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Beneficial Microorganisms and Water Stress Influence *Quercus ilex* Seedlings' Response to *Phytophthora cinnamomi* Rands

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Abstract: Root rot affecting holm oak is a cause of high ecological and economic losses in the Iberian Peninsula, highlighting the relevance of developing disease control methods. The aim of this work was to assess the effect of a biological treatment composed of beneficial organisms (*Trichoderma* complex, T-complex) on holm oak seedlings infected by *Phytophthora cinnamomi* in two contrasted holm oak ecotypes, one considered highly susceptible (HU) and another considered tolerant to the pathogen (GR). For this purpose, a complete multifactorial test was carried out in a greenhouse, and seedlings were monitored for survival analysis and morphological and physiological attribute evaluation. Mortality began earlier in the susceptible (HU) than in the tolerant (GR) ecotype, and survival showed different trends due to the inoculation with beneficial microorganisms depending on the ecotype of the plants. The tolerant ecotype showed a high survival rate and better response to the treatment with beneficial microorganisms. GLM showed that the main reason for differences between treatments was ecotype, followed by T-complex and irrigation, and a weak interaction between ecotype and *P. cinnamomi* was found. The linear relationship between photosynthesis (A) and transpiration (Tr) showed an increase in the A/Tr rates for infected and inoculated plants under drought conditions for the GR ecotype. The tolerant ecotype was benefited more by the beneficial microorganism treatment. The understanding of the genetic diversity of *Q. ilex* and water stress influence on the efficacy of biological treatments against root rot provides useful information to develop environmentally friendly disease control methods to address the holm oak decline.

Keywords: beneficial bacteria; integrated pest management; oak decline; root rot; *Trichoderma* spp.

1. Introduction

Holm oak (*Quercus ilex* subps. *ballota*) is the most relevant forest species in the Iberian Peninsula [1]. Its plasticity allows it to thrive in contrasting environmental conditions, including soil and climate, being distributed from semi-arid (<300 mm average precipitation) to sub-humid climates (2500 mm) [2]. This ecological plasticity, together with its monoecious–allogamous tendency and its ability to hybridize with other *Quercus* spp., gives a high interpopulation and intrapopulation variability to the holm oak [2], covering a multitude of ecological niches throughout its distribution. Holm oak savanna-like forests (*dehesa* in Spain or *montados* in Portugal) occupies over 2 million ha in the Iberian Peninsula [3], and constitutes an ecosystem of high ecological and socio-economic value. The traditional management of these agrosilvopastoral systems leads to a great variability in ecological micro-niches, increasing both biodiversity and site conditions. On the other hand, the socioeconomic relevance of the *dehesas* is largely sustained by the woodland, including multiple secondary products such as wood fuel or fungi, shelter for cattle, and production of acorns for Iberian pig breeding [4]. Moreover, the main distribution area of *dehesas* and *montados* covers marginal bioclimatic zones, also considered highly exposed to climate change effects. Therefore, these ecosystems are very sensitive to sudden changes such as pests and diseases or extreme climatic events, which lead to forest decline [5].

Oak decline is currently one of the challenges in the management of Spanish *dehesas* [1]. This syndrome is influenced by many factors, including management (overgrazing, intensive cultivation, lack of tree regeneration, land abandonment) [6] and climatic events, including heavy rainfalls and long intense drought periods [5]. However, it is considered that the main factor causing holm oak mortality in *dehesas* is the root rot caused by plant pathogens, in particular, the oomycete *Phytophthora cinnamomi* Rands [7–10]. Several studies have shown that the inter- and intrapopulation levels of genetic diversity observed in holm oak generate a high diversity level in their response to root rot [11], also linked with the environmental influence. The soil microbial community is a key element in this equation, regulating the intensity of the disease either due to the improvement in site conditions, or their effect on the tree health status. It has been demonstrated that mycorrhizal fungi and some endophytic organisms improve the nutrient and water uptake of tress [1,4]. Moreover, high microbial diversity in soil decreases the ability of the pathogen to cause damage, due to competition [10]. Other specific fungal taxa such as some *Trichoderma* species can control the inoculum levels through inhibition or even mycoparasitism [12]. Other works have shown similar results of biologic agents controlling several soil-borne plant pathogenic fungi [13,14] including *Phytophthora cinnamomi* [15].

Trichoderma spp. is one of the most-studied filamentous fungi [16,17]. The genus includes mostly non-pathogenic soil-borne fungi, considered as avirulent opportunistic plant symbionts and root colonizers, which, in several cases, produce compounds that stimulate plant protection and growth [18–20]. Some *Trichoderma* spp. provide benefits such as control of pathogenic and competitive microbiota, and improvement in plant health and root growth. These organisms can rapidly colonize plant rhizospheres by inserting themselves into stable, pre-existing microbial communities [21]. Regarding *P. cinnamomi*, in vitro experiments have shown effects of different *Trichoderma* species inhibiting the mycelial growth of the pathogen, but also showing hyphal penetration and mycoparasitism in some cases [12]. Other studies have shown that treatment with several *Trichoderma* spp. reduced the mortality rate of holm oak seedlings in a nursery [22,23]. Specific bacteria are other promising organisms for the biological treatment of root rot. They are being widely used in agriculture to enhance plant growth and suppress plant pathogens either through direct beneficial effects on the plant and displacing certain pathogens through ecological niche occupation, or by creating a mutualism with the plant that improves its survival capabilities. Some bacteria genera commonly used in agriculture are *Azospirillum*, *Rhizobium*, *Pseudomonas*, and *Bacillus* [24–27].

