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Biostimulant Effects of an Aqueous Extract of Duckweed (*Lemna minor* L.) on Physiological and Biochemical Traits in the Olive Tree

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Abstract: Biostimulants are becoming increasingly popular in agriculture for their ability to induce beneficial effects in crops, paving the way towards the identification of new materials with biostimulant potential. This study evaluated the potential of different concentrations of an aqueous extract (0.25%, 0.50%, and 1.00%, dry weight/water volume, respectively) obtained from duckweed (*Lemna minor* L.) to stimulate olive plants. Leaf net photosynthesis (Pn), leaf transpiration rate (E), stomatal conductance (gs), sub-stomatal CO_2 concentration (Ci), chlorophyll content and other plant growth parameters were investigated. As a result, the extract improved Pn, gs, Ci, chlorophyll content and plant biomass production (leaf fresh and dry weight). Furthermore, the duckweed extract generally increased the uptake of nitrogen (N), potassium (K), calcium (Ca), magnesium (Mg), iron (Fe) and zinc (Zn), while it did not influence the content of sodium (Na), manganese (Mn) and copper (Cu). The untargeted metabolomic profiling of the extract revealed the presence of signalling compounds (including phytohormones), phenolics and glutathione. Such broad diversity of bioactives may support the stimulatory potential observed in olive. In summary, this study revealed for the first time that duckweed could be seen as a promising species to obtain extracts with biostimulant properties in olive trees.

Keywords: biostimulant; aquatic species; photosynthesis; plant growth; plant nutrition; bioactive metabolites

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1. Introduction

The use of different formulations of certain organic materials and microorganisms, defined with the term biostimulants, aims to stimulate nutrition and crops growth, increase their tolerance to environmental stress, and improve the efficiency in the use of natural resources of agroecosystems [1,2]. In addition, biostimulants are gaining attention for the possibility of reducing chemical inputs as fertilizers [3]. The first definition of biostimulants can be found in a web journal of 1997 called "Ground Maintenance", in which Zhar and Schmidt, from the Department of Crop Science and Soil Environment at Virginia Polytechnic Institute, defined biostimulants as "materials that, in minute amounts, promote plant growth" [1]. In the following years, the use of the term "biostimulant" has become increasingly popular in the literature, expanding the range of substances and the inherent modes of action.

Biostimulants are successfully utilized in both cereal and horticultural crops, as they are materials capable of promoting plant growth without being fertilizers, soil conditioners

or pesticides [4,5]. Currently, biostimulants are seen as an interesting and innovative technology to increase the ability of crops to cope with some adverse environmental conditions [4]. These materials can exert beneficial effects on crops both when applied to plants grown under optimal environmental conditions and when administered to species exposed to abiotic and biotic stresses [4,5]. In this context, the effectiveness of biostimulants does not only derive from increased crop yields under stressful environmental conditions, but also from their ability to maintain high product quality traits [6]. In this sense, biostimulants are the object of intense research to ascertain their capacity to improve fruit quality and nutraceutical value [7]. For instance, in a recent study, it was found that a commercial biostimulant increased tomato fruit yield and size, nutritional composition and antioxidant properties [8]. Additionally, the effect of an extract of *Moringa oleifera* L on two genotypes of *Brassica* was investigated [7]. The authors of this study found that the biostimulant improved some nutraceutical aspects, depending on the species treated.

Given the high number of materials capable of stimulating crops, biostimulants are usually grouped into different families depending on the raw materials from which they are derived: humic substances, complex organic materials, beneficial chemical elements (e.g., silicon), inorganic salts, algae and plant extracts, protein hydrolysates, chitin and chitosan derivatives, antiperspirants (e.g., kaolin), amino acids and other compounds [1,9,10].

When applied to plants or soil, biostimulants can regulate and/or improve crops' physiological and biochemical processes, increasing their productivity and quality [3,9,11]. Biostimulants can also promote plant growth by modifying root development and architecture, thus predisposing the treated crop to absorb and translocate nutrients more efficiently [12]. In addition, these materials can increase photosynthetic efficiency, promote the accumulation of sugars in fruits, fruit set and storability. Particularly interesting is also the ability of biostimulants to make crops less sensitive to abiotic stresses, such as extreme temperatures, drought, salinity, excessive moisture in the rhizosphere, or over-or under-exposure to light [13].

