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Role of an Aqueous Extract of Duckweed (*Lemna minor* L.) in Increasing Salt Tolerance in *Olea europaea* L.

Luca Regni *, Ciro Tolisano, Daniele Del Buono *, Dario Priolo and Primo Proietti

Department of Agricultural, Food, and Environmental Sciences, University of Perugia, Borgo XX Giugno, 06121 Perugia, Italy; ciro.tolisano@studenti.unipg.it (C.T.); dario.priolo@unipg.it (D.P.); primo.proietti@unipg.it (P.P.)

* Correspondence: luca.regni@unipg.it (L.R.); daniele.delbuono@unipg.it (D.D.B.)

Abstract: Salt stress is one of the preeminent abiotic stressors capable of strongly impacting crop productivity and quality. Within the array of strategies garnering interest in safeguarding crops against abiotic stresses, the use of plant biostimulants is emerging as a noteworthy avenue. For the above, there is an increasing interest in finding new plant extracts showing biostimulating effects in crops. In the present study, the efficacy of an aqueous extract from an aquatic species, the duckweed (*Lemna minor* L.), was assessed in olive plants (cv. Arbequina) grown in hydroponics and exposed to severe saline stress (150 mM NaCl). Salt stress caused considerable diminutions in biomass production, leaf net photosynthesis (Pn), leaf transpiration rate (E), and stomatal conductance (gs). The application of the duckweed extract resulted in a notable plant functionality recovery and counteracted the detrimental effects of the NaCl stress. Indeed, the plants stressed with NaCl and treated with the extract showed enhanced physiological and biometric traits compared to samples treated with NaCl alone. In particular, the duckweed extract improved photosynthetic activity and stomatal conductance, reduced the intercellular CO_2 concentration, and ameliorated other physiological and morphological parameters. All these benefits influenced the whole plant growth, allowing samples treated with the extract to maintain a similar performance to that exhibited by the Control plants.

Keywords: biostimulants; aquatic species; photosynthesis; NaCl stress; olive

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1. Introduction

Due to ongoing climate change, soil salinity stress is an increasing worldwide issue for cropping systems and its impact is particularly detrimental for most fruit-tree crops cultivated in the countries of the Mediterranean basin [1,2]. In these zones, many agricultural coastal areas are experiencing the harmful effects of extreme events caused by climate change, such as flooding and rising sea levels [3–5], which are provoking the progressive degradation of the primary natural resources, soil and water. This is due to the uncontrollable land salt intrusion, which determines the gradual salinization of soils and saltwater dispersion into freshwater aquifers [3]. In addition, it should be considered that salinity stress often occurs along with high temperature and drought stress. Nowadays, it has been estimated that salinity is affecting about 800 million hectares of arable land worldwide, strongly decreasing crop yields. The situation has worsened over the last 20 years due to increased irrigation requirements in arid and semi-arid regions [4]. In this context, it should also be considered that the global demand for food is constantly increasing due to the continuously growing world population. These issues are particularly challenging to address, but they require effective solutions that could be implemented quickly.

Salinity severely impairs crop productivity because its harmful effects can cause a variety of different physiological, morphological, and biochemical changes [5]. In particular, salt stress can influence plant establishment, cause stunted growth, and give rise

to oxidative perturbations, hindering pivotal metabolic processes such as photosynthesis and, in turn, biomass production [6]. In addition, it has often been reported that salinity impacts plants by provoking osmotic and ionic alterations [7], negatively affecting nutrient acquisition and translocation, and interfering with enzyme activities [6]. Salinity can also cause the death of the leaves due to salt accumulation in the cell wall or cytoplasm since the vacuole cannot sequester high amounts of salt [8]. Under salt stress, plants usually show restricted root extension, a higher root-to-shoot ratio, as well as altered root morphology [9]. Moreover, reductions in the total leaf area have been recorded that decrease the photosynthesizing surface, which probably represents an attempt by the plant to minimize water loss by transpiration, or could be a consequence of hampered plant nutrition [10].

