

Article

Impacts of Olive Pomace Stress on *Vicia faba* L.'s Growth, Secondary Metabolism, and Nutrient Uptake

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Abstract

Olive pomace (OP), an olive mill byproduct, poses environmental risks if mismanaged due to its high phenolic content, acidic pH, organic load, and electrical conductivity. This study evaluated the impact of olive pomace filtrate (OPF) at varying doses (OP-5, OP-10, OP-15) on broad bean (*Vicia faba* L.) growth, secondary metabolites, and nutrient accumulation. The highest OPF dose (OP-15) exhibited a clear negative, dose-dependent phytotoxic effect, causing stem discoloration, reduced root growth, necrosis, and chlorosis, while untreated controls showed vigorous growth. This significantly ($p < 0.05$) reduced leaf development, average number of leaves, and total leaf area, even at the lowest concentration (5%). Consequently, OP-15 reduced dry and fresh biomass by over 50% and shoot/root lengths by up to 61.55% compared to the control. Liquid chromatography mass spectrometry (LC-MS/MS) analysis revealed a positive dose-dependent effect of OPF on beneficial phenol and flavonoid accumulation, with significantly higher amounts of ferulic, isoferulic, caffeic, chlorogenic, and 4-hydroxybenzoic acids, as well as luteolin-4'-rutoside and 4,7-dihydroxyflavone. OP application significantly ($p < 0.05$) decreased relative water content and increased electrolyte leakage and malondialdehyde, indicating stress. Furthermore, OP decreased the uptake of K, P, Fe, S, Zn, and Cu. Therefore, the intrinsic phytotoxicity of OPF suggests that mitigation measures are essential before considering environmental application to prevent potential adverse effects on sensitive crops and the wider ecosystem.

Keywords: broad bean; nutrients uptake; olive pomace; polyphenols; secondary metabolism



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1. Introduction

In the Mediterranean, olive oil production generates substantial amounts of lignocellulosic waste, such as olive pomace (OP) each year, the disposal and treatment of which remain a significant challenge for the olive-processing industry [1,2]. OP is characterized by its high organic load, acidic pH, and phytotoxicity, which is linked to the presence of polyphenolic compounds, residual oils, and organic acids. If not properly managed, these components may negatively impact soil and water quality [3,4]. Furthermore, its high organic load, reflected in elevated biological and chemical oxygen demand, can lead to oxygen depletion in receiving waters, thereby threatening aquatic life [5,6].

Its characteristic dark coloration also reduces light penetration, hindering aquatic photosynthesis [7]. Moreover, OP poses significant toxicological risks to a wide range of organisms including animals, humans, microorganisms, and plants. Several studies have highlighted its harmful effects on aquatic life, including fish [8], crustaceans, e.g., *Daphnia magna* [6], and algae [9], affecting their survival, growth, and reproduction. Its disposal into the environment may also disrupt soil and water microbial communities, affecting bacteria (including nitrogen-fixing bacteria) and fungi, with potential consequences for nutrient cycling [10]. Moreover, negative influences on earthworms (*Eisenia fetida*) [11] and seed germination and growth of various plant species are also reported [3]. According to Černe et al. [12], OP exhibits potential phytotoxic characteristics due to its complex composition, including elevated levels of phenolic compounds (from 566 to 4632 mg/kg f.w.), high salinity (from 2.1 to 7.2 mS/cm), acidity (from pH 4.2 to pH 5.6), and higher C/N (from 43 to 94). These constituents negatively impact plant growth [13,14]. For instance, high phenolic concentrations can inhibit seed germination in *Lactuca sativa* (lettuce) and *Raphanus sativus* (radish) [15]. Similarly, high salinity can hinder water uptake, leading to reduced root and shoot growth in crops like *Triticum aestivum* (wheat) [16] and *Hordeum vulgare* (barley) [17].

OP typically has high acidity (low pH), which, when added to soil, can lower overall soil pH. This decrease in pH can disrupt nutrient availability, making essential macronutrients like phosphorus less accessible to plants while increasing the solubility of potentially toxic elements such as aluminum and manganese [18]. The degree of phytotoxicity also depends on several factors, including OP concentration (with higher concentrations being generally more toxic), olive-processing technology (e.g., three-phase systems produce highly polluted wastewater), plant species (some species, like *Olea europaea* itself, are more tolerant than others) [19], and soil type [20].

Although research has been conducted, important gaps remain in our understanding of how plants physiologically respond to and absorb nutrients in soils treated with raw, acidic OP. To address this, our research provides the first comprehensive physiological and metabolomic assessment of *V. faba* L. under raw, acidic OP stress, uniquely quantifying both its impact on essential nutrient uptake and its specific role in altering the plant's secondary metabolite defense system (phenolics and flavonoids). This study examines the effects of OP application on *V. faba* L. (broad bean), a widely used model in plant stress physiology. It focuses on four key areas: (1) growth parameters, i.e., assessing OP's impact on plant development; (2) nutrient uptake, evaluating the absorption of essential macro (P, K, Mg, S) and micronutrients (Fe, Zn, Cu, Mn, B); (3) accumulation of major phenolic compounds (e.g., hydroxytyrosol, caffeic, vanillic, ferulic, and p-coumaric acids); and (4) flavonoid biosynthesis, analyzing changes in key flavonoids (e.g., rutin, luteolin, quercetin, apigenin). This approach aims to clarify how *V. faba* L. responds to OP, providing insight into its potential as a sustainable agricultural amendment.

2. Materials and Methods

2.1. Experimental Design

Seeds of *V. faba* L. (Superaguadulce variety) were obtained from Semenarna, Ljubljana, Ltd, Ljubljana, Slovenia. The seeds were sterilized by immersing them in a 2.5% sodium hypochlorite (NaOCl) solution for 15 min, then thoroughly rinsing them with distilled water to remove any residual disinfectant. The seeds were germinated in Petri dishes maintained at 25 °C. Upon germination (5 to 7 days), healthy seedlings were transferred to individual pots (1 kg) containing a mixture of sterile sand and vermiculite (1:1, v/v) as the growth medium. The experiment was arranged as a completely randomized design (CRD) with $n = 3$ biological replicates for each treatment. Pots were randomly positioned within

the growth chamber, and their positions were systematically rotated every three days to account for potential positional effects. Ten days after planting, pots were divided into four experimental groups. The control group received standard Hoagland's solution, while the other three were irrigated with olive pomace filtrate (OPF), diluted in Hoagland's solution at different concentrations.