As for the olive tree (*Olea europaea* L.), the application of biostimulants results in some beneficial effects, as these substances can enhance the leaf area and the total chlorophyll content [14], the nutritional status, the olive production and some olive oil quality parameters [15]. Differently, contrasting results can be found in the literature in young olive. Molina Soria [16] reported no significant effects of biostimulants on the growth of young trees, whereas Saour [17] found that the use of a combination of biostimulant/kaolin particle film enhanced growth, resulting in the production of higher quality olive seedlings. Positive effects on photosynthetic activity and growth of young olive trees were also observed by Almadi et al. [18], who applied a biostimulant consisting of a complex of amino acids (glycine, proline, hydroxyproline, etc.). Furthermore, during their production cycle, olive trees can often be subjected to environmental stress conditions, whose frequency and intensity are increasing due to climate change, which could lead to a lower yield and, in some cases, provoke plant death. For this, it is necessary to implement agronomic techniques, including cultivation operations, to increase and encourage those physiological mechanisms of tolerance to stress triggered by processes activated at the molecular level. Among the techniques that can be used to enhance the tolerance of the olive trees to abiotic stress, there is also the biostimulant application.

In this context, the research is also interested in finding new substances with biostimulant activity from a wide range of starting materials; to this end, particular interest is shown in plant extracts showing biostimulatory potential for their bio- and eco-compatibility [19,20]. Furthermore, some plant extracts obtained from terrestrial species can improve crop growth and productivity, dry matter, nutrient concentration and antioxidant activities, thus representing a sustainable and effective tool for crop systems [20–22]. As for aquatic species, the ability of extracts obtained from seaweeds on crop growth and stress resistance has been demonstrated in many studies [23–25].

In general, no studies have investigated the effects of plant extracts obtained from freshwater aquatic species except for a recent one on *Lemna minor* L. (duckweed), which

showed the biostimulant potential of this plant in maize [26]. In particular, the beneficial effects of the duckweed extract, found in this study, were attributed to the high abundance of phytochemicals with bioactive properties [26].

Duckweed is a free-floating plant of *Lemnaceae*, widely distributed in lagoons, wetlands, and ponds, which shows rapid growth and adaptability to adverse environmental conditions [27]. Duckweed is also excellent in removing toxic substances from polluted water, and its ability to tolerate toxicants has been attributed to its antioxidant activities, which can be easily induced by some compounds [28]. *Lemnaceae* are plants rich in metabolites that exhibit antioxidant and antibacterial properties [29]. In addition, duckweed has been recently demonstrated to possess a high content of phenolic acids, phenols and flavonoids [26,30]. It is well known that certain compounds could benefit plants when exogenously applied [31]. In light of the above, duckweed can be seen as a biological stock of metabolites with potential bioactive properties.

Based on these premises, this research aimed at ascertaining whether an aqueous extract obtained from duckweed could exert biostimulatory activity on olive plants. To this scope, a duckweed aqueous extract was administrated at different concentrations to olive plants (cv. Arbequina) grown in hydroponic, and some physiological and nutritional aspects were investigated in treated samples compared to untreated controls. The cv. Arbequina was chosen for the experiment since its use in the world is rapidly increasing due to its adaptability to new high-density olive planting [32–35].

Finally, this research aimed to identify the metabolites with biostimulant potential in the duckweed extract by an untargeted metabolomic approach.

2. Materials and Methods

2.1. Olive Material and Growing Conditions

Rooted olive cuttings cv. Arbequina (about 18 cm height) were removed from the perlite substrate of the mist propagation system. After root washing with distilled water, the plants were placed in 800 mL pots containing expanded clay (10 g per pot) and put in a hydroponic system for an adaptation period of 60 days. Clay is an inert and commonly used substrate for hydroponic [36].

The hydroponic system was maintained in a growing chamber (Figure 1), and plants were exposed to light with an active photosynthetic radiance by a system equipped with lamps (PHILIPS SON-T AGRO 400 W) producing 200 µmol m^{-2} s^{-1} photon flux density, under a photoperiod of 16 h d^{-1}. The temperature was constantly set at 23 °C (±1 °C) and relative humidity at about 60%. The hydroponic system consists of PVC containers comprising five plastic hydroponic pots and five plants each. Each container is connected to a tank (volume 3.5 L) containing the nutrient solution (half strength Hoagland solution, pH 7.5). An automated system due to pressurized air ensured the flux of the nutrient solution from the tank to the PVC containers with the plants three times per day. The nutrient solution was replaced every 30 days, while the evapotranspirated water was reintegrated every 2 days.

2.2. Lemna Minor Growth Conditions and Preparation of the Extract

Duckweed was harvested from a freshwater basin near the city of Perugia (Italy). First, the plants were sterilized with a 0.5% sodium hypochlorite solution for 2 min. After that, the plants were copiously rinsed twice with distilled water. Duckweed plants were then transferred to polyethylene trays (35 × 28 × 14 cm) and grown according to a published protocol [28]. The culture media were renewed every two weeks.

Ten grams of duckweed were collected, rinsed with water, and dried at 40 °C until constant weight. After that, 1 g of dried plant material was extracted with a mortar and pestle and 100 mL water (pH 7.0). The resulting suspension was maintained in an orbital shaker (100 rpm) for 24 h. After this time, the extract was vacuum filtered on a Buchner filter, and the liquid phase was brought to 100 mL. This solution was the most concentrated (1.00% duckweed extract).