Mortality of holm oak trees due to root rot threatens the *dehesa* ecosystems, causing incalculable ecologic and economic losses. The development of disease control methods is key for the sustainability of these ecosystems and their environmental services, including landscape structure, biodiversity refuge, and rural population support in vast areas of the south-west of the Iberian Peninsula. On the other hand, the ecological relevance of these agroecosystems makes it necessary to search for alternative eco-friendly methods of pest and disease control, looking for strategies to reduce the use of synthetic products [14]. For this purpose, we tested the ability of a biological treatment composed of two *Trichoderma* spp. and several beneficial bacteria species to reduce *Phytophthora cinnamomi* damage on holm oak roots. Two holm oak seedling ecotypes from contrasting origins were used for this test, one from a highly susceptible parental and another from parentals considered tolerant to the pathogen [28], and drought stress was included as additional abiotic factor. To achieve our goal, three specifics objectives were addressed: (i) to assess the differences in susceptibility to drought and *P. cinnamomi* infection of the two selected ecotypes; (ii) to evaluate the effect of biological treatment on seedling survival when subjected to intense drought, *P. cinnamomi* infection, and a combination of both factors, and (iii) to assess the effect of the biological treatment on seedling physiology when subjected to intense drought, *P. cinnamomi* infection, and a combination of both factors. The understanding of the role of genetic diversity and water stress in the effect of beneficial microorganisms on pathogen damage in *Q. ilex* seedlings will provide useful information to focus on environmentally

friendly control methods for holm oak decline. This information could be used in the development of integrated pest management protocols, considering direct treatments with biological control products or the adequate selection of parental materials for afforestation practices in degraded lands.

2. Materials and Methods

2.1. Experimental Site and Plant Material

The experiments were carried out in the facilities of the Campus of Rabanales, at the University of Córdoba, Spain (37°54′53.30″ N, 4°43′00.92″ W, 136 m.a.s.l.), in a greenhouse with glass cover and natural roof and side wall aeration. Seedlings were cultivated under semi-controlled conditions, with a range in maximum temperature between 30 and 42 °C and minimum between 10 and 25.2 °C. Air humidity was maintained above 40%.

The experiment was carried out using 6-month-old seedlings obtained from acorns collected from two selected holm oak ecotypes with different susceptibility to *Phytophthora cinnamomi* root rot, as tested in previous works [28,29]. The first ecotype, considered more susceptible, was collected from a seed collection stand located in Paymogo (Huelva, Spain, 37°44′52.08″ N, 7°19′56.28″ W, HU) and the second ecotype, considered as more tolerant, from a seed collection stand located in Arenas del Rey (Granada, Spain, 37°01′27.12″ N, 3°56′44.16″ W, GR). In both cases, parentals were in a low-density wooded pasture (*dehesas*). Acorns were collected from five trees per ecotype previously used in the tolerance tests, and 80 acorns were germinated from each tree (400 seedlings per ecotype). Acorns were sterilized by immersing them in 2.5% NaOCl for 5 min, then rinsed with sterile water and, after drying, stratified at 4 °C for 15 days. Seedlings were cultivated in 3.5 L plastic containers (150 mm wide, 150 mm long, 180 mm deep) filled with standard growth media (1:1:1, *v*/*v*, siliceous sand, vermiculite, and black peat) previously subjected to a sterilizing treatment in an autoclave at 120 °C for 45 min at 1.5 atmospheres. Seedlings were grown in optimal growth conditions (25 °C, RH 80%, two irrigations per week, 250 mL per plant, 12 h of light) for six months, and then divided into treatment groups, aiming for a homogeneous distribution of their morphological characteristics.

2.2. Experimental Design

A complete factorial experiment was carried out with four factors and two levels for each factor: ecotype (Paymogo population—HU and Arenas del Rey population—GR), inoculation with beneficial organisms (*T-complex*—T), inoculation with *Phytophthora cinnamomi* (Ph), and watering treatments (irrigated—W, and non-irrigated—D), generating a combination of sixteen treatments. To ease interpretation, the results were grouped considering ecotype susceptibility (HU as susceptible and GR as tolerant) (Table 1).

Table 1. Experimental design for each ecotype tested (Granada—GR and Huelva—HU). Treatments were individually *T-complex* inoculation (T) *Phytophthora cinnamomi* inoculation(Ph) and watering treatment during the trial (irrigated—W and non-irrigated—D), generating a combination of eight combined treatments per ecotype (N = 50 per treatment).

Treatment	Trichodema-Based Compound	*P. cinnamomi* Inoculation	Water Supply (Irrigated)
W	No	No	Yes
W + T	Yes	No	Yes
W + Ph	No	Yes	Yes
W + T + Ph	Yes	Yes	Yes
D	No	No	No
D + T	Yes	No	No
D + Ph	No	Yes	No
D + T + Ph	Yes	Yes	No

Groups of fifty seedlings per treatment were randomly selected and identified as a block, and each block rotated its position in the greenhouse to ensure that seedlings grew under the same environmental conditions. Three months after germination, treatments W + T, D + T, W + T + Ph, and D + T + Ph for both the HU and GR ecotypes were inoculated with *Trichoderma* complex (T-complex) produced and supplied by Agrogenia Biotech SL (https://www.agrogenia.es/; accessed on 28 February 2023). The T-complex included two different species of *Trichoderma* spp. ($2–4 \times 10^5$ colony-forming units CFU mL^{-1}) and a complex of rhizobacteria and plant growth-promoting bacteria (PGPB) (108–109 UFC mL^{-1}) (Supplementary Materials, Table S1). The treatment was applied through irrigation with 200 mL per pot at the manufacturer's recommended concentration (10.5% *v/v* of commercial product). The treatment was repeated after 45 days to ensure the success of the treatment. Non-inoculated treatment plants were subjected to mock inoculation with 200 mL of water without the T-complex.

After an additional 45 days of growth (when seedlings reached 6 months old), the treatments W + Ph, D + Ph, W + T + Ph, and D + T + Ph for both the HU and GR ecotypes were inoculated with *P. cinnamomi* by adding 200 mL of liquid inoculum concentrate prepared in carrot broth at 22 °C [30]. The colony-forming units (CFU) of *P. cinnamomi* in the solution were quantified using a Thoma chamber (Brand, Blaubrand, Germany) and adjusted to 5×10^3 CFU mL^{-1} of inoculum. Plants belonging to the treatments without *P. cinnamomi* (W, D, W + T, and D + T) were mock-inoculated with 200 mL of medium without *P. cinnamomi* inoculum.

