± 199.17
L × C	62.97 ± 16.74	3535.8 ± 437.61	25.5 ± 4.32	18.6 ± 0.91 ab	5168.28 ± 571.53
L × B	7.73 ± 0.83	5262.93 ± 136.15	12.45 ± 0.93	21.11 ± 0.84 ab	7227.3 ± 153.84
R × C	84.62 ± 20.58	3631.32 ± 367.27	52.68 ± 9.5	21.29 ± 0.89 ab	4809.29 ± 406.91
R × B	24.58 ± 3.39	4826.06 ± 119.08	32.34 ± 3.18	21.54 ± 0.82 a	6062.76 ± 134.44
Cv. × SP					
IB × I	7.4 ± 0.96	4437.18 ± 172.14	14.58 ± 2.44 bc	15.43 ± 0.66 d	5664.86 ± 192.74
IB × II	77.96 ± 19.08	3691.27 ± 452.97	17.78 ± 2.71 bc	17.09 ± 1.13 cd	4810.09 ± 526.63
L × I	8.98 ± 1.11	5030.81 ± 146.16	12.86 ± 1.03 c	20.32 ± 0.79 ab	7036.79 ± 159.55
L × II	61.71 ± 16.99	3767.92 ± 484.72	25.09 ± 4.38 bc	19.4 ± 1.04 bc	5358.79 ± 610.18
R × I	38.12 ± 5.69	4475.23 ± 209.56	28.34 ± 5.66 b	19.57 ± 0.59 bc	5666.72 ± 223.5
R × II	71.09 ± 22.06	3982.15 ± 380.3	56.68 ± 7.44 a	23.26 ± 0.82 a	5205.33 ± 423.06
T × SP					
C × I	21.09 ± 4.74 b	4444.64 ± 168.06 b	21.19 ± 4.24 b	18.09 ± 0.7 b	5953.41 ± 227.76
C × II	130.54 ± 13.46 a	2431.92 ± 280.02 c	46.16 ± 5.89 a	17.85 ± 1.04 b	3500.84 ± 336.58
B × I	15.25 ± 3.03 b	4850.84 ± 125.83 ab	16 ± 1.62 b	18.79 ± 0.72 b	6292.17 ± 175.76
B × II	9.97 ± 1.37 b	5195.64 ± 105.38 a	20.2 ± 3.32 b	21.98 ± 0.64 a	6748.64 ± 149.43
Cv. × T × SP					
IB × C × I	9.09 ± 1.62	4271.64 ± 273.49	18.25 ± 4.49	14.9 ± 0.47	5537.47 ± 320.46 bcd
IB × C × II	150.62 ± 7.19	2023.81 ± 217.69	27.45 ± 1.82	13.13 ± 0.63	2870.14 ± 246.48 e
IB × B × I	5.71 ± 0.72	4602.71 ± 210.64	10.91 ± 1.24	15.96 ± 1.25	5792.24 ± 227.79 abcd
IB × B × II	5.3 ± 0.56	5358.72 ± 193.81	8.11 ± 1.22	21.06 ± 0.74	6750.04 ± 229.32 abc
L × C × I	8.89 ± 1.73	4889.61 ± 199.93	12.76 ± 1.68	19.77 ± 1.19	6947.39 ± 236.23 abc
L × C × II	117.05 ± 19.02	2181.98 ± 507.06	38.24 ± 5.56	17.43 ± 1.31	3389.17 ± 663.13 e
L × B × I	9.08 ± 1.51	5172 ± 214.2	12.96 ± 1.32	20.86 ± 1.1	7126.2 ± 225.84 ab
L × B × II	6.38 ± 0.37	5353.86 ± 176.62	11.93 ± 1.37	21.37 ± 1.33	7328.41 ± 217.95 a
R × C × I	45.29 ± 9.48	4172.66 ± 347.15	32.55 ± 11.14	19.59 ± 0.99	5375.38 ± 373.16 cd
R × C × II	123.96 ± 35.81	3089.98 ± 611.43	72.8 ± 12.12	22.99 ± 1.27	4243.21 ± 691.97 de
R × B × I	30.95 ± 5.86	4777.8 ± 203.79	24.12 ± 2.86	19.55 ± 0.73	5958.07 ± 224.89 abc
R × B × II	18.22 ± 1.84	4874.32 ± 136.31	40.55 ± 3.97	23.53 ± 1.1	6167.46 ± 154.12 abc
CULTIVAR (Cv.)	0.094	0.27	<0.001	<0.001	<0.001
TREATMENT (T)	<0.001	<0.001	<0.001	<0.001	<0.001
SAMPLING PERIOD (SP)	<0.001	<0.001	<0.001	0.014	<0.001
Cv. × T	0.53	0.256	0.538	**0.017**	0.165
Cv. × SP	0.108	0.168	**0.004**	**0.008**	0.044
T × SP	**<0.001**	**<0.001**	**0.001**	**0.005**	<0.001
Cv. × T × SP	0.354	0.051	0.58	0.094	**0.024**