Figure 1. Hydroponic system used for the experiments.

2.3. Olive Treatments with Duckweed Extract

At the end of the adaptation period to hydroponic conditions, plants were treated with different biostimulant concentrations (1.00, 0.50 and 0.25% *w*/*v*, named BIO 1, BIO 0.5 and BIO 0.25, respectively) through the foliar application (time 0 days after the treatment—0 DAT). In particular, for each biostimulant concentration, 15 plants were treated, using 15 mL of solution for each plant. Another 15 plants were left as control and not treated with the biostimulant. After 10 days (10 DAT), the biostimulant treatment was repeated.

2.4. Olive Leaf Gas Exchange, Chlorophyll Content and Growth

Leaf net photosynthesis (Pn), leaf transpiration rate (E), stomatal conductance (gs) and sub-stomatal CO_2 concentration (Ci) were determined for each treatment at 7, 14 and 21 DAT. Leaf gas exchange rates were measured using a portable IRGA (ADC-LCA-3, Analytical Development, Hoddesdon, UK) and a Parkinson-type assimilation chamber. Leaves were enclosed in the chamber and exposed to the same light as in the hydroponic system. The flow rate of air passing through the chamber was kept at 5 cm^3 s^{-1}. During gas-exchange measurements, the external CO_2 concentration was about 375 cm^3 m^{-3}, and the air temperature inside the leaf chamber was about 1 °C higher than the hydroponic room temperature. The chlorophyll content was measured on the middle part of 15 leaves for each treatment, using a SPAD-502 Chlorophyll Meter (Minolta Camera Co. Ltd., Osaka, Japan) at 7, 14 and 21 DAT.

At the end of the experiment, 30 DAT, five plants from each treatment were selected, and roots, shoots, stems and leaves of each plant were weighed fresh (FW) and then oven-dried at 95 °C until constant weight to determine dry weight (DW). Moreover, the number of leaves was also determined.

2.5. Nutrient Determination in Olive Leaves

The nutrient determination in olive leaves was run in triplicate in samples dried in an oven at 60 °C until a constant weight had been reached. Nitrogen quantification was carried out on leaf samples (1.0 g) digested with 12.5 mL H_2SO_4 96% (*v*/*v*), 7.0 mL H_2O_2 30% (*v*/*v*) and a Kjeldahl tablet. Then, digested tissues were left to cool and added with 80.0 mL of NaOH 32.5% (*w*/*v*). Total nitrogen was determined by titration with H_2SO_4 0.1 N [37].

Furthermore, 0.25 g of dried leaves were added with 7.0 mL of HNO_3 65% (*v*/*v*) and 3.0 mL of H_2O_2 30% (*v*/*v*) and left at 90 °C for 90 min. The acid digested samples were filtered, and K, Ca, Mg, Na, Fe, Mn, Zn and Cu were quantified by Inductive Coupling Plasma spectrometry (ICP) [38].

2.6. Duckweed Extract Profiling

The phytochemical profile of the duckweed extract was characterized through an untargeted metabolomics approach, based on ultrahigh-pressure liquid chromatography quadrupole-time-of-fight mass spectrometry (UHPLC-ESI/QTOF-MS) as reported by Del Buono et al. [26]. Briefly, the chromatographic separation was achieved using an Agilent Zorbax eclipse plus column (50 × 2.1 mm, 1.8 µm) and a binary mixture of water and acetonitrile (4–96% in 33 min linear gradient). QTOF-MS acquisition used positive polarity and full scan mode (100–1200 m/z, 1 Hz scan rate, absolute peak height threshold 3000 counts), and the injection volume was 4 µL. Triplicate samples were analysed.

The annotation of raw mass features was performed as previously reported using the software Profinder B.07 (from Agilent Technologies, CA, USA), according to monoisotopic accurate mass and the whole isotopic pattern [26]. The subsequent annotation was carried out using the plant metabolome database PlantCyc (https://plantcyc.org/ access date: 14 October 2021), and only the compounds annotated within 100% of replications were retained and annotated.

2.7. Statistical Analysis

The trials were organized according to a randomized block design, with 4 treatments and 15 plants for each treatment. The experiments were carried out in triplicate. Statistical analysis was performed by analysis of variance (one-way ANOVA). Significant differences between the values were determined at $p \leq 0.05$, according to the Tukey test. The statistical environment R was used to perform the analysis [39].

