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Responses and Differences in Tolerance to Water Shortage under Climatic Dryness Conditions in Seedlings from *Quercus* spp. and Andalusian *Q. ilex* Populations

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Abstract: Analyzing differences in tolerance to drought in *Quercus* spp., and the characterization of these responses at the species and individual population level, are imperative for the selection of resilient elite genotypes in reforestation programs. The main objective of this work was to evaluate differences in the response and tolerance to water shortage under in five *Quercus* spp. and five Andalusian *Q. ilex* populations at the inter- and intraspecies level. Six-month-old seedlings grown in perlite were subjected to drought treatments by withholding water for 28 days under mean 37 °C temperature, 28 W m^{-2} solar irradiance, and 41% humidity. The use of perlite as the substrate enabled the establishment of severe drought stress with reduction in water availability from 73% (field capacity) to 28% (dryness), corresponding to matric potentials of 0 and −30 kPa. Damage symptoms, mortality rate, leaf water content, photosynthetic, and biochemical parameters (amino acids, sugars, phenolics, and pigments) were determined. At the phenotypic level, based on damage symptoms and mortality, *Q. ilex* behaved as the most drought tolerant species. Drought caused a significant decrease in leaf fluorescence, photosynthesis rate, and stomatal conductance in all *Quercus* spp. analyzed, being less pronounced in *Q. ilex*. There were not differences between irrigated and non-irrigated *Q. ilex* seedlings in the content of sugar and photosynthetic pigments, while the total amino acid and phenolic content significantly increased under drought conditions. As a response to drought, living *Q. ilex* seedlings adjust stomata opening and gas exchange, and keep hydrated, photosynthetically active, and metabolically competent. At the population level, based on damage symptoms, mortality, and physiological parameters, the eastern Andalusian populations were more tolerant than the western ones. These observations inform the basis for the selection of resilient genotypes to be used in breeding and reforestation programs.

Keywords: *Quercus* spp.; *Quercus ilex*; drought; climate change; abiotic stress; biodiversity; perlite

1. Introduction

The genus *Quercus* comprises around 300 species, 25 of which are native in Europe, and 9 in the Iberian Peninsula [1]. *Quercus* spp. are distributed in a wide variety of habitats such as temperature and subtropical forests, varying from wet to extremely dry habitats [2]. Within this genus, *Quercus. ilex* constitutes the most representative species in the Mediterranean forest and agrosilvopastoral ecosystem “*dehesa*” with a high environmental and economic relevance [3]. However, the current situation of the species is worrying due to increased tree mortality observed in recent decades [4]. Tree mortality is associated with several factors of an anthropogenic or natural origin, most pertinent being biotic and abiotic stresses [5–7]. Although Holm oak (*Q. ilex*) is considered a species that is well-adapted to a dry climate [8–10], drought stress is the main cause of *Q. ilex* seedling mortality in forest plantations, especially in Andalusia, where this stress is a limiting factor in its regeneration [11–14]. Moreover, the situation could become even worse in a climate change scenario [4,15], in which simulation models predict an increase both in temperature and frequency of severe drought episodes [16,17].

Plant responses to drought stress at the physiological and molecular levels have been widely studied in a wide variety of forest species [18–20]. Some studies have been carried out to determine the response to drought in some species of the genus *Quercus* from a morphological and physiological point of view [14,21,22]. Under drought conditions, species of the genus *Quercus* have developed drought-tolerance mechanisms such as a deep and well-structured root system to maintain a relatively high predawn potential, resistance of the xylem to cavitation and embolism, and stomatal closure [23,24]. The acorn size and reserve nutrients are also related to survival and growth under adverse conditions. Quero et al. [25] reported beneficial effects of larger seed in *Quercus* seedlings, such as seedling establishment in nutrient poor soils and longer roots that favor the survival of seedlings in the first summer drought. Regarding acorn reserve nutrients, Villar-Salvador et al. [26] revealed that most of the nitrogen (N) accumulated in holm oak seedlings came from the acorn at the end of the second shoot flush of growth (three months after shoot emergence), being only 25–38% of the N taken up by the roots. In contrast, acorn N reserved in *Quercus robur* are reduced at the end of the first shoot flush of growth [27].

Studies with Mediterranean *Quercus* species indicate that genetic variation may have contributed to the large differences in drought tolerance and within populations [28–30]. Ramírez-Valiente et al. [30] reported a high degree of inter- and intrapopulation variability for traits related to drought tolerance, and Arend et al. [28] showed similar results in *Q. robur*, *Quercus petraea*, and *Quercus pubescens.* However, the selection of drought tolerant individuals based on the intraspecies variability is still limited. For instance, the identification and characterization of plus or elite *Q. ilex* genotypes, which are more resilient and tolerant to drought, is a priority in plant breeding, reforestation, management, and conservation programs in the Mediterranean area. To enable this, ecophysiological and molecular studies are required to have a better understanding of both the variability in this species and the selection of morphometric, physiological, and molecular markers [31].

Thus, in this study, we have evaluated the effect of drought stress in six-month-old seedlings to determine differences in the response and tolerance to this stress in a total of five species of the genus *Quercus*, and five Andalusian *Q. ilex* populations. Six-month-old seedlings were selected due to the negative impact of first summer drought in the seedling mortality. Differences from previous published studies are four-fold: (i) The experiments were conducted under severe drought conditions representative of the summer conditions of southern Spain, and those predicted in a climate change scenario, characterized by high temperatures, radiation, and low humidity; (ii) The use of seedlings grown in perlite which enables the imposition of a rapid and severe drought stress; (iii) the simultaneous comparison, in a single experiment, of five *Quercus* spp. and five Andalusian *Q. ilex* populations; and (iv) the identification and characterization of drought tolerant individuals within species variability from a physiological and biochemical point of view.