Results are expressed as means ± standard errors (n = 8). Different letters for each variable indicate significant differences between mean values for treatment, sampling period, cultivar, and their interaction ($p \leq 0.05$) using three-way ANOVA and Tukey's post hoc test ($p \leq 0.05$). The absence of letters indicates either non-significant differences or the presence of a higher-order interaction. DW—dry weight.

3.4. Simple Phenols and Total Phenols Determined with LC-MS/MS

There were no significant Cv × T × SP interactions regarding hydroxytyrosol concentrations (p = 0.094) (Table 3). Hydroxytyrosol increased slightly but significantly in 'Istarska bjelica' for treated plants. In 'Rošinjola', it significantly increased between SPs, while T × SP interactions showed a treatment effect on hydroxytyrosol between SPs, as well as a treatment-positive influence on its concentration in SP-II compared to the control.

When monitoring statistically significant Cv × T × SP interactions for total phenols determined by LC-MS/MS (p = 0.024) (Table 3), their concentration decreased in control plants only in 'Istarska bjelica' and 'Leccino' but remained stable within B+ treatment across the SP regardless of cultivar. However, boron foliar treatment in SP-II increased total phenol levels across cultivars (Table 3).

4. Discussion

Boron is essential for phenolic metabolism in plants, influencing the synthesis and stabilisation of phenolics involved in cell wall formation and plant defence mechanisms [17]. Adequate boron nutrition has been shown to enhance the accumulation of individual phenols such as oleuropein in olive leaves [12] and total phenols in olive oil [18]. On the other hand, opposite findings have also been reported, such as increased total phenols in boron-deficient olive leaves [19] and decreased total phenols with increasing boron rates [14]. This suggests that the relationship between boron supplementation and phenol concentration is not always linear and may be influenced by growth conditions, timing, cultivar, and initial plant boron status. Therefore, a more detailed study of the effects of foliar boron supplied as boron ethanolamine on phenolic metabolism, including both temporal and genetic variability, was warranted. In total, 37 different phenolic compounds were studied: 19 flavonoids, 13 phenolic acids, three secoiridoids, and two simple phenols. Having assumed a more critical role in plant physiology, as well as in the potential nutraceutical value, only the results of the most abundant of each subgroup have been discussed.

Among phenolics, flavonoids represent a group of over 9000 compounds, present ubiquitously in the plant kingdom [20]. In olives, however, a much smaller subset of flavonoids was found, with most studies reporting 8–20 different compounds, depending on the cultivar and analytical method used [21–23]. Flavonoids protect plants from various biotic and abiotic stresses; act as unique UV filters; and function as signal molecules, allopathic compounds, phytoalexins, detoxifying agents, and antimicrobial defence compounds [24]. The majority of flavonoids are present as glucosides under natural conditions [25]. Previously, boron foliar application has been shown to increase total flavonoid content in apple peel [26], bush leaves [27], and pomegranate juice [28]. In this work, two major groups of flavonoids are discussed: flavones and flavonols.

