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*Tuber aestivum* as an alternative to *Tuber melanosporum* for *Quercus ilex* truffle plantations under climate change scenarios: tolerance to high summer temperatures and drought

Máster en Restauración de Ecosistemas



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## Abstract

Agricultural abandonment is nowadays one of the largest land use changes in Europe, especially in Mediterranean areas. However, this abandonment can be an opportunity for ecological restoration. Truffle plantations, made up of a forest tree species mycorrhized with a fungi of the genus *Tuber*, are a restoration strategy with a great potential to obtain ecological services together with economic benefit. *Tuber melanosporum*, one of the most valuable truffles in Europe, is susceptible to extreme climate conditions and has decline its production due to climate change. *Tuber aestivum* can develop under harsh climate conditions. The objective was to compare the performance of *Quercus ilex* seedlings mycorrhized with *T. melanosporum* to those with *T. aestivum*, under present and future summer thermic environments and three drought intensities. Seedlings were evaluated periodically for visual damage index (VDI) through the summer. In the middle of summer, maximum stress moment, physiological performance of the seedlings was measured (water stress and photosynthetic performance). Increment of summer temperatures was the most negative factor on seedling performance, independently of the mycorrhizal species or the drought intensity. High temperatures strongly incremented VDI over the time and decreased leaf water potential, but did not damage the photosynthetic machinery ( $F_v/F_m$ ) and capacity ( $\Phi_{PSII}$  and  $A_{net}$ ) nor produced mortality. Drought had minor effects under present temperature environment but increased synergistically the negative effects of the future increased temperatures. Seedlings mycorrhized with *T. aestivum* had a better performance than those with *T. melanosporum*. In general, *T. aestivum* seedlings had lower VDI and higher leaf water potential, than those with *T. melanosporum*. Consequently, results demonstrate that *T. aestivum* is a potential alternative for truffle plantations to *T. melanosporum* for the drier and hotter regions or for areas where harsh conditions are predicted in the future due to climate change.

**Keywords:** ecosystem restoration, hotter drought, fluorescence of Photosystem II, leaf water potential, photosynthesis, VDI.

## Resumen

El abandono agrícola es actualmente uno de los mayores cambios de uso del suelo en Europa, especialmente en zonas mediterráneas. Este abandono también puede suponer una oportunidad para la restauración ecológica. Las plantaciones trufas, plantaciones de una especie arbórea forestal micorrizada con hongos del género *Tuber*, son una estrategia de restauración con un gran potencial para obtener servicios ecosistémicos y beneficio económico. *Tuber melanosporum*, una de las trufas más preciadas de Europa, es susceptible a condiciones climáticas extremas y ha disminuido su producción debido al cambio climático. *Tuber aestivum* puede desarrollarse en condiciones climáticas adversas. El objetivo fue comparar el desempeño de plantas de *Quercus ilex* micorrizadas con *T. melanosporum* frente a micorrizadas con *T. aestivum*, en ambientes térmicos estivales presentes y futuros y tres niveles de sequía. Se evaluó periódicamente el daño visual (VDI) de las plantas durante el verano. A mitad de verano, momento de máximo estrés, se midió su desempeño fisiológico (estrés hídrico y capacidad fotosintética). El incremento de las temperaturas estivales fue el factor más negativo sobre el desempeño de las plantas, independientemente de la especie de micorriza o de la intensidad de la sequía. Las altas temperaturas incrementaron el VDI a lo largo del tiempo y disminuyeron el potencial hídrico, pero no dañaron la capacidad fotosintética ( $F_v/F_m$ ,  $\phi_{PSII}$  and  $A_{net}$ ) ni produjeron mortalidad. La sequía tuvo efectos menores bajo temperatura presente, pero aumentó sinérgicamente los efectos negativos del aumento de temperaturas. Las plantas micorrizadas con *T. aestivum* presentaron mayor desempeño. En general, las plántulas de *T. aestivum* tuvieron menor VDI y mayor potencial hídrico foliar que aquellas con *T. melanosporum*. Los resultados demuestran que *T. aestivum* es una alternativa potencial para las plantaciones trufas para las regiones más secas y cálidas o para áreas donde se prevén condiciones futuras estresantes por el cambio climático.

**Palabras clave:** restauración de ecosistemas, sequía más cálida, fluorescencia del Fotosistema II, potencial hídrico de la hoja, fotosíntesis, IDV.

## Introduction

Agriculture has currently become the most extensive terrestrial biome, representing approximately 40% of the earth's surface (Rey Benayas et al., 2007; Levers et al., 2018) and it will have an even greater increase in the next decades (Laurance et al., 2014; Ceddia, 2019; Williams et al., 2021). Despite this increase, large part of agricultural land will be abandoned in the coming years, particularly in developed countries (Levers et al., 2018; Isbell et al., 2019; Williams et al., 2021). Agricultural abandonment is currently one of the largest use of land changes within Europe (van der Zanden et al., 2017; Perpiña Castillo et al., 2020; Zavalloni et al., 2021), where abandonment of around 3-4% of agricultural areas is expected by 2030 (Keenleyside and Tucker, 2010; Zavalloni et al., 2021). The extension of these abandoned areas is not homogeneous, being greater in Mediterranean areas (between 10 and 14%) compared to the rest of Europe (around 1%). In addition, this process can have negative consequences, such as soil erosion, reduction of local biodiversity, and loss of soil quality (Rodrigo-Comino et al., 2018). However, this agricultural abandonment can be an opportunity for ecological restoration (Navarro and Pereira, 2015; Löf et al., 2019).

Ecological restoration of abandoned agricultural fields can be carried out through various strategies: passive restoration or ecological succession, and active restoration (Rey Benayas, 2005). The first strategy is the most widespread. It consists of spontaneous, or no assisted regeneration of the vegetation, in an area previously degraded, once the disturbance has ceased (Chazdon, 2008; Lozano-Baez et al., 2019; Lisboa et al., 2021). Its biggest advantage is its low economic cost and little or no intervention is needed. However, the evolution is non-controlled and it can sometimes give rise to a new ecosystem that is very different from the initial ecosystem (Chazdon et al., 2020). Nevertheless, passive restoration requires a huge time scale to obtain results, and it does not give satisfactory results on areas with vast degradation problems. The second strategy requires direct human intervention, where vegetal species are planted following a strategic intervention to obtain a new ecosystem (Jones et al., 2018; Lozano-Baez et al., 2019; Chazdon et al., 2021; Lisboa et al., 2021). It is used in areas exposed to continuous degradation, without the possibility of recovering by passive regeneration. In this way, the main bottlenecks are overcome. However, it has added economic costs and requires a large work and time investment (Löf et al., 2019). Due to the fact that a large part of the abandoned agricultural fields present a marked degradation, a classic ecological

restoration would be inefficient, caused by the impossibility of recovering the forest and original composition, and the restoration by active restoration would imply an exacerbated economic cost (Navarro and Pereira, 2015).

Truffle plantations are made up of an autochthonous forest tree species, mycorrhized with the fungus of interest of the genus *Tuber*. Truffles are hypogeous fruiting bodies of fungi belonging to the genus *Tuber*, that being edible have economic value (Thomas and Büntgen, 2019). For this reason, plantations of mycorrhized plants with truffles had been extended in the last decades, in particular in those locations where farmers can obtain economic benefits (Perez et al., 2020). Forest trees mycorrhized with *Tuber* also present an ecological added value, being able to grow in relatively harsh conditions, highly degraded areas, even those areas that have suffered fires (Martínez de Aragón et al., 2012), such as in Mediterranean areas (Bonet et al., 2006). Moreover, these plantations can change the whole agricultural field, providing not only a new economic service, but also enhancing biodiversity and avoiding soil loss and/or erosion, and requiring low or no maintenance (Domínguez-Núñez et al., 2005; Bonet et al., 2006). In this context, truffle plantations would represent an ideal strategy: ecosystem services are recovered and local biodiversity is improved together with an economic benefit (Chazdon, 2008).