2. Materials and Methods

2.1. Study Area

Drought experiment was conducted on five *Quercus* spp. ((*Q. pyrenaica*, *Q. faginea*, *Q. robur*, *Q. suber*, and *Q. ilex*) and five *Q. ilex* populations from Andalusia (Gr: Arenas del Rey (Granada); Ja: Sierra de Segura (Jaen); Se: Almaden de la Plata (Seville); Co: Pozoblanco (Cordoba) and Ca: Behamahoma (Cadiz)) in Cordoba, Andalusia, Southern Spain at 37°54′46″ N, 4°43′15″ O in July 2018 for 28 days (Table 1). Cordoba is under Mediterranean climate with 36 °C and 19 °C maximum and minimum temperatures, respectively, 28 W m-2 solar irradiance, and 42% relative humidity in July over the period 2005–2019.

Table 1. Environmental features of the *Quercus* spp. and Andalusian *Q. ilex* populations used. Altitude (Meters Above Sea Level–MASL), average temperature of the coldest month (T_{min}), average temperature of the warmest month (T_{max}), and average annual rainfall (P) [29]. ES code indicates the region of origin as detailed in "*Centro Nacional de Recursos Genéticos Forestales, El Serranillo*"). Localization of all species is included in Figure S1.

Species	Location	MASL (m)	T_{max} (°C)	T_{min} (°C)	P (mm)
Quercus robur	ES01	488	24.8	2.2	1375
Quercus faginea	ES10	1032	27.6	−0.1	577
Quercus pyrenaica	ES08	823	24.5	−1.8	792
Quercus suber	ES01	456	33.7	2.4	824
Quercus ilex	ES11	506	34.5	2.6	635
Quercus ilex (Jaen)	38°17′ N, 2°36′ W	643	23.1	4.4	795
Quercus ilex (Granada)	36°57′ N, 3°54′ W	489	24.7	11.5	489
Quercus ilex (Cordoba)	38°22′ N, 4°54′ W	618	26.8	8.1	613
Quercus ilex (Cadiz)	36°45′ N, 5°27′ W	649	24.9	9.8	1264
Quercus ilex (Seville)	37°52′ N, 6°05′ W	482	26.4	9.5	722

2.2. Plant Material, Growth Conditions, and Drought Treatment

Acorns from different *Quercus* spp., and Andalusian *Q. ilex* populations from were used. The populations were chosen according to Valero-Galván, et al. [9] and Fernández i Marti, et al. [32]. Healthy acorns were selected, germinated, and sown leaving acorns in the development of seedlings, in black plastic pots (3L, 14.5 × 14.5 × 22 cm) containing perlite (Gramoflor GmbH & Co. KG Diepholzer Strabe 173, Vechta, Germany), and grown in a greenhouse as previously reported [33]. All the seedlings were germinated in January 2018 and irrigated every two days with approximately 200 mL of tap water per pot and once a week with a Hoagland nutrient solution [34]. To achieve a severe and rapid drought stress, perlite was used as the substrate, an alumino-silicate of volcanic origins characterized by a closed cellular structure. Severe drought was imposed by withholding water for 28 days under the following environmental conditions: mean values of 46 °C and 22 °C maximum and minimum temperatures, 28 MJ m^{-2} per day^{-1} solar irradiance, and 41% relative humidity. The experiment was performed based on a completely randomized design with ten biological replicates per treatment to analyze damage symptoms and mortality rate caused by drought (in total 200 seedlings). Out these biological replicates, three individuals were randomly selected for measuring relative leaf water content, physiological parameters, or biochemical analyses (in total 60 seedlings). *Quercus ilex* was considered as the mean of all the Andalusian populations analyzed.

2.3. Perlite Water Content and Matric Potential

Perlite water content (PWC) was estimated by weighing pots containing dry or totally wet (field capacity) perlite as well as the pots corresponding to the different days of the experiment. The following formula was employed:

$$\text{PWC}_t\ (\%) = (\text{pot wet weight}_t - \text{pot dry weight}/\text{pot wet weight}_0 - \text{pot dry weight}) \times 100 \quad (1)$$

where t corresponds to the different days, and 0 to the initial, maximum value.

Perlite matric potential (Ψ_m, kPa) was measured by using a tensiometer (Soil Moisture Equipment corp, Santa Barbara, CA, USA) at 15-cm depths. Both parameters were plotted, and a correlation equation obtained.

2.4. Damage Symptoms and Seedling Mortality

Damage symptoms (leaf chlorosis, wilting, and senescence) caused by the drought treatment was visually observed throughout the whole experiment. Photographs were taken with a digital camera to register damages. Visual damage symptoms were quantified every three days according to a 0–5 scale where 0 = no leaves showing symptoms; 1 = one or two leaves showing slight drought symptoms (necroses along edges and/or veins; changes in the color of foliage (light green, yellow and brown); and/or irregular spot changes); 2 = most leaves showed slight levels of drought symptoms however one or two leaves still showed no symptoms; 3 = all leaves showed drought symptoms but these were not severe; 4 = all leaves showed severe drought symptoms (leaves showed a totally dry-yellow aspect); and 5 = the whole seedling showed wilting and/or fall of leaves. The number of dead seedlings was also recorded when they were classified in 4–5 scale and Fv/Fm values near 0.

2.5. Leaf Water Status

Relative leaf water content (RLWC) was calculated at day 25 from fresh (FW), turgid (TW), and dry (DW) weights. Upon leaf removal (three living seedlings per treatment and one non-damaged leaf per seedling), FW was determined, then leaves were soaked in distilled water at 25 °C for 24 h and weighted again to record the TW. For dry weight measurements, leaves were kept in an oven at 65 °C for five days until weight remained constant. RLWC was calculated according to the formula:

$$\text{RLWC}\ (\%) = ((\text{FW} - \text{DW})/(\text{TW} - \text{DW})) \times 100. \quad (2)$$

2.6. Quantum Yield of Photosystem II, Net Photosynthesis Rate, and Stomatal Conductance

Leaf fluorescence and derived photochemical efficiency of photosystem II (F_v/F_m) in dark-adapted seedlings was measured regularly with a portable fluorometer (FluorPen FP100, Photon Systems Instruments, Drásov, Czech Republic) [35]. Three values per seedling in the youngest fully expanded leaves were taken every three days in the early morning, when the leaves were adapted to darkness throughout the night according to Strasser et al. [36]. All measurements were carried out in the same seedlings throughout the experiment. The net photosynthesis rate (A, µmol CO_2 m^{-2} s^{-1}) and stomatal conductance (Gs, mol H_2O m^{-2} s^{-1}) were measured in three fully-expanded leaves in the *Quercus* spp., at day 9, and in Andalusian *Q. ilex* populations at day 21 by using a portable infrared CO_2 gas analyzer (LiCor Li6400XT, Li-Cor, Inc.; Lincoln, NE, USA) fitted with a 6-cm^2 leaf cuvette. The measurements were taken using a CO_2 concentration of 400 ± 1.7 ppm, a flow of 300 ± 1.2 cm^3 min^{-1}, and PPFD > 1000 mol (photons) m^{-2} s^{-1}. All the measurements of physiological variables were taken between 12:00–14:00 h UTC (Universal Time Coordinates), considering a 2 h window around the solar noon (12:00–14:00 h CET—Central European Time) [7].