The OP samples were obtained from a local two-phase olive mill company located on the Adriatic coast of the Istria region, Croatia. OPF was chosen for its homogeneity and ease of application via irrigation water, and was prepared using filter paper. All chemical and physical analyses of the OPF solution were conducted at the Institute of Agriculture and Tourism. The basic chemical and physical characteristics of OPF are as follows: pH: 4.61 ± 0.15 ; Electrical conductivity: 12.68 ± 0.27 mS/cm; Total carbon: 40 ± 1.20 g/L; Organic matter: $7.76 \pm 0.46\%$; Total nitrogen: 0.4 ± 0.20 g/L; Ash: $14.08 \pm 0.71\%$; Nitrate $\text{NO}_3\text{-N}$: < 0.079 mg/L; Ammonium $\text{NH}_4\text{-N}$: 0.93 ± 0.05 mg/L; Total phenols: 483.63 ± 53.21 mg/L. Three OPF dilutions (v/v) in Hoagland's solution were prepared: 0.5:10 (OP-5), 1:10 (OP-10), and 1.5:10 (OP-15), representing increasing OPF concentrations.

All plants were cultivated for 40 days in a controlled growth chamber (GC 401, Nuve, Ankara, Turkey) maintained at a 16 h/8 h light/dark photoperiod with a light intensity of $350 \mu\text{mol m}^{-2} \text{s}^{-1}$, a 25/15 °C day/night temperature cycle, and 60% humidity. After harvest, the plants were rinsed with distilled water and separated into two groups. One group was used to determine growth parameters, including length, fresh weight, and dry weight (measured after oven-drying at 80 °C for 48 h) of roots and shoots and leaf area using ImageJ software v1.54. The second group was reserved for further analysis.

2.2. Relative Water Content and Malondialdehyde Content

The relative water content (RWC) of plant leaves, an indicator of their water status, was determined using a protocol based on [21] and quantified with [22] formula. This involved measuring the fresh weight (FW) of excised leaves, followed by their saturation in distilled water for 24 h in darkness to obtain the turgid weight (TW), and finally oven-drying at 60 °C for 48 h to record the dry weight (d.w). The RWC was then calculated as:

$$\text{RWC (\%)} = \frac{\text{FW} - \text{DW}}{\text{TW} - \text{DW}} \times 100$$

To determine Malondialdehyde (MDA) content, a protocol adapted from [23] was employed. Briefly, dry plant samples (30 mg) underwent extraction with 1.5 mL of 0.1% trichloroacetic acid (TCA). Following homogenization, the resulting extract was centrifuged to eliminate cellular debris. Subsequently, a 400 μL aliquot of the supernatant was combined with a 0.5% thiobarbituric acid (TBA) solution. This mixture was then subjected to heating at 95 °C to facilitate the formation of a colored complex with MDA. After cooling, the mixture was centrifuged again at $16,000 \times g$ for 10 min. Absorbance readings of the resulting colored solution were taken at 532 nm and 600 nm using a Tecan Infinite 200 Pro M Nano+ microplate reader (Männedorf, Switzerland). The MDA concentration was ultimately expressed as nanomoles per gram of dry weight.

2.3. Electrolyte Leakage

To assess electrolyte leakage, leaf discs (6 mm diameter) were excised from young leaves of each experimental unit. Following a thorough rinse with distilled water, five such discs were immersed in 10 mL of distilled water within test tubes. These tubes were then incubated in a 25 °C water bath for a duration of 24 h [24]. After this period, the electrical conductivity of the bathing solution (C1) was measured using a calibrated

conductivity meter (VWR MU 6100 H pH-enomenal) (VWR International Srl, Milano, Italy). The conductivity meter was calibrated daily using a standard KCl solution. Furthermore, the meter was set to automatically apply temperature compensation (ATC) to a reference temperature of 25 °C to ensure accurate and comparable readings. Subsequently, the samples were subjected to a 20 min boiling treatment (100 °C) and allowed to cool to room temperature. A second conductivity reading (C2) was then taken at 25 °C. The percentage of electrolyte leakage (EL) was calculated using the formula: $EL (\%) = (C1/C2) \times 100$, as described by Tripathy et al. [25].

2.4. Determination of Elemental Content

Mineral analysis was performed according to Vidović et al. [26]. Briefly, 250 mg of dried broad bean leaf samples were weighed into microwave pressure vessels along with 6 mL of concentrated nitric acid (HNO₃) and 2 mL of 30% hydrogen peroxide (H₂O₂). Samples were digested using a microwave digestion system (Milestone ETHOS UP, Bergamo, Italy) at 1800 W (200 °C) for 40 min. Once cool, the digestates were diluted to 25 mL with deionized water and quantitatively transferred to plastic vials (10 mL). The concentrations of boron (B), calcium (Ca), copper (Cu), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), sodium (Na), phosphorus (P), sulfur (S), silicon (Si), and zinc (Zn) were measured by inductively coupled plasma-optical emission spectrometry (ICP-OES, Shimadzu, Kyoto, Japan). The WEPAL (Wageningen Evaluating Programme for Analytical Laboratories) reference material was also analyzed for quality control. All measured values fell within $\pm 10\%$ of the certified reference values.

2.5. Preparation of Plant Samples for HPLC Analysis

Leaf samples (approximately 30 mg) were lyophilized using a Labogene Coolsafe 95-15 Pro (Allerød, Denmark) following the method of Major et al. [27]. Co-extraction was then performed by sonicating the lyophilized material in 80% aqueous methanol for 10 min, followed by centrifugation at 16,000 rpm for 10 min. A 300 μ L aliquot of the supernatant was transferred to a clean Eppendorf tube and evaporated to dryness under lyophilization conditions. The resulting residue was reconstituted in 600 μ L of the starting mobile phase (methanol with 0.1% acetic acid), filtered through a 0.22 μ m nylon filter, and transferred to HPLC vials. All extracts were stored at -80 °C until further analysis.

2.6. Determination of Phenolic and Flavonoid Content

Phenolic and flavonoid profiles were analyzed using an LC-MS/MS system (Shimadzu, Kyoto, Japan) consisting of an autosampler (Shimadzu Nexera SIL-40CX3, Kyoto Japan), two solvent delivery units (Shimadzu Nexera SIL-40CX3, Kyoto, Japan), a thermostatic column compartment (Shimadzu Nexera CTO-40C, Kyoto, Japan), and a triple quadrupole mass spectrometer (LCMS-8045), as described by Major et al. [28]. Separation was achieved on a C18, 2.1 mm $\times$ 150 mm, 2.7 μ m core-shell column (Advanced Materials Technology, Wilmington, DE, USA) maintained at 37 °C. A 1 μ L sample injection was used with a linear gradient elution at 0.35 mL/minute, employing mobile phase A (water + 0.1% acetic acid) and mobile phase B (methanol + 0.1% acetic acid). The gradient program 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. Polyphenolic compounds were identified and quantified using authentic analytical standards.