Olive is generally considered mildly tolerant to salinity [11], but different cultivars showed great differences in salt tolerance [12]. All the researchers agree on the negative influence of moderate and high salinity stress on plant growth (mainly due to the leaf area reduction), even if the extent of the latter varies with the length of salt exposure and the cultivar [13].

The cv. Arbequina, investigated in the present study, has been described in the literature as a plant species that is medium-tolerant to salt stress [14]. However, Arbequina is of great interest for cropping systems since it is one of the most suitable species for very high-density planting systems thanks to its low vigor, high branching density, and high fruit-bearing capacity [15,16]. For its adaptability to super-intensive planting systems, Arbequina cultivation is spreading rapidly in most of the olive-growing areas of the world.

Given the strong impact of biotic and abiotic stress on olive and other crops, there is an ever-increasing focus on finding, developing, and implementing eco-friendly solutions to help plants counteract the detrimental effects of salinity and other environmental stressors. Among the strategies that can be adopted to increase plant resistance to salt stress, biostimulants are gaining increasing attention for their capacity to prompt benefit in crops [17,18]. In general, biostimulants increase crop productivity by inducing plant nutrition and nutrient use efficiency, improving photosynthetic machinery and increasing crop resistance to various abiotic stresses [7]. In addition, they can also positively affect plants by stimulating primary and secondary metabolism [7]. Biostimulants can be natural products, but given the wide range of raw materials from which they can be obtained, they have been grouped into two main classes according to their origin [19]. The first classification concerns the distinction between biostimulants of microbial and non-microbial origin [19]. Thus, the latter include plant and algae extracts, protein hydrolysates (mainly plant-derived), fulvic and humic substances, chitosan, and some inorganic compounds [20].

In recent years, researchers have been directed at finding new natural substances with biostimulant effects. Particular attention is being paid to finding plant extracts with relevant bioactive properties and also characterized by eco-compatibility [21,22]. Indeed, biostimulants deriving from plant extracts can contain active molecules (protein, small peptides, amino acids, phytohormones, and antioxidants) that can activate biochemical, physiological, and metabolic responses in crops, thus improving their productivity in normal conditions and their ability to cope with some abiotic or biotic environmental stressors.

In this perspective, very recent studies have been conducted on the biostimulatory effects of extracts obtained from the freshwater aquatic species *Lemna minor* L. (duckweed) [23–25]. Duckweed is widespread in freshwater basins, found on several continents, and is characterized by fast growth and a remarkable ability to adapt to even very different and unfavorable environmental conditions [23]. It is also easy to grow under controlled conditions and has a high content of metabolites with stimulatory properties [23]. Concerning the extracts obtained from this species, it has been shown that they can promote benefits in maize [23,24] and olive plants [25], even when the former crop was grown on a copper-polluted substrate [24]. Metabolomic studies have revealed that this species contains a broad spectrum of bioactives, including signaling compounds, phenolics, flavonoids, and many different antioxidants that can be responsible for the beneficial effects recorded [23–25]. To date, no studies have been conducted on using duckweed aqueous

extracts to ascertain their eventual beneficial effect on plants grown under salt-stress conditions. Therefore, this work aims to evaluate the ability of an aqueous duckweed extract to increase the tolerance of olive trees to salt stress (cv. Arbequina). The experimentation was conducted in a hydroponic growing system since, even though it is different from the open field conditions, it allowed the study of the effects of the duckweed extract on olive plants, avoiding the interferences of the types of soil and microbiota.

2. Materials and Methods

2.1. Olive Material and Growing Conditions

Olive plantlets of the cv. Arbequina, approximately 20 cm in height, were transplanted in 200 mL pots containing rock wool for an acclimatization period of 60 days and were grown in a hydroponic system under controlled conditions. The olive plants were allocated in PVC containers comprising five plastic pots for hydroponics, with one plant for each pot. A tank containing the nutrient solution (3.5 L of half-strength Hoagland solution, pH 7.5) was connected to each container. The flux of the nutrient solution from the tank to the PVC containers containing the olive plants was ensured by an automated system three times per day. The nutrient solution was replaced twice per month, while the water lost due to evapotranspiration was replenished every 2 days.