2.7. Photosynthetic Pigment and Anthocyanin Analyses

Leaves from the *Q. ilex* Se population (three biological replicates) were used to quantify the total content of chlorophylls (a and b), carotenoids, and anthocyanins according to Sims and Gamon [37], at day 25 of the experiment. Briefly, they were extracted by using two solutions, the first being acetone:Tris (1M) pH 7.8 (80:20 *v*/*v*) and the second methanol:1% HCl:water (90:1:1, *v*/*v*/*v*)). Supernatants, after homogenization and centrifugation, were used to read absorbance at 663, 647, 537, and 470 nm (Thermo Scientific Evolution 201 UV–Visible Spectrophotometers), from which the pigment and anthocyanin contents were calculated.

2.8. Total Sugar, Phenolic, and Amino Acid Analysis

Leaves from seedlings of the *Q. ilex* Se population (three biological replicates) were used to quantify total sugar, phenolic, and amino acid content at day 25 of the experiment, by using, respectively, 3, 5-dinitrosalicylic acid (DNS) [38], Folin-Ciocalteu [39] and ninhydrin [40] methods. Briefly, all samples were extracted using chloroform/methanol/water (1:2.5:0.5, *v*/*v*/*v*). Reference standards of glucose, chlorogenic acid, and glycine were used for quantitation, respectively. Supernatants, after homogenization and centrifugation, were used to read absorbance at 570 nm (sugars and amino acids) and 765 nm (phenolics) (Thermo Scientific Evolution 201 UV–Visible Spectrophotometers).

2.9. Statistical Analyses

All the statistical analyses were performed using STATISTIX 10.0 software (Analytical Software, Tallahassee, FL, USA). The following statistical tests were employed: Student's t ($p < 0.05$) was used for total phenolic, sugar, amino acid, and pigment values and for the photosynthesis rate and stomatal conductance data. When the homogeneity of variance was not satisfied, the Kruskal–Wallis´ test was used; Kruskal–Wallis ($p < 0.05$ and means separated by the Dunn test at $p < 0.05$) for damage symptoms; One-way ANOVA (analysis of variance) ($p < 0.05$ and means separated by the post hoc least significant difference, LSD, test at $p < 0.05$) for F_v/F_m and photosynthesis rate and stomatal conductance parameters in non-irrigated seedlings at species and population levels. Both in the Kruskal–Wallis and the one-way ANOVA tests, the area under the curve for damage symptoms and F_v/F_m was calculated. Two-way ANOVA ($p < 0.05$ and means were separated by the LSD test at $p < 0.05$), using species/populations and treatment as factors, for RLWC. The Levene´s and Folded F tests were used to determine the homoscedasticity of the variables for ANOVA and *t*-test, respectively.

3. Results

3.1. Perlite Water Content and Matric Potential

Perlite water content (PWC) percentages decreased from 73% (field capacity) at zero time to 28% at day 28, which corresponded to matric potentials, as determined by tensiometry, of, respectively, 0 and −30 kPa. A linear regression was obtained for both parameters with equation

$$\Psi_m \text{ (kPa)} = -51 + 0.68 \text{ PWC (\%) } (R^2 = 0.981) \tag{3}$$

Drought caused a linear decrease in perlite water content, with experimental data fitting in the equation

$$\text{PWC (\%)} = (0.71 - 0.015 \text{ days}) \times 100 \; (R^2 = 0.992) \tag{4}$$

or

$$\Psi_m \text{ (kPa)} = 1.48 - 1.20 \text{ days } (R^2 = 0.990) \tag{5}$$

3.2. Evaluation of Damage Symptoms and Seedling Mortality

Neither chlorosis nor wilting was observed in irrigated seedlings. Leaf damage symptoms occurred to differing degrees in non-irrigated seedlings depending on the surveyed species and populations. The first *Quercus* species displaying visible stress symptoms in the foliage were *Q. faginea* and *Q. pyrenaica*, beginning with yellow-brown necrosis at the edges and tips of the youngest fully expanded leaves, followed by leaf wilting and fall. *Quercus robur* displayed an intermediate behavior, while *Q. ilex* and *Q. suber* had both individuals with slight (most leaves showed drought symptoms but these were not severe) or serious (all leaves showed a totally dry-yellow aspect and wilting) damage symptoms at the end of the experiment (Figure 1A, Figure S2). At day 12 (57% PWC and −13 kPa Ψ_m values), 100% mortality was recorded for *Q. faginea* and *Q. pyrenaica*, and at day 28 (28% PWC and −32 kPa Ψ_m values), Q. *robur* had 100% and, *Q. suber* and *Q. ilex* 40% mortality rates. In the Andalusian *Q. ilex* populations analysed, from seedlings without symptoms to seedlings with all leaves showed a totally dry-yellow aspect and wilting were observed in leaf symptoms, and the number of dead seedlings observed varied between 40% and 80% in Ja and Ca populations, respectively, at day 28 (Figure 1B).