2.7. Statistical Analysis

Statistical analysis of all data, which consisted of $n = 3$ biological replicates per treatment group, was conducted using GraphPad Prism 10 (GraphPad Software, LLC, San Diego, CA, USA). Analysis of variance (ANOVA) was used to assess the significance of

variations among groups. Where significant differences were detected, a Tukey's post hoc test was used to delineate specific group differences. All results are presented as the mean $\pm$ Standard Error of the Mean (SEM). Graphical representation and Pearson's correlation analysis were performed using GraphPad Prism 10.

3. Results

3.1. Effect of OPF on Plant Growth of *V. faba* L.

Figure 1 shows the effects of treatments (OP-5, OP-10, and OP-15) on plant length and biomass compared to the control. The OPF treatment significantly affected ($p < 0.05$) biomass production (shoot fresh biomass, root fresh biomass, shoot dry biomass, and root dry biomass) and plant growth (shoot length and root length). The control plants recorded the highest shoot fresh biomass, whereas OP-5 and OP-10 showed moderate reductions. OP-15 recorded the lowest value, with a 51% reduction compared to the control (Figure 1a,c). Root fresh biomass followed the same trend: The control plants recorded the highest values, OP-5 and OP-10 declined moderately, and OP-15 recorded the lowest biomass, with a 61.55% reduction relative to the control (Figure 1b,d).

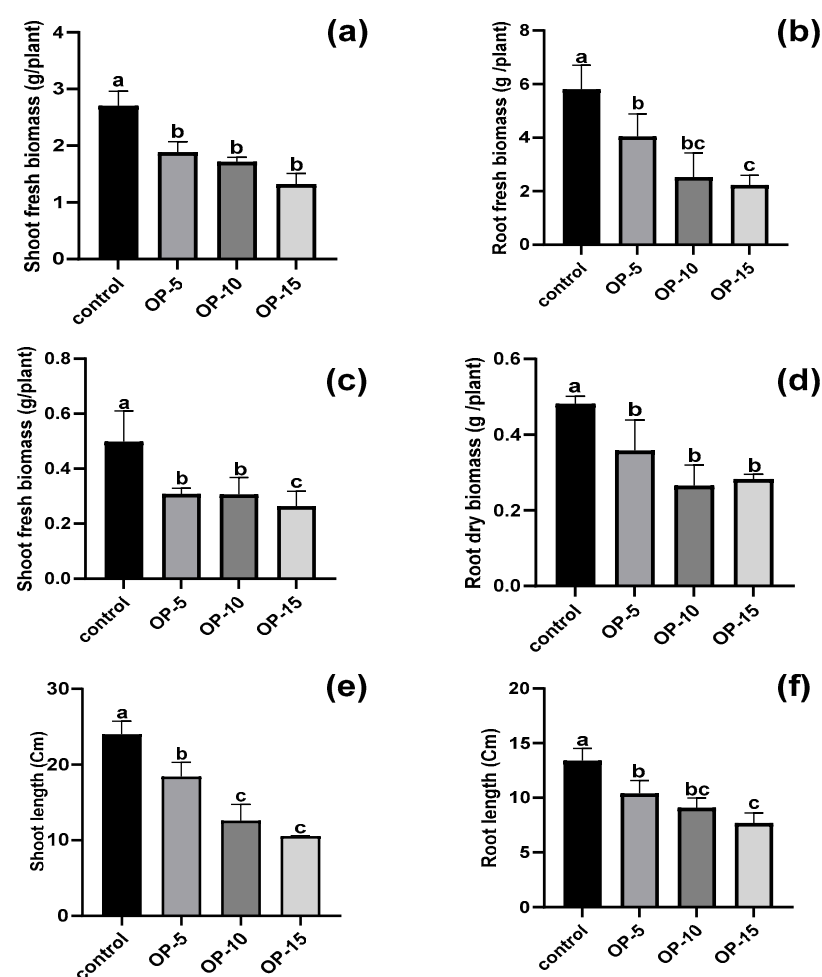


Figure 1. The effect of concentrations of OPF on (a) fresh shoot biomass, (b) fresh root biomass, (c) dry shoot biomass, (d) dry root biomass, (e) shoot length, and (f) root length of *V. faba* L. Data are presented as the mean $\pm$ Standard Error of the Mean (SEM) ($n = 3$ biological replicates). Different letters indicate a significant difference at $p < 0.05$, using Tukey's post hoc test following a one-way ANOVA.

Shoot dry biomass exhibited a comparable pattern, showing with reductions of 38.55% and 47.18% under OP-10 and OP-15, respectively. Similarly, root dry biomass showed

44.90% and 41.37% reductions under OP-10 and OP-15, respectively, with the control group maintaining the highest values, followed by OP-5, while OP-10 and OP-15 progressively reduced biomass accumulation (Figure 1c,d). Shoot length exhibited a similar trend, with control plants recording the greatest length, followed by OP-5, whereas OP-10 and OP-15 significantly reduced height of 47.62% and 56.04%, respectively. Root length decreased, with the largest reduction observed in OP-15, which declined by 42.5% decline compared to the control (Figure 1e,f).

Figure 2A clearly shows a dose-dependent phytotoxic effect of OPF on broad bean plant development. The untreated control plant exhibited vigorous growth with healthy roots and foliage, showing no signs of necrosis or chlorosis. Application of a 5% OPF solution did not induce any visible phytotoxic symptoms. Conversely, treatment with a 10% solution induced stress responses, characterized by stem discoloration and reduced root growth, accompanied by necrosis on lower leaves and potential early chlorosis. The highest concentration tested, 15% OPF, resulted in severe damage, characterized by extensive necrosis and chlorosis across the leaves, significant stem deterioration, and a severely underdeveloped root system. These findings indicate that while low concentrations of OPF may be tolerated, higher concentrations are increasingly detrimental to bean plant health and development.