All the plants were subjected to flooding for 48 h to encourage *P. cinnamomi* infection in inoculated seedlings. Then, the watering treatment plants (W, W + Ph, W + T, and W + T + Ph) were irrigated at soil capacity twice a week for the whole experiment duration, and the rest did not receive any more water after the 48 h of waterlogging until the end of the experiment (D, D + Ph, D + T, and D + T + Ph treatments).

2.3. Data Collection

Seedling mortality was registered daily after *P. cinnamomi* inoculation. Seedlings were considered to be dead when they lost all their leaves, or the leaves were completely desiccated, and the plant did not present any active/fresh buds. Physiological and morphological measurements of seedlings were taken before inoculation with *P. cinnamomi*, and then every 15 days on ten seedlings chosen randomly for each treatment. Physiological measurements were carried out around solar noon (considering a window of ± 1.5 h) using a portable infrared gas analyzer (LiCor Li6400XT, Li-Cor, Inc., Lincoln, NE, USA) and fluorescence with a Handy PEA+ fluorimeter (Hansatech Instruments Ltd., Norfolk, United Kingdom) (Table 2).

Table 2. Morphological and physiological traits measured in the trial.

Abrev.	Trait	Unit
Morphological traits		
Ø	Root collar diameter	Mm
DrMS	Stem dry matter	g $\times$ 100 g^{-1} (%)
DrMR	Root dry matter	g $\times$ 100 g^{-1} (%)
DrMA	Aboveground dry matter (stem and leaf)	g $\times$ 100 g^{-1} (%)
Physiological traits		
A	Photosynthetic rate	μmol CO$_2$ m^{-2} s^{-1}
Gs	Stomatic conductance	mol H$_2$O m^{-2} s^{-1}
Tr	Transpiration rate	mmol H$_2$O m^{-2} s^{-1}
WUE	Water use efficiency	μmol CO$_2$ mol^{-1} H$_2$O
Fv'/Fm'	Maximum photosynthetic efficiency of PSII	Adim

Before inoculation with *P. cinnamomi*, a random subsample of ten seedlings per treatment was collected (160 seedlings) to characterize the initial above- and belowground

biomass (Table 2). Forty-five days after inoculation, another ten seedlings were randomly selected for each treatment, and the measurement of biomass allocation was repeated. The dry weight of the root, stem, and leaves were determined for each plant, following the procedure described in the EN ISO 18134-3:2015, drying the samples at 105 °C until constant weight was reached [31] in a conventional air-dryer oven (JP Selecta Conterm, Barcelona, Spain).

The presence or absence of microorganisms in each treatment group was corroborated by isolation in selective culture media, seeding root segments randomly chosen from 3 plants for each treatment in a PARPBH selective medium for *P. cinnamomi* [32] and *T-complex* selective medium (TSM) [33] for *Trichoderma* spp. Colonies growing on the selective medium were transferred to carrot agar (CA) plates and identified through microscopic observation under a visible microscope equipped with 10×, 20×, 40×, and 100× lenses (Eclipse 50i; Nikon Instruments Inc., Melville, NY, USA).

2.4. Statistical Analysis

The normality of the data was tested using the Kolmogorov–Smirnov test with the correction of Lilliefors ($p < 0.05$), and Levene's test of variance was also used to analyze homoscedasticity ($p > 0.05$). The diameter did not fit normality ($p < 0.05$) until transformed by the inverse function ($1/x$). Parametrical analyses of this variable were carried out using this transformation.

Seedling survival at the end of the trial was analyzed using the Kaplan–Meier survival test (95% confidence intervals). Chi-squared tests and the log-rank test were used to statistically compare seedling mortality across different treatments [34]. The collinearity and correlations between variables were tested using a Pearson correlational matrix, and linear regression analysis (using the least squares method) was conducted only in cases where correlations between seedling attributes were significant ($p < 0.05$; Figure S1, Supplementary Materials).

Multifactorial ANOVA was used to test significant differences between and within groups. First, a preliminary analysis was carried out to test block influence over morphological or physiological variables, proving uniform nursery growth. Therefore, the block was eliminated as a factor, considering only irrigation (W and D) and inoculation (T and Ph) and their interactions as sources of variation in the analysis. A generalized linear model (GLM) was fitted. Tukey's post hoc test for multiple comparisons was applied where the differences were significant ($p < 0.05$), paying attention to homogeneous groupings within ecotype.

The data analysis was conducted in the R environment [35] using the RStudio user interface [36] and the libraries "survival" and "ranger" [37].

3. Results

3.1. Mortality

Mortality began earlier in treatments with *P. cinnamomi* (W + Ph, D + Ph, W + T + Ph, and D + T + Ph) for the Huelva (HU) ecotype, exceeding the T50 threshold before the 25th day after inoculation (Figure 1). The Granada (GR) ecotype showed lower mortality rates than HU for all treatments, as well as less abrupt mortality behavior for non-irrigated and *P. cinnamomi* treatments. The HU seedlings with non-irrigated and inoculated with *P. cinnamomi* treatment exceeded 75% mortality at the end of the trial. Regarding T-complex inoculation, differences appeared in the HU ecotype, where the non-irrigated control (D) presented higher mortality and early events compared with the non-irrigated inoculated plants (D + T).