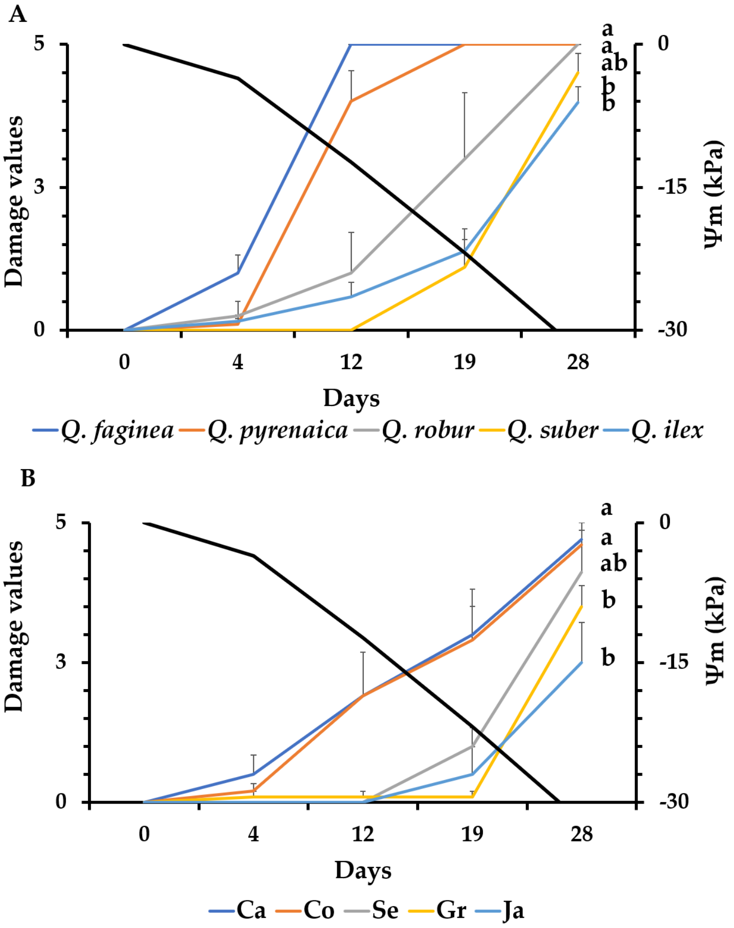

Figure 1. Damage symptoms in drought-stresses seedlings of *Quercus* spp. (**A**) and *Q. ilex* Andalusian population (**B**) throughout the experiment. Symptoms were quantified in a 0–5 scale based on visual evaluation (see material and methods section). Data are mean of 10 biological replicates ± standard error (SE). The same letter indicates that there is no statistical difference between populations ($p = 0.05$). The black line corresponds to the theoretical Ψ_m values (Ψ_m (kPa) = −51 + 0.68 PWC (%)). Ca: Behamahoma (Cadiz); Co: Pozoblanco (Cordoba); Se: Almaden de la Plata (Seville); Ja: Sierra de Segura (Jaen).

3.3. Relative Water Content in Q. ilex Leaves

Mean values in the irrigated and non-irrigated seedlings ranged between 76.6–86.3% and 43.1–70.7%, respectively (Figure 2). Drought caused a statistically significant reduction in RLWC ($F = 51.93$; $p = 0.0000$), with differences among populations ($F = 3.38$; $p = 0.0313$) (Figure 2). Under drought conditions, Gr and Ca populations showed the highest and lowest RLWC.

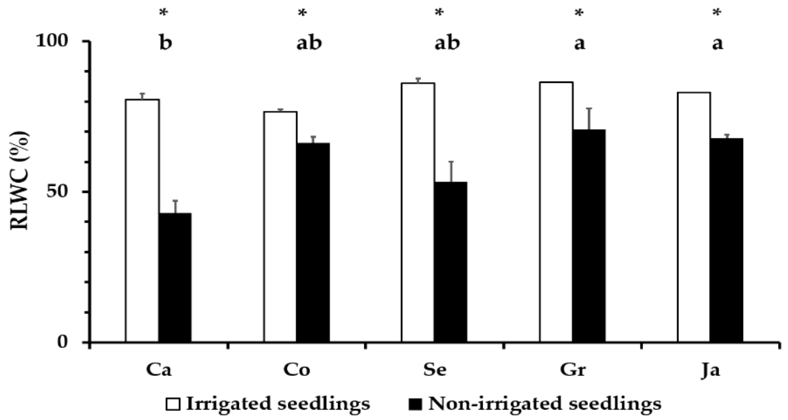

Figure 2. RLWC (Relative leaf water content) determined in seedlings of the Andalusian *Q. ilex* populations at day 25. Values are mean ± SE of three biological replicates. Statistically significant differences were observed between irrigated and non-irrigated seedlings. Different letters indicate that there is significant difference among populations ($p = 0.05$). Asterisk indicates significant differences between irrigated and non-irrigated seedlings (* $p < 0.05$).

3.4. Quantum Yield of Photosystem II (F_v/F_m)

Leaf fluorescence and derived PS II quantum yield at dark-adapted state (F_v/F_m) remained nearly constant (between 0.60 and 0.80) throughout the experiment in irrigated seedlings both in *Quercus* spp., and Andalusian *Q. ilex* populations (Figure S3).

In non-irrigated seedlings, the general tendency observed was a progressive reduction in F_v/F_m values throughout the drought period (Figure 3). This occurred first in *Q. faginea* and then in *Q. pyrenaica which both* showed a sudden and rapid decline to zero values at day 6 (PWC and Ψ_m values of 66% and −6 kPa, respectively), and 10 (PWC and Ψ_m values of 60% and −10 kPa, respectively), respectively (Figure 3A). A less pronounced and slower decrease was observed for *Q. robur* and *Q. suber*, with values of 0.15 and 0.18 determined at day 27, respectively (PWC and Ψ_m values of 29% and −31 kPa, respectively). In the case of *Q. ilex*, significant differences were observed between irrigated and non-irrigated seedlings ($F = 0.48$; $p = 0.0007$); however, no significant differences were observed between populations ($F = 0.65$; $p = 0.9986$). *Quercus ilex* showed the highest F_v/F_m values in non-irrigated seedlings, with values in the range of 0.31–0.49 measured at day 27 (PWC and Ψ_m values of 29% and −31 kPa, respectively), and depending on the population, with the lowest and highest values corresponding to the Ca and Ja populations, respectively (Figure 3B).