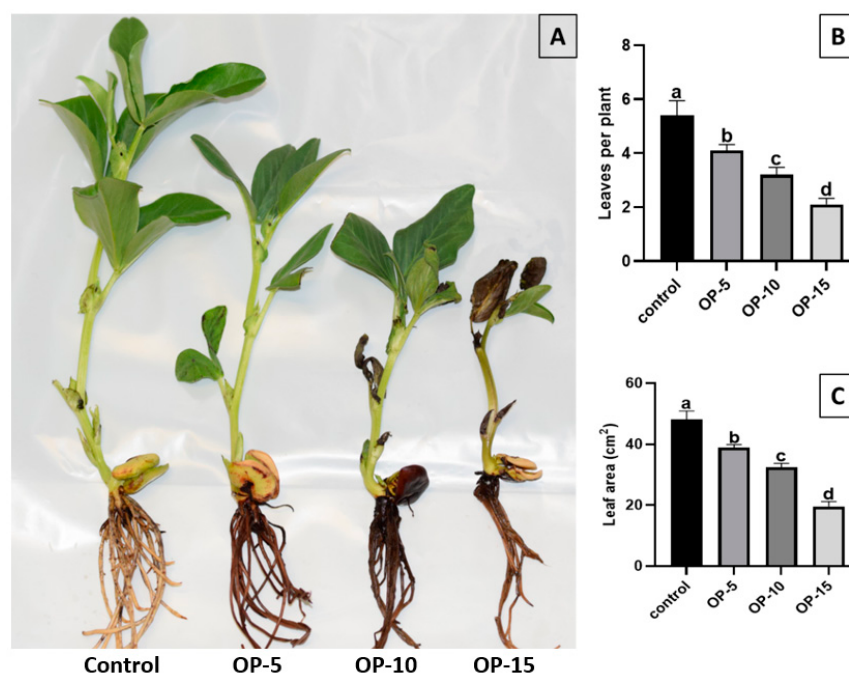


Figure 2. Effects of 0, 5, 10 and 15% of OPF on *V. faba* L. plants. (A) Plant phenotypes, (B) leaves number and (C) leaf area per plant. Data are presented as the mean $\pm$ Standard Error of the Mean (SEM) ($n = 3$ biological replicates). Different letters indicate a significant difference at $p < 0.05$, using Tukey's post hoc test following a one-way ANOVA.

Figure 2B,C show that the OPF significantly ($p < 0.05$) impacted leaf growth of broad bean plants. Figure 2B presents the average number of leaves per plant. The control group, without any filtrate application, had the most leaves. The average number of leaves significantly ($p < 0.05$) decreased with increasing OPF concentration. Even the lowest concentration (5%) resulted in a slightly smaller, but still statistically significant ($p < 0.05$), reduction in the number of leaves compared to the control. Figure 2C shows the average total leaf area per plant. Similar to the leaf count, the control plants recorded the largest leaf area. Total leaf area progressively and significantly ($p < 0.05$) declined with increasing OPF concentrations, with the highest reduction observed in the 15% treatment.

3.2. Effect of OPF on Relative Water Content, Electrolyte Leakage and Malondialdehyde Content

Figure 3 shows the effects of increasing OPF concentrations (0%, 5%, 10%, and 15%) on *V. faba* L. plants. Relative water content (3a) significantly decreased ($p < 0.05$) from approximately 92% in the control to around 58% at 15% OPF. Conversely, electrolyte leakage (b) significantly increased ($p < 0.05$), from 70.1% in control to 90.65% at 15% OP. Similarly, malondialdehyde content (c) significantly increased ($p < 0.05$), from 0.274 nmol MDA g⁻¹ d.w in control to 0.491 nmol MDA g⁻¹ d.w at 15% OP.

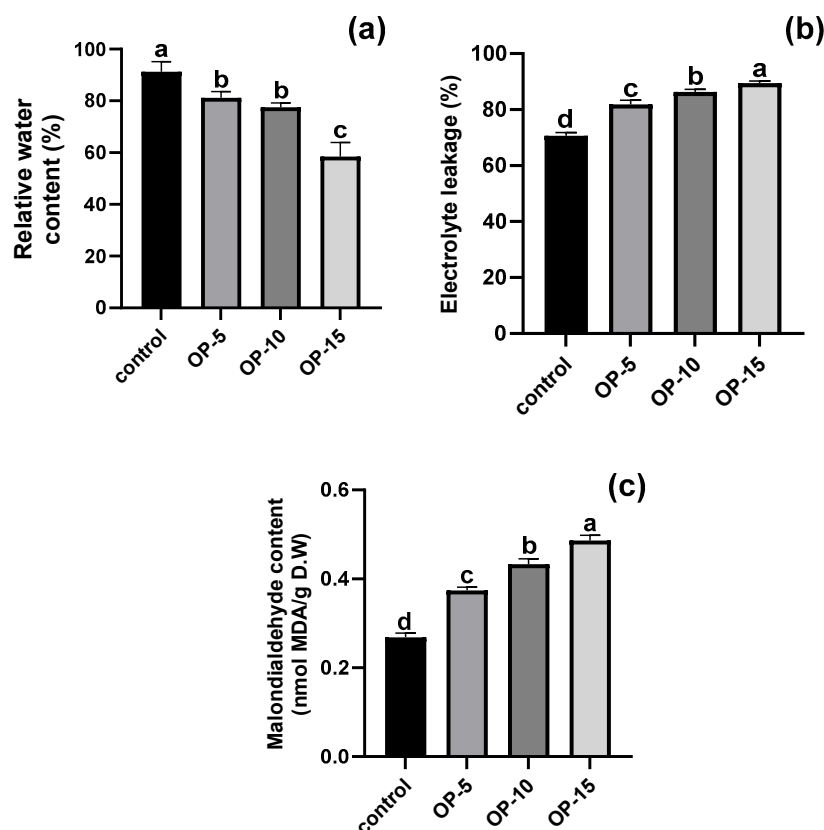


Figure 3. Effects of 0, 5, 10 and 15% of OPF on *V. faba* L. plants. (a) Relative water content, (b) Electrolyte leakage and (c) Malondialdehyde content. Data are presented as the mean $\pm$ Standard Error of the Mean (SEM) ($n = 3$ biological replicates). Different letters indicate a significant difference at $p < 0.05$, using Tukey's post hoc test following a one-way ANOVA.

3.3. Effect of OPF on the Phenolic Content of *V. faba* L.

The biochemical analysis shows significant ($p < 0.05$) variations in the concentrations of phenolic compounds across different OPF treatments (OP-5, OP-10, and OP-15) compared to the control (Figure 4). Levels of Hydroxytyrosol and protocatechuic acid increased, reaching peak values of 2.33 $\mu\text{g/g}$ dry weight and 3.10 $\mu\text{g/g}$ dry weight, respectively, under the OP-15 treatment. Chlorogenic and vanillic acid levels increased under OP-10 and OP-15 treatment. Further, caffeic acid and syringic acid levels also increased under OP-10 and OP-15 treatment. Caffeic acid reaching approximately 9 $\mu\text{g/g}$ d.w and syringic acid reaching 0.18 $\mu\text{g/g}$ d.w at OP-15. A slight increase was observed for both acids at OP-5. P-coumaric and ferulic acid levels increased, reaching 8.05 $\mu\text{g/g}$ d.w and 19.92 $\mu\text{g/g}$ d.w under OP-15 treatment. Isoferulic acid displayed a moderate increase across OP treatments, while levels of CAPE (Caffeic Acid Phenethyl Ester) significantly declined under OP-10 and OP-15 exposure.