Non-irrigated treatments with *P. cinnamomi* (D + Ph) showed the fastest and earliest mortality. *P. cinnamomi* in irrigation treatment (W + Ph) and with *T-complex* (W + T + Ph) showed a lower mortality rate (50% at the end of the trial) than non-irrigation treatment in GR, while the HU ecotype seedlings with the same treatments died before the 30th day after inoculation. Although the treatments W + T + Ph and D + T + Ph did not present

significant differences compared with Ph and D + Ph treatments at the end of the test, survival was significantly different in GR at day 30 after inoculation, with plants treated with the T-complex presenting a significantly higher survival rate than W + Ph treatments, both with (χ^2 = 12.8, df = 3; $p < 0.01$) and without irrigation (χ^2 = 43.1, df = 3; $p < 0.005$).
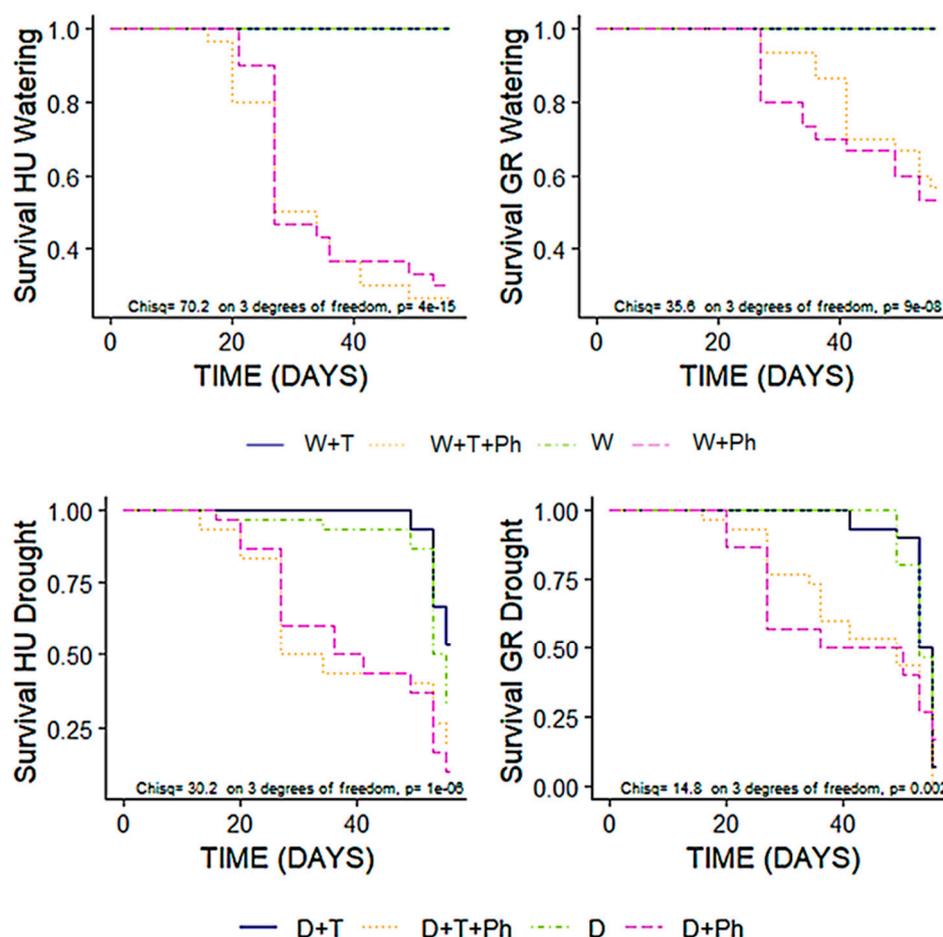

Figure 1. Kaplan–Meier holm oak seedling survival for organism and watering analysis by ecotype (Granada, GR; and Huelva, HU). Survival analyses were categorized by treatment (N = 50): inoculated with Trichoderma (T) and/or *Phytophthora cinnamomi* (Ph) and water supply (non-irrigated—D and irrigated—W). Chi square and P value of Kaplan–Meier survival curve are included ($p < 0.05$).

3.2. Morphology and Growth

Ecotype expressed the higher variability in the GLM model, followed by T-complex inoculation and irrigation, with a weak interaction between ecotype and *P. cinnamomi* (Table 3). Differences were observed in root collar diameter (Ø) for non-irrigated treatment and there was an interaction between drought and T-complex inoculation for the Granada ecotype, but in the Huelva ecotype, no differences or interactions were observed (Table 3). Root dry matter (DrMR) was the most influenced by irrigation treatment, followed by T-complex inoculation. The strongest interaction was found between the ecotype and the T-complex, followed by non-irrigated and *P. cinnamomi* infection, but with lower statistical differences (Tables 3 and 4). At the end of the experiment, the root collar diameter (Ø) in the GR plants was significantly higher for plants treated with the *T-complex* (W + T, W + T + Ph, D + T, and D + T + Ph). However, in the case of the HU ecotype, only non-irrigated plants inoculated with *P. cinnamomi* (D + Ph) differed significantly from the control and T-complex treatments (W, W + T) (Table 4).

Table 3. Effects of watering and inoculation treatments and their interaction on holm oak seedlings according to the generalized linear regression model (GLM). Variables that do not appear in Table 3 did not present significant fitting in the GLM. Treatments: *T-complex* (T) and *Phytophthora cinnamomi* (Ph) and water treatment (D). Values bold-highlighted were statistically significant at $p < 0.05$.

Sources	Ø		DrMS		A		Tr		WUE	
	F Value	Pr (>F)	F Value	Pr (>F)	F Value	Pr (>F)	F Value	Pr (>F)	F Value	Pr (>F)
Granada										
D	**8.31**	**0.004**	**106.011**	**<0.001**	**104.724**	**<0.001**	**114.331**	**<0.001**	**5.185**	**0.026**
Ph	2.22	0.137	1.139	0.289	0.003	0.954	**2.002**	**<0.001**	0.205	0.652
T	0.02	0.885	**16.88**	**<0.001**	0	0.984	0.152	0.697	0.198	0.658
D × Ph	2.22	0.137	2.963	0.089	3.291	0.073	**8.301**	**0.005**	0.701	0.405
D × T	**5.78**	**0.017**	**11.473**	**<0.001**	1.381	0.243	2.825	0.097	0.549	0.461
Ph × T	0.02	0.883	0.143	0.706	0.259	0.612	1.721	0.193	**6.935**	**0.010**
D × Ph × T	0.13	0.748	2.745	0.101	2.449	0.121	0.354	0.553	**7.266**	**0.009**
Huelva										
D	1.89	0.169	**42.721**	**<0.001**	**34.832**	**<0.001**	**36.845**	**<0.001**	**36.845**	**<0.001**
Ph	0.60	0.436	1.672	0.200	**8.537**	**0.004**	**0.235**	**<0.001**	**0.235**	**<0.001**
T	0.06	0.795	0.192	0.662	1.18	0.281	0.208	0.650	0.208	0.65
D × Ph	0.49	0.482	**11.534**	**<0.001**	0.475	0.492	1.477	0.228	1.477	0.228
D × T	3.51	0.062	0.407	0.525	0.015	0.901	0.691	0.409	0.691	0.409
Ph × T	2.95	0.087	0.028	0.867	3.645	0.060	0.639	0.427	0.639	0.427
D × Ph × T	0.46	0.495	1.936	0.168	**4.656**	**0.034**	0.436	0.511	0.436	0.511

