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Ectomycorrhizal Fungi Modulate Biochemical Response against Powdery Mildew Disease in *Quercus robur* L.

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Abstract: In light of climate change, pedunculate oak (*Q. robur* L.) was marked as the most threatened European tree species. Pedunculate oak is particularly jeopardized by powdery mildew disease caused by *Erysiphe alphitoides.* We hypothesized that priming of this tree species with ectomycorrhizal fungi could mitigate biotic stress and produce bioprotective properties against the disease. In this study, we have compared oaks' foliar physiological and biochemical responses upon infection with *E. alphitoides* in the presence and absence of ectomycorrhizal fungi (ECM). The main aim of this study was to inspect how ECM modulate an oak's biochemical response to infection with *E. alphitoides*, particularly at the level of the accumulation of the main polyamines (putrescine, spermidine, and spermine), soluble osmolytes (proline and glycine betaine), and phenolics (total phenolic content, flavonoids, and condensed tannins). A polyamine quantification was performed after derivatization by using high-performance liquid chromatography (HLPC) coupled with fluorescent detection. Oak seedlings inoculated with ECM fungi exhibited significantly higher levels of putrescine, spermine, and proline compared to non-inoculated seedlings, indicating the priming properties of the ECM. *E. alphitoides* caused an increase in individual and total polyamine content and lipid peroxidation in oak leaves regardless of the effect of ECM, while causing a decrease in physiological and antioxidative parameters and water use efficiency (WUE). Common biochemical parameters may contribute to understanding the underpinning plant defense mechanisms in three-way interactions among plants and pathogenic and ectomycorrhizal fungi and can be used as reliable adaptability descriptors in the context of climate change.

Keywords: pedunculate oak; ectomycorrhizal fungi; powdery mildew; polyamines; proline; condensed tannins; climate change

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1. Introduction

Climate change (CC), characterized by the increasing trend of green-house gasses (GHGs) emission and the amplified frequency and amplitude of heat waves, as well as altered precipitation patterns, has emerged as one of the most important issues of our time [1]. Except for affecting abiotic stress factors, CC also alters plant–pathogen interactions, which results in the modification of trees' resilience to biotic stress [2]. Furthermore, CC affects the mechanisms of pathogens' virulence, epidemiology, reproduction, and survival [2].

According to RCP climate scenarios [3], in the territory of the Republic of Serbia, pedunculate oak (*Quercus robur* L.) has been marked as the most affected tree species due to ongoing, intensive climate change. The decline of oak forests can cause great economical losses since oaks are considered highly valuable tree specimens due to their long history of exploitation for wood production and construction [4]. Powdery mildew fungi represent

an important group of biotrophic fungal pathogens that cause powdery mildew, which is a common foliar disease of many tree species, including pedunculate oak. Recent molecular studies have shown the causal agents of the disease on *Q. robur* in Southern Europe are *Erysiphe alphitoides* [(Griffon and Maubl.) U. Braun and S. Takam.] and *E. quercicola* (S. Takam. and U. Braun). The disease can be severe on young oak seedlings and saplings and in natural regenerations, nurseries, and plantations [5,6]. In mature trees, the disease is less damaging, but in combination with other biotic and abiotic factors, it can contribute to a tree's decline [7,8]. Symptoms primarily appear on the upper side of young leaves and on soft shoots as white pustules containing mycelium and oidia on the conidiophores. Over time, these pustules increase in size and coalesce, covering the entire surface. The affected developing leaves become distorted and necrosis and defoliation can occur, especially in combination with other stress-triggering factors [9].

In agriculture, several fungicides with different modes of action have been registered for application in the management of powdery mildews (demethylation inhibitors, carboxamides, anilino-pyrimidines, quinone outside inhibitors, phthalimides, and inorganic sulphur), but the application of fungicides in forests is challenging due to the difficulties associated with their application and the development of resistant fungal strains [10]. Therefore, the search for alternative ways for preventing infection with this pathogen, including biopesticides, presents one of the hottest scientific topics [11]. Regarding oak species, the only efficient protection of *Q. robur* against powdery mildew has been reported in the case of an application with an antagonistic fungus, *Trichoderma asperellum* (Samuels, Lieckf. and Nirenberg), which caused the activation of defensive volatile organic compounds and positive effects with respect to photosynthetic parameters [12]. To the best of the authors' knowledge, there are still no reports on the effects of ectomycorrhizal fungi as bioprotectors against powdery mildew in *Q. robur*.

Mycorrhizal fungi have a pivotal role in linking the aboveground and belowground components of biogeochemical cycles through a complex network of mycelium, popularly called the '*wood wide web*', which facilitates communication between trees in the forest [13]. Under CC, the stability and health of the entire forest ecosystem and the carbon and nutrient balance depend on the trees' intimate root–soil interactions with mycorrhizal fungi in the soil [13]. However, arbuscular mycorrhizal fungi (AMF) are well known to improve plant health, boost plant immunity, and induce plant defenses through mycorrhiza-induced resistance (MIR) in the plants, thereby significantly mitigating biotic stress and increasing resistance against various pathogens [14–16]. There are significantly fewer reports about the effects of ectomycorrhizal fungi (ECM) on plant defenses upon infection. Mycorrhizal fungi represent the key players in carbon dynamics and fluxes among plants, soil, and the atmosphere due to their well-branched hyphae system that they use to deliver water, nitrogen, and phosphorus to the plant in exchange for photosynthetically produced carbohydrates and even lipids from the host plant [17,18]. Mycorrhizal fungi have a beneficial effect with respect to the mitigation of drought stress by enhancing secondary metabolites' production [19], the modulation of osmolytes (sugars, amino acids, citric acid intermediates, etc.), phenolics, and plant hormone levels in plants, as well as by causing a reduction in reactive oxygen species (ROS) [20,21], but it has not been completely elucidated yet how mycorrhizal fungi in particular affect a plant's osmolytes in the presence of biotic stress factors such as powdery mildew. The overlapping of plant responses to inoculation with the pathogenic fungus (powdery mildew) and beneficial fungi (mycorrhizae) at the same time makes this experimentation quite complex, since both the mycorrhizal and the pathogenic fungi activate pattern-triggered immunities (MAMP and PAMP, respectively), which affect plant defense hormone networks [22].

One of the most remarkable groups of biomolecules regarding the improvement of plants' resilience to both abiotic and biotic stress factors is the group of polyamines (PAs). PAs as ubiquitous low-molecular-weight organic polycations displaying high biological activity exert different effects that could help plants to deal with stressful conditions due to their antioxidant, osmoprotective, and antimicrobial activity against plant pathogens [23].

The common Pas in plants are spermidine (SPD), spermine (SPM), and their diamine precursor, putrescine (PUT). Besides being actively involved in plant tolerance to abiotic stress factors, such as drought, heavy metals, and salt stresses [24–26], polyamines, as molecules synthesized from amino acids, are one of the main biomolecules acting in plant signaling during biotic stress, both in terms of plant immunity and disease [27,28]. Although the mechanism is still not elucidated, Pas, as biological amines, regulates the biosynthesis of NO radicals [29]. The NO radical plays an important role in conveying and amplifying signals during biotic stress (e.g., beneficial and pathogenic microbes, fungi, insects, and other herbivores) and plays a key role in the activation of the hypersensitive reaction (HR) and the establishment of systemic defense and disease resistance mechanisms in plants [30]. Interestingly, due to polyamines' ubiquitous nature, plants' interactions with both beneficial (including mycorrhiza) and pathogenic microbes are followed by changes in PA metabolism in both the host and the microbes [31]. On the other hand, PAs play an important role in AMF and plant interactions as well as the root system architecture [32,33]. The effect of powdery mildew and/or ectomycorrhiza on the levels and profiles of PAs in *Quercus* species has not been investigated so far.

Since an infected plant generates a high concentration of ROS during HRs to protect itself from the pathogen, the plant employs additional mechanisms to prevent secondary stresses—such as oxidative and osmotic stress—by increasing the production of compounds with antioxidant and osmoprotective properties. However, the most referred osmolytes, such as glycine betaine and proline, are confirmed in a large embodiment of the literature to alleviate the oxidative and osmotic stress caused by abiotic stress (drought, heat, or heavy metals); thus, there is a lack of knowledge about the effects of biotic stress factors on these osmolytes [26,34–36]. Furthermore, it has been proven that proline functions as a molecular chaperone as well as an efficient scavenger of ROS and an activator of ROS-scavenging enzymes [37]. Besides these compounds, reduced GSH plays multiple roles in redox control, but is also involved in signaling cascades, antioxidant defense, detoxification processes of different xenobiotics, and responses to pathogen attack [38,39]. Considering the facts, i.e., that biotic stress usually facilitates secondary metabolites' production, and that oak species are particularly abundant with constitutive polyphenols and tannin equally strong antifungal agents, it is still an open question how an infection with powdery mildew affects the tannin levels in *Q. robur* knowing that tannase is a ubiquitous enzyme in the entire microbial world [40–42].

The main objective of this paper is to explore the bioprotective properties of ectomycorrhiza with respect to the mitigation of biotic stress in *Q. robur* infected by powdery mildew (*E. alphitoides*), and to track the alternations in the plant at the physiological and biochemical levels caused by either the pathogenic or/and ectomycorrhizal fungi. We hypothesized that an inoculation with ectomycorrhizal fungi affects the *Q. robur* seedlings during an infection with *E. alphitoides* by causing changes such as:

i. Those at the physiological level (net rate of photosynthesis, stomatal conductance, and transpiration);
ii. The differential pathogen or ectomycorrhiza-specific accumulation of osmotically active substances such as glycine-betaine and proline;
iii. The differential regulation of polyamine metabolism resulting in different foliar polyamine profiles;
iv. The differential activation of the total antioxidant (ABTS) and reducing (FRAP) capacity and accumulation of various antioxidants (i.e., non-protein thiols, phenolics, flavonoids, and tannins).

2. Materials and Methods

2.1. Experimental Design

The tripartite experiment plant–ectomycorrhiza–pathogen was designed and established in the greenhouse under (semi)controlled conditions to test how ectomycorrhizal fungi affect polyamine metabolism, osmoprotectants, phenolic compounds, antioxidant

properties, and overall oak fitness and physiology under biotic stress caused by powdery mildew.

The acorns of pedunculate oak species (*Quercus robur* L.) were collected from a natural oak population from Serbia. Seeds were soaked in tap water at room temperature for 24 h and germinated in vermiculate in a climate chamber at 25 °C under 80% humidity. Prior to infection with the powdery mildew, half of the seedlings were inoculated with the commercially available inoculant Ectovit®, which contains a mixture of propagules of ECM [43]. Commercial product Ectovit (Symbiom, s.r.o., Lanškroun, Czech Republic) contained the mycelium of 4 species of ECM fungi, namely, *Amanita muscaria* (L.) Lam., *Hebeloma crustuliniforme* (Bull. ex St. Amans.) Quél., *Laccaria proxima* (Boud.) Pat., and *Paxillus involutus* (Batsch) Fr., and the spores of 2 species of ECM fungi: *Pisolithus arrhizus* (Scop.) Rauschert and *Scleroderma citrinum* Pers. We have chosen to work with this inoculum as *H. crustiliniforme, L. proxima, P. involutus,* and *S. citrinum* are well-known ECM fungi of *Q. robur* [44–46]. Inoculation was performed according to the producer's instructions. The inoculum was applied as a slurry prepared by mixing the dry component of the product and an adequate amount of water. Two to three-month-old oak plants were pulled out from vermiculite, their roots were dipped into the slurry, and then they were planted into a new substrate (mixture of sterilized forest soil, vermiculite, and perlite, 3:2:1). One week later, each plant was watered with 50 mL of the slurry. The soil used for the substrate mixture was collected from the ILFE Experimental Forest Estate and sterilized by autoclaving at 121 °C for 35 min using the autoclave decontamination program (Lab Companion ST-G) in order to prevent the occurrence of other common soil-inhabiting fungi. Seedlings were watered with tap water every second day to the field capacity and no fungicides were applied. At the beginning of the experiment, seedlings were supplied with autoclave-sterilized Hoagland solution.