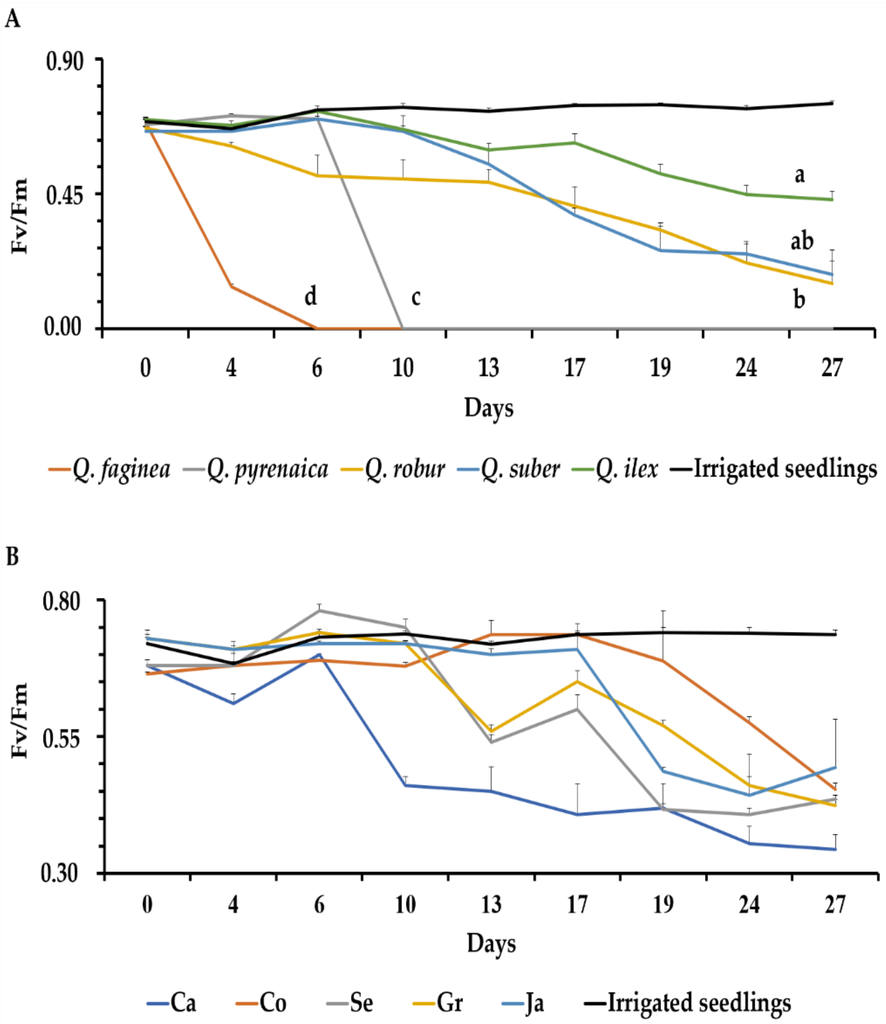

Figure 3. Measurements of quantum yield of photosystem II (F_V/F_m) in dark adapted leaves from *Quercus* spp., (**A**) and *Q. ilex* interpopulation species (**B**) (non-irrigated seedlings) during drought progression. Values are mean ± SE of three biological replicates. In *Quercus* spp., the same letter indicates no significant difference between species ($p = 0.05$). The black line indicates the mean values of irrigated seedlings shown in Figure S3. The lack of letters in Andalusian *Q. ilex* populations indicates no significant differences between populations.

3.5. *Leaf Photosynthesis Parameters*

Living *Q. ilex* non-irrigated seedlings showed higher A and Gs values than the rest of the species of the genus *Quercus* (Figure 4A,B). Significant differences in A and Gs were observed between irrigated and non-irrigated seedlings in all the species analyzed, except *Q. ilex*, with significant differences for Gs (Figure 4; Table S1) but none for A. As for Andalusian *Q. ilex* populations, at day 9, significant differences were observed between treatments in the Co and Ja populations in *A* and in the Co, Gr, and Ja populations in Gs (Figure 4C,D; Table S1). Under drought conditions, Se and Ca populations showed the highest values in A and Gs, respectively (Figure 4C,D). At day 21, drought caused a decrease in A and Gs values in all populations (Figure 4D,E; Table S1). In A and Gs, under drought conditions, Ca showed the highest values in both parameters, and Se gave the lowest A values of the populations (Figure 4D,E; Table S1).