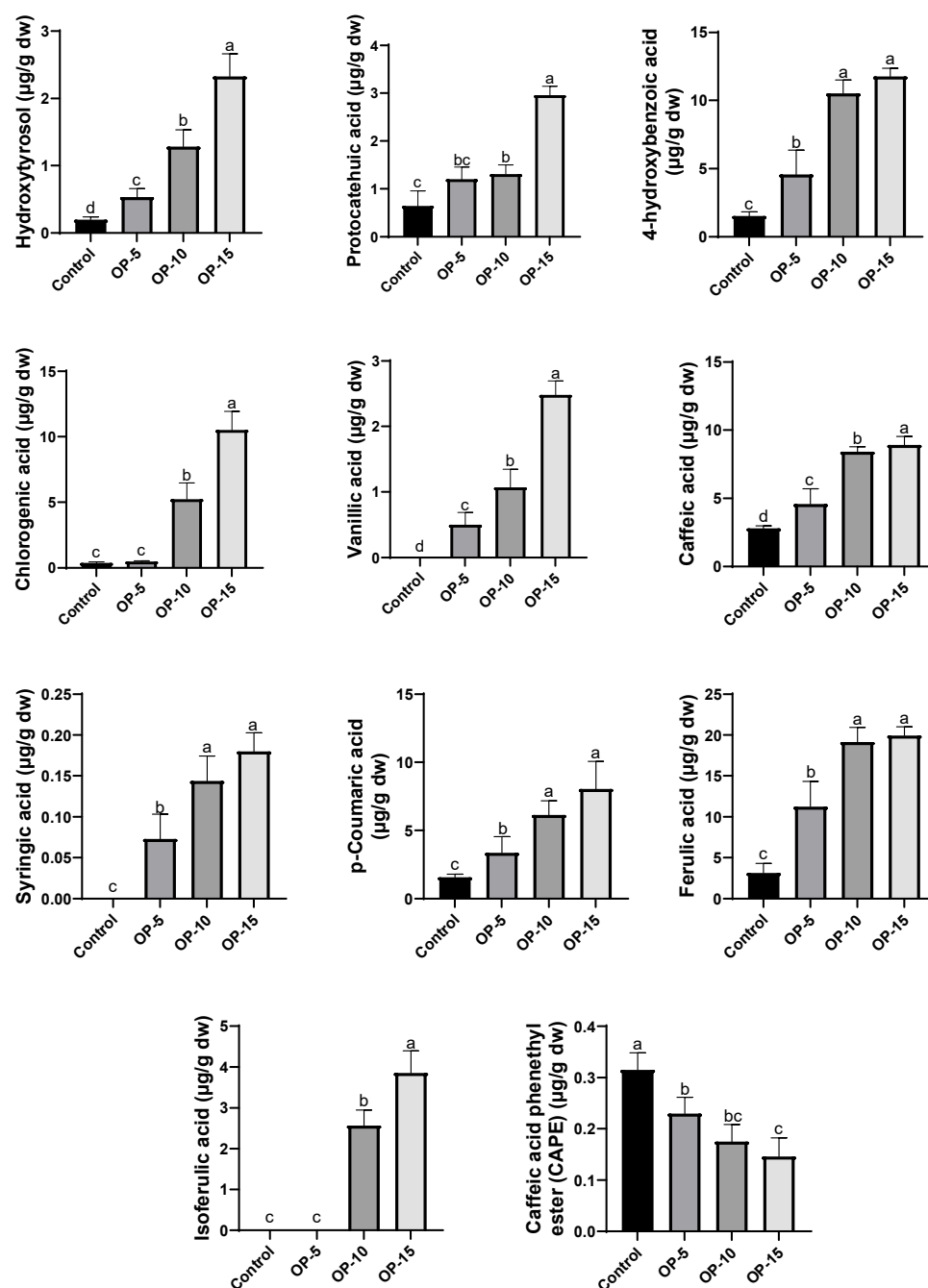


Figure 4. Effect of OPF concentrations on phenolic compounds in leaves (µg/g d.w) of *V. faba* L. Data are presented as the mean ± Standard Error of the Mean (SEM) ($n = 3$ biological replicates). Different letters indicate a significant difference at $p < 0.05$, using Tukey's post hoc test following a one-way ANOVA.

3.4. Effect of OPF on the Flavonoid Content of *V. faba* L.

Figure 5 presents the accumulation of various flavonoid compounds in the samples. Most compounds showed peak concentrations in the OP-10 or OP-15 treatments. OP-10 consistently led to higher concentrations of key bioactive compounds. Delphinidin-3-glucoside (myrtillin) was significantly elevated in OP-10 (1.92 µg/g d.w) compared to the control (1.10 µg/g d.w). Similarly, cyanidin-3-glucoside peaked in OP-10 at around 7.02 µg/g d.w, whereas the control recorded a lower concentration of 3.11 µg/g d.w. Luteolin concentrations varied across treatments, ranging from 0 (<detection limit) to 6.07 µg/g d.w, with the highest levels observed in OP-5. Apigenin, however, remained at relatively low levels

across all treatments, peaking at 0.22 $\mu\text{g/g d.w.}$ Quercetin derivatives displayed distinct trends, with quercetin-3-rutinoside (rutin) reaching its highest concentration (10.56 $\mu\text{g/g d.w.}$) in OP-15, while in the control it was much lower (1.26 $\mu\text{g/g d.w.}$). Quercetin-3-rhamnoside was more abundant in plants treated by OP-10, reaching around 6.77 $\mu\text{g/g d.w.}$, compared to the control, which remained close to zero (Figure 5).