3. Results

3.1. Leaf Net Photosynthesis (Pn), Leaf Transpiration Rate (E), Stomatal Conductance (gs) and Sub-Stomatal CO_2 Concentration (Ci)

The photosynthetic activity was recorded at 7, 14 and 21 DAT in plants treated with the duckweed extract at three concentrations (BIO 0.25, BIO 0.5 and BIO 1). Significant Pn increases were observed at 21 DAT for all the duckweed extract concentrations used in biostimulated olive leaves compared to untreated controls (Figure 2). Furthermore, an increase in gs for samples treated with the two highest dosages, BIO 0.5 and BIO 1 was observed (Figure 2). All the duckweed extract concentrations used significantly increased Ci with respect to the control. In contrast, the leaf transpiration rate (E) resulted unaffected by the treatment with the duckweed extract. Therefore, the biostimulated plants at 21 DAT exhibited greater Pn, gs, and Ci values than untreated plants (control).

The olive treatment with the duckweed extract enhanced the leaves chlorophyll significantly compared to untreated samples throughout the experimental period (Figure 3). In particular, inductive effects on chlorophyll were found for all the dosages applied BIO 0.25, BIO 0.5 and BIO 1 at 14 and 21 DAT. The biostimulated plant leaves, at all the duckweed extract concentrations applied, showed higher SPAD values than those of control plants.

3.2. Plant Growth and Biomass Development

Regardless of the concentration applied, the treatment with the duckweed plant extract prompted significant increases in the leaves number, fresh and dry weight and shoot fresh and dry weight compared to the untreated controls (Table 1). The BIO 0.25, BIO 0.5, and BIO 1 treatments showed no significant difference between them regarding the effect on the above parameters. Finally, the duckweed extract did not affect the growth and development of the other plant organs such as roots and stem (Table 1).

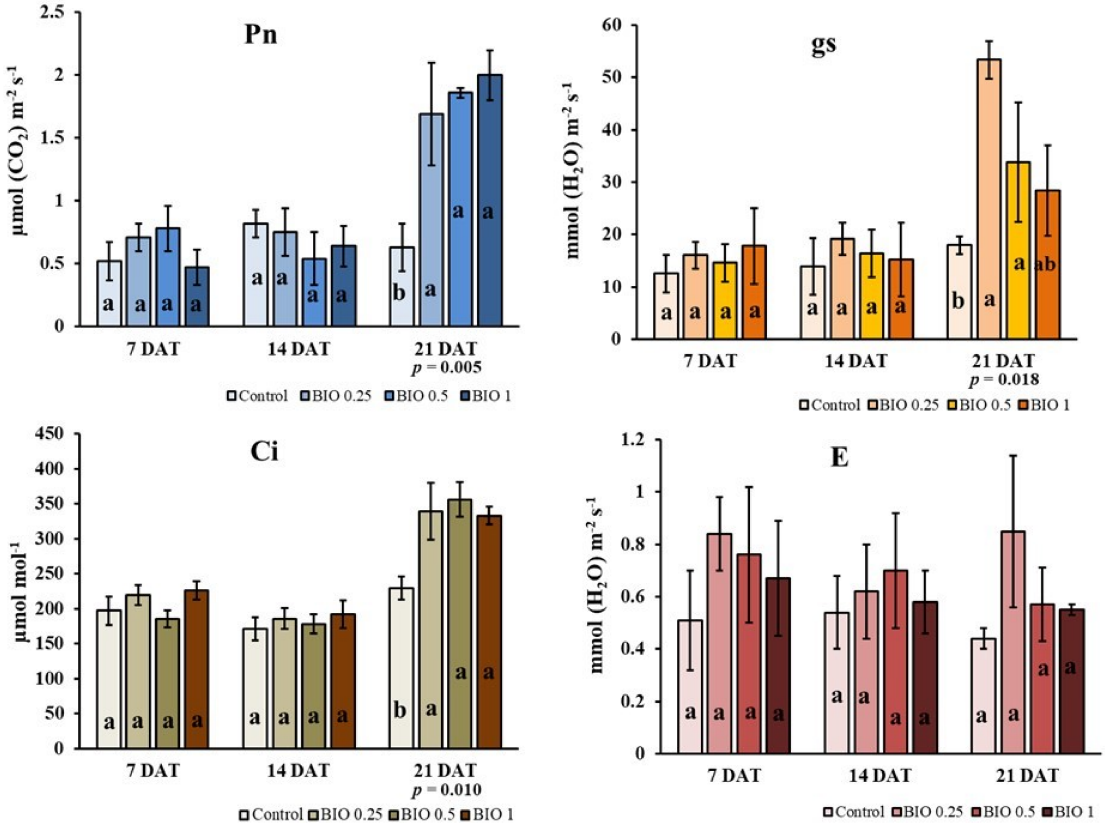

Figure 2. Leaf net photosynthesis (**Pn**) (µmol (CO_2) m^{-2} s^{-1}), stomatal conductance (**gs**) (mmol (H_2O) m^{-2} s^{-1}), sub-stomatal CO_2 concentration (**Ci**) (µmol mol^{-1}) and leaf transpiration rate (**E**) (mmol (H_2O) m^{-2} s^{-1}) measured at 7, 14 and 21 days after duckweed extract treatment (DAT). For each DAT and for each parameter, means with different letters are significantly different ($p < 0.05$) as indicated by one-way ANOVA followed by Tuckey test. The bars reported SD (standard deviation).