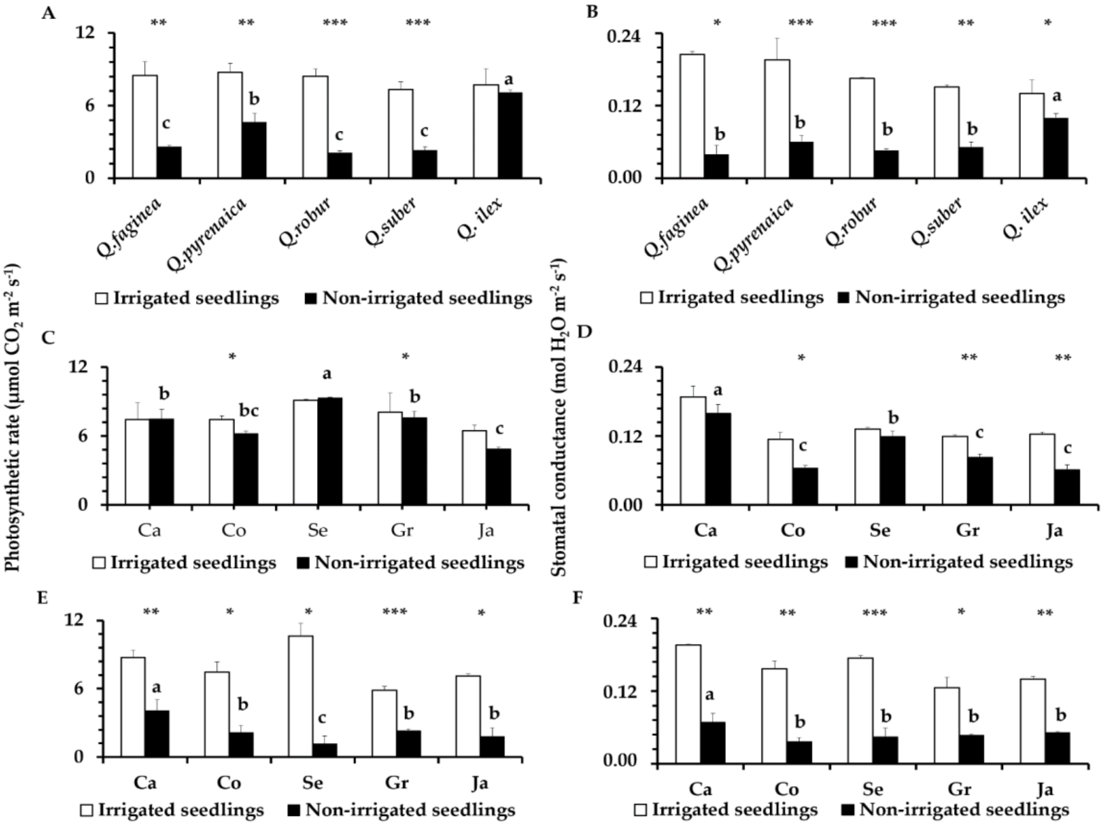

Figure 4. Photosynthesis rate, A, and stomatal conductance, Gs, in seedlings of the surveyed *Quercus* spp. and Andalusian *Q. ilex* populations, taken at day 9 (species (**A** and **B**, respectively) and populations (**C** and **D**, respectively) and day 21 (populations (**E** and **F**, respectively)). Values are mean ± SE of three biological replicates. Asterisk indicates significant differences between treatments (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). In the non-irrigated seedlings, the same letter indicates that there is no significant difference between species or populations ($p = 0.05$).

3.6. Pigment Content and Anthocyanins Analysis

Figure 5A shows both photosynthetic pigment and anthocyanin content in *Q. ilex* Se seedlings at day 25 under drought conditions. No significant differences were observed between irrigated and non-irrigated seedlings in photosynthetic pigments, with mean value ranges being 0.21–0.22 μmol/g (chlorophyll a), 0.06–0.07 μmol/g (chlorophyll b), and 0.13–0.15 μmol/g (carotenoids). Anthocyanin content was significantly lower in the non-irrigated than the irrigated seedlings, with mean values of 0.07 and 0.09 μmol/g, respectively ($T = 3.50$; $p = 0.0249$) (Figure 5A).

3.7. Amino Acid, Total Phenolic Compound, and Sugar Content Analysis

Figure 5B shows the primary metabolism in *Q. ilex* Se seedlings at day 25 under drought conditions. Mean values ranged between 4.21 and 7.90 μmol/g dry weight (amino acids), 33.14 and 37.29 μmol/g dry weight (phenolics), and 84.66 and 131.19 μmol/g dry weight (sugars). Drought caused a significant increase in the three parameters (Figure 5B, $T = -2.92$; $p = 0.0434$, $T = -3.73$; $p = 0.0202$, and $T = -3.74$; $p = 0.0201$, respectively).

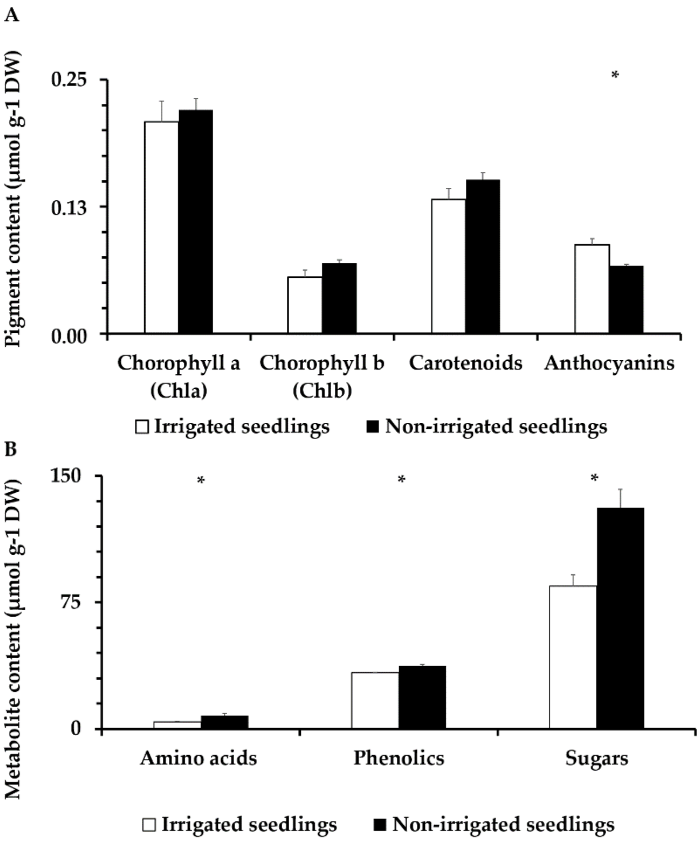

Figure 5. Photosynthesis pigment (chlorophyll (a and b) and carotenoids) and anthocyanins (**A**) and metabolite (phenolics, sugars, amino acids) (**B**) contents in leaves of the *Q. ilex* Se population. Values are mean ± SE of three biological replicates at day 25. Asterisk indicates significant differences between irrigated and non-irrigated seedlings (* $p < 0.05$).

4. Discussion

By utilizing a successful germination procedure [33] with an innovative use of perlite under typical summer conditions reached in Southern Spain, a quick and severe drought stress was achieved in this study. The imposition of these stress conditions in a reasonable experimental time (1 month) is necessary for species that like *Q. ilex* are highly tolerant and essential for subsequent molecular analysis.