Twenty-four weeks after inoculation, seedlings were assessed for mycorrhizal colonization levels by ECM using Olympus SZX10 stereo microscope (Olympus Co., Tokyo, Japan), and an Olympus BX53 F light microscope with differential interference contrast levels and an accompanying software as described by Agerer [47]. The fine roots were defined as mycorrhizal when the root tips were covered with fungal mantles, and fresh and turgescent mycorrhizal root tips were selected for morpho-anatomical characterization and classification. The mycorrhizal root tips were classified into morphotypes according to the criteria of the color atlas of ectomycorrhizas [48]. Three representative root tips of a single morphological group were placed in separate, sterile microcentrifuge tubes and stored at −20 °C until further use for molecular identification. The ECM colonization levels were assessed using a visual approach by estimating the percentage of ECM root tips present in the root system under a stereo microscope [48].

Each of the selected root tips were grounded using a micro pestle (Carl Roth, Karlsruhe, Germany) in a 1.5 mL microcentrifuge tube. Thereafter, the DNA was extracted using Qiagen DNeasy plant mini kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The PCR mixture contained 3 µL of undiluted DNA and other ingredients as described in Karličić et al. [49]. Initially, we tried to amplify the ITS region of the rDNA using fungal-specific primer pairs ITS1F and ITS4 [46], but since this did not result in amplification, the PCR was performed using primers ITS1F (fungal-specific) and ITS 4B (Basidiomycota-specific) [50,51]. The PCR was performed in an Eppendorf Mastercycler epgradient S thermal cycler (Eppendorf AG, Hamburg, Germany) and the cycling parameters were set as previously described by Milović et al. [46]. The PCR products were separated by electrophoresis on 1.75% (*w*/*v*) agarose gels (SeaKem LE agarose, Lonza, Belgium) in 1xTBE buffer, stained with Roti-GelStain (Carl Roth GmbH + Co. KG, Karlsruhe, Germany), and gel images were captured using E-box gel documentation system (Vilber Lourmat, France). The DNA molecular weight standard O'GeneRuler 100 bp DNA ladder (Thermo Scientific, Vilnius, Lithuania) was used to estimate the size of the amplified products. The PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Sequencing was performed in both

directions, using the same primers as used for the amplification, and it was performed by Macrogen Europe (Amsterdam, The Netherlands). The sequence was deposited in GenBank (accession number: ON239799) (Table S1). Sequence alignment and maximum likelihood phylogenetic analyses were performed as described in Zlatković et al. [51].

After the oak seedlings inoculated with ectomycorrhizal fungi were left to develop mycorrhiza-induced resistance for twenty-four weeks, both mycorrhizal and non-mycorrhizal oaks were infected with the fungal pathogen *E. alphitoides*, the causative agent of the oak powdery mildew. A year prior to experiment, a single leaf with sporulating mycelium was collected from mature *Q. robur* tree severely infected with a powdery mildew disease grown in the ILFE Experimental Forest Estate near Novi Sad, Serbia. The sporulating mycelium was scraped off the surface of the leaf using a sterile scalpel, resuspended in 10 µL of sterile distilled water, and the leaves of a single plant were inoculated using a thin paintbrush. The plant was then used as a source of natural inoculum of the powdery mildew for healthy *Q. robur* plants grown in proximity to the inoculated plant in the same chamber in the greenhouse (n = 10, 20 cm distance between plants). In order to confirm that the disease was caused by *E. alphitoides*, sporulating mycelium was scraped away from the leaf fragment and the DNA was extracted using Plant/Fungi DNA isolation kit (Norgen Biotek Corp., Thorold, ON, Canada) following the manufacturer's instructions. The PCR mixture was prepared as described in Karličić et al. [49]. The ITS rDNA was amplified using the fungal-specific primer pair ITS1F/ITS4 as described in [46]. Gel electrophoresis and PCR clean-up were performed as described above. Sequencing was performed by Macrogen Europe, and the sequence was deposited in GenBank (accession number: OM009776) (Table S2). Bioinformatic analyses were conducted as described by Zlatković et al. [51]. The morphological features of the pathogen were examined using Olympus SZX10 stereo microscope, an Olympus BX53F light microscope, and accompanying software.

E. alphitoides population maintained on *Q. robur* seedlings in the greenhouse was then used as inoculum source for infections of mycorrhizal and non-mycorrhizal *Q. robur* seedlings. The sporulating mycelium was scraped and each plant was inoculated using a paintbrush as described above. Both mycorrhized and non-mycorrhized seedlings inoculated with *E. alphitoides* were incubated (25 °C, 80% Rh) until the average disease intensity reached > 75% in treatment with the highest incidence of powdery mildew. The evaluation was performed based on a scale from 0 to 3 (Table S3). Disease intensity (%) was calculated based on scores of 10 randomly selected leaves in three replications (a total of 30 leaves per treatment) using the standard formula [52]. Forty-five days after inoculation, all physiological measurements were performed and plant leaf material for biochemical analysis was sampled, frozen, and lyophilized.

The whole experiment consisted of four groups of treatments (each treatment consisted of 10 pots with one oak seedling per pot), with a randomized block design, as follows (Figure 1):

a. **ECM-PM—**Oak plants infected with powdery mildew, whose roots were previously primed with ectomycorrhiza;
b. **NM-PM—**Oak plants infected with powdery mildew with no mycorrhiza on their root tips;
c. **ECM-C—**Oak plants that were not infected by powdery mildew but were primed and inoculated with ectomycorrhizal inoculum;
d. **NM-C—**Healthy plants that were neither infected with powdery mildew nor inoculated by ectomycorrhizal inoculum.

2.2. Physiological Measurement

Relative leaf water content in oaks was measured and expressed in percentages [53]. CIRAS-3 Portable Photosynthesis System (PP Systems International, Amesbury, MA, USA) was used for measurements of gas exchange parameters, namely, net photosynthesis rate (A, µmol CO_2 m^{-2} s^{-1}), transpiration rate (E, mmol H_2O m^{-2} s^{-1}), stomatal conductance (gs, mmol m^{-2} s^{-1}), and intracellular CO_2 concentration (Ci, mmol CO_2 m^{-2} s^{-1}) [53]. Mea-

surements were conducted in clear, sunny weather between 9 and 11 a.m. The irradiance level inside the leaf chamber was set to 1000 µmol m^{-2} s^{-1}, while CO_2 concentration, air temperature, and humidity were taken from the ambient atmosphere. Water-use efficiency (WUE, µmol CO_2 $mmol^{-1}$ H_2O) was calculated as the ratio of net photosynthesis and transpiration rates.

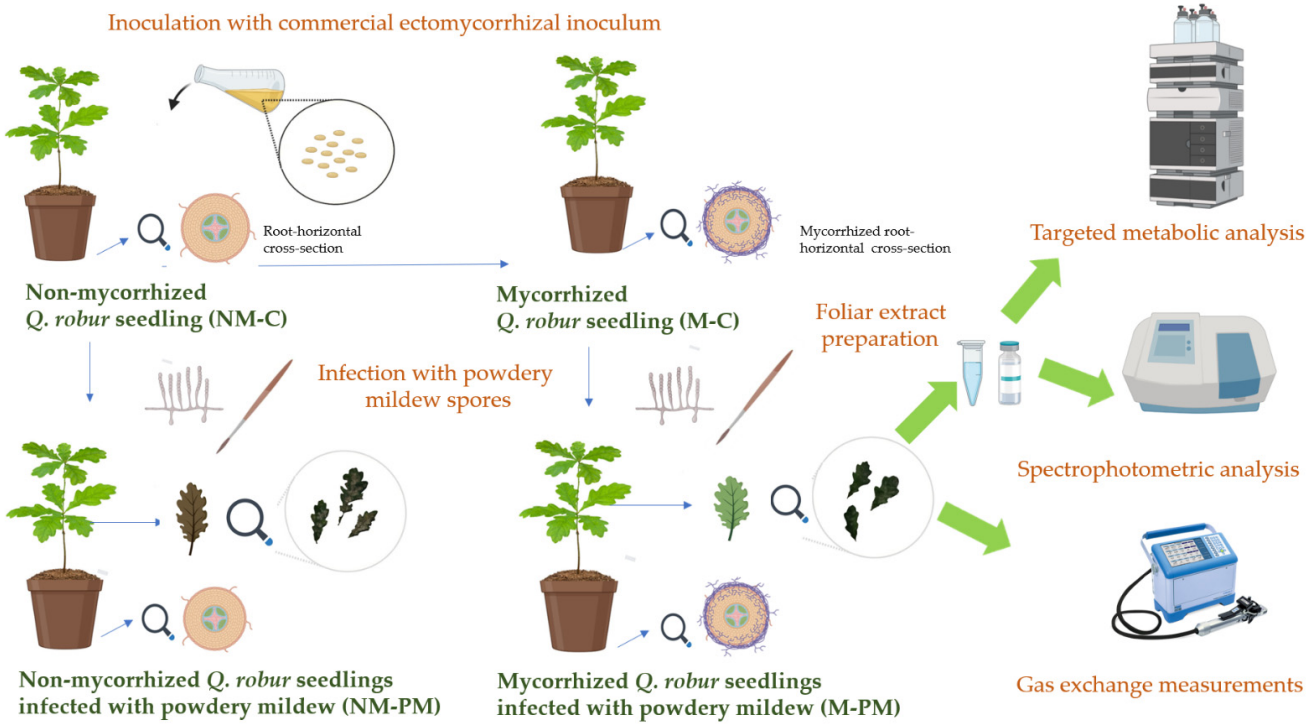

Figure 1. Illustration of the overall experimental design, treatments, and applied analysis (performed using biorender.com accessed on 13 July 2022).

2.3. Measurements of Osmolytes' Accumulation

a. Polyamine profile and content of three main polyamines in plants—putrescine (PUT), spermidine (SPD), and spermine (SPM)—were determined in oak leaves via HPLC method after derivatization with dansyl-chloride as a pre-treatment [54]. Plant tissues (approx. 20 mg DW of freeze-dried material) were extracted with 10 volumes of 4% perchloric acid (PCA); the homogenate was kept on ice for 1 h and then centrifuged at 15,000× *g* for 30 min. Aliquots of the supernatants and standard solutions of dansylated derivatives were extracted with toluene, dried, and resuspended in acetonitrile prior to HPLC analysis. PAs were separated and quantified by HPLC (Şchimadzu, Kyoto, Japan) using a reverse phase C_{18} column (Spherisorb ODS2, 5-µm particle diameter, 4.6 × 250 mm, Waters, Wexford, Ireland) and a programmed acetonitrile-water step gradient, as previously described [54].

b. Proline (PRO) concentration was estimated following the well-established ninhydrin method [55]. Data were calculated on a DW basis.

c. Glycine-betaine (GB), as predominant quaternary ammonium compound (QAC), was quantified using the precipitation method of QAC-periodide complexes in acid medium [56]. Data were calculated on a DW basis.