The plants were exposed to light provided a system equipped with PHILIPS SON-T AGRO 400 W (Koninklijke Philips N.V., Amsterdam, The Netherlands) delivering a photon flux density of 200 µmol m^{-2} s^{-1}, with a photoperiod of 16 h d^{-1}. The temperature was maintained constantly at 23 °C (±1 °C) and relative humidity was maintained at about 60%.

2.2. Lemna Minor Growth Conditions and Preparation of the Aqueous Extract

Duckweed was harvested from a freshwater basin near Perugia (Italy). Initially, the plants underwent sterilization with a 0.5% sodium hypochlorite solution for 2 min. Following the sterilization, the plants were copiously rinsed twice with distilled water. Subsequently, duckweed plants were transferred to polyethylene trays (35 × 28 × 14 cm) and cultivated following a previously published protocol [26]. The culture media was replaced every two weeks.

Ten grams of duckweed were collected, washed, and dried at 40 °C until a constant weight was achieved. After that, 1 g of dried plant material was ground with a mortar and pestle and mixed with 100 mL water (pH 7). The resulting suspension was maintained in an orbital shaker (100 rpm) for 24 h. After this time, the extract was filtered under vacuum using a Buchner filter, and the liquid phase was brought to a volume of 100 mL, resulting in a 1.00% concentration of duckweed extract.

2.3. Salt Stress and Treatments with Duckweed Extract

Following the 60-day adaptation period to hydroponic conditions, 30 plants were exposed to salt stress by introducing 150 mM NaCl into the solution (Stress), whereas 15 olive plants continued to grow in the same nutrient solution but without NaCl (Control). The NaCl concentration was chosen because for the cultivar Arbequina can be considered a sublethal dosage. Furthermore, at the dosage of 150 mM NaCl for the cultivar Arbequina using a commercial biostimulant, good results were obtained in terms of enhanced tolerance [17]. Among the thirty stressed plants, fifteen were treated through foliar application twice (at 7 and 14 days after the beginning of salt stress) with 2.0 mL per plant of the duckweed extract at a concentration of 1% in volume (Stress + Bio). The dosage of the duckweed used in a previous study [25] on olive plants under non-stress conditions was effective in improving leaf gas exchange, chlorophyll content, plant biomass (leaf fresh and dry weight), and uptake of nitrogen (N), potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), and zinc (Zn).

2.4. Olive Leaf Gas Exchanges and Plant Growth

Leaf net photosynthesis (Pn), leaf transpiration rate €, stomatal conductance (gs), and sub-stomatal CO_2 concentration (Ci) were assessed for each treatment at 7, 15, and 30 days after the treatment with the duckweed aqueous extract (DAT). Leaf gas exchange rates were measured utilizing a portable IRGA (ADC-LCA-3, Analytical Development, Hoddesdon, UK) coupled with a Parkinson-type assimilation chamber. Leaves were enclosed within the chamber and exposed to the light of the hydroponic system. The airflow through the chamber was maintained at a rate of 5 cm^3 s^{-1}. During the gas exchange measurements, the external CO_2 concentration was approximately 375 cm^3 m^{-3}, and the temperature of the air inside the leaf chamber was around 1 °C higher than the temperature in the hydroponic room.

At 45 DAT (end of the experiment), six plants from each treatment were selected. The number of leaves, lateral shoots, and total lateral shoot lengths were assessed. Additionally, destructive measurements were carried out on the selected plants. In particular, the roots, shoots, stems, and leaves of each plant were weighed fresh (FW). Finally, root analysis was performed on root-scanned images using RhizoVision Explorer v2.0.3.0 to investigate root length, number of root tips, diameter, surface area, and volume [27].