*Tuber* spp. can have different host plants, so it is necessary to take into account the conditions that not only truffles need, but also the hosts. Despite *Tuber* spp. has several host plants, the genus *Quercus* stands out (Zambonelli et al., 2016). Furthermore, *Tuber* spp. are mainly produced in orchards where *Quercus ilex* is planted. On the other hand, *Q. ilex* is an endemic Mediterranean species, a key element on many ecosystems, so its study can give a wide amount of information. Within the genus *Tuber*, *T. melanosporum* stands out owing to its high economic value. Its distribution is limited, being found in Mediterranean areas, highlighting northeastern Spain, north and central Italy, and southern France (Ceruti et al., 2003; Thomas and Büntgen, 2019) where, despite being distributed in different environments, it is demanding regarding climatic and edaphic conditions, requiring calcareous soils with basic pH (Bonet et al., 2006; Mello et al., 2017). Despite being adapted to Mediterranean climate, *T. melanosporum* is susceptible to extreme drought conditions, being able even to stop the fruiting process if conditions are truly extreme (Zambonelli et al., 2014; Tacon, 2016). However, plantations of *T. melanosporum* are currently facing the problem of climate change: an increase in

temperatures in conjunction with xericity are reducing the productivity and/or viability of truffle plantations (Thomas and Büntgen, 2019). In fact, productions of *T. melanosporum* have already been negatively impacted since the middle of the 20th century, with a decrease in production due to the increasingly drier and warmer climate (Büntgen et al., 2015; Thomas and Büntgen, 2019). In addition, due to climate change, new threats appear to these plantations, such as the risk of fires, heat waves or pests, so alternatives must be sought in these future scenarios (Thomas and Büntgen, 2019; Perez et al., 2020). In this context, solutions must be proposed based on an adequate choice of both, forest species and mycorrhizal fungus, thus creating a new system that is resilient to both climate change and anthropogenic activities (Chazdon, 2008).

There are other *Tuber* species such as *T. aestivum* which, unlike *T. melanosporum*, is a highly distributed species in Europe, with a great ecological breadth and with a more extensive harvest period owing to its morphological diversity (Zambonelli et al., 2016; Mello et al., 2017). *Tuber aestivum* is able to complete the fruiting process even in harsh conditions, like drought, high temperatures, winter frost or rainy climate (Zambonelli et al., 2016; Ori et al., 2020). Thus, it is a widely distributed species due to its climatic, edaphic and ecological breadth. Besides having a lower economic value compared to *T. melanosporum*, it still has a remarkable monetary value and higher production. Consequently, it becomes an ideal species for truffle plantations and to replace *T. melanosporum*, particularly in the areas where it cannot be cultivated or does not grow naturally. Currently there are hardly any studies on *T. aestivum*, despite its importance and interest. For this reason, the comparative study of these two *Tuber* species in different climatic conditions is essential to know the different geographical territories where they can be cultivated, allowing to establish different use recommendations.

Productivity of truffles is also conditioned by climate change, which can modify the distribution and fruiting zones of truffles. Although this effect is also conditioned by the plant-fungus pair, depending on the ecology of plant-fungi species. The effects of climate change, such as drought or temperature increase, produce negative effects on the growth of truffles, reducing their production (Zambonelli et al., 2016; Perez et al., 2020). The increasing effects of these abiotic factors are well studied separately, especially drought studies, but there is no much known about the combination of them acting at the same time (Gargallo-Garriga et al., 2015). Mediterranean ecosystems are characterized by a summer xericity, along with high temperatures that coincide in this period. Summer



stress had been pointed out as the main reason that limits the success of revegetation, and plant survival (Ramón Vallejo et al., 2012). Furthermore, temperatures and drought are expected to increase both in frequency and intensity over the years at a global level. The increase of temperatures enhances evapotranspiration, leading to water stress, resulting in an effect of amplified xericity, that act synergistically with the precipitation reduction due to climate change known as hotter drought (Elliott et al., 2020). This effect alters forest systems at the level of structure and composition, affecting at the same time the viability and future production on truffle orchards (Sperlich et al., 2019; Misson et al., 2011; Tognetti et al., 2019; Ogaya et al., 2020). Thus, is mandatory to evaluate the effect of both factors of climate change, increased temperatures and drought, on truffle plantations to maintain their viability in the future.

The objective of this work is to evaluate the effects of climate change, particularly the increase in temperatures and drought during summer, in two plant-fungus pairs: *Q. ilex* - *T. melanosporum* and *Q. ilex* - *T. aestivum*, which have different climatic and edaphic requirements. The following hypotheses will be evaluated: (1) either drought or increased temperatures will have a negative effect in the performance of the plants irrespective of the fungi partner; (2) the combined effect of increased temperatures and drought (hotter drought) will have an increased negative effect; and (3) *Q. ilex* mycorrhized with *T. aestivum* will have a higher performance than *Q. ilex* mycorrhized with *T. melanosporum* under climate change conditions.

## **Material and methods**

A full-factorial experiment was carried out with mycorrhizal partners (*Tuber melanosporum* and *T. aestivum*), summer temperatures (actual temperatures -present-, and ~ 2.5°C above actual temperatures -future-), and drought intensity (control, moderate and severe).

### ***Plant and Tuber material***

A total of 279 two-years-old *Q. ilex* seedlings were used. Seedlings were produced under nursery seedling production standard protocols at the Real Jardín Botánico Juan Carlos I (40°30'22" N; 3°20'31" O, 600 masl). Seedlings from inland provenance ES10 were grown in 350 cm<sup>3</sup> forest containers filled with 5:1 (v:v) peat and calcium carbonate stones. Calcium carbonate was added to correct the acid pH of the peat to basic values as

recommended for *Tuber* species (García-Montero et al., 2006; Hilszczańska et al., 2019). During the first year, the seedlings were fertilized at 220 mg N seedling<sup>-1</sup> (1:0.5:1 NPK), while in the second year they were not fertilized. Inoculation was carried out in the spring of the first year with inland sporocarps of each *Tuber* species. Additionally, on 9 and 17 November 2020, calcium powder (Dolokal, Sibelco, Spain) was added with 25 ml of 1:100 (g CaCO<sub>3</sub>: ml water) solution to adequate the pH.

On 9 December 2020, seedlings were transplanted to 3500 cm<sup>3</sup> forest containers filled with a 5:1 (v:v) mixture of unfertilized-peat, and calcium carbonate stones (97.5% CaCO<sub>3</sub>, granulometry of 3 mm). On 22 December, 2020 each seedling received 250 ml of the calcium carbonate solution described above. At transplant, mycorrhization levels were 2.25 and 1.9 for *T. melanosporum* and *T. aestivum*, respectively, according to the scale of Chevalier and Grente (Andrés-Alpuente et al., 2014).