Ø = Root collar diameter, DrMS = stem dry matter, A = photosynthetic rate, Tr = transpiration rate, WUE = water use efficiency.

All dry matter variables differentiated the non-irrigated treatments for GR. GLM showed the greatest statistical inference for non-irrigated plants, with interactions with *P. cinnamomi* infection, followed by the interaction between population and the *T-complex* (Table 4).

3.3. Physiological Response

The GLM model showed significant differences considering watering for A, Tr, and WUE. The factor inoculation with *P. cinnamomi* (W + Ph) showed differences in the Granada ecotype only in transpiration (Tr), while for the Huelva ecotype, it showed differences for all physiological variables (Table 3).

The Huelva ecotype only showed multiple interaction (D + Ph + T) for the variable photosynthesis (A), while the Granada ecotype showed an interaction of drought and *P. cinnamomi* factors for transpiration (Tr), while water use efficiency (WUE) interacted for *P. cinnamomi* and the T-complex (W + Ph + T) and multiple interaction (D + Ph + T) (Table 3).

Photosynthesis grouped the values of control, control with T-complex for GR, and *T-complex* for HU from non-irrigated control treatments (D) of both ecotypes (Table 4). Stomatic conductance (Gs) showed significant differences between control treatments for the two ecotypes and non-irrigated treatments. Water use efficiency (WUE) also significantly differentiated non-irrigated control (D) and the *T-complex* with no irrigation (D + T) for HU (Table 4). Transpiration (Tr) grouped the control (W) and *T-complex* (T) treatments of GR with the *T-complex* and *T-complex* and *P. cinnamomi* (W + T + Ph) treatment of HU. It also perfectly grouped non-irrigated control (D), non-irrigated with *T-complex* (D + T), and non-irrigated–*T-complex*–*P. cinnamomi* (D + T + Ph) treatments (Table 4). Fluorescence variables (Fv/Fm) only showed differences for GR, grouping *P. cinnamomi* (W + Ph) and *T-complex*–*P. cinnamomi* (W + T + Ph) treatments with non-irrigated control (D) (Tables 3 and 4).

Table 4. Morphological and physiological traits (mean ± SE, N = 50) of holm oak seedlings in the eight different watering and inoculation treatments at the end of the 40-day experiment. Means followed by the same letter(s) do not differ significantly at the 95% level of probability, Tukey's test. Treatments: *T-complex* (T) and *Phytophthora cinnamomi* (Ph) and water treatment (irrigated—W and non-irrigated—D).