Reduced soil water content and reduced matric potential were directly associated with plant drought stress; factors associated with plant growth [41,42]. Aliniaeifard et al. [43] reported better vegetative and physiological characteristics (root fresh weight, root dry weight, leaf number shoot:root ratio, among others) in perlite compared to a sandy loam farm soil. In another study, a comparison of several substrates (tuff, sand, peat moss, lightweight expandable clay aggregate (LECA), cocopeat) determined that tuff, LECA, and perlite have a higher ability to tolerate drought [44]. Perlite retains most water superficially, and releases it slowly at a relatively low tension, which therefore requires frequent irrigation to prevent a fast-developing water stress [45]. Thus, in our experiments, water content decreased from 73% (field capacity) at day 0 to 28% at day 28, corresponding to matric potentials of, respectively, 0 and −30 kPa, with high correlation between both parameters (Ψ_m (kPa) = −51 + 0.68 PWC (%) ($R^2 = 0.981$)). Moreover, this substrate could be used as an alternative to hydroponics as it may mimic a higher mechanical impedance to root growth and allow a higher control of water and nutrient conditions than in soil [46]. This substrate is more commonly used in herbaceous and horticultural species (barley [47], gerbera [48], and tomato [49]), and in other woody (citrus [50,51] and shrub species (rose [52])). However, we show that perlite could be also considered as a useful substrate to induce a drought stress in forest trees, as it enables good control of treatments [53].

4.1. Drought Visual Symptoms and Mortality Rate as Indicators of Drought Tolerance

Both leaf damage and seedling death were higher and more rapid in the deciduous oak species (*Q. pyrenaica*, *Q. faginea*, and *Q. robur*) than in the evergreen oak species (*Q. suber* and *Q. ilex*), as previously reported by [54]. Both leaf morphology and structure may be related to different mechanisms to cope with tolerance to drought. Previous studies have demonstrated that the reduction in leaf size is associated with dry habitats [55,56]. This fact could explain the delay in leaf damages and lower number of dead seedlings observed in *Q. ilex* and *Q. suber* since both species have the smallest leaf sizes of all *Quercus* spp., analyzed in this study. In fact, leaf reduction is proposed as one the most relevant traits to withstand water deficit [56]. On the other hand, the feature of sclerophyllous leaves is considered as a functional adaptation to tolerate water stress under drought conditions [57,58]. This feature is not present either *Q. pyrenaica* or *Q. faginea* or *Q. robur*, which could also justify a higher survival in *Q. ilex* and *Q. suber* under drought conditions.

Regarding *Q. ilex* interpopulation variability, eastern populations (Ja and Gr) showed a lower damage and mortality than western ones (Ca, Co, and Se) (Figure 1), in agreement with the results observed by [29]. At plant individual level, not all the *Q. ilex* seedlings from the same population had a homogeneous behavior, and both live and dead individuals in different percentages were observed at the end of the experiment (around 70% in Ca, Co, and Se; and 50% in Gr and Ja). These data show the existence of neighboring individuals with different phenotypes in terms of abiotic stress tolerance. Thus, the percentage of live and dead individuals differing between populations should be used as an indication of the response to this abiotic stress in this species. The use of this indicator together with the quality of microhabitats would increase the survival of *Q. ilex* seedlings in the field. Seedlings located under the canopy of oaks, pines, or adult pines in afforestation stands showed higher seedling growth than tall shrub and open sites, indicating that sheltered microhabitats are more suitable for oak establishment [59].

4.2. Leaf Water Status in Droughted Q. ilex Seedlings as a Physiological Parameter of Tolerance Differences

RLWC as a parameter of plant water status, has been related to drought tolerance in *Quercus* [60]. We did not observe differences in irrigated seedlings among populations. Water withholding caused a decrease in RLWC determined at day 25 to 43.1–70.1% depending on the population. Gr and Ca populations showed the highest and lowest RLWC, which in agreement with Valero-Galván, et al. [9]. However, our results differ from those by Villar-Salvador, et al. [12] who found no changes in RLWC in non-irrigated *Q. ilex*. As stated above, the climate conditions beyond the watering regime will also determine the effect and the response to drought. In any case, it has been reported that *Q. ilex* has a high capacity of maintaining leaf turgor under severe drought [14].

4.3. Photosynthesis Parameters in Droughted Quercus Seedlings and Tolerance Differences

The F_v/F_m parameter reflects the amount of light energy required for photosynthesis that is used as a parameter of response to drought stress and tolerance as different studies have revealed [60,61]. As expected, irrigated seedlings showed optimal values for different plant species of around 0.83 [62]. Under water deficit, a decrease in photosynthesis—and hence demand in ATP and NADPH—occurs, which is accompanied by a decrease in electron flux from PSII to the quinone acceptor, thus reducing F_v/F_m value [63]. This is the pattern observed in the present work and reported in previous publications for *Q. coccifera*, *Q. pubescents*, and *Q. ilex* [60,64–66]. Clear differences among *Quercus* spp. were observed. Thus, *Q. faginea* and *Q. pyrenaica* seedlings showed F_v/F_m values near zero at, respectively, days 6 and 10, as was also observed above in the damage appearance and dead seedlings. As expected, dead oaks grouped in 5 scale did not show F_v/F_m values. At the end of the experiment, a 70% and 40% reduction in F_v/F_m took place in *Quercus robur*/*Q. suber* and *Q. ilex*. F_v/F_m has been employed as a parameter of *Quercus* performance under drought conditions [60,67], although in some cases different results when comparing species, e.g., *Q. ilex* and *Q. suber*, have been reported [68]. This can be due to either the severity of the stress, as well as the genotype, developmental stage, or the climate conditions employed. Thus, Méthys, et al. [65] determined that F_v/F_m was only affected when oak seedlings are subjected to an intense drought, with leaf predawn water potential < −4 MPa, a value that is rarely observed on mature trees, and Quero, et al. [69] have clearly shown that irradiance affects photosynthesis performance under drought conditions. By using the F_v/F_m parameter, we did not find statistically significant differences among *Q. ilex* populations. This can be interpreted as alive non-damaged seedlings, independently of its geographical origin showed similar responses to drought conditions, at least at the photosynthetic level.

Lack of water generally induces stomata closure, causing a decrease in the photosynthesis rate and stomatal conductance [70], factors related to drought tolerance [71]. The quantitative response depends on the species analyzed [72]. In the present study, at day 9, A and Gs decreased in the non-irrigated seedlings for all the *Quercus* spp., except in *Q. ilex*, where A values did not show significant differences between irrigated and non-irrigated individuals. *Quercus ilex* recorded higher values of A and Gs than the rest of the species analyzed in this study, indicating that *Q. ilex* delays the stomatal closure under drought conditions [73,74], correlating with the highest level of tolerance for this species observed in visual damages, dead seedlings, and F_v/F_m values.