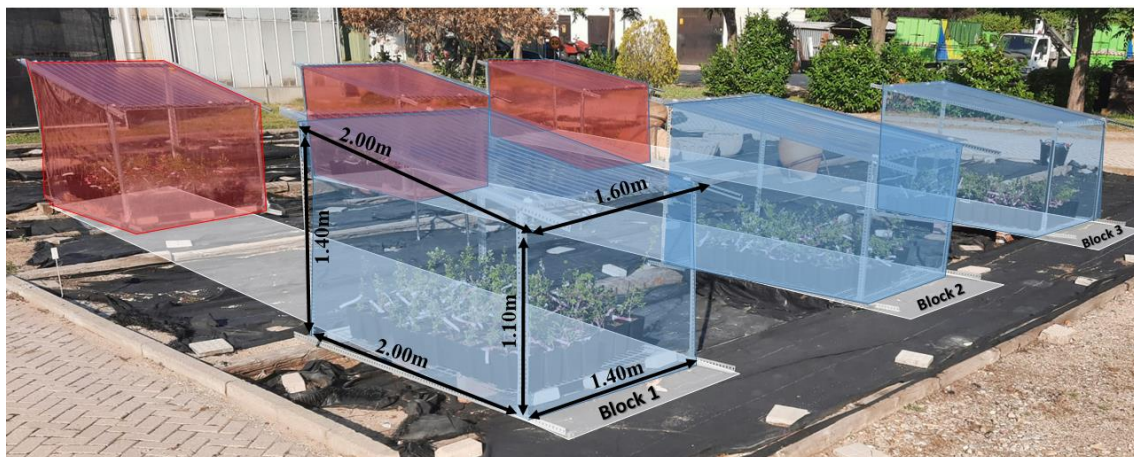
On 13 March 2021, despite the seedlings were already mycorrhized, a second inoculation with the respective *Tuber* species was carried out with 1:60 and 1.5:60 (FW:v, truffle:deionized water) suspensions for *T. melanosporum* and *T. aestivum*, respectively. This inoculation was carried out to avoid any lack or limitation of propagules (either spores or hyphae from the pre-existent mycorrhizae) for mycorrhizal colonization of the new roots and, consequently, any detected effect on mycorrhization would be exclusively due to the experimental conditions.

On 8 and 17 June 2021, seedlings were fertilized. On each fertilization event, each seedlings received 200 ml of 1 mM N solution containing nitrate, ammonium and glycine in equimolar proportion (i.e. 0.33 mM KNO<sub>3</sub>, 0.165 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and 0.33 mM glycine) and 1:0.5:1 (N:P:K) ratio. Nitrogen concentration applied is similar to those found in natural Mediterranean forest soils, usually below 2 mM N (Serrasolses et al., 1999).

### ***Experimental design: temperature and drought treatments***

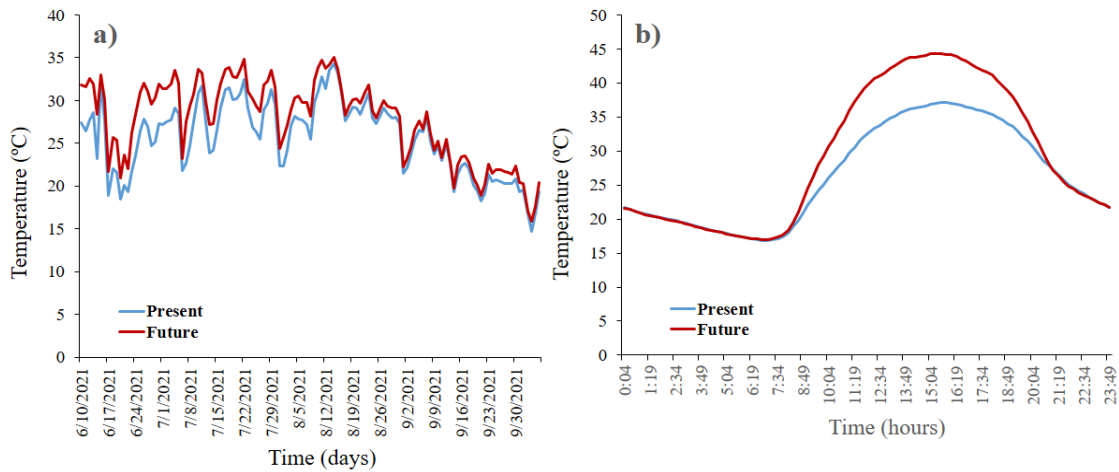
On 28 May 2021, initial height and diameter of each plant was measured. On 1 June 2021, half of the seedlings, twenty-one seedlings per plant-fungus pairs (84 seedlings in total) were moved to an area with a rain exclusion system (1.4×2×1.1/1.4 m, width×length×height). Rain exclusion was obtained by a sloped ceiling covering made with a transparent corrugated polycarbonate panel (0.8 mm thickness and 76×17.3 mm

width×height of the wave dimensions, 95% light transmittance), sides were opened and, consequently, temperatures inside the rain exclusion area were the actual temperatures during the summer period of the year of the experiment and considered as present summer temperature conditions (present temperatures, hereon). A second rain exclusion system was used but with the sides closed with transparent plastic to increase the temperature (future temperatures, hereon). This second greenhouse had the same number of seedlings as the first one. Temperatures inside this second rain exclusion system emulate the expected increment of temperatures in 2100 due to climate change in the average scenarios according to IPCC (Díaz et al., 2019; Tollefson, 2020; Mosadegh and Babaeian, 2021). The pair formed by the present and future greenhouses was considered a block that was repeated 3 times (Figure 1). Temperature in each greenhouse was monitored every 15 min with temperature recorder sensors (Hobo Pendant Temperature 64K Data Logger, Onset, USA).



**Figure 1.** Rain exclusion systems in the experiment: future and present (red and blue, respectively). Each pair formed by present and future greenhouses formed a block for a total of three blocks (represented in white).

During the whole experiment, the mean daily temperature of the future greenhouse was 2.5 °C above the temperature of the present greenhouse (Table 1, Figure 2a). This difference was especially notorious during July, when maximum average temperatures were 7.3°C above the present greenhouse. Maximum difference in temperatures between present and future greenhouses were reached in the central hours of the day (between 12:00 and 17:00), being 7°C higher in the future greenhouse respect to the present (Figure 2b). Minimum temperatures were similar between both greenhouses.



**Figure 2.** Mean daily temperatures during the experiment (m/d/y) (a) and mean hourly temperatures (b) from 10 June 2021 to 30 September 2021 in present and future temperature environments (n=3).

**Table 1.** Maximum and minimum temperature values, both mean and absolute during experiment since 10 June 2021 to 30 September 2021 in present and future scenarios (n=3). Data are mean  $\pm$  standard error.

	<i>Present</i>	<i>Future</i>
Mean temperature (°C)	25.54 $\pm$ 0.40	27.88 $\pm$ 0.44
Mean minimum temperature (°C)	15.60 $\pm$ 0.29	15.78 $\pm$ 0.28
Absolute minimum temperature (°C)	6.63	6.76
Mean maximum temperature (°C)	38.63 $\pm$ 0.47	44.73 $\pm$ 0.53
Absolute maximum temperature (°C)	50.36	55.60

Once moved, seedlings were irrigated to field capacity during 2 weeks to promote the acclimation to temperature scenarios before starting the drought treatments. Seedlings were surrounded with a file of empty pots to avoid edge effects.

Drought treatments were initiated on 16 June 2021. Whiting each greenhouse, one third of the seedlings per plant-fungus pairs were randomly assigned to each of three drought levels: control, moderate and severe. The control seedlings were watered to field capacity, and the moderate and severe seedlings were watered to 85 and 60% of container weight, respectively (50, 35 and 15% of container water content for control, moderate and severe drought treatments, respectively). Each seedling was weighed and watered to target weight every 3-4 days from the beginning of the drought treatments to 18 September 2021. In each watering event, containers were randomly rearranged to avoid edge effects.

In each watering event, the visual damage index (VDI) in the shoots of each seedling was recorded by one visual observer (Figure 3) to evaluate the evolution of the damage on each seedling along the experiment. Visual damage was considered as proportion of shoot with symptoms of leaf senescence, dry tips and/or shoot die-back. The VDI had a value from 0 to 100% (0% when there was not any damage, and 100% when there was a complete damage).