2.4. Assays of Antioxidant Defense Systems

Fully developed leaves of *Q. robur* were sampled from each treatment, frozen, and ground in liquid nitrogen and later lyophilized in a freeze dryer at −80 °C prior to analysis. For the different chemical analyses, either the freeze-dried material was used directly, or extracts in ethanol or phosphate-buffered saline (PBS; 0.1 M KH_2PO_4, KOH, pH = 7)

were prepared. Ethanolic extracts were prepared in 2 mL test tubes by mixing around 0.1 g of powdered freeze-dried leaf material with 2 mL of ethanol (96%). Samples were vigorously vortexed and then centrifuged for 30 min at 13,200 rpm at 40 °C. The supernatant was used for the determination of total phenolic and total flavonoid contents and for the quantification of the antioxidant activity and reducing capacity of plant extracts (see below). Extracts obtained by mixing 0.1 g of plant material with 2 mL of PBS buffer were used for the determination of total non-protein thiol and malondialdehyde (MDA).

Therefore, to investigate the antioxidant capacity of the selected plant specimens, the following non-enzymatic biochemical parameters were measured:

i. Lipid peroxidation was quantified by using a thiobarbituric assay where the level of lipid peroxidation corresponds to the amount of accumulated end-product malondialdehyde (MDA). Results are expressed as nmol MDA equivalents on a DW basis.
ii. Total non-protein thiol compounds were measured according to a modified colorimetric assay based on measuring the absorbance of yellow Ellman's reagent (5,5′-dithiobis-(2-nitrobenyoic acid; DTNB) reduced by sulfhydryl compounds at 413 nm. After construction of the calibration curve, where we used reduced glutathione (GSH) as standard, total non-protein thiol compounds were expressed as GSH equivalents on a DW basis [57].
iii. Trolox® Equivalent Antioxidant Capacity (TEAC) was estimated with the 2,2′-azinobis-(3-ethylbenzothiozoline-6-sulfonic acid) (ABTS) assay based on the capability of the ethanolic extract to scavenge ABTS radicals [58]. Data were calculated on a DW basis.
iv. The Ferric Reducing Antioxidant Power (FRAP) assay provided additional measurement of the antioxidant activity level of ethanolic extracts. In this assay, the ability of the plant extract to reduce the ferric 2, 4, 6-tripyridyl-S-triazine complex $[Fe^{3+}—(TPTZ)_2]^{3-}$ to the intensely blue-colored ferrous complex $[Fe^{3+}—(TPTZ)_2]^{2-}$ in acidic medium was estimated [59]. Data are expressed as TEAC on a DW basis.
v. Total phenolic content (TPC) was determined according to reactivity with Folin–Ciocalteu reagent [60]. Data are expressed as mg of Gallic Acid Equivalents (GAE) on a DW basis.
vi. Total flavonoid content (TFC) was measured by the aluminum chloride colorimetric method [61]. Data are expressed as mg of Quercetin Equivalent (QE) on a DW basis.
vii. Condensed tannins (CT) content was determined from methanolic extracts using butanol-HCl-Fe (III) method [62]. Data were expressed as leucocyanidin equivalents (LE) on a DW basis.

2.5. Elemental Analysis of Nitrogen and Carbon Content

The total contents of inorganic nitrogen (N) and carbon (C) were determined from freeze-dried and powdered oak leaf samples (25–30 mg) with a CHN analyzer, model Elemental VARIO EL III, according to manufacturer's instruction.

2.6. Statistical Analysis

Descriptive statistics, two factorial ANOVA with Tukey posthoc test, principal component (PCA) analyzes, and Pearson correlation statistical techniques were used. Powdery mildew and mycorrhiza were used as factors in two-way ANOVA, which was interpreted via Fisher (F) test and their statistical significance levels. Results of the Tukey post hoc test were visually presented on bar-chart figures with standard deviation (SD) bars. All statistical data processing was conducted in the R program environment (R Core Team). The "rstatix" R package [63] was used to calculate descriptive statistics and conduct two-way ANOVA with Tuckey HSD post hoc test, while other graphical interpretations were provided via "ggplot2" R packag [64]. Across the whole paper, we used the same statistical significance level ($p < 0.05$).

3. Results

Ectomycorrhizal assessment and molecular phylogenetic identification of ectomycorrhizal root tips and powdery mildew

All seedlings inoculated with ECM became mycorrhizal, with root tips covered by a fungal mantle, and a single ECM morphotype was observed (Figures 2 and S1). The morphotype was covered with a white mantle sheath, a monopodial-pyramidal and dichotomous branching pattern, numerous white rhizomorphs, an outer mantle layer with a loose plectenchymatous hyphal arrangement of the type A, and hyphae with clamp connections, which according to the color atlas key [65–67] resembled those of a *Scleroderma citrinum*. After PCR amplification, only one DNA band was observed in each selected sample indicating that a single fungal species was associated with the ECM root tips. Based on molecular phylogenetic analyses of the internal transcribed spacer region (ITS) of the ribosomal RNA, the identity of an ECM morphotype was confirmed: *S.citrinum* (Figure S2). Regarding ECM colonization levels, there were no significant differences between ECM-C and ECM-PM treatments, whereas the percentages of fine roots' colonization with ECM were of 62.22% and 58.42% for the ECM-C and ECM-PM treatments, respectively. Ectomycorrhiza was absent in the roots of the negative control seedlings (NM-PM, NM-C) (Table S4).

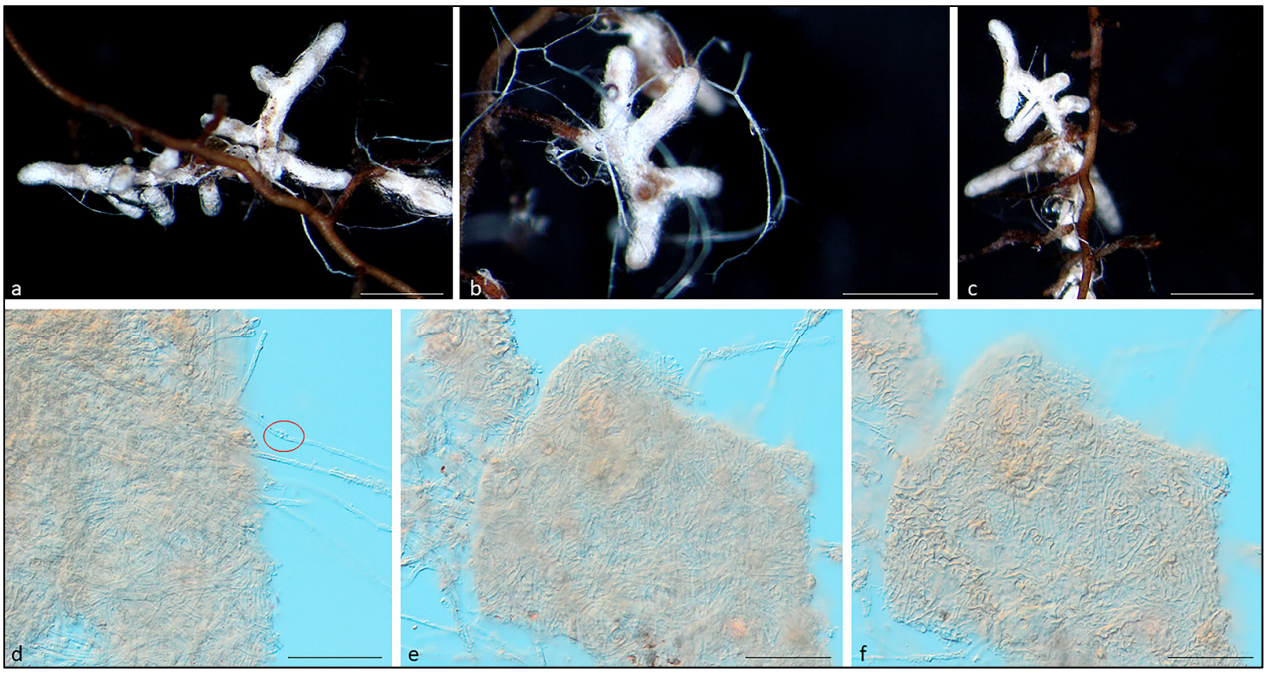

Figure 2. The morphological and anatomical features of *Scleroderma citrinum* ectomycorrhizae observed under the dissecting and compound microscopes. (**a**–**c**) ECM root tips with white mantle sheath, monopodial-pyramidal and dichotomous branching pattern, and numerous white rhizomorphs; (**d**) outer mantle and hyphae with clamp connections; (**e**) outer mantle plectenchymatous A type; (**f**) inner mantle plectenchymatous type with pseudoparenchymatous nests of cells; Scale bars = (**a**–**c**)—1mm; (**d**–**f**)—50 µm.

The morphological and molecular phylogenetic analyses confirmed that *E. alphitoides* was the causal agent of the powdery mildew disease in this trial (Figures 3 and S3). Symptoms of a powdery mildew disease occurred only in treatments that were inoculated with *E. alphitoides* (ECM-PM and NM-PM) (Table S4). The presence of the ectomycorrhiza affected the PM disease intensity, whereas the percentages of disease incidence in the NM-PM and ECM-PM treatments were 93.3 and 42.89%, respectively (Tables S3 and S4).