2.5. Statistical Analysis

The experimental design was organized according to a randomized block design, with 3 treatments (Control, Stress, and Stress + Bio), 3 replicates, and 15 plants for each treatment. Statistical analysis was performed by analysis of variance (one-way ANOVA) and significant differences were determined according to the Tukey HSD test ($p \leq 0.05$). The statistical environment R-4.3.2 was used to perform the analysis [28].

3. Results

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

At 7 DAT, Pn reductions were observed in Stress and Stress + Bio plants (Figure 1). However, at 15 and 30 DAT, the Pn values recorded for the Stress + Bio plants increased, reaching those shown by the Control plants, while the values of the Stress samples remained significantly lower. Furthermore, severe decreases in E and gs were recorded in plants stressed with NaCl alone throughout the experimental time. On the contrary, the plants biostimulated with the duckweed extract showed significant increases in the above parameters until reaching values similar to those observed for the Control plants at 30 DAT. Finally, at 7 DAT, Ci was significantly higher in the Stress and Stress + Bio plants compared to the Control samples. Despite this, at 15 and 30 DAT, the Ci values of the Control and Stress + Bio samples were lower than those of the Stress plants. Therefore, the values recorded for the plants treated with the biostimulant tended to reach the values observed for the Control plants, as further observed for the other parameters.

3.2. Plant Growth and Biomass Development

Salt stress caused a severe decline in plant growth (Table 1). Indeed, at 45 DAT, the samples treated with salt alone showed significant decreases in the number of leaves, lateral shoots, and length. On the contrary, the treatment of plants grown in salt stress conditions with the duckweed extract counteracted the reduction in plant growth caused by NaCl, and the values recorded were not statically different from those found for the Control plants (Table 1). In particular, in the plants treated with the extract, the number of lateral shoots and their length were not significantly different from the Control plants.