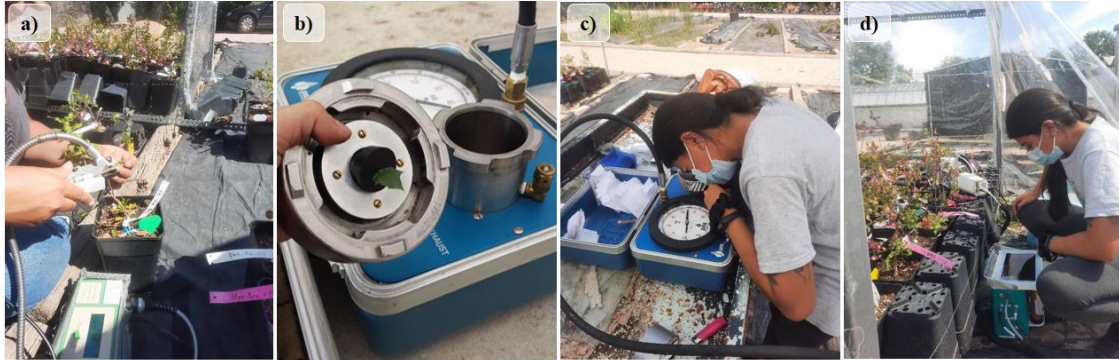


**Figure 3.** Visual damage scale (VDI) on *Q. ilex* seedlings, (from left to right) 0, 20, 50, 70, 90% VDI, respectively.

### ***Physiological characterization of the seedlings***

Physiological characterization of the seedlings was carried out in mid-summer (2 – 6 August 2021) when plant stress is assumed to reach a maximum. Three randomly selected seedlings per treatment (plant-fungus pair, greenhouse or thermic environment and drought treatment) and block (for a total of 9 seedlings per treatment, and 108 seedlings in total) were measured for: (a) plant water status by (1) leaf water potential at dawn ( $\Psi_{\text{dawn}}$ ), and (2) at midday ( $\Psi_{\text{midday}}$ ) with a Scholander pressure chamber (PMS70, PMS Corvallis, OR, USA); (b) fluorescence parameters-light use efficiency: (3) the maximum efficiency of photosystem II ( $F_v/F_m$ ) at dawn ( $F_v/F_{m\text{dawn}}$ ) and (4) at midday ( $F_v/F_{m\text{midday}}$ ); and (5) the effective quantum yield of photosystem II ( $\Phi_{\text{PSII}}$ ) at midday with a fluorometer (FMS2, Hansatech Instruments LTD, United Kingdom), and (c) gas exchange capacity: (6) net photosynthesis rate ( $A_{\text{net}}$ ) with an infrared gas analyzer (LI-

6800 Portable Photosynthesis System, LI-COR, USA) (Figure 4). Dawn measurements were taken between 6:00 and 7:30 am, midday measurements were taken between 2:00 and 3:00 pm, and gas exchange activity was measured between 9:00 and 10:30 am. Measurements were done on mature leaves of the upper third of each individual. Thus, we ensure that the measured leaves have grown during the experiment.



**Figure 4.** Measured variables during experiment. Measurement of the effective quantum yield of photosystem II ( $\Phi_{PSII}$ ) with a fluorometer (a); measurement of leaf water potential with a Scholander chamber (b, c); measurement of net photosynthesis rate ( $A_{net}$ ) with an infrared gas analyzer (d).

The pressure chamber or Scholander chamber is used to determine rapidly the water potential of the plant, as an index of drought stress in the seedlings. It increases the pressure around the leaf, until xylem sap starts to bubble at the cut done previously at the end of the petiole. The pressure needed to obtain this condition is the negative pressure in the stem that forces the water of the leaf cells going out and, consequently, indicates the hydration status of the plant (Boyer, 1967; Yegappan and Mainstone, 1981). The more negative the water potential, the more dehydrated or water stressed is the seedling. The water potential values are higher at midday than at dawn due to consumption in light processes such as photosynthesis and transpiration. Consequently, dawn values indicate the chronic water stress due to soil availability, while midday values include the chronic water stress but also the climatic daily stress. Measurements were made immediately after cutting the petiole.

To estimate the maximum quantum yield ( $F_v/F_m$ ), the fluorescence is measured in dark preadapted leaves for a half an hour with a light saturation pulse method. Thus, the PSII reaction centers are in an open state ( $F_o$ ), so the photochemistry reactions are not limited when the light pulse is sent, and reach the maximal fluorescence value  $F_m$  (Méthy,

Damesin and Rambal, 1996; Baquedano and Castillo, 2007). With this method we can estimate the intrinsic ability of the plant to capture light ( $F_o-F_m/F_m$  or  $F_v/F_m$ ) (Kim et al., 2019). The  $F_v/F_m$  value of a healthy plant is 0.8, and decreases when it is damaged.  $F_v/F_m$  values are higher at dawn than at midday, in response to excess radiation and other stress factors that reduce the amount of operative reaction centers and/or the efficient functioning of them (Björkman and Demmig, 1987; Méthy, Gillon and Houssard, 1997; Kim et al., 2019). On the contrary, the quantum yield of photosystem II ( $\Phi_{PSII}$ ), is the measurement of fluorescence in light acclimated leaves. Thus, only part of the PSII reaction centers are in an open state due to actual functioning of the photosynthetic machinery (Méthy, Damesin and Rambal, 1996; Baquedano and Castillo, 2007). In this sense,  $\Phi_{PSII}$  is a surrogate of the rate of electron transport through PSII reaction centers, or the real operative efficiency of the light capture process. It is related to the efficiency of carbon assimilation (Jin et al., 2020). This last measurement was done at midday under direct sunlight on fully sunny days.

Net photosynthesis ( $A_{net}$ ) is the total amount of carbon fixation rate minus the  $CO_2$  loss in respiration and photorespiration (Sullivan et al., 1996). The  $A_{net}$  was measured at an air  $CO_2$  concentration set at 400 ppm, vapor pressure deficit (VPD) at 1.5 kPa, and light at saturating values of  $1500 \mu mol m^{-2} s^{-1}$ . Temperature was set as environment and corresponded with the ambient temperature of each greenhouse.

### ***Statistical analysis***

The best fitted model in base to AIC criterion (see table S1 in supplementary material) to evaluate the effect on the VDI of the thermic environment, the drought and the mycorrhizal fungi over time was a generalized linear mixed-effects model (*glmer*) with data distribution binomial and link cloglog (logarithmic transformation of both VDI and time). Drought was considered as a nested factor in the thermic environment, and two random factors were included: the block and the seedling identity (ID), that was nested in the block in which it was located, using the package *lme4* (Bates et al., 2015). ID was included due to the repeated measures on the same seedling.

The effect on the  $\psi_{dawn}$  and  $F_v/F_m_{midday}$  of the mycorrhizal fungi, environment and irrigation was analyzed with generalized linear mixed-effects model (*glmer*), considering mycorrhizal fungi and thermic environment as independent factors, with drought nested in the thermic environment. The block was considered as a random variable. A *gamma*

data distribution was used after verifying that data distribution was not normal, using a Shapiro test.

The effect on  $\psi_{\text{midday}}$ ,  $F_v/F_{m\text{dawn}}$ ,  $\phi_{\text{PSII}}$  and  $A_{\text{net}}$  of the mycorrhizal fungi, thermic environment and drought was analyzed with linear mixed-effect model (*lmer*), after using a Shapiro test and verifying that the distribution was normal. Mycorrhizal fungi and thermic environment were considered as independent factors, with drought nested in the thermic environment. The block was considered as a random variable. Normal distribution was checked using a Shapiro test.

In all the previous analyses, when there were statistically significant effects of the factors, a Tukey's post hoc test was performed with the *lsmeans* package (Lenth, 2016), establishing a level of significance at  $\alpha = 0.05$ . All analysis were performed using RStudio software Version 2021.09.0+351 (RStudio Team, 2021).