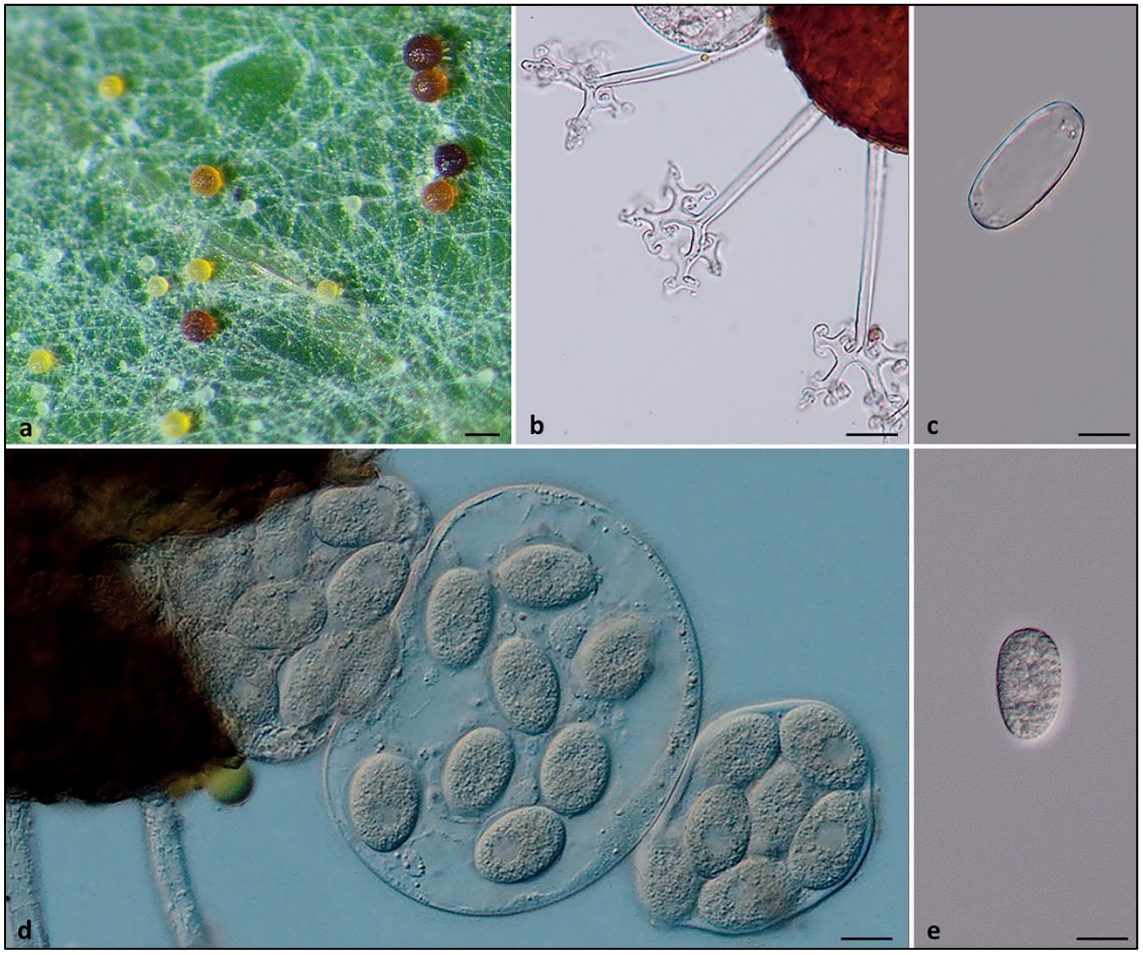

Figure 3. Morphological features of *Erysiphe alphitoides* used for inoculation of *Q. robur* plants: (**a**) Epiphytic hyphae and chasmothecia on the leaf surface; (**b**) Chasmothecium with appendages dichotomously branched at the tip; (**c**) Conidium; (**d**) Ruptured chasmothecium showing several asci containing ascospores; (**e**) Ascospore. Scale bars: (**a**)—100 µm; (**b**)—30 µm; (**c**–**e**)—10 µm.

The relative water content (RWC) measured in the leaves of the oaks' one year old seedlings varied from 56.91 to 88.72% depending on the treatment. The plants that were inoculated with ectomycorrhizal inoculum showed a slightly increased leaf relative water content in comparison to those grown in the absence of ectomycorrhiza (ECM). In both cases, in the presence and absence of mycorrhization, an infection with powdery mildew induced a reduction in the RWC in oak leaves. Plants that were inoculated with ectomycorrhiza and that were not infected with powdery mildew had a slightly higher leaf RWC than those without ECM (Figure 4a).

The oak leaves of the plants inoculated with ectomycorrhiza—both infected and not infected with a powdery mildew—had a slightly higher leaf nitrogen content than those plants that were not inoculated. An infection with powdery mildew caused an inconsiderable decrease in the leaf nitrogen content in both groups of plants, i.e., inoculated and non-inoculated ectomycorrhizal inoculum. The leaves' nitrogen content ranged from 2.43 to 3.29% of DW (Figure 4b). Powdery mildew did not cause any changes in the oak leaf carbon content, neither in the plants that were inoculated with ECM, nor those that were grown in absence of these beneficial microbes. It is notable that an inoculation with ectomycorrhiza only faintly increased the leaf carbon content in the oak plants (Figure 4c).

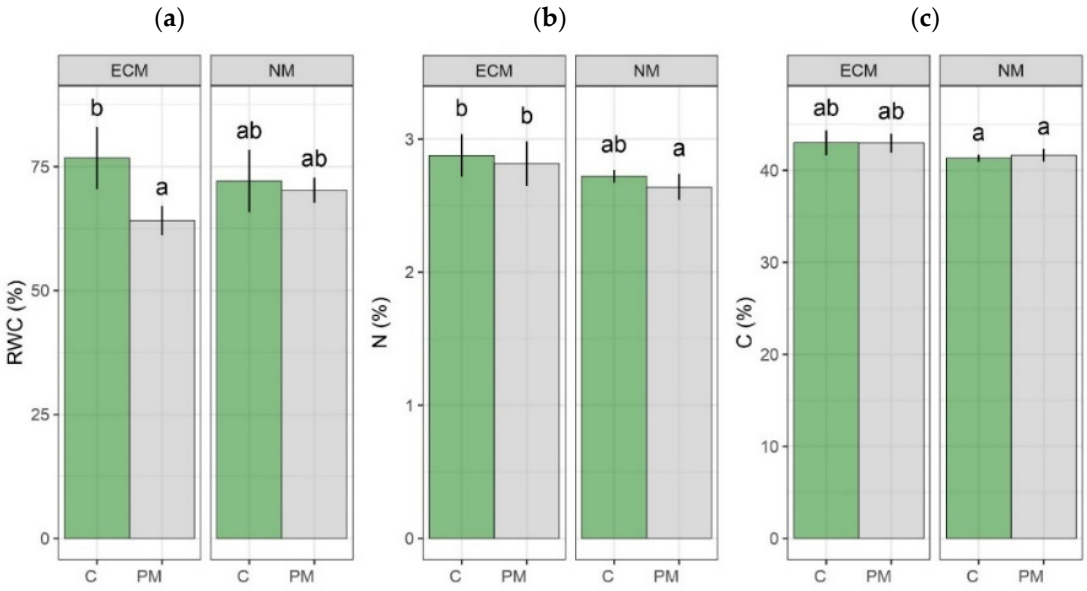

Figure 4. Changes in: (**a**) Relative water content (RWC; %), (**b**) Nitrogen content (N; %), and (**c**) Carbon content (C; %) in leaves of *Q. robur*. Treatments: C—control non-infected oak seedlings, PM—oak seedlings infected with powdery mildew spores, ECM—oak seedlings inoculated with ectomycorrhizal inoculum, and NM—oak seedlings not inoculated with ectomycorrhizal inoculum. Different small letters indicate significant differences across the different treatments; Tukey's honestly significant difference (HSD) post hoc test ($p \leq 0.05$). Data represent the mean ± standard deviation (SD).

Considering physiological parameters, we found that oak seedlings infected with powdery mildew (PM) showed reduced values of A, gs, and WUE under both treatments, ECM and NM, compared to the controls that were not infected (Figure 5). This trend was particularly pronounced in the oak seedlings not inoculated with ectomycorrhizal inoculum (NM), since these parameters, including Ci, were around twice lower in the PM compared to the control (C) group. Contrary to this, although the majority of gas exchange parameters (except E) were slightly reduced in the infected seedlings, no significant differences were observed between the control and PM-infected seedlings, within those that were inoculated with ectomycorrhizal inoculum (ECM). The alternations in all the gas exchange parameters caused by the infection with powdery mildew were more prominent in the seedlings that were not inoculated with mycorrhiza. The levels of statistical significance of the inspected effects (ECM, PM, and ECMxPM) with respect to all physiological parameters are presented in Table S5.

The foliar free proline content in the oak seedlings was significantly inconsistent among the different treatments. The effect of mycorrhization was obvious and caused ambiguous oak responses to the infection with powdery mildew. Therefore, in the leaves of the oak seedlings previously inoculated with ectomycorrhizal fungi, the infection with powdery mildew induced a significant decline (for 83.3%) in the free proline content compared to non-infected controls, while the infection with powdery mildew of the non-inoculated seedlings caused a significant enhancement (ca., 3.5-folds) in the foliar free proline content. Interestingly, the free proline content in the leaves of the control seedlings inoculated with ectomycorrhiza was almost threefold higher than the proline content in the control plants (not infected with PM) in the absence of ectomycorrhizal fungi (Figure 6a). On the contrary to the free proline content, the foliar glycine betaine content in the oak seedlings was not affected significantly either by the powdery mildew infection or by an inoculation with ectomycorrhizal fungi (Figure 6b). The detected amounts of proline content varied from ca., 4 to 16 μmol g^{-1} DW, while the quantified amounts of glycine betaine ranged from ca., 20 to 23 μmol g^{-1} DW.

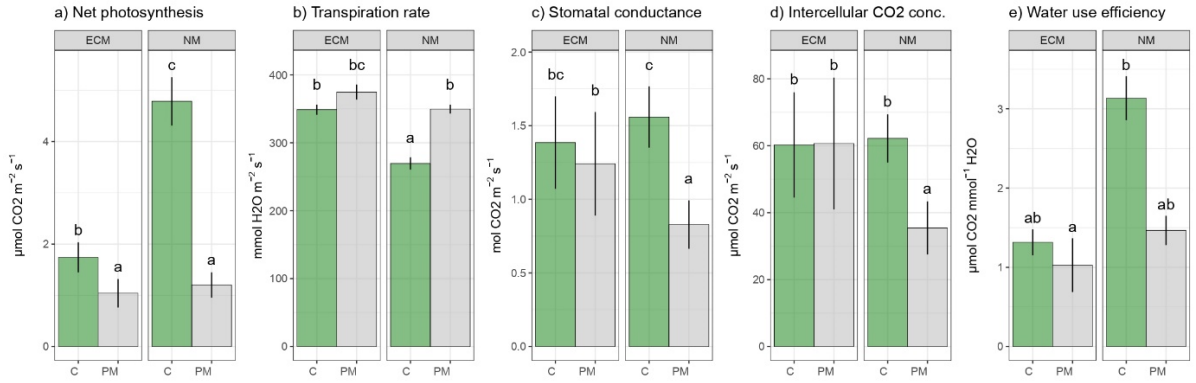

Figure 5. Changes in: (**a**) net photosynthetic rate (A; mmol CO_2 m^{-2} s^{-1}), (**b**) Transpiration rate (E; mmol H_2O m^{-2} s^{-1}), (**c**) Stomatal conductance (gs, mmol CO_2 m^{-2} s^{-1}), (**d**) Intracellular CO_2 concentration (Ci; mmol CO_2 m^{-2} s^{-1}), and (**e**) Water use efficiency (WUE; mmol CO_2 $mmol^{-1}$ H_2O) of leaves of *Q. robur*. Treatments: C—control non-infected oak seedlings, PM—oak seedlings infected with powdery mildew, ECM—oak seedlings inoculated with ectomycorrhizal inoculum, and NM—oak seedlings not inoculated with ectomycorrhizal inoculum. Different small letters indicate significant differences across the different treatments; Tukey's honestly significant difference (HSD) post hoc test ($p \leq 0.05$). Data represent the mean ± SD.