Apigenin-7-glucoside is a flavone that plays several vital roles in plants, including protection against abiotic stress. Like other flavones, its synthesis is regulated by environmental signals and developmental stages. Flavonoids, in general, tend to peak during the early vegetative or flowering stages [29,30]. In our study, cultivars exhibited distinct temporal patterns: 'Rošinjola' maintained stable apigenin-7-glucoside levels across treatments and sampling periods, while the other two cultivars showed clear seasonal dynamics. Boron application stabilised apigenin-7-glucoside only in 'Istarska bjelica', whereas in 'Leccino', both control and treated plants experienced a significant decline between sampling periods. It is also noteworthy that apigenin showed a slight but significant increase only in the control plants of these two cultivars ('Istarska bjelica' and 'Leccino'). In contrast, no such change was observed under boron treatment. Since our sampling occurred 64 and 118 days after treatment, the steady decrease in apigenin-7-glucoside might reflect reallocation of resources toward structural components or reproduction, a phenomenon

well documented in ripening fruits. It has been reported that the maximum level of flavone synthesis is generally observed around 16 weeks after flowering, which aligns with our first sampling period [31]. Thus, it can be hypothesised that boron treatment influences the balance between apigenin glycosylation and its downstream metabolism. These findings underscore pronounced cultivar-specific differences in both temporal dynamics and boron responsiveness.

Luteolin derivatives showed different behaviour, with Cv × T × SP interactions not being statistically significant. However, most notable interactions were Cv × T for luteolin-4-rutinoside and luteolin-7-rutinoside in 'Leccino', and/or 'Istarska bjelica', showing a significant increase with +B treatment. When monitoring SP × T, their concentrations remained unchanged across SPs in the absence of boron treatment. This indicates that late-season environmental or physiological changes alone did not stimulate their additional synthesis. Interestingly, there appears to be a subtle interaction between luteolin derivatives: luteolin-7-glucoside showed a specific pattern: its concentration decreased significantly between sampling periods in control plants, whereas boron treatment prevented this seasonal decline, leading to considerably higher levels at SP-II. This stabilising effect was mainly observed in 'Istarska bjelica' and 'Leccino', whereas 'Rošinjola' remained unaffected. In our previous research, a significant increase in this glucoside was observed in leaves of the 'Leccino' cultivar during October, compared to August and September [32]. However, similar patterns in this study for the 'Leccino' cultivar were apparent only for luteolin-4-rutinoside, which showed significant responses across all two-factor interactions (Cv × T, Cv × SP, and T × SP). Additionally, we previously found no difference in luteolin-7-glucoside concentrations between the harvest, dormancy, and pruning periods [33]. Other authors reported that luteolin-7-glucoside concentration may remain essentially unchanged throughout the year. The only exception was observed in October, when luteolin-7-glucoside reached its lowest concentration in aged leaves [34]. The increase in luteolin-4-rutinoside and luteolin-7-rutinoside, and the maintenance of luteolin-7-glucoside in +B leaves at harvest, possibly reflects boron's role in preserving cellular integrity and antioxidant balance [35]. When monitoring Cv × T and Cv × SP in 'Rošinjola', no differences in concentrations of either of the three major luteolin derivatives were observed.

Remarkably, quercetin and its derivatives seemed to demonstrate the protective effect of +B treatment in 'Istarska bjelica' and 'Leccino'. Namely, a decrease in their concentrations was seen between SPs without boron treatment, but not within +B. This behaviour was significant in either Cv × T × SP interactions or T × SP interactions. The late-season decrease in quercetin concentration has also been previously observed [36], with leaf quercetin peaking in August and gradually decreasing over the next 4 months. It is likely that, regardless of the lack of direct influence on the synthesis of flavonols, boron may induce a combined effect by improving membrane stability and by reducing oxidative stress [37], which possibly depletes quercetin. The improved membrane stability helps maintain regular transport and metabolic fluxes needed for flavonoid synthesis [17]. Similar trends have been observed in buckwheat, where adequate boron supply preserved or enhanced quercetin and rutin levels [38]. On the other hand, cultivar 'Rošinjola' remained impervious to the effects of +B treatment and different SPs, as none of the investigated interactions showed significant changes in quercetin and quercetin derivatives concentrations. It is possible that 'Rošinjola' possesses inherently stable leaf metabolism either due to constitutive gene expression or limited boron responsiveness, resulting in a buffered flavonoid profile.