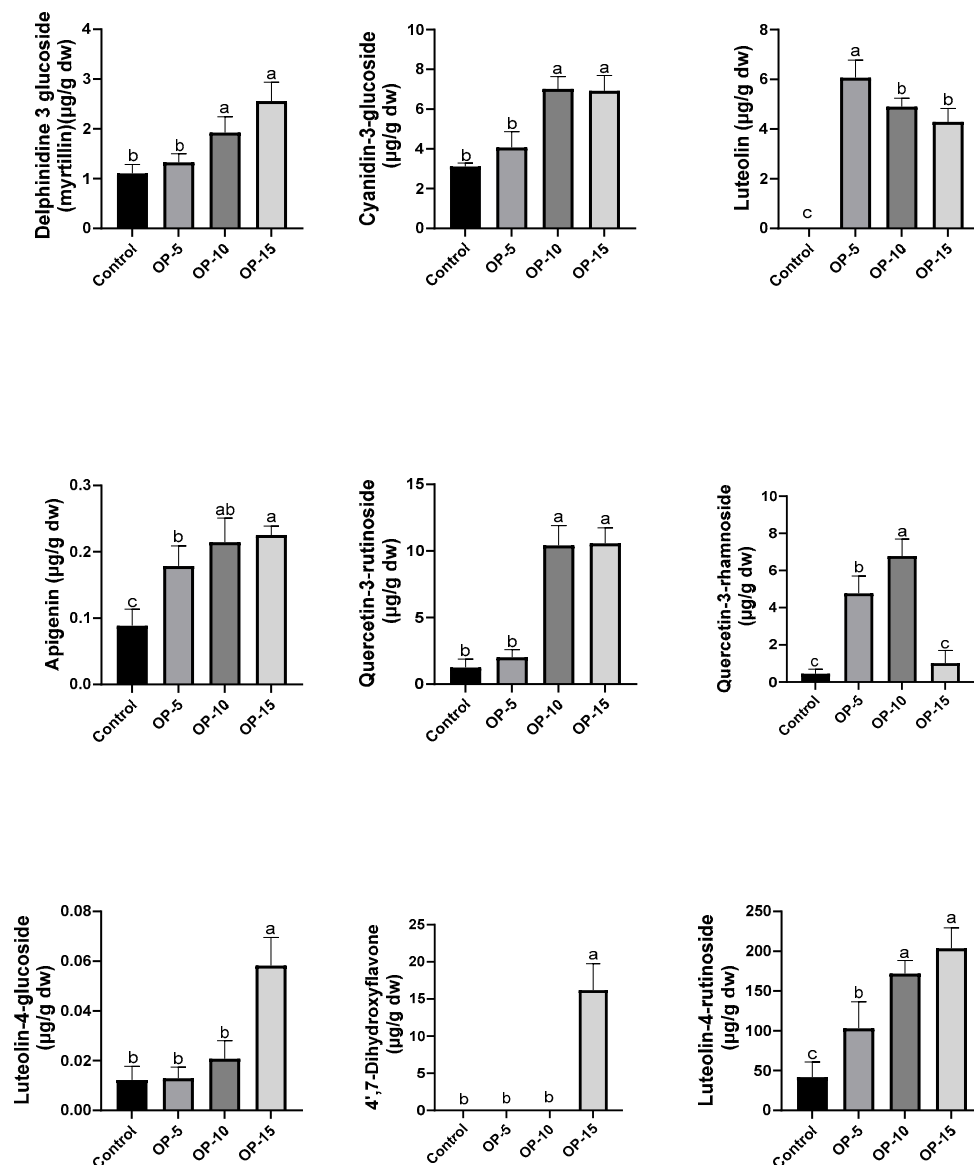


Figure 5. Effect of OPF concentrations on flavonoid compounds in leaves ($\mu\text{g/g d.w.}$) of *V. faba* L. Data are presented as the mean $\pm$ Standard Error of the Mean (SEM) ($n = 3$ biological replicates). Different letters indicate a significant difference at $p < 0.05$, using Tukey's post hoc test following a one-way ANOVA.

However, Luteolin-4-glucoside exhibited minimal variation, with values remaining around 0.05 $\mu\text{g/g d.w.}$ across treatments. In contrast, 4',7-dihydroxyflavone showed a progressive increase with higher OP applications, reaching approximately 20 $\mu\text{g/g d.w.}$ in OP-15. The most striking trend was for luteolin-4-rutinoside, which exhibited a sharp increase (203 $\mu\text{g/g d.w.}$) in the OP-15 treatment compared to the control (41.70 $\mu\text{g/g d.w.}$).

3.5. Effect of OPF on Tissue Nutrient Concentration in *V. faba* L.

Figure 6 presents the accumulation of various nutrients. The boron content increased with OPF application, ranging from 27.43 mg/kg d.w in the control to 30 mg/kg d.w

in plants exposed to OP-15. The calcium content also gradually increased compared to the control (4.48 g/kg d.w), with OP-5 the treatment resulting in the highest amounts (5.77 mg/kg d.w.) Copper levels were highest in the control (12.30 mg/kg d.w), compared to the OP-15 treatment (7 mg/kg d.w). Moreover, iron followed a decreasing trend, i.e., 74 mg/kg d.w. in the control compared to 65 mg/kg d.w in OP-15 treatment. Potassium showed only minor variations compared with the control plants, while magnesium levels increased from 2.23 g/kg d.w in the control to 3.10 g/kg d.w. under OP-15 treatment. Although initially increasing, manganese levels sharply declined from 34.37 mg/kg d.w (OP-10) to 27.91 mg/kg d.w at OP-15.