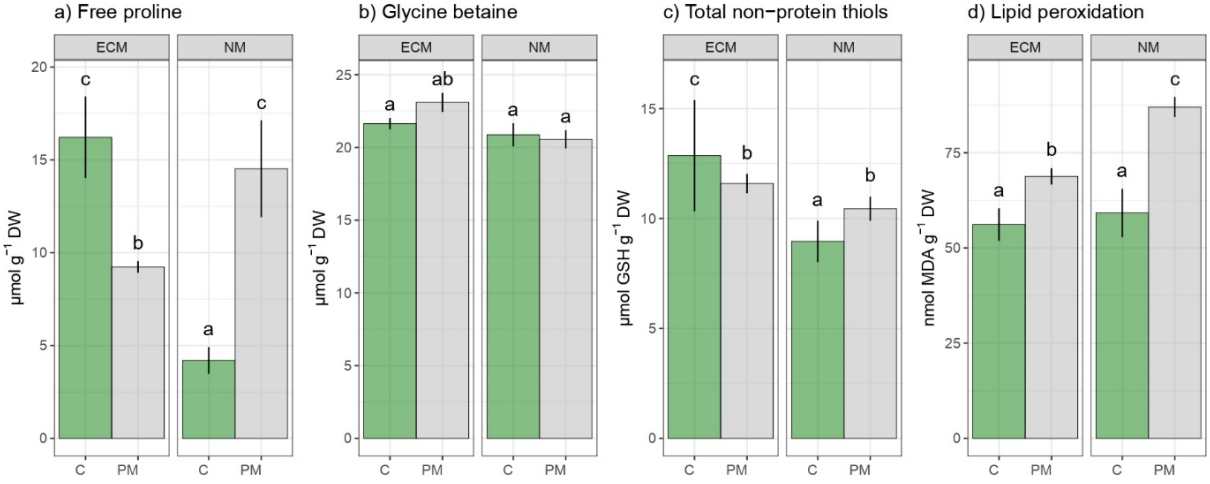

Figure 6. Changes in: (**a**) Proline (PRO; mmol g^{-1} DW), (**b**) Glycine betaine (GB; mmol g^{-1} DW), (**c**) Total non-protein thiols (mmol GSH g^{-1} DW), and (**d**) Lipid peroxidation intensity (nmol MDA g^{-1} DW) content in leaves of *Q. robur*. Treatments: C—control non-infected oak seedlings; PM—oak seedlings infected with powdery mildew, ECM—oak seedlings inoculated with ectomycorrhizal inoculum, and NM—oak seedlings not inoculated with ectomycorrhizal inoculum. Different small letters indicate significant differences across the different treatments; Tukey's honestly significant difference (HSD) post hoc test ($p \leq 0.05$). Data represent the mean ± standard deviation.

The content of the total non-protein thiols was higher in the plants inoculated with ectomycorrhizal fungi in comparison to non-mycorrhizal plants, with significant differences between the plants infected with powdery mildew and the control group (Figure 6c). In the plants inoculated with ectomycorrhizal fungi, the control group (not infected with PM) showed a significantly higher concentration of non-protein thiols (ca., 13 µmol GSH g^{-1} DW) than the plants infected with powdery mildew (ca., 11.5 µmol GSH g^{-1} DW). Contrary to this, in the non-mycorrhized plants, the treatment with powdery mildew induced an increase in the foliar non-protein thiol content in the oaks (Figure 6c).

Powdery mildew infection induced lipid peroxidation in both the mycorrhized and non-mycorrhized plants, which was detected by a significantly higher concentration of

malondialdehyde (MDA) in the infected leaves than in the leaves of controls. A higher intensity of lipid peroxidation was observed in the plants that were not previously primed with the ectomycorrhizal inoculum (ca., 95 and 65 nmol MDA g^{-1} DW in non-mycorrhized and mycorrhized plants, respectively) (Figure 6d).

The content of diamine putrescine was significantly higher within the plants inoculated with ectomycorrhizal fungi than in non-mycorrhized plants (Figure 7a). In both groups, the plants infected with powdery mildew showed a significantly higher concentration of putrescine (ca., 280 nmol g^{-1} DW in mycorrhized vs. 230 nmol g^{-1} DW in non-mycorrhized plants) than in the control plants (ca., 260 nmol g^{-1} DW in mycorrhized vs. 210 nmol g^{-1} DW in non-mycorrhized plants). The biosynthesis of spermidine in the plants was stimulated by the infection with powdery mildew, since significantly higher concentrations of spermidine were detected in the infected plants than in the control groups in both the mycorrhized and non-mycorrhized experimental groups (Figure 7b). Furthermore, it seems that mycorrhization did not have any significant effects on the oak plants' spermidine biosynthesis, since plants infected with powdery mildew showed similar values of spermidine regardless of mycorrhization (ca., 380 and 375 nmol SPD g^{-1} DW in mycorrhized and non-mycorrhized, respectively). The spermine content in the plants increased in a similar manner. As for spermidine, the plants infected with powdery mildew showed significantly higher concentrations of spermine in both-mycorrhized and non-mycorrhized plants (Figure 7c). Interestingly, the plants that were previously inoculated with ectomycorrhizal fungi were more abundant in spermine content than those that were not inoculated, so it seems that ectomycorrhizal fungi have a priming effect on oak plants regarding the spermine content. The total polyamine profile in the oak leaves of the plants that were not infected with PM was followed the sequence PUT > SPD > SPM, whereas after the infection with powdery mildew the polyamine profile changed, so that spermidine became the dominant polyamine in oak leaves (SPD > PUT >SPM). The foliar putrescine content in the oaks ranged between, ca., 154–383 nmol g^{-1} DW, while foliar amounts of SPD and SPM that were detected in oak varied between, ca., 169 and 471 nmol g^{-1} DW and, ca., 22 and 171 nmol g^{-1} DW, respectively.

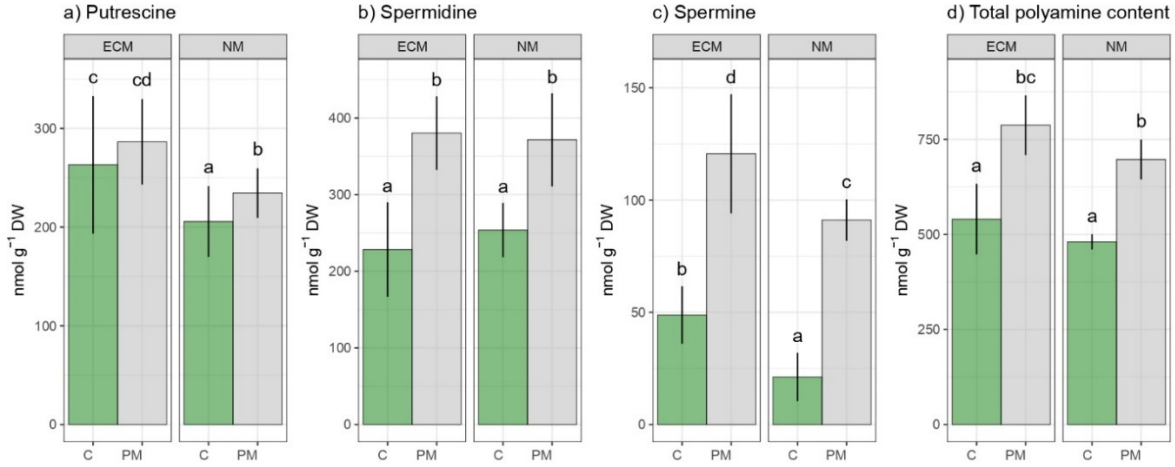

Figure 7. Changes in: (**a**) Putrescine (PUT; nmol g^{-1} DW), (**b**) Spermidine (SPD; nmol g^{-1} DW), (**c**) Spermine (SPM; nmol g^{-1} DW), and (**d**) total polyamine content (PAs; nmol g^{-1} DW) in leaves of *Q. robur*. Treatments: C—control non-infected oak seedlings, PM—oak seedlings infected with powdery mildew, ECM—oak seedlings inoculated with ectomycorrhizal inoculum, and NM—oak seedlings not inoculated with ectomycorrhizal inoculum. Different small letters indicate significant differences across the different treatments; Tukey's honestly significant difference (HSD) post hoc test ($p \leq 0.05$). Data represent the mean ± SD.

The total polyamine content showed a similar pattern as that of the individual polyamines; the infection with powdery mildew also induced the biosynthesis of the

total polyamines in both mycorrhized and non-mycorrhized compared to the control (not infected) groups, but there were no significant differences in the concentrations of total polyamines in the control groups between mycorrhized and non-mycorrhized plants.

Regarding the antioxidant properties estimated by radical scavenger capacity (RSC) against the ABTS radicals within the mycorrhized plants, the group infected with powdery mildew showed a significantly higher radical scavenging capacity against the ABTS (ca., 75 mmol TE g^{-1} DW) than the plants in the control group (ca., 65 mmol TE g^{-1} DW). In the non-mycorrhized plants, the opposite pattern was observed: non-infected plants had a significantly higher RCS than plants infected with powdery mildew (ca., 85 mmol TE g^{-1} DW) (ca., 67 mmol TE g^{-1} DW) (Figure 8a).

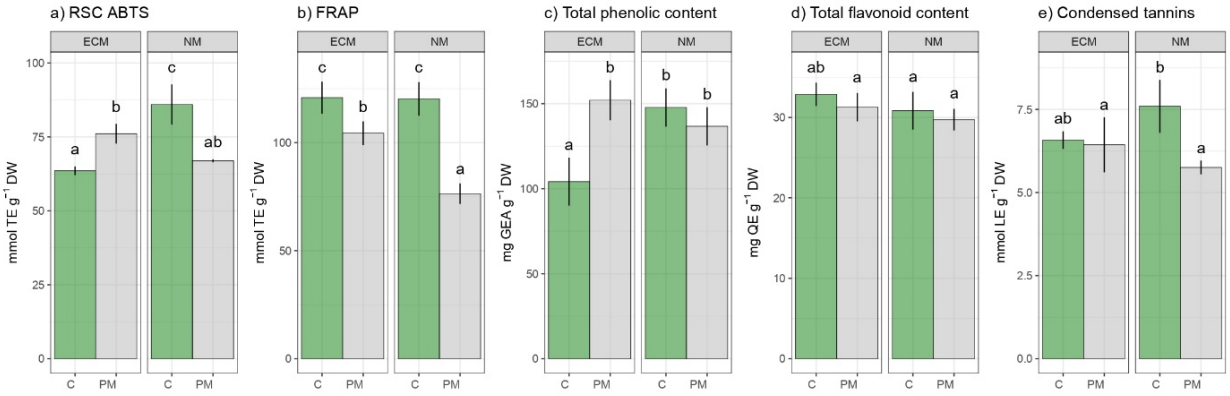

Figure 8. Changes in: (**a**) Radical scavenger capacity against ABTS (ABTS; mmol TE g^{-1} DW), (**b**) Ferric reducing ability of extract (FRAP; mmol TE g^{-1} DW), (**c**) Total phenolic content (TPC; mg GAE g^{-1} DW), (**d**) Total flavonoid content (TFC; mmol QE g^{-1} DW), and (**e**) Condensed tannins (CT, mmol LE g^{-1} DW) content in leaves of *Q. robur*. Treatments: C—control non-infected oak seedlings, PM—oak seedlings infected with powdery mildew, ECM—oak seedlings inoculated with ectomycorrhizal inoculum, and NM—oak seedlings not inoculated with ectomycorrhizal inoculum. Different small letters indicate significant differences across the different treatments; Tukey's honestly significant difference (HSD) post hoc test ($p \leq 0.05$). Data represent the mean ± SD.

The antioxidative potential of oak leaves infected with powdery mildew and the controls in the absence and presence of ectomycorrhizal fungi was estimated using also the FRAP assay (Figure 8b). In both mycorrhized and non-mycorrhized experimental groups, the antioxidative potential estimated by FRAP assay was significantly lower in the plants infected with powdery mildew (ca., 110 and 75 nmol TE g^{-1} DW in mycorrhized and non-mycorrhizal plants, respectively) than in the control groups (ca., 150 nmol TE g^{-1} DW in both mycorrhized and non-mycorrhizal plants). There were no significant differences in antioxidant power between the mycorrhized and non-mycorrhizal plants controls (Figure 8b).