## Results

### *Evolution of the damage on seedlings along the experiment: visual damage index*

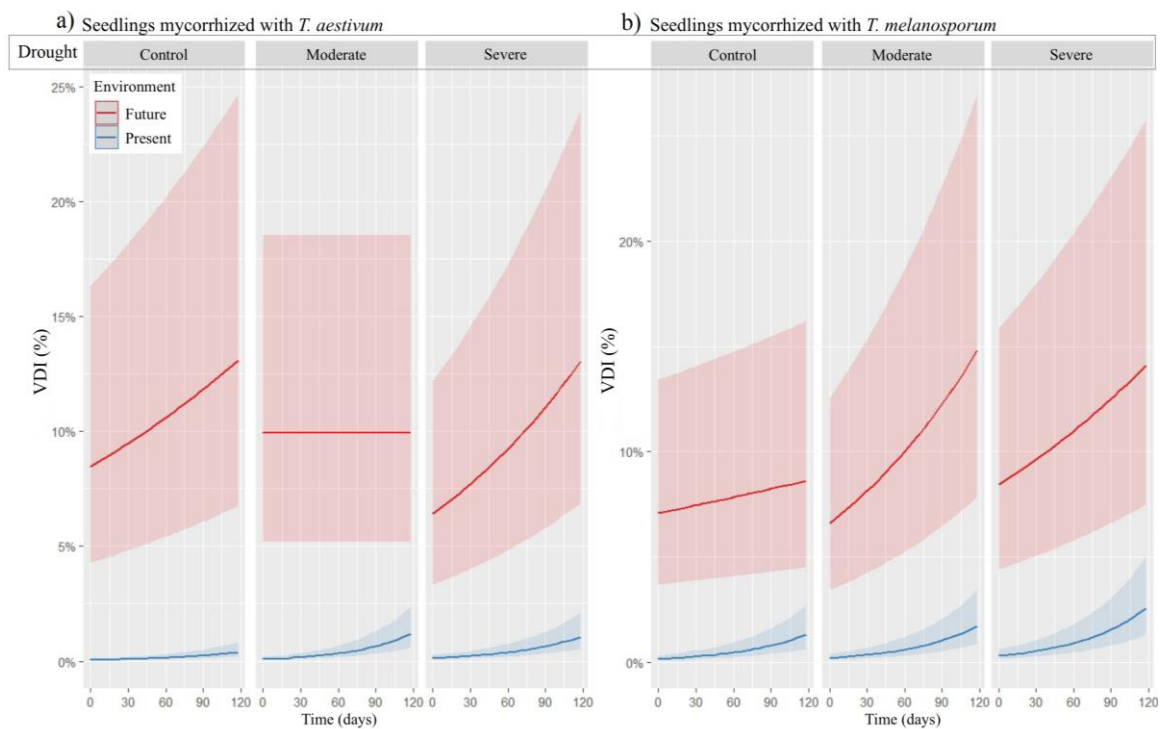
In general, rising temperatures was the factor that most increased VDI through the time, independently of the mycorrhizal fungi and the drought intensity. However, the VDI response to thermic temperature of the environment differed depending on the drought intensity and the mycorrhizal fungi (interaction environment temperature  $\times$  drought  $\times$  mycorrhizal fungi  $\times$  time;  $\chi^2=45.78$ ;  $p<0.001$ ; Figure 5).

In the present greenhouse, the increase of VDI was progressive and mild under all drought levels, and similar for seedlings mycorrhizized with either *T. aestivum* (Figure 5a, blue lines) or *T. melanosporum* (Figure 5b, blue lines). However, seedlings mycorrhizized with *T. melanosporum* showed greater VDI than the ones with *T. aestivum*. VDI increases throughout time, but never exceeded 5% in seedlings mycorrhizized with *T. melanosporum* and 3% in those with *T. aestivum*, regardless the drought intensity. While in seedlings mycorrhizized with *T. aestivum* drought hardly increased VDI, in seedlings mycorrhizized with *T. melanosporum* it increased VDI but only slightly.

Due to the increase of temperatures in the future greenhouses, the VDI increased 4 times in seedlings mycorrhizized with *T. aestivum* (Figure 5a, red lines) and only twice in those with *T. melanosporum* (Figure 5b, red lines) with respect to present temperatures.



Despite this VDI increase, under drought stress, VDI was higher at the end of the experiment in seedlings mycorrhized with *T. melanosporum* (around 15%) than in the ones with *T. aestivum* (around 12%). However, at high temperatures but in the absence of drought, seedlings mycorrhized with *T. melanosporum* showed less VDI than those with *T. aestivum*. The increase of VDI in the seedlings mycorrhized with *T. melanosporum* over time was progressive and intense under both moderate and severe drought levels, but very mild under control treatment. In seedlings mycorrhized with *T. aestivum*, the increase of VDI was progressive but accelerated under control and severe drought treatment, but negligible under moderate drought treatment.



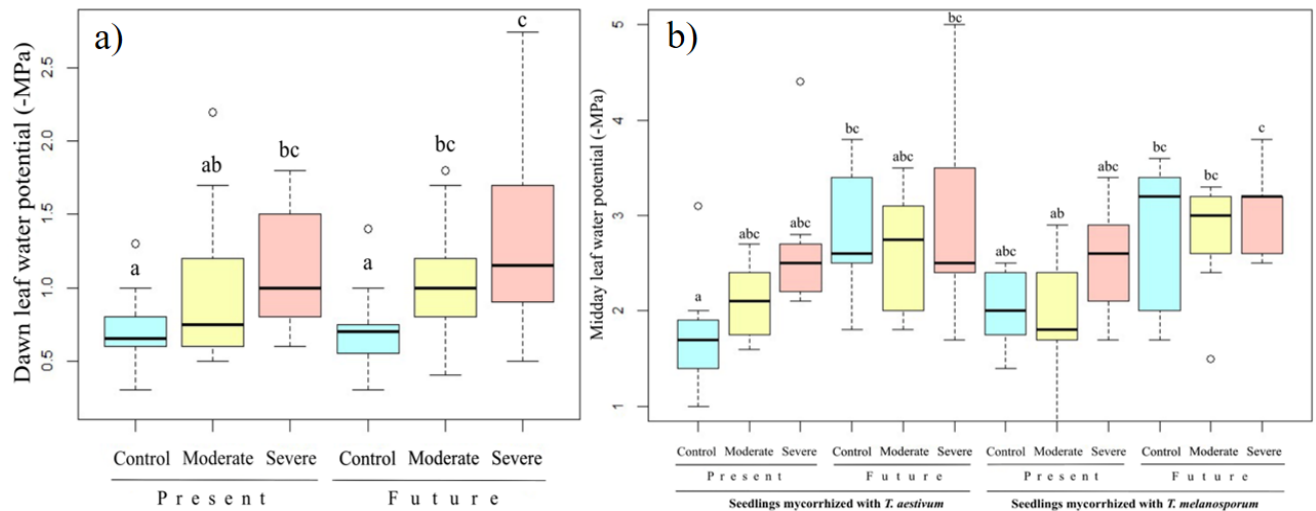
**Figure 5.** Visual Damage Index (VDI) during time on *Quercus ilex* shoots, mycorrhized with *Tuber aestivum* (a) and *Tuber melanosporum* (b) growing during summer under two thermic environments: present (blue, actual temperatures) and future (red, 2.5°C above actual temperatures) and under three drought intensities: control (watered to field capacity), moderate (85% container weight) and severe (60% container weight). Lines indicate predicted values for the adjusted models and shaded areas the 95% confidence intervals.

### *Plant water stress of the seedlings*

The  $\psi_{\text{dawn}}$  showed a reduction as the level of drought increased, but this reduction was more noticeable in the future thermic environment (interaction thermic environment  $\times$  drought;  $\chi^2=20.2121$ ;  $p<0.001$ ; Figure 6a). Furthermore, under present temperatures, only

severe drought stress induced lower  $\psi_{\text{dawn}}$  than control seedlings, with moderate drought stress seedlings having intermediate  $\psi_{\text{dawn}}$  without differences with the previous drought treatments. On the contrary, when seedlings were exposed to increased temperatures both drought stress levels decreased  $\psi_{\text{dawn}}$  below the control seedlings, without differences between them. In any case  $\psi_{\text{dawn}}$  was below -1MPa. The mycorrhizal fungi did not affect  $\psi_{\text{dawn}}$  ( $\chi^2=0.0017$ ;  $p =0.97$ ) nor interact with the other factors (interaction thermic environment  $\times$  mycorrhizal fungi;  $\chi^2=0.088$ ;  $p =0.77$  and interaction thermic environment  $\times$  drought  $\times$  mycorrhizal fungi  $\times$  time;  $\chi^2=3.02$ ;  $p=0.55$ ).