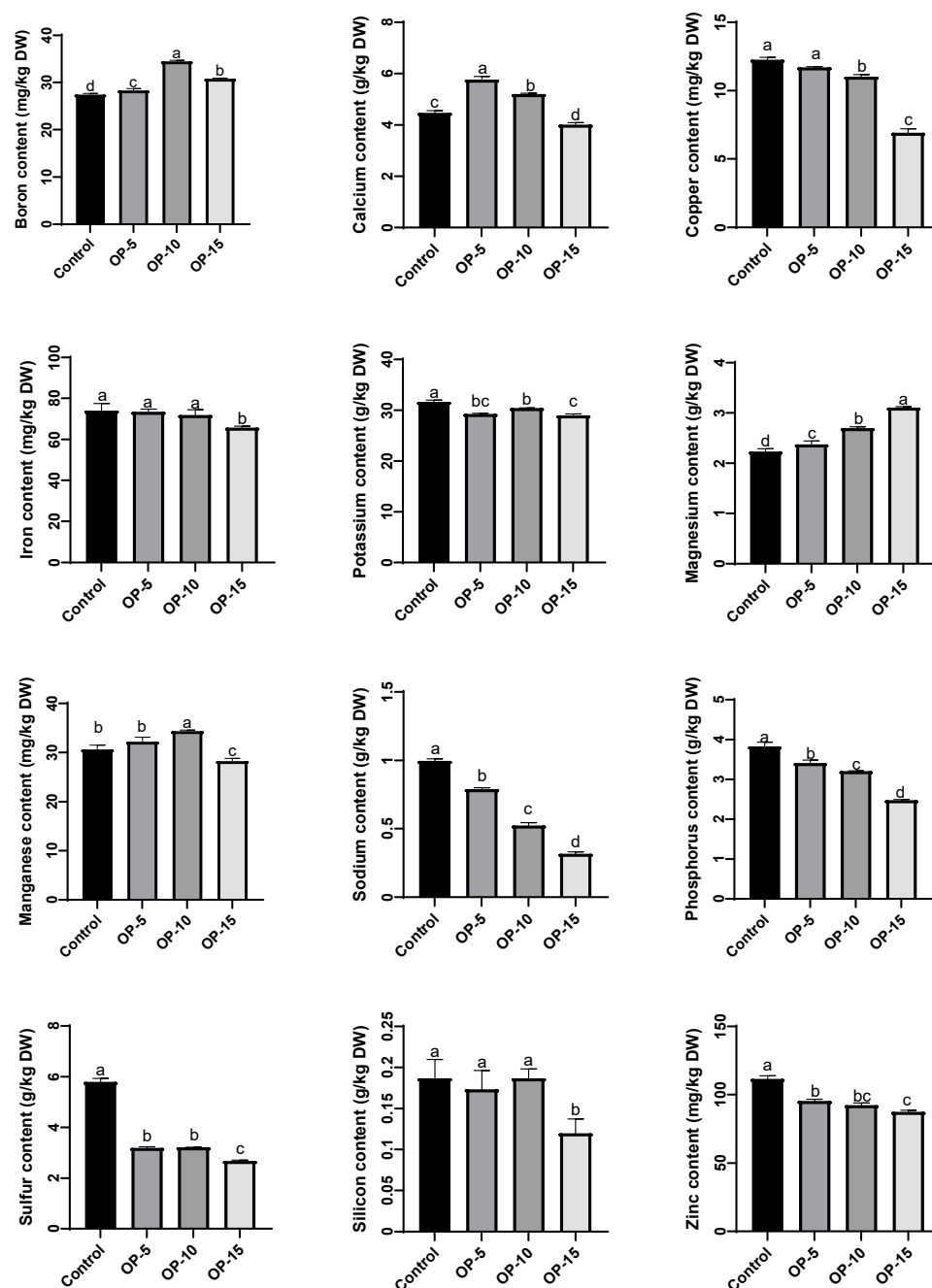


Figure 6. Effect of OPF concentrations on shoots nutrient content (mg/kg d.w) of *V. faba* L. Data are presented as the mean $\pm$ Standard Error of the Mean (SEM) ($n = 3$ biological replicates). Different letters indicate a significant difference at $p < 0.05$, using Tukey's post hoc test following a one-way ANOVA.

Levels of sodium, phosphorus, and sulphur steadily decrease across all treatments. Silicon concentrations also decreased with increasing OPF from 0.18 g/kg d.w in the control to 0.12 mg/kg d.w in OP-15. Zinc also significantly decreases with OPF application: 112 mg/kg d.w in the control to 93 mg/kg d.w in OP-15 treatment.

Figure 7 presents a strong positive correlation between morpho-physiological parameters (shoot/root biomass, length, leaves area and number, RWC), and certain mineral contents (K, P, and Ca). This indicates that the increased presence of these essential elements is associated with enhanced broad bean growth under OPF treatment. Conversely, most phenolic and flavonoid compounds (Chlorogenic acid, Quercetin-3-rutinoside), electrolyte leakage and MDA content correlate negatively with morpho-physiological parameters and mineral content. Notably, Mg also shows a negative correlation with growth parameters, contrary to the overall trend for other minerals. However, caffeic acid phenethyl ester is an exception, showing positive correlations.

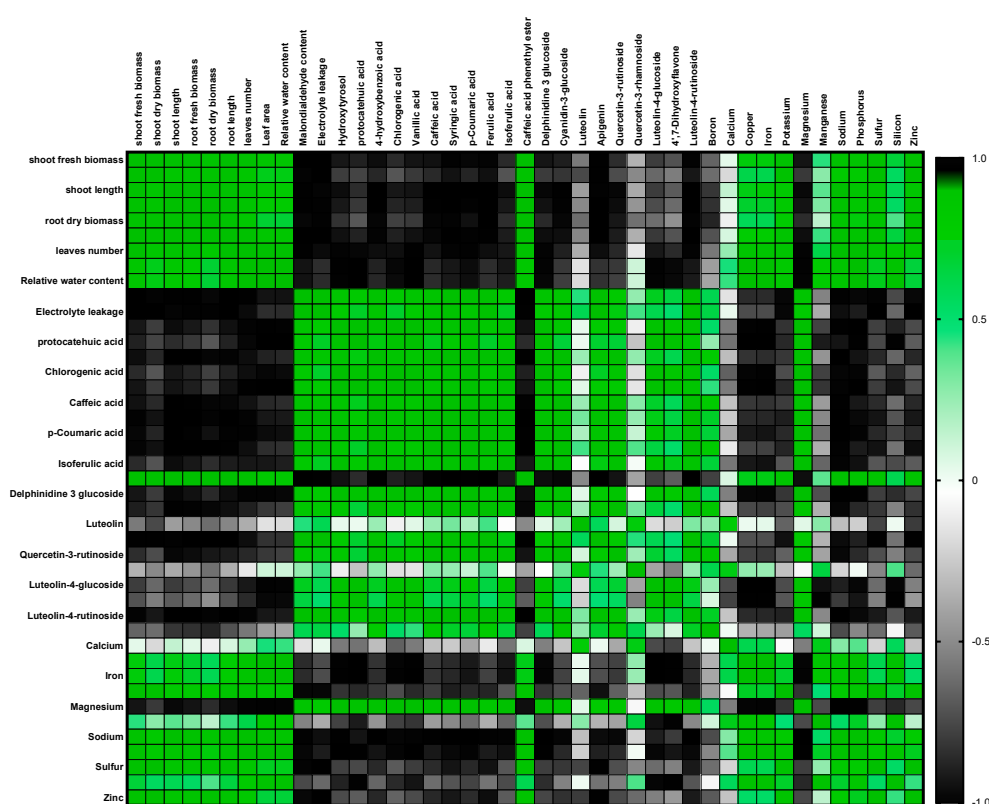


Figure 7. Pearson's r Correlation analysis of broad bean response (growth, phenolics, flavonoids, minerals).

4. Discussion

This study revealed that increasing OPF doses generally inhibited biomass yield and plant growth while significantly influencing the production of secondary metabolites (phenolic and flavonoids) and nutrient dynamics. This decline in plant biomass production and growth with increasing OPF application rates suggests that high OPF exerts phytotoxic effects on *V. faba* plants. The reductions in shoot and root biomass, as well as plant height, were most pronounced under OP-15, which aligns with the study of Killi and Kavdir [29], who found that the direct application of large amounts of OP to the soil adversely affects seed germination and plant growth due to the high phenol and organic acid content of untreated OP. Similarly, Bouhadi et al. [30] report that the OP exhibited severe phytotoxicity in *V. faba* plant, causing an 80% reduction in plant biomass and concurrent oxidative stress. Furthermore, OP application demonstrated potent genotoxic effects, significantly reducing

the mitotic index and inducing chromosomal aberrations in root cells. Javed et al. [31] further support this phytotoxicity, reporting that raw olive OP at concentrations $\geq 10\%$ significantly reduced or completely inhibited seed germination in sensitive crops like radish and barley. This decline can also be attributed to the accumulation of toxic substances, such as phenolics and tannins, which can interfere with nutrient uptake and enzymatic activity [32,33].