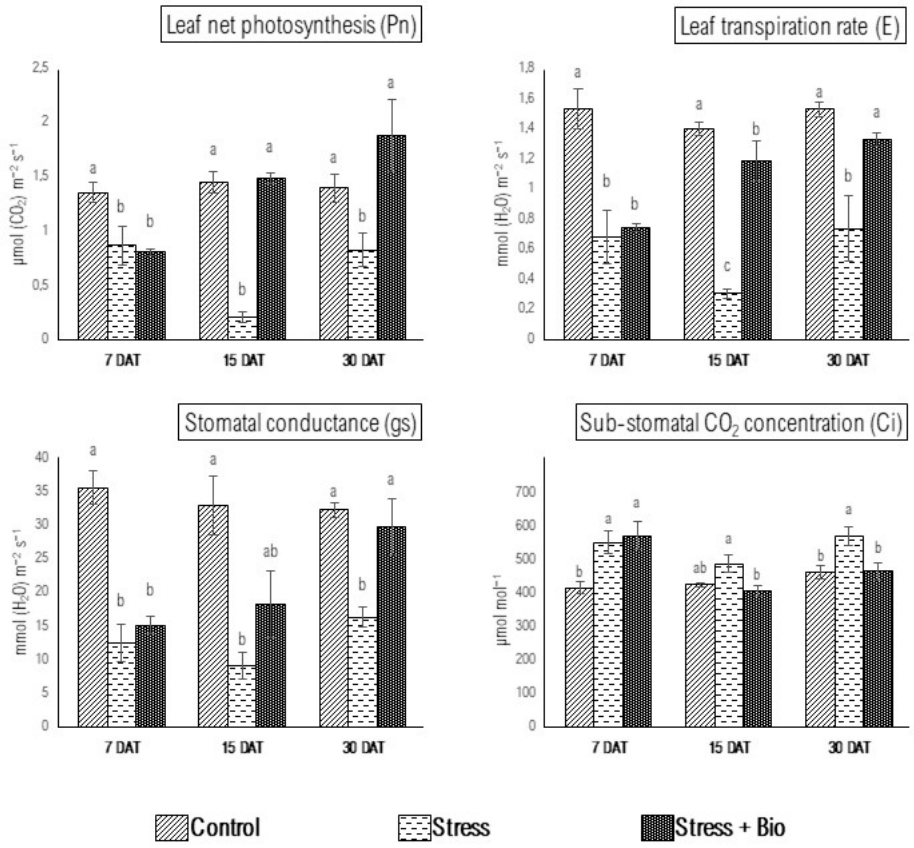

Figure 1. Leaf net photosynthesis (Pn) (µmol (CO_2) m^{-2} s^{-1}), stomatal conductance (gs) (mmol (H_2O) m^{-2} s^{-1}), sub-stomatal CO_2 concentration (Ci) (µmol mol^{-1}) and leaf transpiration rate (E) (mmol (H_2O) m^{-2} s^{-1}) measured at 7, 15 and 30 days after duckweed extract treatment (DAT). For each DAT and each parameter, means with different letters are significantly different ($p = 0.05$), as indicated by one-way ANOVA followed by Tukey's HSD multiple comparison test. The bars report SE (standard error).

Table 1. Number of leaves, lateral shoots, and total length of lateral shoots.

Treatment	Number of Leaves (n)	Number of Lateral Shoots (n)	Lateral Shoots Length (cm)
Control	31 ± 1.4 ^{a}	2.0 ± 0.5 ^{a}	2.80 ± 0.72 ^{a}
Stress	15 ± 1.8 ^{b}	1.0 ± 0.2 ^{b}	0.87 ± 0.33 ^{b}
Stress + Bio	26 ± 1.0 ^{a}	3.0 ± 0.3 ^{a}	3.91 ± 0.72 ^{a}

In each column, mean values ± SE followed by different letters are significantly different ($p < 0.05$) as indicated by one-way ANOVA followed by Tukey HSD test.

Moreover, as a consequence of the reduced growth (Table 1), the fresh weight of the plant components of the stressed plants, treated with NaCl alone, was lower than that observed for the Control samples (Table 2). In contrast, the plants treated with the duckweed extract and subjected to salt stress showed biomass values that did not statistically differ from those of the control samples (Table 2), marking the same trend as for the parameters in Table 1.

The root biomass analysis and morphology showed that salt stress caused a severe decrease in the number of root tips, length, diameter, surface, and volume. The duckweed extract prevented these detrimental effects. In fact, duckweed extract induced in samples under salt stress higher values for the number of root tips, root length, diameter, surface, and volume (Table 3). In addition, the Stress + Bio plants showed values similar to the Control samples for the abovementioned parameters.

Table 2. Fresh weight (FW) of leaves, roots, and stems and shoots.

Treatment	Leaf FW (g)	Stem and Lateral Shoots FW (g)	Root FW (g)
Control	3.81 ± 0.17 [a]	1.33 ± 0.08 [a]	3.23 ± 0.35 [a]
Stress	1.49 ± 0.20 [b]	0.83 ± 0.04 [b]	2.29 ± 0.16 [b]
Stress + Bio	3.36 ± 0.21 [a]	1.21 ± 0.06 [a]	4.54 ± 0.38 [a]

In each column, mean values ± SE followed by different letters are significantly different ($p < 0.05$) as indicated by one-way ANOVA followed by Tukey HSD test.

Table 3. Root analyses.

Treatment	Root Tips (n)	Total Length (cm)	Diameter (mm)	Root Area (mm^2)	Volume (mm^3)
Control	434 ± 19 [b]	138.1 ± 12.5 [a]	0.68 ± 0.02 [a]	3026 ± 342 [a]	850 ± 65 [b]
Stress	348 ± 10 [c]	88.1 ± 6.3 [b]	0.57 ± 0.03 [b]	1612 ± 151 [b]	469 ± 210 [c]
Stress + Bio	503 ± 32 [a]	146.3 ± 11.9 [a]	0.72 ± 0.04 [a]	3225 ± 328 [a]	1294 ± 108 [a]

In each column, mean values ± SE followed by different letters are significantly different ($p < 0.05$) as indicated by one-way ANOVA followed by Tukey HSD test.