Ecotype	Treatment	Ø	DrMS	DrMR	DrMA	A	Gs	WUE	Tr	Fv/Fm
GR	W	3.84 ± 0.12 ab	54 ± 1.08 de	47 ± 1.48 c	55 ± 0.43 d	5.82 ± 0.52 a	0.09 ± 0.01 ab	3.41 ± 0.23 ab	1.73 ± 0.13 a	0.57 ± 0.11 abc
	W + T	4.05 ± 0.13 a	53 ± 3.02 e	46 ± 2.39 c	50 ± 0.92 d	6.94 ± 1.11 a	0.12 ± 0.02 a	3.27 ± 0.24 ab	2.15 ± 0.35 a	0.48 ± 0.13 bc
	W + PH	3.59 ± 0.13 ab	53 ± 1.75 e	52 ± 2.32 c	54 ± 0.94 d	5.41 ± 0.68 a	0.07 ± 0.01 ab	4.13 ± 0.47 a	1.41 ± 0.23 a	0.82 ± 0.00 a
	W + T + PH	3.72 ± 0.17 ab	57 ± 4.69 cde	40 ± 1.42 c	57 ± 3.25 cd	5.48 ± 0.82 a	0.07 ± 0.01 b	4.69 ± 0.66 a	1.33 ± 0.21 ab	0.81 ± 0.01 ab
	D	3.62 ± 0.20 ab	68 ± 2.40 abc	73 ± 4.48 b	70 ± 3.99 b	1.18 ± 0.76 b	0.01 ± 0.00 b	4.84 ± 2.00 a	0.23 ± 0.09 c	0.11 ± 0.08 d
	D + T	3.16 ± 0.25 b	88 ± 4.75 a	69 ± 2.92 a	86 ± 2.06 a	−0.01 ± 0.16 b	0.00 ± 0.00 b	0.04 ± 2.33 b	0.05 ± 0.02 c	0.44 ± 0.11 cd
	D + PH	3.64 ± 0.21 ab	67 ± 5.91 bcd	66 ± 2.89 b	68 ± 3.13 bc	1.02 ± 0.66 b	0.02 ± 0.01 b	−0.03 ± 1.65 ab	0.52 ± 0.14 abc	0.78 ± 0.02 abc
	D + T + PH	3.25 ± 0.17 ab	78 ± 1.04 ab	63 ± 3.36 ab	78 ± 4.56 ab	1.48 ± 0.53 b	0.01 ± 0.01 b	3.31 ± 2.30 ab	0.15 ± 0.11 c	0.75 ± 0.04 abc
HU	W	3.66 ± 0.15 a	54 ± 1.06 c	47 ± 1.90 c	54 ± 0.89 d	4.10 ± 0.67 ab	0.07 ± 0.01 ab	3.02 ± 0.29 ab	1.34 ± 0.21 ab	0.36 ± 0.12 a
	W + T	3.23 ± 0.15 a	54 ± 0.73 c	47 ± 2.09 c	55 ± 0.63 d	4.69 ± 0.72 ab	0.08 ± 0.02 a	3.21 ± 0.26 ab	1.60 ± 0.28 a	0.64 ± 0.09 ab
	W + PH	3.15 ± 0.20 a	62 ± 4.69 Bc	69 ± 3.45 bc	64 ± 3.28 bcd	5.26 ± 0.82 ab	0.07 ± 0.01 ab	4.20 ± 0.23 ab	1.26 ± 0.19 abc	0.79 ± 0.02 a
	W + T + PH	3.36 ± 0.23 a	56 ± 3.35 c	83 ± 1.77 c	58 ± 1.47 cd	5.99 ± 0.88 a	0.08 ± 0.01 ab	4.28 ± 0.24 ab	1.45 ± 0.22 a	0.77 ± 0.03 ab
	D	3.03 ± 0.24 a	80 ± 1.24 a	50 ± 4.22 a	81 ± 4.58 a	−0.07 ± 0.17 c	0.00 ± 0.00 c	−5.24 ± 1.87 c	0.09 ± 0.03 d	0.38 ± 0.14 b
	D + T	3.27 ± 0.21 a	76 ± 1.04 ab	48 ± 3.88 a	6 ± 4.41 ab	2.11 ± 0.48 bc	0.01 ± 0.00 c	7.32 ± 1.09 a	0.29 ± 0.07 d	0.49 ± 0.12 ab
	D + PH	3.89 ± 0.23 a	65 ± 3.53 bc	65 ± 4.17 ab	67 ± 4.73 abc	3.67 ± 1.07 ab	0.03 ± 0.02 bc	0.71 ± 2.91 abc	0.67 ± 0.37 cd	0.74 ± 0.04 ab
	D + T + PH	3.38 ± 0.22 a	69 ± 2.80 abc	74 ± 3.68 ab	70 ± 3.61 abc	2.00 ± 0.84 bc	0.01 ± 0.01 c	1.36 ± 2.11 c	0.35 ± 0.35 d	0.78 ± 0.02 ab

Ø = Root collar diameter, DrMS = stem dry matter, DrMR = root dry matter, DrMA = stem and leaf dry matter, A = photosynthetic rate, Gs = stomatic conductance, Tr = transpiration rate, WUE = water use efficiency, Fv'/Fm' = maximum photosynthetic efficiency of PSII.

The main physiological differences were related to water stress but depended on ecotype (Table 4). All the physiology variables were similar for the irrigated plants independently of treatment, except for the Fv/Fm parameter, which showed a significantly lower value for plants treated with the T-complex and not inoculated with *P. cinnamomi* (T treatment). The susceptible ecotype (HU) presented lower photosynthesis rates and TR compared with the GR plants, but interestingly, plants of HU subjected to drought but treated with the T-complex presented a very high WUE, comparable to the WUE of the control irrigated plants, together with a heavy reduction in the other physiological parameters. The lower values of Fv/Fm corresponded to the drought control (D) and the plants treated with the T-complex.

However, when relationships between variables were carefully studied, some interesting trends appeared. The linear relationship between photosynthesis (A) and transpiration (Tr) rates presented differences between treatments and ecotypes (Figure 2).
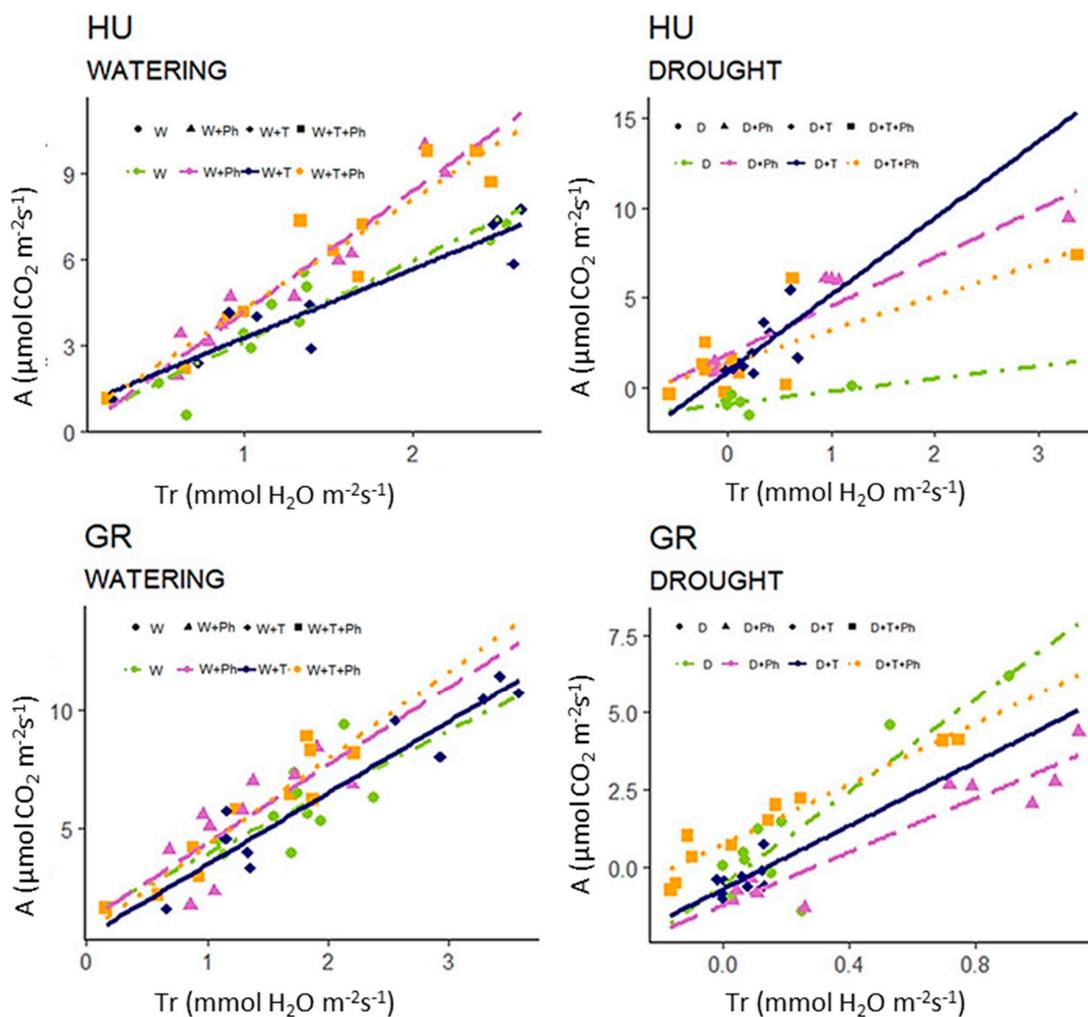
Figure 2. Photosynthesis vs. transpiration rate (A/Tr) for holm oak seedlings as response to inoculation with *T-complex* (T) and *P. cinnamomi* (Ph). Four different treatments of control (green), *T-complex* (blue), *P. cinnamomi* (red), and *T-complex* × *P. cinnamomi* (orange) were used to show the relationship between two different south Spain ecotypes (Granada "GR" and Huelva "HU").