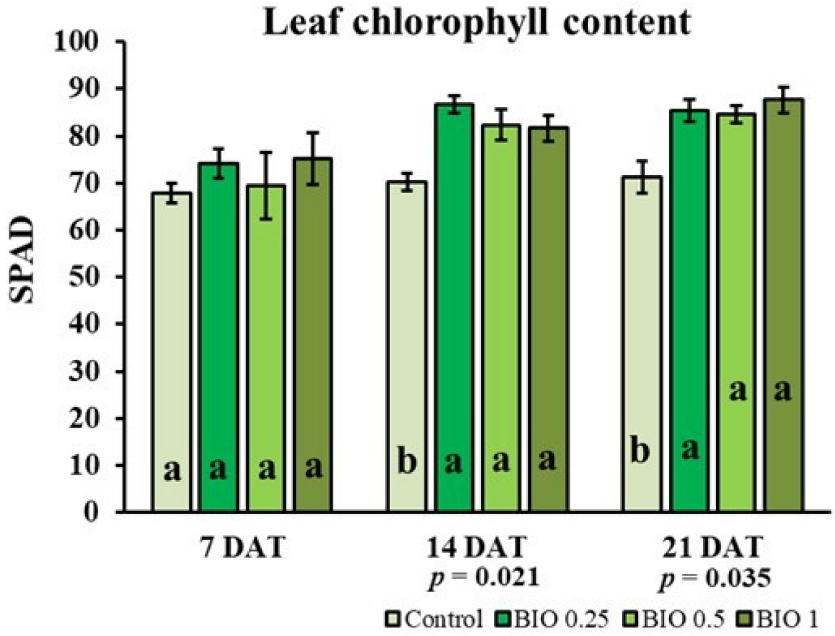

Figure 3. Leaf chlorophyll content was measured by SPAD at 7, 14 and 21 days after duckweed extract treatment (DAT). For each DAT, means with different letters are significantly different ($p < 0.05$) as indicated by one-way ANOVA followed by Tuckey test. The bars reported SD (standard deviation).

Table 1. Fresh weight (FW) and dry weight (DW) of leaves, roots, stem and lateral shoots and total number of leaves at 30 days after duckweed extract treatments (DAT).

	Leaves FW	Leaves DW	Roots FW	Roots DW	Stem FW	Stem DW	Lateral Shoots FW	Lateral Shoots DW	Number of Leaves
	(g)	(g)	(g)	(g)	(g)	(g)	(g)	(g)	(*n*)
Control	1.19 (0.27) b	0.59 (0.08) b	20.61 (3.23) a	3.19 (0.34) a	2.46 (0.25) a	1.37 (0.10) a	0.28 (0.04) b	0.09 (0.03) b	18.4 (3.11) b
BIO 0.25	3.68 (0.30) a	1.48 (0.10) a	19.23 (4.43) a	3.24 (0.62) a	2.95 (0.41) a	1.48 (0.21) a	0.72 (0.19) a	0.24 (0.02) a	46.0 (7.48) a
BIO 0.5	1.76 (0.27) a	0.81 (0.10) a	22.18 (4.93) a	3.80 (0.85) a	3.02 (0.30) a	1.62 (0.13) a	0.47 (0.04) a	0.13 (0.03) a	24.8 (2.52) a
BIO 1	2.62 (0.58) a	1.17 (0.21) a	17.86 (4.00) a	3.06 (0.54) a	2.55 (0.10) a	1.36 (0.08) a	0.45 (0.09) a	0.21 (0.04) a	33.4 (7.32) a
	$p = 0.0015$	$p = 0.0013$					$p = 0.0034$	$p = 0.0042$	$p = 0.0027$

In each column and for each parameter, mean values followed by different letters are significantly different ($p < 0.05$) as indicated by one-way ANOVA followed by Tuckey test. In parenthesis, SD (standard deviation) is reported.

3.3. Effect of the Duckweed Extract, Applied at the Three Different Concentrations, on Olive Nutrient Content

The content of some macro- and micronutrients was investigated in olive leaves treated with the duckweed extract applied at the three different concentrations and compared with untreated control samples (Table 2). Regarding the N content, it was found that the samples treated with the duckweed extract, regardless of the concentration applied, showed significant increases in the content of this element with respect to the control samples. However, the different concentrations of the duckweed extract showed no significant difference in the N content between them. With regard to K, BIO 0.25, BIO 0.5 and BIO 1 significantly increased the content of this element compared to the control samples. However, as with N, no significant differences were found between the different concentrations of duckweed applied to olive samples. Concerning Ca, the BIO 1 was the only treatment effective in raising the content of this element in the biostimulated olive compared to the control samples. Differently, all the treatments with the duckweed extract significantly stimulated the Mg content, but no significant differences were found between BIO 0.25, BIO 0.5 and BIO 1. Finally, the last macronutrient investigated, Na, was unchanged in olive leaves following the treatments with the duckweed extracts.