Net photosynthesis rate and stomatal conductance were reduced under drought conditions at different degrees on the surveyed *Q. ilex* populations, with statistically significant differences among them. At day 21, Ca showed the highest A and Gs, and Se the lowest A one (Figure 5E,F). The other populations had intermediate values for both parameters. The different behavior previously discussed of eastern and western populations was not observed while using these two parameters. The Ca population, where most dead plants were observed, correlated with the lowest leaf water content and the highest stomatal conductance, thus suggesting it to be the least tolerant population amongst the studied ones.

4.4. Leaf Chemical Composition in Se *Q. ilex* Seedlings

Under drought conditions, all the compounds analysed remained in the range of those corresponding to irrigated seedlings (Figure 5), indicating a correct function of the metabolism and metabolic homeostasis. There were no statistically significant differences in the content of photosynthetic pigments, chlorophylls, and carotenoids between treatments, suggesting little or no damage to the photosynthesis apparatus [75,76]. This observation could be species, genotype and/or experiment dependent, as in a previous study, a decrease in chlorophyll and an increase in carotenoids was reported for three *Quercus* spp., *robur, coccifera, and ilex* [77].

An increase in the content of sugar, amino acids, and total phenolics was observed, which is considered an expected effect in those species prone to drought [78]. These compounds contribute to plant responses to biotic and abiotic stresses and to the survival of the seedlings, preventing water loss and enhancing osmoprotection under drought conditions [78]. The increase in the sugars, which act as osmolytes, could be explained by the mobilization of seed starch [79,80]. Previous studies showed that starch reserves were depleted in the conversion to soluble sugars during drought [81]. Amino acids, such as proline and glycine, have been described as active osmotic compounds, and their increase in response to drought has been previously reported in *Q. ilex* [79].

Several phenolic compounds with an antioxidant function have been described in *Q. ilex*, such as gallic acid, isoloquiritigenin, or catechin [82]. Our study found a significant increase in the content of phenolics in the non-irrigated seedlings, as previously reported [78]. This induction could be directly related to a direct response to scavenge the increase in the levels of reactive oxygen species caused by drought. However, anthocyanins displayed a significant decrease under drought conditions that was contrary to previous published results [77], although a direct relationship between anthocyanin accumulation and drought tolerance does not always happen [83].

4.5. Management Implications

These results highlight the relevance of identifying drought tolerant seedlings for reforestation purposes in the Mediterranean area. The eastern Andalusian *Q. ilex* populations would be considered as better candidates for the selection of plant material as they were more tolerant to drought than the western ones. By using the described methodology, a quick screening for tolerance with a high number of individuals can be done at the nursery 6 months after acorn harvesting (June-July, when temperature is high) and a few months before transplanting to the field (October-November). At the same time, a hardening effect can be possible. In addition, it is used in our research group for analyzing multi-stress responses (e.g., drought and *Phytophthora*), and collecting plant tissue (root and leaf) for molecular studies in order to identify key genes and gene products implicated in the response.

5. Conclusions

Summer environmental conditions reached in Southern Spain (water withholding, high temperature, solar irradiance, and low humidity) together with the novel use of perlite as the growth substrate), allowed the imposition of a rapid and severe drought stress, which enabled us to establish the differences in drought tolerance among *Quercus* spp. and the variability among *Q. ilex* populations. Out of the species evaluated, *Q. ilex* behaved as the most tolerant species to drought, as deduced from the analysis of its visual symptoms, leaf water status, photosynthesis parameters, and leaf chemical composition. The *Q. ilex* seedlings that survived under intense drought stress for 28 days adjusted its physiology and metabolism to cope with arid conditions. These seedlings responded with a delay in stomata closure, well hydrated leaves, and less pronounced damages in the photosynthetic apparatus that allowed to be photosynthetically active. Even under severe drought stress, the photosynthetic machinery was not altered, as demonstrated by no significant changes in the concentration of photosynthetic pigments. Also, an increase in the levels of sugars and amino acids were observed, supporting active metabolism and metabolite homeostasis. Differences in tolerance among *Q. ilex* populations were deduced from visual estimation of damage and seedling mortality, with the eastern being more tolerant than the western ones. This difference was not clearly manifested at the leaf water status and photosynthesis parameters, thus indicating that living, non-damaged seedlings showed a similar pattern, independently of their geographical origin. Therefore, either inter- or intrapopulation variability in drought tolerance do exist, with differences among populations determined by the percentage of tolerant individuals. This present study suggests several indicators (damage symptoms, mortality rate, leaf water content, photosynthetic, and biochemical parameters) that can be used in the selection of resilient genotypes in breeding and reforestation programs, especially under climate change conditions. The probability of a seedling to survive under drought conditions will depend largely on the intraspecies genetic variability. Thus, in a reforestation program, a previous analysis of survival in 6-month-old seedlings under drought conditions will allow to know those individuals that will tolerate the first summer drought since *Quercus* species are very vulnerable to stressed conditions during their early stages of life.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/11/6/707/s1, Figure S1: Localization of all *Quercus* spp., and Andalusian *Q. ilex* population used in this study, Figure S2: Visual evaluation of damage symptoms in the two most contrasting species: *Q. pyrenaica* (A) and *Q. ilex* (B). Pictures were taken at days 1 and 9 in *Q. pyrenaica* and at days 1, 9, and 27 in *Q. ilex*; whereas all the seedlings for *Q. pyrenaica* were dead, no clear damage symptoms were observed in any of the *Q. ilex* seedlings, Figure S3: Measurements of quantum yield of photosystem II (Fv/Fm) in dark adapted leaves from *Quercus* spp., (A) and *Q. ilex* interpopulation species (B) (irrigated seedlings) during drought progression. Values are mean ± SE of three biological replicates, Table S1: Statistical values obtained in leaf photosynthesis parameters in *Quercus* spp. (A) and Andalusian *Q. ilex* populations (B) at day 9, and Andalusian *Q. ilex* population (C) at day 21. Asterisk (*) indicates that the Kruskal–Wallis test was carried out obtaining F values.

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