Intra and interspecific variability of *Quercus suber* and *Quercus canariensis*, an intrinsic water-use efficiency approach for differentiation

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Abstract

In Tunisia, the oak forest is essentially restricted to the north-west (Kroumirie and Mogods), which is the wettest zone of the country. It forms ecosystems of great ecological and socio-economic interest. This is a significant source of income for local population. In addition, it contributes to soil conservation and is home to a rich and diversified fauna and flora and offers the region a picturesque landscape. This precious and fragile forest capital is currently threatened under the effect of various pressures, mainly the browsing of young plants being reconstituted and vegetative competition regarding resources of the environment. Hence the interest in studying the intra and interspecific variability of the oak, this study is essentially based on the morphological and physiological comparison of the two species of oak, *Quercus suber* and *Quercus canariensis* with their cohabitation in the same area, this variability was carried out by multivariate biostatistical analyzes using a set of data including morphological traits, isotopic analysis of the leaves, water use efficiency, as well as the phenolic composition and antioxidant activities of the leaves extracts. The results showed that leaf morphology and water use efficiency are discriminating factors for differentiation to choose the right specie that will be used for reforestation. The *Q. canariensis* specie growing in the Bni Mtir region was characterized by a high intrinsic water-use efficiency and high biomass recovery. These results let us suppose that *Q. canariensis* was the perfect specie for reforestation given its ability to produce a large amount of biomass with less water, which is what environmentalists demand.

1. Introduction

The mountains of Kroumirie and Mogods in Tunisia constitute an important potential in terms of biodiversity and offer a remarkable opportunity to enhance sustainable development. The landscape diversity characterizing this region includes a significant number of different types of habitats, such as coastal sand dunes covered by a mosaic of vegetation, freshwater, peatland habitats and numerous forest and shrub formations, distinguished by a significant preserved forest of dominant oaks; *Quercus suber* (Cork oak) and *Quercus canariensis* (Zeen oak) [1].

The forests of Kroumirie and Mogods have significant diversity in terms of species and habitats, which are nowadays threatened by anthropogenic pressure and changes in land use. These lands gave way to large forest plots following reforestation policies resulting from the forest ecosystem restoration strategy [2]. The new formations from plantations are mainly Aleppo pine, pinion pine, maritime pine, eucalyptus, and acacia [3]. These landscape changes correspond to the evolution of the vegetation cover, which is related to the activities of...
local users (clearing, deforestation, etc) and the policies of reforestation and restoration of ecosystems undertaken by the Tunisian Forest Department between the 1960s and 1990s [4]. Montgolfier [5] shows that Zeen oak covers an area of 100,000 hectares. The author concluded at the time that the Zeen oak forest in northwestern Tunisia appears to be in good condition and poses no major regeneration problems. The main question was that of the valuation of its wood. It is indeed a rather difficult wood to work and dry. Its traditional uses, railway sleepers, and vine stakes are in decline. Mohamed, Guy [6] showed that demographic pressure, silvo-pastoral practices and inappropriate management methods have contributed to the modification of land use. The spatial configuration of the landscape has changed over 50 years. The Zeen oak forest has lost, after 40 years, about 40% of its area. That of Zeen oak and Cork oak gave way to stands of low density. However, new forest species have occupied the landscape since the end of the 1980s, such as acacia, Aleppo pine, eucalyptus, pinion pine and maritime pine. This appearance of new forest formations in the Cork oak stands is the result of reforestation. The oak wood is of great interest to North Africa, the high quality of resistance and the high adhesion of these fibers being suitable for several uses (fine carpentry, furniture and high-quality woods of high mechanical resistance). Likewise, they are of great interest from an ecological, biological, aesthetic, landscape and socioeconomic point of view [7]. The wood of Zeen oak presents large multi-seriate ligneous rays and small uniseriate rays, at the origin of a beautiful mesh of the wood when it is cut into quarters [8]. This forest, although still productive, producing 80,000 to 100,000 quintals of Cork per year, remains under constant threat. Currently, it is experiencing severe degradation, accentuated over the past two decades by various factors: aging of stands, stands facing south, fires, attacks by insects or fungi, human overexploitation, and recently, by the climatic changes characterized by these recent years by periods of marked summer drought [9]. Currently, the reconstitution of Cork oak forests poses a delicate problem and its solution is delicate, especially since it was approached very late. It is hampered by socioeconomic and technical difficulties, including the choice of the appropriate oak species by its ability to adapt to stressed environments and its tolerance to climatic changes. In the present work, we propose to present the results of the comparison of three oak populations from two different climates. These results are obtained after the evaluation of the interactions between the physiological (the isotopic composition of carbon 13 and intrinsic water-use efficiency), morphological and biochemical parameters of Q. canariensis (Zeen oak) and Q. suber (Cork oak) to choose the appropriate species for reforestation.