Table 2. Content of mineral elements (N, K, Ca, Mg, Na, Fe, Mn, Zn, Cu) determined in olive leaves at 30 days after duckweed treatment (DAT).

	N	K	Ca	Mg	Na	Fe	Mn	Zn	Cu
	(mg g^{-1} DW)						(µg g^{-1} DW)		
Control	1.84 (0.60) b	59.8 (7.1) b	3.68 (0.15) b	0.52 (0.07) b	0.50 (0.10) a	23.8 (2.4) b	13.7 (3.5) a	9.1 (0.10) b	16.8 (4.8) a
BIO 0.25	3.10 (0.15) a	78.9 (4.5) a	3.70 (0.07) b	0.70 (0.01) a	0.51 (0.12) a	25.0 (2.0) b	17.8 (4.4) a	8.8 (0.6) b	15.6 (3.0) a
BIO 0.5	2.80 (0.10) a	81.6 (5.7) a	4.56 (0.86) ab	0.76 (0.03) a	0.65 (0.11) a	43.2 (7.7) a	12.6 (5.6) a	9.3 (0.3) b	18.2 (2.0) a
BIO 1	3.00 (0.01) a	95.7 (15.4) a	5.30 (0.3) a	0.71 (0.01) a	0.64 (0.07) a	51.8 (7.8) a	14.0 (5.4) a	11.9 (0.3) a	17.5 (1.3) a
	$p = 0.0051$	$p = 0.0093$	$p = 0.0079$	$p = 0.0004$		$p = 0.0006$		$p = 0.00004$	

In each column and for each parameter, mean values followed by different letters are significantly different ($p < 0.05$) as indicated by one-way ANOVA followed by Tukey test. In parentheses, SD (standard deviation) is reported.

Regarding micronutrients, significant effects were found for Fe and Zn, while the treatments did not influence the Cu and Mn content. (Table 2). In particular, BIO 1 and BIO 0.5 significantly increased the Fe content in olive leaves compared to the control sample; in contrast, the lowest duckweed dosage was ineffective in eliciting such an effect. The Zn content was stimulated only in samples subjected to the highest duckweed concentration (BIO 1), while the other two dosages, BIO 0.5 and BIO 0.25, were ineffective in stimulating the content of this element in olive leaves.

3.4. Duckweed Extract Phytochemical Profile

In a previous study [26], duckweed extract was quantitatively analysed to determine phenolics and glucosinolates content and identify other bioactive compounds. In the present study, a broader screening of metabolites of the aqueous duckweed extract was performed using a plant metabolome database to comprehensively highlight the molecules that may help explain the extract biostimulant capacity (Supplementary Table S1). This

untargeted approach revealed the presence of several compounds belonging to phytohormones (auxins, cytokinins, brassinosteroids), amino acids, phenylpropanoids and their glycosides (mainly flavonoids such as hesperidin, kaempferol and quercetin, and phenolic acids such as caffeic acid), and glucosinolates, as previously reported [26]. Besides glucosinolates, other nitrogen-containing secondary metabolites (namely alkaloids) were found in the extract. Moreover, isoprenoids were well represented, including triterpenoids, sesquiterpenes, and terpene hormones (gibberellins and their precursors, abscisic acid derivatives and brassinosteroids). Tetraterpenes (carotenes and xanthophylls) could also be detected in the extract, with pigments such as chlorophylls and related compounds. The duckweed extract showed an accumulation of several molecules involved in plant signalling and communication. For instance, the results indicated the presence of choline and phosphatidylcholine related compounds, jasmonates, dopamine and L-dopa, methylsalicylate and proline. Finally, compounds related to plant stress and detoxification (ascorbates or glutathione) were identified in the plant extract.

4. Discussion

Currently, plant biostimulants are gaining increasing attention, as this category of materials is considered an innovative agronomic tool for improving crop productivity [40]. In particular, it has been reported that biostimulants can act in plants at different levels, showing the main effects in increasing plant metabolic and photosynthetic activities, nutrient absorption, growth, biomass production and yield [3,41–43].