The effect on the  $\psi_{\text{midday}}$  showed significant differences in response to the thermic environment, being lower under future temperatures, with values closer to -3 MPa, compared to the present temperatures with values around -2 MPa, three and two times, respectively, lower than at dawn ( $\chi^2=11.7203$ ;  $p<0.001$ ; Figure 6b). Additionally, the drought intensity had a marginally significant effect on  $\psi_{\text{midday}}$  that was dependent on the thermic environment and the mycorrhizal fungi (interaction of thermic environment  $\times$  mycorrhizal fungi  $\times$  drought;  $\chi^2=15.08$ ;  $p=0.057$ ). Under present conditions, there were no statistical differences on  $\psi_{\text{midday}}$  among drought intensity levels. In the future thermic environment,  $\psi_{\text{midday}}$  did not indicate significant differences among drought treatments on seedlings mycorrhized with *T. aestivum*, that were only statistically lower than control seedlings under present conditions. However, no differences were found on  $\psi_{\text{midday}}$  in either drought treatments on seedlings mycorrhized with *T. melanosporum* or between thermic environments. Furthermore, in general, seedlings mycorrhized with *T. melanosporum* showed a lower water potential than seedlings mycorrhized with *T. aestivum*, especially under future temperatures.

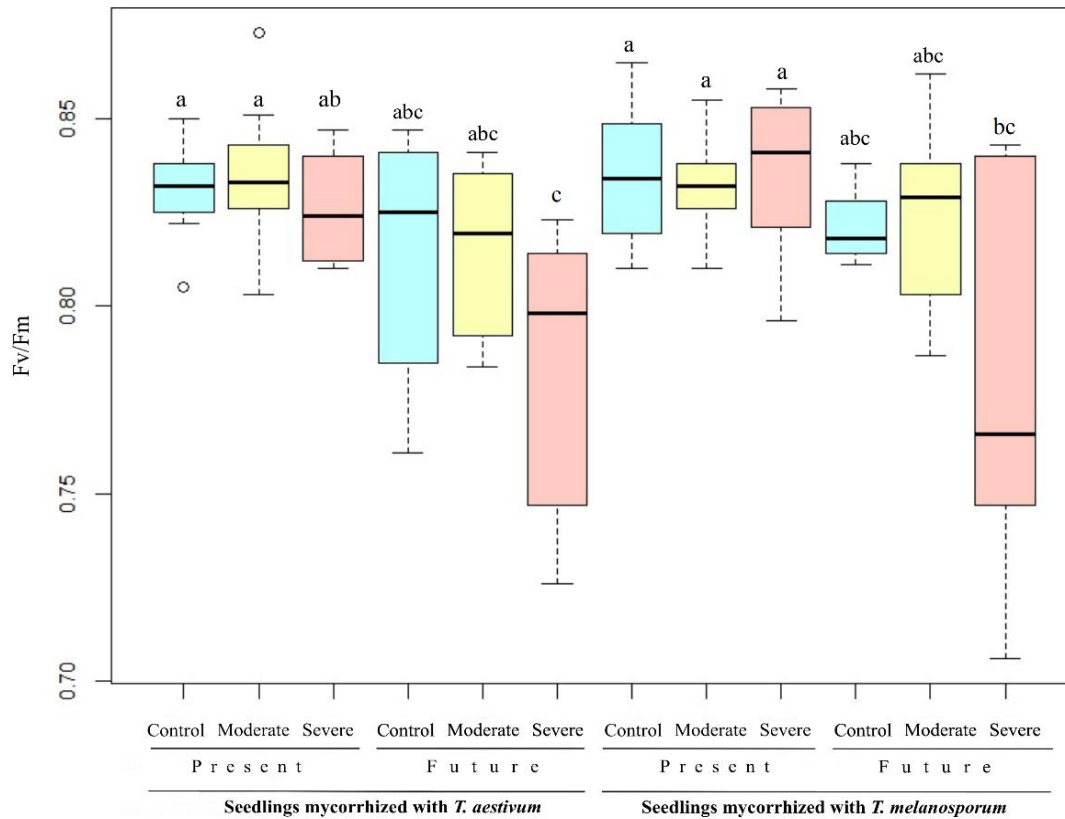


**Figure 6.** Box plots of leaf water potential at dawn ( $\Psi_{\text{dawn}}$ , a) and midday ( $\Psi_{\text{midday}}$ , b) on *Quercus ilex* seedlings mycorrhizized with *Tuber aestivum* and *Tuber melanosporum* growing during summer under two thermic environments: present (actual temperatures) and future ( $2.5^{\circ}\text{C}$  above actual temperatures) and under three drought intensities: control (watered to field capacity), moderate (85% container weight) and severe (60% container weight).

### Photosynthetic performance of seedlings

The effect of drought on  $F_v/F_{m_{\text{dawn}}}$  varied with the thermic environment but depending on the mycorrhizal fungi (interaction mycorrhizal fungi  $\times$  thermic environment  $\times$  drought;  $\chi^2=19.89$ ;  $p=0.011$ ; Figure 7).  $F_v/F_{m_{\text{dawn}}}$  in the present temperature environment did not show differences among drought levels on seedling mycorrhizized with *T. aestivum*. Also, under present temperatures, seedlings mycorrhizized with *T. melanosporum* had no significant differences among drought intensity levels at present temperatures.

With the increase of temperatures in the future thermic environment,  $F_v/F_{m_{\text{dawn}}}$  tended to decrease progressively with the drought intensity below present values, independently of the mycorrhizal fungi. However, this pattern only generated statistical differences between seedlings under present environmental temperatures and the highest drought intensity, severe drought, under future temperatures, and having the remaining treatments intermediate values without differences with the extremes. This pattern was intensified in seedlings mycorrhizized with *T. melanosporum*.