Within the group of mycorrhized plants, the infection with powdery mildew induced a significant increase in the total phenolic content (TPC) (ca., 150 mg gallic acid equivalents-GEA g^{-1} DW) compared to the control group (ca., 110 mg GEA g^{-1} DW). In the non-mycorrhized plants, there was no significant difference in the total phenolic content between the infected plants (ca., 145 mg GEA g^{-1} DW) and the control group (ca., 135 mg GEA g^{-1} DW) (Figure 8c). The total flavonoid content (TFC) ranged from, ca., 30 to 33 mg quercetin equivalents QE g^{-1} DW and none of the applied treatments had a significant effect on the foliar flavonoid concentration in experimental plants (Figure 8d).

The inoculation of the experimental plants with ectomycorrhizal fungi did not have effects on the number of condensed tannins. On the other hand, the infection with powdery mildew significantly reduced (ca., −26%) the accumulation of condensed tannins in the group of plants that were not inoculated with ectomycorrhizal fungi. Oak species are especially abundant in condensed tannins, and their concentration in the tested seedlings ranged from 5.47 to 7.56 mmol leucocyanidin equivalents per gram of dry weight (LE g^{-1}

DW). The levels of statistical significance of the inspected effects (ECM, PM, and ECMxPM) for all biochemical parameters are presented in Table S5.

Principal Component Analysis (PCA) and Correlation Matrix

The effects of mycorrhiza and the powdery mildew (PM) infection on pedunculate oak seedlings' analyzed biochemical and physiological parameters in the controlled conditions were tested using principal component analysis (PCA; Figure 9) and Pearson correlation's matrix for $p < 0.05$ (Figure 10). Both inspected variables (mycorrhization factor and PM infection) affected the sample distribution similarly across both principal components (PC1 and PC2) in such a way that all treatments were placed in different quadrants and no grouping was observed. The first two PCs in the PCA describe 81.18% of the total variance (Figure 9), whereas PC1 explains 47.42%, while PC2 explains 33.76% of total sample variance. High scores in PC1 (which primary define PM effect) correspond to high loadings of total polyamines (PAs), spermidine (SPD), net photosynthesis (A), Water Use Efficacy (WUE), and condensed tannins (CT). On the other hand, PC2 strongly relates to the mycorrhization effect, and highly corresponds with high loadings of total phenolic content (TPC), total flavonoid content (TFC), and nitrogen content (N). The samples that were exposed to mycorrhization (● mark) highly correspond with Ci, PRO, GB, PUT, GSH, C, N, and TFC parameters. On the other hand, the PM effect could be tracked across PC1, whereas the non-mycorrhized plants (yellow ▲ mark)—depending on the PM infection—mostly dissociate across PC1.

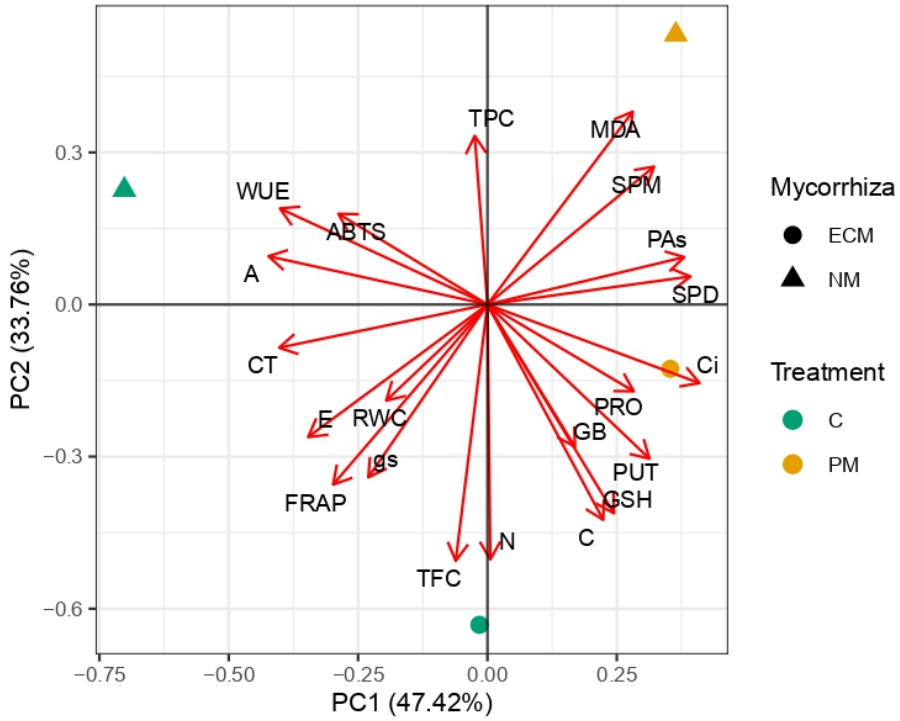

Figure 9. Loadings and the scores of examined treatments at the level of interaction for polyamines, compatible solutes, antioxidant capacities and physiological parameters for *Q. robur* for the first two principal components. Treatments: C—control non-infected oak seedlings, PM—oak seedlings infected with powdery mildew, ECM—oak seedlings inoculated with ectomycorrhizal inoculum, and NM—oak seedlings not inoculated with ectomycorrhizal inoculum. Abbreviations present examined parameters: WUE—water use efficiency, A—net photosynthetic rate, E—transpiration rate, gs—stomatal conductance, ci—internal CO2 concentration, CT-condensed tannins, ABTS—Trolox equivalent antioxidant capacity assay against ABTS radical, TPC—Total phenolic content, FRAP—Ferric reducing antioxidant power, GB—Glycine betaine, LP—Lipid peroxidation, PRO—Proline, SPD—spermidine, SPM—spermine, and PUT—putrescine.

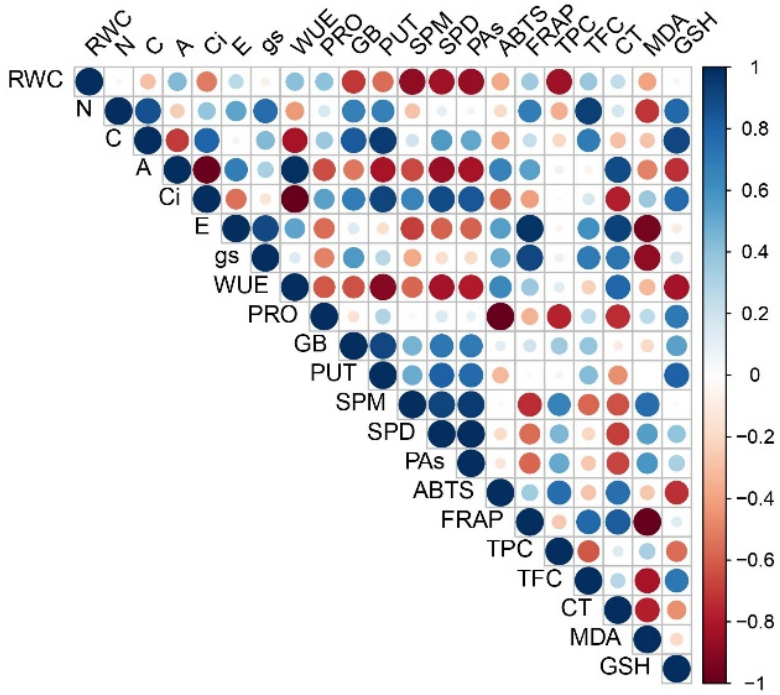

Figure 10. Pearson's correlation matrix of analyzed physiological and biochemical parameters. Abbreviations present examined parameters: WUE—water use efficiency, A—net photosynthetic rate, E—transpiration rate, gs—stomatal conductance, ci—internal CO_2 concentration, CT-condensed tannins, ABTS—Trolox equivalent antioxidant capacity assay against ABTS radical, TPC—Total phenolic content, FRAP—Ferric reducing antioxidant power, GB—Glycine betaine, LP—Lipid peroxidation, PRO—Proline, SPD—spermidine, SPM—spermine, and PUT—putrescine.

The spatial arrangement on the PCA was also confirmed on the correlation matrix (Figure 10). We noted different correlation patterns among the analyzed metabolites. For example, strong correlations were noted among polyamines (SPM, SPD, and PUT) and their total concentrations (PAs) and leaf gas-exchange parameters (A, Ci, E, gs, and WUE) and with RWC. In addition, polyamines (except PUT) strongly correlated with stress indicators such as FRAP, TPC, and MDA, while only polyamine putrescine strongly correlated with C and N. Following the results for all three polyamines analyzed based on their correlation proxies with all other analyzed metabolites, PUT strongly deviated from SPM and SPD, while the other two had very similar responses. Likewise, we confirmed well-known and expected patterns among the parameters from the same group of parameters, e.g., the parameters of photosynthesis (A-WUE; A-E; Ci-WUE; gs-E relations), polyamines (total PAs to individual separated polyamines, PUT-SPD, and SPM-SPD), and biochemical stress indicators, for example, that PRO's relation to ABTS, TPC, CT, and GSH content under high temperatures is associated with a decrease in flavonoid content.

4. Discussion

Quercus species have been widely studied with respect to their responses to different abiotic [8,68] and biotic stresses [12]. However, the effects of ectomycorrhizal fungi upon infection with powdery mildew at the physiological and biochemical levels (e.g., antioxidants and osmoprotectants) have not been studied in the oak species in a comprehensive way.

4.1. Effects of Ectomycorrhiza and/or Powdery Mildew on Oaks' Nitrogen, Carbon, and Leaf Water Content

In this study, the findings that indicate a slightly increased leaf relative water content and nitrogen content in the oak seedlings that were inoculated with ectomycorrhizal fungi could be associated with the fact that mycorrhizae, due to their well-branched

hyphae system, facilitate water delivery as well as nitrogen and phosphorus to the plant in exchange for photosynthetically produced carbohydrates from host trees [17]. During drought stress, plants employ strategies to invest their photosynthate carbon (around 20%) in the development of the hyphae rather than fine roots, due to the hyphae's higher efficacy with respect to the provision of inaccessible water since small and profuse hyphae have 60 times as many absorptive areas than fine roots [17]. At the same time, dry conditions and drought differently affect biodiversity and the abundance of mycorrhiza, so on the one hand drought increases arbuscular mycorrhizas abundance, while on the other drought has variable effects on ectomycorrhizas [69]. Ectomycorrhiza has been proven to be very beneficial in protecting poplars from water deficits [70]. Through a complex network of mycelium popularly called the "wood wide web", which facilitates communication between trees in the forest, mycorrhizal fungi have a pivotal role in linking aboveground and belowground components of biogeochemical cycles [71]. Beside affecting the water and nitrogen supply of the plant, ectomycorrhiza—as a highly differentiated interface between soil, fungi, and tree rootlets—also represent the key players in carbon dynamics and carbon fluxes among plants, soil, and the atmosphere [17]. Due to the lack of photosynthetic pigments, mycorrhizal fungi use plants' photosynthates as a main carbon source. Although mycorrhizal fungi are not saprotrophic but biotrophic, in cases when the amounts of a plant's photosynthates are low, some ectomycorrhizal fungi are prone to enzymatically decompose large organic molecules (e.g., proteins, chitin, pectin, hemicellulose, cellulose, and polyphenols) as an alternative carbon and energy source, which is not specific for arbuscular mycorrhizal fungi (AMF) [72]. In this study, the presence of ectomycorrhiza only slightly affected the leaf carbon content, while powdery mildew did not affect the leaf carbon content. Some authors outlined that carbon starvation triggers an increased susceptibility of oaks towards the attacks of pathogens and insects [73,74]. Furthermore, recent investigations have revealed that nitrogen metabolism is closely related to a plant's defense by discovering that membrane transporters for nitrates (NRT1 and NRT2 family members) act not only as the transporters but are also related to plant defenses such as working as stress receptors—so called "transceptors". An up-regulated expression of these genes (particularly NRT2.1 and NRT2.6) occurs under nitrogen limiting conditions in plants [75] and the enhanced expression of the same genes occurs during pathogen (bacterial or fungal) infection, indicating their role in pathogen resistance [76,77]. Nitrogen availability affects mycorrhiza-induced resistance (MIR); therefore, plants are better protected from pathogens by mycorrhizal fungi at low nitrogen conditions [77].