This study suggests a significant potential of the duckweed extract in promoting beneficial effects in olive in terms of nutritional status, leaf photosynthetic activity and chlorophyll content and, consequently, on the plant growth. On this account, it has been well documented that biostimulant treatments often increase leaf chlorophyll content [44]. In particular, different biostimulants such as a *Moringa oleifera* extract, Actiwave®, the commercial product ONE® and borage extracts enhanced chlorophyll and carotenoid contents in some horticultural crops such as rocket, lettuce and endive [44–46]. Photosynthesis is an integrated and symptomatic result of the general status of the plant [47]. In particular, this activity can give important information on the productive potential of plants and their capacity to react to environmental factors [47]. The increase in photosynthetic activity found in our experiments was associated with increased stomatal conductance (gs) and intercellular CO_2 concentration (Ci), suggesting that the duckweed extract enhanced photosynthesis also by positively affecting the stomatal aperture (Figure 2). Our results agree with Kuluzewicz et al. [48] and Almadi et al. [18], who found that in broccoli and in olive tree, the use of biostimulants can increase stomatal conductance and photosynthetic activity. In vine, humic acids improved physiological parameters related to the whole plant photosynthesis, such as the increased leaf net CO_2 and chlorophyll concentration and total leaf area [49]. The greenhouse jute treated with a commercial vegetal-derived biostimulant from a tropical plant extract (PE; Auxym ®, Italpollina, Rivoli Veronese, Italy) enhanced photosynthetic activity, SPAD index, and especially the nutritional status [50]. The use of borage extracts increased the net photosynthesis in lettuce, while Actiwave® increased the photosynthetic activity by 27% in strawberries [51]. An increase in net photosynthesis was also observed by treating hibiscus and *Euphorbia* × *lomi* plants with a biowaste [52,53].

Furthermore, the stimulatory effects exerted by the duckweed extract on the photosynthetic activity can explain the increased leaf fresh and dry weights (Table 1). These results agree with other studies [41,46,54] that report as biostimulant treatments can enhance plant growth, determining higher dry matter accumulation in vegetable and ornamental crops. In particular, an in vitro experiment with an extract of brown marine algae evidenced significant stimulatory effects on the growth of spinach [55]. In the same way, the use of Bio-algeen S-90 determined an increase of about 30% on the aboveground biomass of lettuce 'Four Seasons' compared to control plants [56]. In addition, a biomass increase in lettuce was reported when the crop was treated with a mixture of extracts from different plant species associated with *Lactobacillus* and yeast [44]. Finally, in olive trees (cv. Arbe-

quina) subjected to severe salt stress, the treatment with a commercial biostimulant Megafol improved plant dry weight and leaf area due to greater photosynthetic activity. Moreover, Megafol caused a reduction in leaf fall and an improvement in the chlorophyll content and antioxidant activities in the salt-stressed olive trees [13]. The improved vegetative activity, due to a higher photosynthetic activity promoted by the biostimulant treatment, deserves attention also for the opportunity of increasing the plant potential to sequester carbon in olive trees [57].

Biostimulants can strongly influence crops ability to acquire nutrients, making their uptake and use more efficient; such an effect, consequently, increases crop productivity and quality [3,10,58]. As already mentioned in the introduction, the potential to act as a biostimulant for a given material is also assessed on its ability to promote plant nutrition without providing nutrients per se [10,58,59]. It has been postulated that biostimulants improve nutrient acquisition by prompting the release from roots of specific substances capable of increasing the mobility and solubility of nutrients [57,58]. In addition, biostimulants can also affect root biomass or modify root architecture and organization [2].

This study showed that the duckweed extract generally increased at the three different dosages the N, K, Mg, Ca, Fe and Zn contents in treated olive (Table 2). Differently, Na, Mn and Cu contents were unaffected by the plant extract.

All the treatments significantly elevated N; this effect could be related to the higher photosynthetic activity, chlorophyll content, and biomass shown by olive samples treated with the extract (Figures 2 and 3, Table 1). The N supply is a key factor that can condition the activities mentioned above [2,60,61]. Generally, the impact of biostimulants on N content is attributed to their ability to stimulate the enzymes of the nitrogen metabolism and upregulate the root nitrate transporters, as shown in recent studies carried out in maize and soybean [62–64].

The duckweed extract also exerted a strong effect on the K acquisition; this can, in turn, stimulate the photosynthetic activity due to the K capacity of inducing the enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase, maintaining high stomatal and gas exchange activities [65,66]. In addition, all the treatments increased the Mg content, making it possible to postulate that the duckweed extract, exerting a beneficial effect on this nutrient acquisition, activated the enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase and stimulated the chlorophyll content [67]. Chloroplasts contain 35% of Mg, and of this, about 25% is bound to the pigment [68]. The effect on Ca was more modest than those found for the other elements mentioned; only the higher duckweed concentration increased its content (Table 2). However, Ca exerts protective and structural functions and affects stomatal conductance and photosynthetic activity [65].

Regarding the micronutrients, the highest dosages of the duckweed extract (BIO 0.5 and BIO 1) affected Fe content. Plant productivity depends on this nutrient for its involvement in photosynthesis, being part of the two photosystems and the Cyt-*b6f* complex [69]. Finally, Zn was slightly increased by the highest dosage, BIO 1. Increases in the content of this element could be of relevance as Zn is involved in chlorophyll biosynthesis and chloroplast development [30,70].