Irrigation treatments showed similar trends for *P. cinnamomi* (W + Ph) and T-complex–*P. cinnamomi* (W + T + Ph) treatments, with increasing A/Tr rates regarding the control (W) and T treatments. This response was consistent for the two ecotypes. On the other hand, trends for non-irrigated treatments differed between ecotypes, differentiating treatments with the T-complex from the rest of treatments in HU. In the case of the GR ecotype, there

was a significant difference in trends for the D and D + T + Ph treatments regarding the rest of the treatments, including regularly watered plants. The plants of this ecotype that were subjected to drought and not inoculated showed lower Tr for similar A rates than D + T and D + Ph treatments, and the combined treatment (D + T + Ph) showed higher levels of A with low levels of Tr. The HU ecotype showed a strong decrease in the A/Tr rate when subjected to drought (D) compared with irrigation (W), while for the Gr ecotype, this same treatment (D) increased its rate compared to the irrigation treatment (W).

4. Discussion

Including biological treatments in integrated pest management plans is a strategy that can improve plant defence against diseases by providing better tree growth conditions. Holm oak has shown a great variability in terms of drought resistance throughout its distribution area due to a great genetic variability [38,39], as previous studies on Andalusian ecotypes have highlighted [10,28,29,39]. Other studies have shown the influence of beneficial microorganisms including *Trichoderma* spp. and pathogenic microorganisms on oak growth and physiology response [10,12,40]. This study corroborates the relevance of ecotype in modulating the effect of inoculation with beneficial microorganisms on drought stress and tolerance to *P. cinnamomi* in holm oak. Although no significant direct effect of T-complex treatment on the pathogen development was identified, our results showed other positive effects of the T-complex on plants, mainly facilitating plant development and reducing physiology stress [9,22,23,41].

4.1. Mortality

The survival analysis confirmed the initial hypothesis of differences in tolerance between ecotypes [11,28] and synergies between inoculation and drought as previously reported [9]. Our results showed more sensitivity of the HU ecotype to *P. cinnamomi* compared to the GR ecotype. This is consistent with mortality records in the areas surrounding the seed collection stands selected, and in concordance with previous studies [28,38,39]. However, the inoculation with the T-complex [42] only showed a positive effect on holm oak seedling survival for the susceptible ecotype (HU) under drought conditions, although mortality was attenuated in the intermediate stages for the tolerant one (GR), with survival at T30 significantly higher in plants infected with *P. cinnamomi* and treated with the T-complex for both irrigation treatments. This might be related to the beneficial effect of microorganisms improving growth and photosynthetic rates more in the GR than in the HU ecotype, leading to a lower impact of root rot in the tolerant ecotype. The lack of differences for the rest of the treatments inoculated with *P. cinnamomi* in HU may be due to the high susceptibility of this ecotype to the pathogen, where the effect of the pathogen was always much higher than the possible effect of the beneficial microorganisms.

On the other hand, *Trichoderma* spp. effects are influenced by the character of the "forma specialis" [43] by which a minimum difference in the host can generate a different reaction. It must be considered that depending on the ecotype of holm oak, the species and even the strain of *Trichoderma* spp. used in the biological treatment must be carefully selected. Previous studies showing different degrees of pathogen inhibition used strains isolated from living trees located in affected areas [12]. In these studies, the possibility of having specific mechanisms between the host and the *T-complex* strain was highlighted [12,42].

4.2. Morphology and Growth

In terms of growth response, the tolerant ecotype presented higher biomass accumulation than the susceptible one and seedlings developed more aerial biomass and roots, allowing the roots to explore a larger soil volume. The increase in root biomass allows plants to absorb more water and nutrients from the soil and accumulate a larger carbon reserve [40]. On the other hand, this larger number of roots is conducive to greater col-

onization by *P. cinnamomi* [44], which can induce worse effects of root rot if there are no additional control mechanisms over the pathogen.