Excessive organic matter application can negatively affect soil structure and water retention capacity, potentially leading to hypoxic conditions that further suppress root development. The presence of phenolic compounds in OP might contribute to allelopathic effects, as described by Li et al. [34], thereby reducing cell division and elongation, ultimately impacting overall plant performance [15,35]. However, a lower OP dose (OP-5) did not significantly impact plant growth, indicating that fresh OP used as a soil amendment at lower application levels might be tolerable and potentially beneficial due to the contribution of organic matter, which can improve both soil fertility and microbial activity [36].

The increase in phenolic compounds, particularly hydroxytyrosol, protocatechuic acid, chlorogenic acid, and caffeic acid, with increasing OPF concentrations, suggests that OPF acts as a stressor, triggering a defensive response in plants. Phenolics play a key role in stress mitigation, acting as antioxidants that scavenge reactive oxygen species (ROS) [37]. The marked accumulation of these compounds, particularly under OP-15, suggests an adaptive response in which the plant increases secondary metabolite synthesis to mitigate oxidative stress. These results align with Cichoński et al. [38], who reported an increased biosynthesis of phenolic compounds in wheat (*Triticum aestivum* L.) exposed to abiotic stress conditions. The accumulation of certain phenolic compounds, such as hydroxytyrosol, in *V. faba* L. may be linked to its potential uptake and translocation from the growth medium, particularly since hydroxytyrosol was prevalent in the OPF medium (321.49 mg/L), as reported by Bouhadi et al. [30]. However, data on the cellular uptake of large molecules, such as phenolic compounds, remain limited.

The decrease in CAPE levels under OP-10 and OP-15 treatments indicates a metabolic shift that prioritizes the synthesis of other critical phenolic compounds under stress conditions. Variations in individual phenolic compounds also suggest differential regulation in response to stress, with certain biosynthetic pathways becoming more active at specific OPF concentrations.

The flavonoid profile also significantly varied with treatment, with OP-15 exhibiting the highest accumulation of delphinidin-3-glucoside, cyanidin-3-glucoside, and others. These findings suggest that moderate OP exposure enhances flavonoid biosynthesis, which has been linked to improved stress tolerance and antioxidant activity [39]. Further, the accumulation of flavonoids such as luteolin-4-rutinoside under OP-15 suggests an adaptive stress response and contributes to ROS scavenging and membrane stability under adverse conditions [40]. Similarly, Hinojosa-Gómez et al. [41], reported that water stress induces anthocyanin production in the roselle calyx. Specifically, cultivar UAN16-2 showed the most significant increases in cyanidin, delphinidin 3-O-glucoside, cyanidin 3-O-glucoside and, most notably, cyanidin 3-O-sambubioside, which showed a 55% increase compared to their control. The observed decline in certain flavonoids under OP-15, particularly quercetin-3-rutinoside, may indicate a threshold effect beyond where stress becomes too intense for optimal biosynthesis. This effect suggests that while moderate OPF application can enhance plant secondary metabolism, excessive OPF amendments may overwhelm the plant's metabolic capacity, leading to altered flavonoid production. Additionally, the contrasting trends between flavonoids indicate that plants selectively regulate their secondary metabolite pathways to optimize stress mitigation strategies [42].

The varying effects of OPF application on nutrient tissue concentration by broad bean plants suggest that it alters nutrient bioavailability and root assimilation. An increase in nutrient shoot concentrations (B, Ca, Mg, and Mn) with increasing levels of added OPF aligns with [43], who found that soil amendments with OP wastewater enhanced nutrient accumulation in wheat (*Triticum aestivum* L.). In the case of B, which plays a crucial role in cell wall formation and stability [44], its increased levels found under moderate OPF application might benefit plant structural integrity [45]. In addition, the enhanced Ca and Mn content at a low OPF dose can be explained by the additional supply of these elements with OPF application. Likewise, Mechri et al. [46] demonstrated that olive trees subjected to low concentrations of olive-processing waste showed increased uptake of Ca, Mg and Mn compared to untreated controls. However, a reduction in the tissue accumulation of Cu, Fe, K, Na, P, S, Si and Zn indicates that OPF treatment may interfere with the elemental mobility, possibly due to the chelating effects of phenolics or changes in growth medium pH [47].

These observations agree with Mechri et al. [43], who found that application of high amounts of olive mill waste ($150 \text{ m}^3 \text{ ha}^{-1}$) decrease N, P, K, Ca, Mg, Fe, Cu, Mn and Zn uptake by olive trees. This inhibition in the elemental uptake could be attributed to factors such as increasing acidity, salinity, TPC, and organic matter levels [48]. This effect is exacerbated by the high organic matter content and low water activity [49], which may lead to the immobilization or reduced solubility of essential elements.

The decline in K and P accumulation under OP-15 suggests nutrient imbalances resulting in reduced plant growth and biomass production [50]. Several studies have shown that limited P availability can reduce photosynthesis, leading to reduced carbon fixation, plant growth and productivity [51–53]. In particular, low P accessibility may cause a decrease in phosphoenolpyruvate carboxylase (PEPC) activity, ATP and NADPH levels and stable chlorophyll a fluorescence [54]. Potassium deficiency can impair stomatal function in many plant species, lowering stomatal conductance, chlorophyll content [55–57], and chloroplast surface area [58].

Notable decreases in shoot Fe and Zn concentrations underscore potential challenges associated with micronutrient deficiencies. Iron and Zn are crucial for the proper function of many plant enzymes involved in vital processes like energy production, growth, and stress defense [59–61]. Deficiencies in either nutrient can impair enzymatic activities, resulting in poor growth, development and plant health [62]. For example, Wang et al. [63], stress caused by Fe deficiency in Chinese cabbage severely stunted plant growth, reduced vital metabolites levels (vitamin C, proteins, sugars), also impaired photosynthesis and nitrate processing, and disrupted the plant's oxidative stress defense, resulting in decreased antioxidant enzyme activity in leaves and roots (except root POD) and increased malonaldehyde, indicating significant cellular damage. Additionally, adding more organic matter could alter nutrient cycling dynamics, increasing competitive inhibition among elements and ultimately limiting their uptake [64].

5. Conclusions

Our study demonstrates that OP application under laboratory-based conditions exerts concentration-dependent effects on *V. faba*. While lower OPF application rates may benefit its growth and development and enhance the production of beneficial secondary metabolites, exceeding a certain threshold can prove detrimental, potentially reducing macro and micronutrient uptake. Therefore, applying fresh OP as a soil amendment will require careful management. Future studies should focus on soil microbial interactions and decomposition dynamics to understand the long-term effects of OP-derived amendments.

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