Phenolic acids are another large group of plant phenols, comprising more than 160 compounds in plants, among which the hydroxycinnamic acids are the most abundant [39]. Their roles in plant physiology are numerous and diverse, including maintenance of struc-

tural integrity, defence and resistance to stress, signalling, symbiosis, and support for growth and development [40].

There were no significant Cv × T × SP interactions for hydroxybenzoic acids, and treatment had no effect either. However, some of the Cv × SP interactions reached statistical significance, namely, protocatechuic acid significantly decreased over time in all cultivars. This is consistent with our recent work, which found a significant decrease in 4-hydroxybenzoic and vanillic acids in Cv × SP interactions [33]. It is interesting to note that the glucosylated form of vanillic acid, vanillic-4-glucoside, which is also the most abundant among the major researched hydroxybenzoic acids, showed an increase in concentration between SPs, regardless of the cultivar and +B application. It seems likely that glucosylation occurs during the later stages of vegetation and is independent of boron supplementation.

Among hydroxycinnamic acids, chlorogenic acid concentrations appeared to be strongly cultivar dependent, and this dependence remained dominant even when observing Cv × T × SP interactions. A significant difference in concentration was observed only in 'Leccino', which had the highest chlorogenic acid concentration, and where +B partly stabilised it between SPs. Hydroxycinnamic acid derivative, verbascoside, showed similar behaviour in 'Istarska bjelica' and 'Leccino', where +B exhibited the most substantial effect specifically at SP-II, resulting in a marked increase compared with the corresponding controls, while no treatment effect was observed at SP-I. Although variability partly masked differences in the Cv × T × SP interaction, our recent work involving three sampling periods [33] clearly demonstrated that part of the seasonal dynamics of verbascoside occurs naturally and is largely boron-independent. Indeed, having observed that verbascoside showed the greatest increase across the three SPs, we previously concluded that it was the most potent differentiator between the collecting periods [1].

Secoiridoids are the signature phenolic compounds in the olive tree. The major secoiridoids include oleuropein, ligstroside and their aglycone and dialdehydic derivatives, such as oleuropein aglycone, oleacein and oleocanthal. These compounds are mainly accumulated in leaves and fruit and play key roles in plant defence and protection against oxidative stress [41].

There was an interesting pattern of behaviour for the two studied minor secoiridoids, oleacein and oleuropein aglycone, which increased in concentration between SPs without B post-anthesis application, but the +B treatment blocked this increase. This was the opposite of the oleuropein concentration, where oleuropein tended to decrease between SPs in the −B treatment. Still, this decrease was prevented with the +B treatment, leading to significantly higher oleuropein levels in B-treated leaves at SP-II compared with untreated ones. Similar results with T × SP interactions for oleuropein were previously observed [12]. It is probable that, between SPs late in fruiting season, untreated leaves exhibited hydrolytic/oxidative conversion of oleuropein to oleuropein aglycone and oleacein, although the enzymatic mechanisms underlying this process were not directly assessed in this study and therefore remain hypothetical. Foliar boron blocked the decrease in oleuropein plausibly by stabilising cell wall/membrane integrity, lowering oxidative stress and possibly by modulating enzymatic activity to retain oleuropein in its glycosylated form [42,43]. In our more recent work [33], oleuropein levels increased between SPs without +B. However, in our previous work, the sampling season occurred between harvest and pruning, when the metabolic flux likely shifted back to oleuropein accumulation, whereas cold and post-harvest stress could trigger phenolic synthesis or redistribution from other tissues. This aligns with the different research, which found the highest concentration of leaf oleuropein between November and March [36].

Hydroxytyrosol is a low molecular weight phenolic alcohol derived mainly from the hydrolytic degradation of oleuropein and other secoiridoids in olive tissues. It is one of the most potent antioxidants, contributing to protection against oxidative stress and pathogen defence [44]. In our experiment, its concentration rose slightly but significantly in 'Istarska bjelica' with treatment (Cv × T), in 'Rošinjola' between SPs (Cv × SP) and with boron treatment between SPs (T × SP). This behaviour points to cultivar-specific activation of hydroxytyrosol synthesis. However, while changes occur, they are small and suggest a stable overall leaf metabolism.