4.2. Perturbations in the Gas Exchange Parameters Caused by Powdery Mildew Infection and Inoculation with ECM Fungi

The results from the present study evidenced that the biotic stress caused by powdery mildew led to a disturbance in leaf gas exchange, particularly in the seedlings not inoculated with ectomycorrhizal inoculum. The significant reduction in A and gs observed in the aforementioned seedlings corresponds to the findings of Pap et al. [78], who reported that even a moderate infection of the leaves with powdery mildew can notably interrupt these processes. However, owing to the fact that leaf physiological traits are quantitatively associated with the leaf infestation intensity [79], gas exchange processes are especially impeded under a higher degree of leaf infection [80–82], as confirmed by our research. On the other hand, our results showed that the presence of ectomycorrhiza alleviated the negative effect of the powdery mildew, since the differences between the controlled and infected seedlings were found to be negative for all the studied traits, except the net CO_2 assimilation rate. This is in agreement with previous studies that demonstrated the enhanced capacity of seedlings inoculated with ectomycorrhizal fungi to cope with different stress factors [83,84]. However, a more pronounced decrease in net photosynthesis in the infected leaves compared to the uninfected ones might be the consequence of destructed palisade cells caused by the pathogen [85] that further triggers a reduced carbon gain in infected regions [79]. Pap et al. [78] reported that in such circumstances, net

photosynthesis can decrease by even 70% in comparison to the completely healthy leaves due to its high sensitivity to the powdery mildew infestation intensity. Similarly, other authors [12,60] reported that net photosynthesis declined between 40–50% in the leaves covered by epiphytic mycelia above 75% and 85%, respectively.

4.3. Erysiphe Alphioides and Ectomycorrhizal Inoculum Alter Amounts of Compatible Solutes

Mycorrhizal fungi improve plants' tolerance to unfavorable abiotic stress factors such as heat, drought, salinity, or the presence of heavy metals, and boost plants' immunity, increase their resistance to pathogens, and provide other ecosystem services [16,86]. During mycorrhizal establishment, there is a modulation of plant defense responses and molecular reprogramming, which leads to an effective activation of the plant's immune responses and the expression of defense genes, very similar to Induced Systemic Resistance (ISR) [14,87]. This kind of induced resistance is called mycorrhiza-induced resistance (MIR) and it has been reported that MIR results in the active suppression of components of salicylic acid (SA)-dependent defense pathways, causing the systemic activation of jasmonate (JA)-dependent defense pathways [88,89]. Furthermore, mycorrhiza induces the modulation of nearly the entire metabolome, including carbohydrates, organic acids, sugar alcohols, amino acids, etc. [90]. To escape infection by pathogens, plants have evolved a battery of defense mechanisms mediated by multiple pathogen-specific signal transduction pathways [91]. The excessive production of ROS in plant cells is potentially harmful to nucleic acids, proteins, and lipids, which can lead to cell injury and death [92]; conversely, ROS are important signal molecules involved in the acclimation of plants to environmental stresses [93]. Aside from being detrimental to biomolecules, such as nucleic acids, proteins, and lipids, leading to cell death [92], ROS are important signal molecules and in combination with NO radicals they can activate a hypersensitive reaction in plants [94].

There is an interesting interplay between ROS and proline metabolism. On the one hand, proline regulates the concentration of ROS, while on the other hand, due to the elevated ROS amounts during biotic stress, the biosynthesis of proline may be upregulated by ROS action. Regardless, proline is a potent nonenzymatic antioxidant capable of scavenging of hydroxyl radicals [95] and is one of the main regulators of the intracellular redox potential [37,96]. On the other hand, proline does not quench $^1O_2$ as it was previously thought [73,97]. During biotic stresses such as plant–pathogen incompatible interactions, ROS can mediate the upregulation of genes that code the biosynthetic enzymes of proline, especially the gene AtP5CS2, one of two genes that code a rate-limiting proline-biosynthetic enzyme, namely, pyrroline-5-carboxylate synthetase (P5CS), and cause enhanced proline accumulation [98]. Similar results were obtained in this study, where in the absence of ectomycorrhizal fungi, the biotic stress induced by powdery mildew induced a threefold increase in proline content; it may be that the ROS released during biotic stressor polyamine catabolism upregulated pyrroline-5-carboxylate synthetase (P5CS), which contributed to the increased proline accumulation in the leaves treated with powdery mildew. Additionally, the high amounts of H_2O_2 that are produced during biotic stress or those produced by polyamine catabolism can also facilitate the expression of P5CS, as it was reported in rice seedling leaves treated with H_2O_2 [99]. Furthermore, it was reported that an elevated H_2O_2 concentration negatively affects the activity of the catalytic enzyme involved in proline metabolism, proline dehydrogenase (ProDH). Nitric oxide (NO), as a concomitant product of polyamine metabolism, can also induce significant proline accumulation, as it was confirmed in wheat when it was treated with a potent NO donor, sodium nitroprusside [100,101]. The protective role of exogenously applied proline against the fungal pathogen *Colletotrichum trifolii* (Bain) prevented ROS-induced PCD during nutritional stress [102]. Besides being an osmoprotectant, proline, as a scavenger of hydroxyl radicals [95], may be important in preventing oxidative damage caused by ROS, especially related to the initiation of lipid peroxidation. In accordance with our findings, where a negative correlation between malonyl dialdehyde (MDA) and proline has been detected, a highly negative relationship between proline and lipid peroxidation has

been recorded in transgenic sugarcane overexpressing the P5CS gene [103]. Furthermore, the opposite responses in proline trends in the absence and presence of ectomycorrhiza during powdery mildew induced biotic stress could be addressed by the dual nature of proline in plants, where proline can act as antioxidant and ROS scavenger, while on the other hand, it can also act as pro-oxidant by the modulation of ProDH activity whereas the P5C proline cycle can generate ROS as a response to pathogen [104]. In our study, neither powdery mildew infection nor inoculation with ECM inoculum caused any significant changes in oaks' foliar glycine betaine level, although it was recently found that arbuscular mycorrhizal fungi with glycine betaine individually or in combination alleviate oxidative stress induced in sorghum by chromium [105]. Contrary to our findings, some authors also reported a significant increase in glycine betaine during drought stress in the presence of mycorrhizal fungi in white grape seedlings [84,106].

4.4. Oaks' Foliar Phenolics Content Is Modulated by Ectomycorrhiza and/or Powdery Mildew

Phenolics, as a large and diverse group of secondary metabolites, are known as important agents in a plant's response to biotic stress and disease progression due to their deterrent and antifungal properties [107,108]. Interestingly, in this study, the presence of ectomycorrhizal fungi changed the oaks' responses to powdery mildew. The evident mitigatory effects of ECM upon induced oxidative stress during the oaks' infection with PM were reflected in the oaks' increasing patterns in terms of total phenolic content and condensed tannins, while in the absence of ECM fungi, the oaks exhibited declining patterns of condensed tannins and total antioxidant properties. The presence of ECM fungi during PM infection did not affect the content of flavonoids nor that of condensed tannins, which remained approximately at the same level before and after powdery mildew infection. The increasing TPC in the oaks in the presence of ectomycorrhizal fungi during PM infection is similar to that found in cucumbers infected by powdery mildew, where elevated patterns of phenolic compounds were reported, confirming the fungitoxicity and high pathogen growth inhibition of hydroxycinnamic acids and their common derivates [109]. Likewise, flax (*Linum usitatissimum* L.) lines with a higher resistance towards powdery mildew contained higher concentrations of total phenols, but also showed increased activities of polyphenyl oxidase (PPO), comparted to more susceptible lines [110]. Similarly, in pea plants (*Pisum sativum* L.) infected with powdery mildew upon inoculation with AMF, a high correlation of phenolic accumulation (phenolcarboxylic, tannic, gallic, ferulic acids, etc.) with mycorrhizal colonization and disease intensity was reported [111]. Regardless of the infection with PM, the non-mycorrhized oak seedlings from this study exhibited a higher phenolic content and tannin level as well as total antioxidant capacity compared to mycorrhized seedlings, which is not in accordance with elevated phenolics, tannins, and antioxidant activity found in the medical plant, *Valeriana jatamansi,* by Jones [112]. In the same study, the authors confirmed that AMF induce increased activities of phenylalanine ammonia lyase (PAL), a key enzyme in phenolics biosynthesis [112]. Furthermore, increased amounts of phenolics, flavonoids, and tannins were detected in melons infected with powdery mildew under different light qualities [113]. Additionally, the mechanisms of the regulation and accumulation of secondary metabolites in plant symbiosis with mycorrhizal and endophytic fungi were thoroughly explained [91,114].

4.5. Reinforcement of Polyamine Metabolism Induced by Ectomycorrhizal Fungi and E. alphitodes

In the present study, the plants inoculated with ectomycorrhizal fungi had higher levels of constitutive putrescine and spermine compared to non-mycorrhized plants, while mycorrhization did not affect spermidine levels.

The effects of ectomycorrhizal fungi on endogenous polyamines have not been elucidated in oak species so far. A great deal of the literature has focused on research related to arbuscular mycorrhizal fungi's interaction with polyamine metabolism, rather than ectomycorrhizal fungi [31,115–118]. Arbuscular mycorrhizal fungi are considered to induce a stronger rhizosphere priming effect in forest trees than ectomycorrhizal fungi, although

interactions with ectomycorrhizal fungi are more prominent in forest trees [15,119,120]. Inoculum of ectomycorrhizal fungi enhanced oaks' putrescine and spermine level compared to non-mycorrhized seedlings, whereas increased levels of putrescine through alternative paths via diamine oxidase could contribute to increased GABA levels [21,121], a substance known to boost the plant immune system and cause excellent priming effects in plants [122,123]. Therefore, the oak seedlings inoculated with ectomycorrhiza in this study could be considered to be primed with putrescine and spermine compared to non-mycorrhized plants.