4. Discussion

Plant biostimulants are recognized as an innovative agronomic tool due to their proven effectiveness in enhancing crop performance [29]. Numerous studies [30,31] have extensively documented their positive impacts on plant biomass production, nutrient utilization efficiency, flowering, and overall growth. Furthermore, the use of these substances is a common practice for boosting crop resistance against various detrimental biotic and abiotic environmental stresses [32,33]. In this context, a growing body of research aims to identify new bioactive substances and plant extracts to promote positive effects in crops cultivated under normal conditions or subjected to abiotic stressors. For these reasons, the present work investigated the capacity of an aqueous extract obtained from an aquatic species, the duckweed (*L. minor* L.), for its richness in bioactive compounds [23–25,34,35], to increase the salt tolerance in *Olea europaea* L. cv. Arbequina.

It is well known that salt stress, among other things, can affect plant photosynthesis, and the extent of this impairment is closely related to the duration and severity of the stress and salt concentration [1,36–38]. However, it is necessary to point out some variability in the ability of crops to tolerate or resist salt stress that also depends directly on the cultivar.

Our experiments showed that salt stress determined decreases in photosynthetic activity which were accompanied by increases in sub-stomatal CO_2 concentration. Such an effect can be considered the cause of the stomatal closure with a consequent decrease in stomatal conductance, as already observed by other authors [39]. The increase in sub-stomatal CO_2 concentration indicates that non-stomatal effects mainly caused a reduction in photosynthesis. Such CO_2 accumulation could result from damage to photosystems due to salt stress, as documented for other abiotic stresses, which can no longer sustain the light phase with a consequent decrease in the dark phase, which uses carbon dioxide to synthesize carbohydrates [40,41]. In general, the most significant inhibition in the photosynthesis rate occurs in olive cultivars characterized by inherently high photosynthesis and stomatal conductance [36]. For instance, six one-year-old olive cultivars subjected to salt stress (200 mM NaCl) for five months exhibited a notable reduction in the carbon assimilation rate by the end of the experiment [37]. In general, a decline in stomatal conductance can precede alterations in photosynthesis in salt-stressed olive plants. A marked reduction in photosynthesis in olive plants treated with some different NaCl concentrations was also observed by other authors [42]. Despite this, they reported a complete recovery of photosynthesis in plants subjected to 50 and 100 mM NaCl concentrations, especially in the salt-tolerant cultivar 'Frantoio,' accompanied by increased stomatal conductance and transpiration. These results suggest that during the initial stages of salinity stress, the plant experiences stomatal limitations that affect the entire photosynthetic process. More recently,

Loreto et al. [36] demonstrated that the primary limitations of photosynthesis in moderately salt-stressed olive plants result from the low chloroplast CO_2 concentration due to both low stomatal and mesophyll conductances.

A reduction in photosynthetic activity in NaCl-stressed crops generally hampers plant growth, as this effect adversely affects the plant's capacity to acquire nutrients, resulting in inadequate plant development, among other things [37,43,44]. In addition, some scientific evidence has demonstrated that plants may decrease biomass production to counteract the impact of certain stresses, and this to reprogram metabolism and activate defensive mechanisms [45]. In particular, in order to cope with oxidative stress due to salinity, some species increase the content of molecules and enzymes with antioxidant activity, regulate ion uptake and distribution, and maintain osmotic balance [45]. Our experiments corroborate the strong impact of salt stress on olive growth, and stressed plants displayed a lower fresh weight than the Control samples due to the reduced development of leaves, shoots, stems, and roots. However, the application of the duckweed extract reverted the detrimental effects on olive samples due to NaCl treatment, especially contrasting the impairments on photosynthesis and plant growth. In particular, our experiments indicated that the extract stimulated a significant recovery of Pn, associated with an increase in gs and a decrease in Ci. This suggests that the bioactives in the extract positively affected photosynthesis and stomatal aperture, although the plants were raised in salinity. Indeed, duckweed extract has been found to exhibit a range of bioactive metabolites and the presence of regulatory and signal molecules that can trigger changes in plant metabolic processes [23]. Our results align with previous studies that have highlighted the benefits of biostimulants in reducing the impact of salt stress. In particular, similar effects on NaCl-stressed olive plants were observed in response to Megafol treatment, a commercial plant biostimulant. In fact, olive plants subjected to salt treatment without Megafol exhibited substantial reductions in biomass production, leaf gas exchange, and relative water content (RWC) [17]. Differently, when the plants were subjected to the biostimulant treatment, they significantly improved despite salt stress.