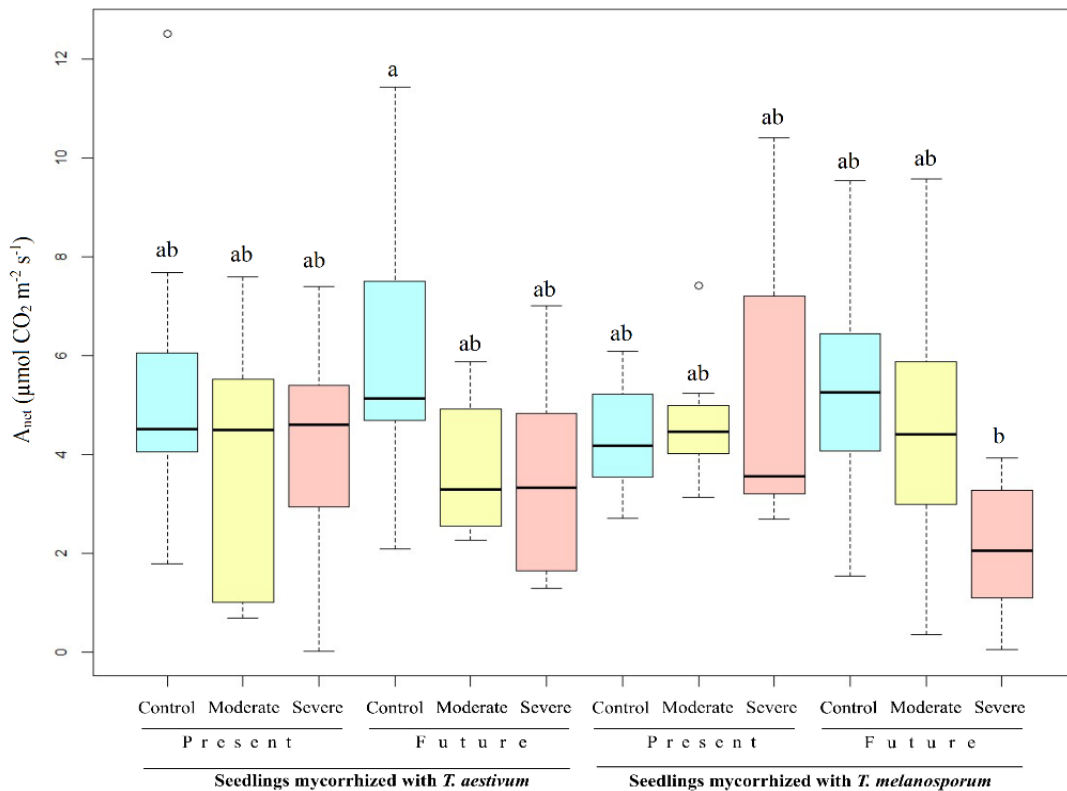
**Figure 7.** Maximum efficiency of photosystem II at dawn ( $F_v/F_{m_{dawn}}$ ) on *Quercus ilex* seedlings mycorrhized with *Tuber aestivum* and *Tuber melanosporum* growing during summer under two thermic environments: present (actual temperatures) and future (2.5 °C above actual temperatures) and under three drought intensities: control (watered to field capacity), moderate (85% container weight) and severe (60% container weight).

$F_v/F_{m_{midday}}$  remained at high values ( $0.819 \pm 0.0029$ ; data not shown) regardless of the thermic environment ( $\chi^2=0.34$ ;  $p=0.56$ ) or the level of irrigation ( $\chi^2=6.83$ ;  $p=0.74$ ). Additionally, mycorrhizal fungi did not affect  $F_v/F_{m_{midday}}$  ( $\chi^2=2.31$ ;  $p=0.13$ ;  $0.817 \pm 0.008$  and  $0.822 \pm 0.008$  for *T. aestivum* and *T. melanosporum*, respectively) nor its interaction with the other factors (interaction mycorrhizal fungi  $\times$  thermic environment  $\times$  drought;  $\chi^2=4.09$ ;  $p=0.39$ ).

Also  $\Phi_{PSII}$  remain constant across treatments ( $0.177 \pm 0.005$ , data not shown), without differences between mycorrhizal fungi ( $\chi^2=1.68$ ;  $p=0.19$ ), nor between thermic environments ( $\chi^2=0.005$ ;  $p=0.94$ ), among drought levels ( $\chi^2=5.99$ ;  $p=0.20$ ) nor of their possible interaction (interaction mycorrhizal fungi  $\times$  thermic environment  $\times$  drought;  $\chi^2=3.33$ ;  $p=0.50$ ).

The effect of drought on  $A_{net}$  depended on the thermic environment and varied depending on the mycorrhizal fungi (interaction thermic environment  $\times$  mycorrhizal fungi

× drought,  $\chi^2=19.9145$ ;  $p=0.01066$ ; Figure 8). At both thermic environments, seedlings did not show  $A_{net}$  differences among drought levels, independently of the mycorrhizal fungi. However,  $A_{net}$  reached a maximum under future temperatures without drought stress in seedlings mycorrhized with *T. aestivum* and a minimum for severe drought level under future temperatures in seedlings mycorrhized with *T. melanosporum*, having the remaining treatments intermediate values. Furthermore, in the present environment, seedlings mycorrhized with *T. aestivum* tended to have a higher  $A_{net}$  than those mycorrhized with *T. melanosporum*, especially at severe drought intensity, even not significant. On the contrary, in the future thermic environment, seedling mycorrhized with *T. melanosporum* tended to have a higher  $A_{net}$  than those mycorrhized with *T. aestivum*, except at severe drought level when seedlings micorrhized with *T. aestivum* had higher  $A_{net}$  values than those with *T. melanosporum*, even not significant.



**Figure 8.** Net photosynthesis rate ( $A_{net}$ ) on *Quercus ilex* seedlings mycorrhized with *Tuber aestivum* (left) and *Tuber melanosporum* (left) growing during summer under two thermic environments: present (actual temperatures) and future ( $2^\circ\text{C}$  above actual temperatures) and under three drought intensities: control (watered to field capacity), moderate (85% container weight) and severe (60% container weight).

## Discussion

### *Climate change effects on mycorrhizal Q. ilex seedlings*

Increment of summer temperature was the most negative factor on the performance of the seedlings independently of the mycorrhizal *Tuber* spp. VDI showed an early and intense increment in the future environment. Furthermore, water stress was much higher in the future environment across drought treatments than under present environment, which denotes increased water stress just due to higher temperatures. Despite  $\psi_{\text{dawn}}$ , chronic water stress in seedlings indicates a tendency to decrease as the drought stress level increases, this pattern was more intense under a future thermic environment. Additionally, and more important,  $\psi_{\text{midday}}$  showed that seedlings under future temperatures were highly dehydrated, independently on the drought level. This effect might be due to an increase evapotranspiration demand to refrigerate the leaves (Gargallo-Garriga et al., 2015). However, the tested increment in temperatures was not enough to generate damage on the photosynthetic machinery. Fv/Fm values (above 0.75) indicate that there was no damage on the photosynthetic machinery, which emphasized that effects on the photosynthetic machinery were very low (Siam et al., 2018; Kunert et al., 2021). Also, under higher temperatures, there was not a notorious reduction in C acquisition capacity, as  $A_{\text{net}}$  or  $\Phi_{\text{PSII}}$  values did not experience a noticeable decrease under high temperatures. The low effect of temperature increase on the photosynthetic machinery and capacity can be explained by the acclimation of the seedlings to the thermic environment (Hinojosa et al., 2019; Vico et al., 2019). However, the increment of VDI never was enough to produce any mortality on seedlings, and, even complete dry shoots were detected on several seedlings, they were able to resprout. This is consistent with the high capacity to regrow of *Quercus ilex* after disturbances like fire, high temperatures, or herbivory (Martín-Forés et al., 2020; Hernando et al., 2021).

Drought intensification under the present thermic environment barely had effects on the VDI and physiological parameters of the mycorrhizal seedlings. Firstly, VDI was almost despicable under the present thermic environment and there was no increment on VDI due to the intensification of drought, with minor effects of the fungi. Although both,  $\psi_{\text{dawn}}$  and  $\psi_{\text{midday}}$ , tended to decrease as the drought intensity level increased under present summer temperatures, only a severe drought significantly increased seedlings water stress above the control level. Additionally, there were no effects on the photosynthetic capacity

(Fv/Fm) nor its performance ( $\Phi_{PSII}$  and  $A_{net}$ ). Furthermore, the small VDI was only notorious at the end of the experiment, after approximately 120 days (prolonged drought). The low effect of drought is consistent with the high tolerance to drought of *Q. ilex*, which can survive after long summer drought periods, characteristic of Mediterranean climate (García de Jalón et al., 2020; Guerrero-Sánchez et al., 2021).

Drought synergistically increased the negative effect of the high temperatures, as initially hypothesized. Consistently with our results, forests have less productivity under the stress of both factors (Lempereur et al., 2017; Oriens et al., 2019; Jin et al., 2020). At increased temperatures but without drought stress, VDI barely increased during time. However, when high temperatures coincided with drought, either moderate or severe drought, the damage increased notably through summer period. Additionally, in the future temperature environment, both severe and moderate drought levels significantly increased the chronic water stress of the seedlings. Despite the increase of VDI under both stress factors, increase in temperature and drought intensity, there were no differences between the VDI of moderate and severe drought treatments. It indicates that an increase in xericity due to climate change barely had effects even associated with the future rise of temperatures. Temperatures increment by itself increase water stress of seedlings, probably promoting acclimation process in seedlings such as increase in osmotic potential (Reddy et al., 2004) or water use efficiency (Tenhunen et al., 1990) among other acclimation mechanisms. This increase tolerance to dehydration of seedlings might contribute to reduce the effect of an increased xericity reducing differences between drought intensity treatments. Furthermore, this indicates within the factors associated with climate change, increased temperatures might play a key role in *Q. ilex* response.