In general, the stimulatory effect of biostimulants on biomass development and plant growth is considered the mechanism which regulates the increased demand for nutrients [71]. On this account, Jannin et al. [72] showed that rapeseed elevates the expression of genes responsible for nutrient acquisition after applying an algal biostimulant. Therefore, the increases in K, Mg, Ca, Fe, and Zn contents in biostimulated olive samples can be seen as a crop response prompted by the biostimulant to support the increased demand for biomass production.

The potential of the duckweed extract in promoting the beneficial effects we observed could be linked to the presence of several bioactive compounds, as suggested by Del Buono et al. [26], but also to the presence of plant regulators and signal molecules that can trigger changes metabolic processes in plants. For instance, the untargeted profiling highlighted the presence of auxins and auxin-related compounds, which might partially ex-

plain the increase in photosynthetic performance and plant growth. Several studies indicate the benefits of applying exogenous auxins to plants and, in particular, the indoleacetic acid (IAA). Li et al. [73] revealed that the addition of exogenous IAA increased photosynthetic capacity in *Zizania latifolia*. These authors reported that exogenous IAA led to significant increases in biomass accumulation in *Z. latifolia* and contributed to higher stomatal conductance and transpiration rate [73]. Moreover, auxins are considered key regulators in plant root development, essential for water and nutrient acquisition [74]. Plant extracts having a biostimulant activity have been reported to contain cytokinins, auxins or hormone-like substances [44]. However, the extracts seemed to be more than just a plant regulator due to the presence of molecules such as phenolic compounds. The addition of exogenous phenolics has been previously reported to enhance plant performance [75]. In particular, Zhang et al. [75] showed that the addition of chlorogenic acid and hesperidin alleviates the impact of salt stress by improving photosynthetic performance. Moreover, Zhang et al. [76] pointed out that the addition of phenolics, including hesperidin, can modulate functional traits in lettuces, also modifying the endogenous phenolic content. In this sense, hesperidin has been found to be the most abundant flavone in duckweed extracts [26]. Exogenous phenolics have been reported to trigger the accumulation of electron carriers, increase stomatal conductance and elicit secondary metabolism in lettuce, both under normal and abiotic stress conditions [75].

On the other hand, the content of amino acids could also explain the enhancement of plant performance. Other authors observed that the effect of biostimulants on plant growth might be linked to the direct incorporation of amino acids used for protein biosynthesis [44]. Moreover, some amino acids found in the duckweed extract (proline) are also related to plant signalling. It has been reported that proline supplementation may ameliorate olive tolerance to salinity by increasing the activity of some antioxidant enzymes, photosynthetic activity, plant growth and plant water status [77]. Likewise, it has been proposed that the action of biostimulants could be linked to the presence of signal molecules, as in the case of protein hydrolysates. In this case, it has been proposed that the stimulatory effect is due to amino acids and small signalling peptides [78].

Besides, other signalling molecules such as L-dopa, dopamine, serotonin or phosphatidylcholine-related compounds could be detected in the duckweed extract. These compounds deserve future investigations in terms of their biostimulant potential. Particular attention should be paid to the presence of glutathione (GSH) in the extracts. GSH has numerous roles in plant cells in both primary and secondary metabolism [79]. Several authors showed that exogenous GSH could enhance abiotic stresses tolerance by restricting the entry of toxic ions, enhancing antioxidant defences, and modifying the photosynthetic parameters and photosystem II efficiency [80].

Further studies are needed since the duckweed extract contains many potential signal compounds. However, although it was not possible to identify a specific bioactive molecule, the biostimulant effects were evident and significant. Noteworthy, given the broad spectrum of bioactive compounds in the duckweed extract, a synergic action of different components can be postulated. This assumption would be in line with what is often observed for plant extracts.

5. Conclusions

In conclusion, this study showed for the first time the potential of an extract obtained from an aquatic species, duckweed (*Lemna minor* L.), to act as a biostimulant in olive for its capacity to improve leaves photosynthetic activity and chlorophyll content, plant growth and nutritional status at all the concentration used.

The metabolomic characterization of the extract evidenced a significant presence of several metabolites, which can support the beneficial effects found. In particular, plant regulators (including auxins) and signalling molecules, among others, were annotated in the extract, as discussed in the precedent section. Similarly, the presence of glutathione

and the broad phenolic profile support the effects observed in olive. However, further investigations are needed to fully understand the stimulatory potential of duckweed.

Furthermore, the results of this research suggest that further studies should be carried out to ascertain the effect of duckweed extracts in mitigating the negative effects that biotic and abiotic stresses can have on plants, especially those related to climate change. Finally, this research highlighted that biostimulants could be found from resources readily available in nature. This aspect is relevant for finding new sustainable solutions to reduce the environmental impact of agriculture.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture11121299/s1, Table S1: untargeted metabolomics of the aqueous duckweed extract.

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