When observing total LC-MS/MS phenols and considering the dominant influence of oleuropein, they mimic oleuropein's behaviour in Cv × T × SP, reaching statistical significance ($p = 0.024$). Treatment with boron (+B) prevents the decrease in total phenols between SPs, which otherwise occurs in −B treatment, and they show significantly higher total phenol levels in SP-II across all studied cultivars, including 'Rošinjola'. This decrease is more pronounced in 'Istarska bjelica' and 'Leccino', while 'Rošinjola' shows no significant seasonal change and therefore exhibits a more stable or buffered phenolic profile.

Overall, the observed cultivar-dependent responses, varying from minimal changes in most individual phenolics in 'Rošinjola' to notable concentration shifts in specific polyphenols in 'Istarska bjelica' and 'Leccino', suggest that boron foliar application can influence both the biosynthetic pathways and the subsequent fate of olive leaf polyphenols. The generally limited response of 'Rošinjola' may reflect inherently stable polyphenol homeostasis or a reduced capacity for boron uptake or redistribution within leaf tissues, although an apparent boron-induced increase in total phenols was observed at SP-II. These patterns imply that boron modulates metabolic fluxes, enhances cellular stability, and mitigates oxidative stress, thereby altering the accumulation profiles of different polyphenol classes.

5. Conclusions

Boron nutrition, with foliar application of boron ethanolamine, modulated phenolic metabolism in olive trees, with clear differences among cultivars and phenolic subclasses. While some compounds, such as certain hydroxybenzoic acids, remained unaffected, most phenolics showed higher or more stable concentrations under boron treatment than the decreases sometimes observed in untreated samples. The response was strongly cultivar-dependent: two cultivars responded to boron, whereas 'Rošinjola' showed a limited response, with a noticeable boron-induced increase only in total phenols at SP-II. Overall, this work provides one of the most detailed cultivar-specific insights to date into how boron modulates phenolic metabolism in olive leaves. These findings suggest that boron contributes to maintaining phenolic biosynthesis and accumulation, as well as antioxidant capacity, in leaves, particularly in responsive genotypes, and should be considered in optimal nutrient management strategies aimed at supporting tree vitality and the bioactive quality of olive-derived products. Overall, these results provide valuable guidance for optimising foliar micronutrient management to maximise the bioactive potential of olive by-products in Mediterranean production systems. Nevertheless, further research is needed regarding the impact of B primary metabolism on other physiological traits, which may contribute to a better understanding of its role in olive physiology.

Author Contributions: Conceptualisation: I.P., P.Ž. and M.P.P.; validation and formal analysis: P.Ž., P.P., N.M. and M.P.P. investigation: I.P., Š.M. and M.P.P.; resources: I.P. and S.G.B.; data curation: I.P., A.B., M.S.B. and M.P.; writing—original draft preparation: P.Ž.; writing—review and editing: I.P., P.Ž., N.M., S.G.B., Š.M., P.P., A.B., M.S.B., M.P. and M.P.P.; visualisation: P.Ž. and M.P.P.; supervision: I.P.; project administration: I.P., P.P. and M.P.P.; funding acquisition: S.G.B., I.P. and P.P. All authors have read and agreed to the published version of the manuscript.

Funding: The open access publication costs of this research were financed by the European Agricultural Fund for Rural Development (EAFRD) within the framework of Intervention 70.05—Support for the conservation, sustainable use, and development of genetic resources in agriculture, under the Strategic Plan of the Common Agricultural Policy of the Republic of Croatia 2023–2027, for the year 2025. The work of Igor Pasković, Paula Žurga, Šime Marcelić, Marija Polić Pasković, and Paula Pongrac was supported by the Croatian Science Foundation (CSF (HRZZ)) and the Slovenian Research Agency (ARIS) under the WEAVE initiative bilateral project nos. HRZZ IP-2022-10-8305 and N4-0346 (PROGRESS) and through core ARIS funding (P1-0212).

Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

SP-I	1st sampling period
SP-II	2nd sampling period
SPs	Sampling periods
Cv	Cultivar
T	Treatment
+B	Boron foliar fertilisation 50 days after anthesis
−B	Control plants—foliar water application 50 days after anthesis
DW	Dry weight

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