2. Materials and methods

2.1. Plant material
The experiments were conducted at 2 sites with a mixed Cork oak/Zeen oak transect in the region of Hammam Bourguiba (HB) and Ben Metir (BM) (Belonging to the forest in Kroumirie and Mogods, Tunisia) with eight populations (table 1, figure 1). Five well-lit and healthy uppermost leaves with similar size and phenological stage were harvested from 10 randomly selected adult trees. The minimum distance between selected trees was 20 meters. This approach makes it possible not to collect material from physically and genetically close individuals [10]. After the measuring of the morphological parameters, the leaves were dried at 40 ± 3 °C and then finely ground for the rest of the analyses.

### Table 1. Characteristics geographical, bioclimatic of oak different populations.

| Populations       | Purity   | Species | Latitude          | Longitude          | Altitude* (m above mean sea level) | Average annual precipitation (Mm) | Average annual temperature (°C) |
|-------------------|----------|---------|-------------------|--------------------|------------------------------------|----------------------------------|---------------------------------|
| HBQcp Pure Cork   | Q. suber | 36.79230567 | 8.63361112        | 462                | 1500                               | 15                               |
| HBQcm Mix Cork    | Q. suber | 36.78969443 | 8.62605555        | 552                |                                    |                                  |
| HBQzm Zeen        | Q. canariensis | 36.78737515 | 8.650867217      | 686                |                                    |                                  |
| HBQzp Pure Zeen   | Q. canariensis | 36.74636114 | 8.7436666667     | 690                | 1563                               | 15.2                             |
| BMQcm Pure Cork   | Q. suber | 36.73030565 | 8.70727778        | 723                |                                    |                                  |
| BMQzm Mix Zeen    | Q. canariensis | 36.79230567 | 8.63361112        | 462                | 1500                               | 15                               |
| BMQzp Zeen        | Q. canariensis | 36.72011112 | 8.68038889        | 800                |                                    |                                  |

Altitude* (m above mean sea level); HB Qcp: Pure Cork oak population in the Hammam Bourghiba site; HB Qcm: Mixed Cork oak population in the Hammam Bourghiba site; HB Qzm: Mixed Zeen oak population in the Hammam Bourghiba; HB Qzp: Pure Zeen oak population in the Hammam Bourghiba; BMQcp: Pure Cork oak population in the Bni Mtit site; BM Qzm: Mixed Zeen oak population in the Bni Mtit site; BM Qzp: Mixed Cork oak population in the Bni Mtit site; BM Qcp: Pure Zeen oak population in the Bni Mtit site.
2.2. Morphological descriptors of leaves

According to Chahidi, El-Otmani [11], intact and unparasitized leaves were harvested and measured using a digital caliper and analytical balance. Foliar surface was measured by a flatbed scanner and image analysis (ImageJ software ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA) [12]. The average leaf mass per area (LMA) of each individual was estimated by the ratio of leaf dry mass/leaf surface from each individual, 5 leaves were taken, i.e. 400 measured units. Vein numbers was performed with phenoVein (A Free Tool for Leaf Vein Segmentation and Analysis) according to [13]. Biomass was the weight of 5 dried leaves. The descriptors that were retained are summarized in table 2.

2.3. Measurement of the isotopic composition of carbon 13, % C and % N

For each population, the leaves were dried for 48 h at 40 °C, weighted, and then ground using a ball mill for 5 min at a rate of 30 oscillations per second (Mixer Mill MM 400, RETSCH, Germany). One milligram (± 50 μg) of the powder thus obtained was placed in a sealed tin capsule and stored in a desiccator. Subsequently, each capsule was introduced into a DeltaS mass spectrometer (Thermo Finnigan, Germany) coupled to an elemental analyzer NA-1500 (Carlo-Erba NA-1500 Elemental Analyser, Rodano, Italy), where it underwent complete combustion. The values of the isotopic composition of carbon 13 (δ13C) were established in relation to the international standard IAEA-CH7 PEF 160 of δ13C equal to 31.8‰ ± 0.2 (International Atomic Energy...