### ***Tuber aestivum* as an alternative to *T. melanosporum* under climate change scenarios**

According to the hypothesis, seedlings mycorrhized with *T. aestivum* had a better performance than seedlings mycorrhized with *T. melanosporum*. In general, at every thermic environment and drought treatment, seedlings mycorrhized with *T. aestivum* had a smaller VDI than those mycorrhized with *T. melanosporum*. Furthermore, seedlings mycorrhized with *T. melanosporum* had a lower water potential than those with *T. aestivum*, and, consequently, seedlings were more dehydrated. The explanation is due to the ecology and morphology of the fungi species. *Tuber melanosporum* requires a longer

period of time to reach a complete symbiosis development in the roots than other *Tuber* spp. (Zambonelli et al., 2016; Ori et al., 2020). This suggest that mycorrhizal colonization of the new roots growth during the experiment was not achieved or it was reached in minor intensity than in *T. aestivum*. A low mycorrhizal colonization can explain lower beneficial effect of *T. melanosporum* than of *T. aestivum*. Furthermore, mycorrhizal hyphae of *T. melanosporum* are smaller than others belonging to the genus *Tuber* (Zambonelli et al., 2016; Ori et al., 2020), like *T. aestivum* (Zambonelli et al., 2016; Nahberger et al., 2021). Consequently, soil explored and water acquisition capacity might be lowered in *T. melanosporum* mycorrhizal seedlings. Finally, both species differ in ecology, being *T. aestivum* more tolerant to drought and high temperatures stress (Zambonelli et al., 2016; Ori et al., 2020). Additionally, *T. melanosporum* has been indicated as extremely sensitive to both stress factors of climate change (Büntgen et al., 2015; Thomas and Büntgen, 2019). In this experiment,  $A_{net}$  on seedlings mycorrhizal with *T. melanosporum* only decreased when both factors coincided, increased temperatures and a severe drought. Those results reaffirms that seedlings mycorrhizal with *T. melanosporum* had a worse performance, especially under water and heat stress of future climate scenarios (Baragatti et al., 2019; Thomas and Büntgen, 2019; Piñuela et al., 2021).

At present conditions, there were minor differences in performance between seedlings mycorrhizal with *T. aestivum* and seedlings mycorrhizal with *T. melanosporum*. At first, the selection of any of those *Tuber* spp. under present conditions would be adequate for truffle plantations. However, it is necessary to highlight that *T. melanosporum* has a higher monetary value than *T. aestivum*, that can reach up to 700-1200€ and 200-600€, respectively (Zambonelli et al., 2016; El Karkouri et al., 2019; Strojnik et al., 2020). Thus, *T. melanosporum* is recommended for truffle plantations under present conditions for most of the Mediterranean areas, but with the exception of the hottest and drier regions. However, under future conditions, performance of seedlings mycorrhizal with *T. melanosporum*, due to the low tolerance to stress factors of the fungus, was worse than *T. aestivum*. This worst performance would be traduced in lower truffle production (Büntgen et al., 2015; Thomas and Büntgen, 2019). Even in zones where the severity of climate is (or would be) a limiting factor, some practices can help to alleviate it, for example, controlled irrigation during summer (Chevalier and Sourzat, 2012; Piñuela et al., 2021), but at the expense of increasing maintenance costs. In the



other hand, *T. aestivum* truffles still have an elevated monetary value, making the proper forest production economically profitable without further maintenance costs. In this sense, *T. aestivum* is one of the most produced fungus in Europe, and is leading and expanding in many countries (Bragato et al., 2021). For this reason, *T. aestivum* would be a remarkable alternative to *T. melanosporum*, especially in areas with current elevated temperatures and high aridity.

### ***Next steps and future research***

The presented results are included on a bigger experiment, where a second measurement of the different variables was taken at the end of the summer. This second measurement would help to analyze the evolution of the seedlings over the time, especially when the temperatures start to decrease. These measures can give more information about the resilience of the seedlings once stress factors cease. Also, there will be a laboratory analysis of other variables that indicate stress or acclimation of the seedlings, such as the concentration compatible solutes like proline (its accumulation on seedlings indicates a tolerance response to environmental stress, like drought or high temperatures), soluble sugars and photosynthetic pigments (Verbruggen and Hermans, 2008; Moradi, 2016; Zandalinas et al., 2018). Besides, it would be necessary to analyze other physiological variables that were measured with LI-COR but not included here, such as stomatal conductance ( $g_s$ ), transpiration rate ( $T_r$ ) and  $CO_2$  concentration in the mesophyll ( $C_i$ ). Furthermore, measuring evapotranspiration of seedlings can give information about the capacity of the seedling to refrigerate, and can complete the information of the water stress in future environments. Finally, to reaffirm and confirm the results, it is necessary to quantify the evolution of mycorrhizal colonization of both fungi across treatments. For this reason, a harvest of the seedlings at the end of the experiment is essential to quantify mycorrhizal colonization but also to quantify the growth of the seedlings.

Moreover, for future research, tolerance to water stress and high temperatures should be investigated in other Mediterranean forest species that are mycorrhized with these *Tuber* species, like *Q. faginea*, or *Pinus halepensis*. Obtaining the information of its performance, we can determine the optimal distribution of different host plants-mycorrhizal *Tuber* pairs. The final aim is to include the largest number of abandoned lands, and promote ecological restoration through truffle plantations maximizing truffle production and minimizing maintenance costs.

Finally, evaluating the survival rate, the establishment and the productivity in a real truffle plantation is fundamental to know the real impact of a restoration. Consequently, it is necessary to carry out a reforestation of abandoned agricultural fields with those different plant- *Tuber* pairs and prove the success of the mycorrhized seedlings, and truffle and economic productivity under real conditions.

## Conclusions

Increment of summer temperatures was the limiting and most negative factor on the performance of the seedlings, independently of the mycorrhizal *Tuber* spp. or the drought intensity. The future temperature environment increased seedlings water stress and shoot senescence and damage (VDI) but not enough to produced seedling mortality or damage on photosynthetic machinery ( $F_v/F_m$ ) and capacity ( $\phi_{PSII}$  and  $A_{net}$ ).

The increase on drought intensity under the present temperature environment did not affect negatively the performance of seedlings but worsen seedling performance under future temperature environment. Nevertheless, the effect of drought intensity increase under the future temperature environment was antagonic, with mayor effects of the high temperatures.

Seedlings mycorrhized with *T. aestivum* had a better performance that seedlings mycorrhized with *T. melanosporum*. At every environment and drought irrigation, seedlings mycorrhized with *T. aestivum* had smaller values of VDI and higher leaf water potential than seedlings mycorrhized with *T. melanosporum*. All together, the results indicate that seedlings mycorrhized with *T. aestivum* are a more adequate alternative than *T. melanosporum* for truffle plantations, considering future climate change scenarios.

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## Supplementary material

Table S1. Fitted models to evaluate the effect on VDI and AIC values.

glmer model	AIC
thermic environment* drought /thermic environment* mycorrhizal fungi *time+(1 block/id)+(1 block)	57011
thermic environment* drought /thermic environment* mycorrhizal fungi *time+(1 block)	124055
thermic environment* drought * mycorrhizal fungi *time+(1 block)	124055
thermic environment* drought * mycorrhizal fungi +time+(1 block)	125718