There are many records that mycorrhizal fungi not only orchestrate the biochemical and physiological pathways that contribute to stress tolerance but boost the inherent tolerance of plants towards many abiotic and biotic stresses, especially through the reinforcement of PA metabolism [124–127]. Recently, a review covered the phenomenon whereby arbuscular mycorrhizal fungi modulate polyamine and aquaporins metabolism to mitigate abiotic stresses in plants such as salt, heavy metals, and drought stress [128]. The pivotal role of polyamines in the establishment of symbiosis with AMF [117,129] and their multifunctionality in the plants was associated with polyamines' ability to regulate plant defense responses through the modulation of phytohormone synthesis [130,131] and the upregulation of osmolyte production [132,133]. AMF ensure the PA homeostasis and root plasticity in the host, which enables stress mitigation by scavenging ROS or by affecting the expression of the ROS-scavenging enzymes or other stress-responsive genes, contributing to the improvement of membrane stability and the maintenance of ion homeostasis and water status [127]. Furthermore, PAs stimulate mycorrhizal growth and development by fostering sucrose's conversion into more fungi-absorbable glucose and facilitate fungal penetration into the host cells trough pectinases' modulation [134]. Recently, the entire mechanism of how ectomycorrhizal fungi promote root colonization through effector-induced modulation of the polyamine biosynthetic pathway in *Eucalyptus obliqua* L'Hér. has been documented [135].

The findings from this study highly agree with the findings of Zou et al. [85] who detected enhanced levels of polyamines (particularly Put, Cad, and Spd, as well as some of their precursors *L*-ornithine and agmatine) in *Poncirus trifoliata* L. Raf. plants colonized with AMF. Furthermore, they found that increased activities of Pas' synthetic enzymes (ADC, ODC, SPMS, and SPDS) and catabolizing (PAO and DAO) enzymes' activities simultaneously contributed to the enhanced drought tolerance of mycorrhized *P. trifoliata* plants.

A thorough metabolomic and transcriptomic study, which inspected the effects of the inoculation of *Quercus suber* with the ectomycorrhizal fungi *Pisolithus tinctorius,* reported an increased expression of the genes *PAO2* and *PAO4* in mycorrhized plants compared to non-mycorrhized controls [136]. These genes encode polyamine-catabolic enzymes that contribute to the increased levels of gamma amino butyric acid (GABA), which is known as a potent priming agent involved in a plant's tolerance to biotic stress [136]. Through untargeted metabolic analysis, the same study proved that mycorrhized plants have different metabolic profiles compared to non-mycorrhized plants at the root level, including changes in different major chemical classes such as organic acids, carbohydrates, phenolics, tannins, fatty acids, and various lipids [136].

Additionally, it has been shown that putrescine has an important role—mostly during initial plant growth—in mycorrhization, while later, during further mycorrhizal development processes, it depends more on higher polyamines, namely, Spm and Spd, as it was shown that the ratio of Spd and Spm to Put was augmented in *Lotus glaber* Mill. following colonization by *Glomus intraradices* (Schenck and Smith) in saline soils [117]. The powdery mildew in this study induced an augmentation in the amounts of higher polyamines, triamine spermidine, and tetraamine spermine, increasing the Spd and Spm to Put ratio. Contrary to the research where the effects of only one mycorrhizal species upon plant polyamine metabolism were inspected, we inspected the effects of a commercial inoculum consisting of dozens of different ectomycorrhizal species that probably contribute in various synergistic ways to hosts' PA metabolism; however, experiments with high microbial

diversity more precisely simulate complex microbial environment present in oaks' natural forest habitat.

Interestingly, polyamines have complex and multiple roles in the plant–pathogen interaction and their involvement seems to be independent of the nature of the pathogen, whether it is biotroph or a necrotroph [137,138]. On the one hand, polyamines play a pivotal role in plant immunity by amplifying both pattern-triggered immunity (PTI) responses and effector-triggered immunity (ETI) via ROS generation through their catabolism [28]. Additionally, due to their ubiquitous nature, PAs are abundant in pathogenic fungi, especially during infection, and they may contribute to the pathogen's growth and development, as well as to the production of virulence factors, and therefore have an important role in pathogenesis [28,139]. For instance, in *Fusarium graminearum* Schwabe [(Schweinitz)Petch], putrescine triggers the expression of virulence factors and acts as inducer of mycotoxin production [140], while in *Magnaporthe oryzae* (T.T. Hebert) M.E. Barr, spermine was responsible for cuticle penetration, thereby enabling tight sealing by the stabilization of the cell wall integrity of the fungal appressorium in the interaction with the leaf surface [141]. Due to its polycationic nature at the physiological pH, polyamines are known to stabilize negative macromolecules such as DNA, proteins, and phospholipids in thylakoid membranes—including those within photosystem complexes, preventing chlorophyll loss [142]—while also being able to inhibit vacuolar ion channels [143]. Furthermore, an increased concentration of polyamines is associated with enhanced pre-penetration and penetration resistance in different oat cultivars upon rust inoculation with crown rust (*Puccinia coronata* Corda *f.* sp. *avenae* Eriks) [144].

The involvement of PA metabolism and their multifaced role in plant–microbe and plant–pathogen interactions at the physiological and molecular levels has been reviewed in detail in [31]. The increasing patterns of all the individual and total polyamines in oak leaves during the infection with *E. alphitoides* regardless of inoculation with ectomycorrhizal fungi found in this study are similar to the polyamine response reported in oats (*Avena sativa* L.) during an infection with powdery mildew (*Blumeria graminis* f. sp. *Hordei)* [144,145]. Increasing patterns of foliar polyamines (Spd and Put) induced by powdery mildew were also previously reported for barley (*Hordeum vulgare* L.) [105], while the rust fungus *P. hordei* induced only putrescine increment in barley [146]. Conversely, abundant putrescine levels induced by *Ralstonia solanacearum* Smith have been found to accelerate wilt disease in tomatoes (*Solanum lycopersicum* L.) [147]. Some authors assumed that pathogens modulate the host's polyamine metabolism to their benefit [28], while some authors assume that pathogens import PAs from the host since an increased expression of PA transporters and biosynthetic *ACD* genes was detected in fungus [139].

The main increments of foliar PA content are commonly detected in the vicinity of the entry point of the pathogen. The total amount of PAs depends on the activities of the main biosynthetic enzymes involved in either arginine- (by ADC) or ornithine- (by ODC) derived pathways, as well as their catabolic enzymes, such as diamine oxidase (DAO) or polyamine oxidase (PAO), whose activities are often disturbed during abiotic and biotic stresses [148]. Polyamines in alliance with their catabolic by-products (GABA, H_2O_2, and NO), represent the main stress messengers and signaling molecules of abiotic and biotic stress in plants [31,138]. The high correlation among total and individual polyamines and MDA, as an end-product of lipid peroxidation in this study, could be attributed to the amplified production of H_2O_2 that occurs during PAs catabolism, owing to the fact that H_2O_2 can induce lipid peroxidation. Through intensive cross-talk with plant hormones (e.g., ABA, JA, and SA) [149], polyamines cooperatively regulate many physiological processes, such as stomatal closure [150], or trigger the hypersensitive reaction (HR) [151], being key regulators of redox homeostasis through the modulation of genes coding antioxidant machinery [152]. Conversely, salicylic acid, as a key molecule in plant defense and immunity, activates the expression of ADC and ODC, thereby upregulating polyamine levels in tomato plants during stress [153].

In this study, a high negative correlation has been detected among the total polyamine content, transpiration rate, and stomatal conductance, which could be explained through

the fine-tuning of ABA by the catabolic products of polyamines (e.g., NO and H_2O_2) and consequential stomata closure [101,151]. Similarly, a high positive correlation between polyamines (especially Put) and the expression genes involved in ABA biosynthesis has been reported during abiotic stress [148,154], since it was demonstrated that putrescine activates the *NCED* gene that codes for the ABA production via zeaxanthin [155]. Additionally, the relatively high correlation between polyamines and some osmolytes (e.g., GB and PRO) could be addressed by the fact that proline and polyamine metabolism share the same precursor, glutamate, and that polyamines indirectly stimulate amino acid metabolism (proline, GABA, and glycine betaine) [156,157].

4.6. Limitation of the Study and Future Perspective

In the present work, we used a commercially available ECM inoculum "Ectovit" (Symbiom, Czech Republic) comprising mycelium and the spores of six fungi including *H. crustiliniforme*, *L. proxima*, *P. involutus*, and *S. citrinum,* which are well known ECM fungi of Q. robur [50,51]. Moreover, fungi from the genera *Scleroderma*, *Hebeloma,* and *Laccaria* have already been found forming ECM symbiosis with the roots of *Q. robur* in stands where acorns of *Q. robur* were collected for the purpose of this experiment [46]. The fungus *S. citrinum* is considered an early-stage species and is quite frequent among transplanted seedlings in the field; thus, it was not surprising that it colonized the roots during our experiment. However, we can only speculate that the ECM fungi from Ectovit used in this study originated from the Czech Republic where the Ectovit was produced. Therefore, to better understand the putative role of symbiosis in protecting a plant against the pathogen, future studies should focus on the use of a native ECM fungal strain that has co-evolved with genotypes of *Q. robur* naturally occurring in the study area and that therefore might be even more efficient in protecting this tree species against the pathogen.

5. Conclusions

This study confirmed the beneficial and bioprotective effects of ectomycorrhizal fungi against the oak powdery mildew disease caused by *E. alphitoides*. The presence of ectomycorrhizal fungi caused a significant alternation in oak leaves at the physiological and biochemical level. Specifically, the plants colonized with ectomycorrhizal inoculum exhibited higher levels of proline, non-protein thiols, putrescine, and spermine compared to the non-mycorrhized seedlings. Due to the proven metabolic crosstalk of proline, glutathione, putrescine, and spermine with an important priming agent, GABA, the possible priming effects of ectomycorrhizal fungi towards oak seedlings could be assumed. Furthermore, this study undoubtedly confirmed that powdery mildew reinforces foliar polyamine accumulation in oaks regardless of mycorrhization, but higher polyamine levels were detected in infected seedlings previously colonized with ectomycorrhiza. The presence of ectomycorrhizal fungi prevented a decline in physiological parameters, phenolics, and antioxidant capacity, which was otherwise noticed under powdery mildew infection in the absence of ECM. The effects of powdery mildew infection were mostly related to parameters grouped around PC1, such as the total PAs, SPD, condensed tannins, and gas exchange parameters, while the effects of inoculation with ectomycorrhizal fungi were dominantly described by the variability of the parameters grouped around PC2, similar to the total phenolic, flavonoid, and nitrogen content. This study confirmed that common biochemical parameters could be useful in defining trees' adaptability towards climate change. The application of these simple biochemical parameters could broaden the paradigm of the concept of climate smart forestry, which strives for the implementation of new measurable parameters that will enable indication of more tolerant tree species, genotypes, and provenances towards upcoming climate change.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f13091491/s1, Figure S1: *Scleroderma citrinum* ectomycorrhiza observed on roots of *Quercus robur* in the ECM-C treatment; Figure S2: Phylogenetic tree generated from a maximum likelihood (ML) analysis based on ITS sequence data showing the posi-

tion of *Scleroderma citrinum* in relation to its closely related *Scleroderma species*. Figure S3: Phylogenetic tree generated from a maximum likelihood analysis (ML) based on ITS sequence data of the *Quercus robur* powdery mildews showing the position of *Erysiphe alphitoides* in relation to closely related *Erysiphe quercicola*. Table S1: Sequences used in the phylogenetic analyses of *Scleroderma* species. Table S2: Sequences used in the phylogenetic analyses of *Erysiphe* species. Table S3: Disease index scale for powdery mildew. Table S4: Description of the treatments-defined by percentages of powdery mildew (PM) disease intensity and percentages of colonization with ectomycorrhiza (ECM). Table S5: A two-way ANOVA results on variable (ECM, PM, ECMxPM) effects on inspected parameters.

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