In addition, a general negative effect of salinity on the number of leaves, lateral shoots, total length of lateral shoots, and main root tissue characteristics was observed, in line with what has already been found for photosynthetic activity. In contrast, olive samples treated with the duckweed extract showed a complete recovery at the shoot and root level for all the characters studied. Regni et al. [25] have already demonstrated the efficacy of an aqueous extract derived from duckweed in enhancing the vegetative activity of olive plants under non-stressing conditions. The phytochemical profile of duckweed showed the presence of compounds with biostimulatory activity, particularly a high content of auxins and related compounds [25] that can explain the benefits we found on photosynthesis, shoot biomass, and root development. In fact, the addition of auxins to stressed plants (e.g., indoleacetic acid) was shown to promote the photosynthetic activity of *Zizania latifolia* [46], thus resulting in increased biomass production. In addition, auxins can help increase stomatal conductance and transpiration, and these results align with what we ascertained in our experiments [46].

Finally, it is to be remarked that the *Lemnaceae* species has a very high content of metabolites with antioxidant properties [47]. For instance, the duckweed extract employed in this study showed a significant content of phenolic compounds [25]. Therefore, the treatments of the olive samples with this extract resulted in the exogenous application of phenolics, which promoted beneficial responses, improving the plant tolerance to salinity. In fact, some phenolic compounds can counteract the impact of salt stress by stimulating photosynthesis and modulating the functional traits of the crops [25]. In this context, the duckweed extract showed abundant amounts of hesperidin [25], which may benefit photosynthesis and shoot and root biomass production in plants grown in salinity [48]. Finally, it is worth mentioning that this extract also has a high content of proline, an amino acid that can effectively improve the tolerance of olive plants to salinity by stimulating the activity of some antioxidant enzymes and biomass production and improving the water

status [49]. In light of the above, the results of our experiment can also be related to the possibility that the duckweed extract could have activated the antioxidant machinery [24], thus resulting in a protective effect against the abiotic stressor.

Finally, regarding the roots, the inductive effect exerted by the extract should be considered of pivotal importance since a functional root system, especially under stress conditions, allows the crop to carry out adequate plant nutrition. Therefore, these results indicate that duckweed extract modulated root development and architecture, thus improving the plant's adaptability to cope with the abiotic stress. In agreement with the above, it has indeed been demonstrated that substances with biostimulatory properties can also improve root biomass production or modify root architecture and organization, thus resulting in more efficient plant productivity and nutrition, water acquisition, and resistance to abiotic stresses [50–53]. In line with such an effect, in addition to the already mentioned high phytohormone content, a considerable presence of glucosinolates is present in duckweed extracts [26,27], and these substances may exert a positive effect on the development of the root portion of the plant.

5. Conclusions

In conclusion, this study demonstrated for the first time the potential of an extract obtained from an aquatic species, duckweed (*Lemna minor* L.), to counteract the detrimental effect of salt stress in olive plants. Indeed, the duckweed extract improved photosynthetic activity and whole plant growth in olive plants exposed to NaCl stress, allowing them to maintain values of the studied parameters similar to those of Control plants not subjected to salt stress. However, further investigations are needed to reach a deeper understanding of the stimulatory potential of the duckweed extract on olive and other crops in salt stress conditions, as well as test this extract against other environmental stressors. In light of the above, this research demonstrated that resources readily available in nature could be sources of bioactive or biostimulant substances effective in increasing the plant's capacity to face salinity, one of the main abiotic stresses, and their eco-friendly properties pave the way towards more sustainable ways to maintain high crops productivity.

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