We observed better plant architecture, with higher biomass accumulation, diameter, and height in seedlings inoculated with the T-complex under drought treatment, once again showing higher sensibility to the T-complex in the tolerant ecotype (GR). Several works have previously described the mechanisms underlying a decrease in sensitivity to *P. cinnamomi* by improving plant architecture (e.g., root length, leaf area, biomass, and proliferation of secondary roots) in trees inoculated with *Trichoderma* spp. [45,46]. Additionally, it has been seen that *Trichoderma* altered physiological functions (e.g., higher photosynthesis and chlorophyll content, more phosphate solubilization) and produced growth-regulating phytohormones such auxin that stimulate plant growth and development [45], enabling plants to use resources such as water and light more effectively. However, growth decreased in the susceptible ecotype (HU) under non-irrigated and *P. cinnamomi* treatments combined with the T-complex. The tolerant ecotype was possibly able to take advantage of the effect of the beneficial microorganisms more efficiently than the susceptible one.

The application of the T-complex had no positive impact on plant morphological or physiological variables under watering conditions. This result agrees with the finding of Soldan et al. [47] that *Trichoderma* spp. had no significant effect on field grown *Myrcianthes pungens* plants. In addition, Filho et al. [48] reported that seedlings of *Eucalyptus urophylla* clones and *Eucalyptus grandis* clones inoculated with *T. atroviride* had a dry biomass accumulation decrease of approximately 17% when compared to the control treatment, showing the high variability of hosts in response to *Trichoderma* spp.

4.3. Physiological Response

The main effect of the beneficial microorganisms in this study was linked to watering conditions, agreeing with previous works in which significant interactions of pathogen inoculation and drought were identified [9]. Regularly watered plants showed lower transpiration rates for the same levels of photosynthetic activity. Nevertheless, under drought conditions, the response of plants inoculated with the T-complex was different from the control plants, but varied depending on ecotype. The ecotype factor influenced photosynthesis with significant differences between the control and T-complex treatments. The tolerant ecotype (GR) showed higher photosynthesis values. Studies have demonstrated that different plant varieties, populations, or individuals can be associated with a particular strain of *Trichoderma* and also with different responses, since the way of interacting may differ between *Trichoderma* strains and the phenotypic characteristics of the plant (including genotype). According to Harman et al. [19], some isolates increase the total surface of the root system, facilitating greater access to soil nutrients, soil nutrients' solubilization, and availability of plant nutrients [49]. They can also optimize the efficiency of absorption and utilization of elements such as nitrogen and phosphorus by the plants [47]. This can explain the differences between our genotypes regarding T-complex influence.

Another response to the inoculation with the T-complex was the lower rates of transpiration for D and D + T treatments in HU. Control plants subjected to drought showed higher levels of transpiration and lower indices of photosynthesis compared to plants inoculated with T-complex and subjected to drought. The D + T treatment showed lower transpiration rates for the same photosynthesis levels, together with high WUE. Increase in the uptake capacity of beneficial microorganisms has been related previously with increases in the hydric status of plants, including WUE [19,50].

The higher indices of A/Tr shown by the susceptible ecotype under drought stress are in accordance with the isohydric behaviour of holm oak [51]. This behaviour can lead to a plant collapse when soil water availability reaches very low levels, agreeing with the differences and trends seen in the survival analysis. As one of the effects of endophytic fungi and beneficial bacteria is to promote better root development and ease the uptake of water and nutrients [52,53], the inoculation with the T-complex reduced the stress of the plant under drought conditions. This was clearer in the susceptible ecotype, while the

tolerant ecotype showed a different response. Plants of the GR ecotype infected with the pathogen and treated with the T-complex under drought conditions showed restrained transpiration rates and better photosynthesis rates. This might be explained by the different behaviour of plants from this ecotype facing drought, reducing physiological activity, as can also be seen in the drought treatment for GR. In previous studies, it has been observed that holm oak seedlings inoculated with *P. cinnamomi* reduced their transpiration rates as a response to the root damage, but increased their metabolic activity [9], which has been related to defence mechanisms and secondary metabolite production [29]. This agrees with the observed response for the less tolerant ecotype under regular watering, with an increase in physiologic activity when infected with the pathogen at the same or lower levels of transpiration. If the tolerance of the GR ecotype was related to the ability of plants to produce this change, the inoculation with the T-complex would be an improvement, facilitating this response under drought conditions. This also would explain why the GR plants seemed to have better survival rates in the middle of the experiment, but reached similar mortality rates by the end, when conditions were extreme.

Seedlings inoculated with the beneficial microorganisms showed high values for the quantum yield of PS II (Fv/Fm) in some cases. Fluorescence variables discriminated T-complex treatment for the GR population. The Fv/Fo ratio also showed that the plant response was largely dependent on ecotype. The fluorescence response to biotic stress in GR had the same trend as the increase due to abiotic stress in HU. The Fv/Fm value ranged between 0.7 and 0.8 in both ecotypes under trial conditions, higher than the Fv/Fm values in the control seedlings. Harman et al. [19] stated that *Trichoderma* spp.'s mechanisms improve the availability of nutrients, enhancing plant physiological activity. This is another reason to conclude that the holm oak seedlings of the tolerant ecotype take greater advantage of the effects of the T-complex treatment.

5. Conclusions

This study has shown the potential of the applications of beneficial microorganisms for increasing both the growth and physiological conditions of holm oak seedlings in combination with watering conditions. We can conclude that the tested bio compound improves plant response to drought conditions, but with differences regarding ecotype. Our results showed that the more tolerant ecotype took advantage of the beneficial effect of the T-complex to defend itself against the pathogen infection. Therefore, ecotype is a fundamental factor when considering the plant responses to treatment and pathogen infection. The strongest effect on growth and development traits was observed in the tolerant ecotype when plants were inoculated with the T-complex.

However, the research on the specific relationships between *Trichoderma* spp. and forest species, including holm oak, is insufficient to assess the biological control measures for improving field conditions in areas affected by *P. cinnamomi*. Thus, although the use of *Trichoderma* spp. and beneficial bacteria is supported by the literature and by our results, the relevance of selecting the right isolates for each case is emphasized. It is necessary to conduct additional research to investigate the influence of genetic variability of the species on the responses to *Trichoderma* and bacteria colonization, as well as methods of inoculation, concentrations, and the identification of different species or even strains of beneficial microorganisms.

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