Table 2. Characteristic morphological traits of oak leaves.

| Abbreviation | Character            | Unit     |
|--------------|----------------------|----------|
| Petiole      | Petiole length       | mm       |
| Thickness    | Leaf thickness       | mm       |
| Vein         | Number of veins per leaf | —         |
| Blade width  | Blade width          | cm       |
| Blade length | Blade length         | cm       |
| Ratio        | Blade length width ratio | —         |
| Lobs         | Number of leaf lobs  | —         |
| Biomass      | Weight of 5 dried leaves | g         |
| Area         | Foliar surface       | mm²      |
| LMA          | leaf mass per area   | g m⁻²    |

Figure 1. Representative structure of Cork oak and Zeen oak populations in the Hammam Bourghiba site (HB) and Bni Mtit site (BM). HB Qcp: Pure Cork oak population in the Hammam Bourghiba site; HB Qcm: Mixed Cork oak population in the Hammam Bourghiba site; HB Qzm: Mixed Zeen oak population in the Hammam Bourghiba site; BM Qcp: Pure Zeen oak population in the Hammam Bourghiba; BM Qzm: Pure Oak population in the Bni Mtit site; BM Qzm: Mixed Zeen oak population in the Bni Mtit site; BM Qzp: Mixed Zeen oak population in the Bni Mtit site; BM Qzp: Pure Zeen oak population in the Bni Mtit site.
The periodic measurement of the internal standard makes it possible to estimate and correct any possible drift of the mass spectrometer. The $^{12}\text{C}_2\text{O}_2$ and $^{13}\text{C}_2\text{O}_2$ produced were separated according to their atomic mass and quantified by the mass spectrometer. The device provides the values of the isotopic composition of carbon 13 per sample as well as the percentage of carbon and nitrogen per unit of leaf mass. The relative abundance of $^{13}\text{C}$ (‰) was expressed as:

$$\delta^{13}\text{C} = \left( \frac{R_s - R_b}{R_b} \right) \times 1000$$  

(1)

Where $R_s$ and $R_b$ refer to the $^{13}\text{C}/^{12}\text{C}$ in the sample and in the Vienna Pee Dee Belemnite standard, respectively [14].

Carbon isotope discrimination ($\Delta^{13}\text{C}$) was calculated as:

$$\Delta^{13}\text{C} = \delta^{13}\text{C}_{\text{air}} - \delta^{13}\text{C}_{\text{sample}}$$  

(2)

Where $\delta^{13}\text{C}_{\text{air}}$ represents the carbon isotope composition of the atmospheric CO$_2$ and $\delta^{13}\text{C}_{\text{sample}} = -8$‰ [15].

### 2.4. Measurement of the intrinsic water-use efficiency (iWUE)

The measurement of the isotopic composition of plant tissues, and the subsequent calculation of the isotopic discrimination, constitutes an effective tool for the estimation of the time-integrated water use efficiency (iWUE). Carbon 13 isotope discrimination is mainly related to the passage and fixation of CO$_2$ in the stomata. A model was proposed by Farquhar and Richards [15] which relates $\Delta^{13}\text{C}$ to the diffusion of CO$_2$ through stomata and carboxylation in chloroplasts according to the following equation:

$$\Delta^{13}\text{C} = a + (b-a)C_i/C_a$$  

(3)

Where $a$ is the discrimination against $^{13}\text{CO}_2$ during CO$_2$ diffusion through the stomata (a = 4.4‰) [16], $b$ is the discrimination associated with carboxylation (b = 27‰) [15], and Ci and Ca are the intercellular and ambient CO$_2$ concentrations, respectively.

The iWUE can be calculated as the ratio of photosynthesis (A) to the conductance for water vapor (gH$_2$O):

$$i\text{WUE} = \frac{A}{g\text{H}_2\text{O}} = \left( \frac{C_a - C_i}{16} \right)$$  

(4)

The combination of equations (3) and (4) results in the formula for iWUE calculating:

$$i\text{WUE} = C_a(b - \Delta^{13}\text{C}/1.6(b-a)$$  

(5)

with $C_a = 378 \mu\text{mol mole}^{-1}$[17].

### 2.5. Extraction and determination of phenolic compounds in oak leaves

Phenolic compounds extraction was carried out according to the method of Msaaed, Tammar [18]. 1 g of dry plant matter is mixed with 10 ml of 80% methanol. The mixture is stirred for 60 min and then kept at rest for 24 h at 4 °C and in the dark. The extracts obtained are filtered with Whatman qualitative filter paper, Grade 4, then stored at 4 °C to be used later for the assays of total polyphenols, flavonoids, condensed tannins and total antioxidant activity. Total phenolic content (TPC) was determined by the Folin-Ciocalteu method according to Yeddes, Chalghoum [19] and expressed as mg Gallic acid equivalents (EGA) per g dry weight (mgEAG.g$^{-1}$DW). Total flavonoid content (TFC) was determined according to Rguez, Papetti [20] and expressed as mg Quercatin equivalents (EQ) per g dry weight (mgEQ.g$^{-1}$DW). Condensed tannin content (CTC) was determined according to Rguez, Msaaed [21] and expressed as mg catechin equivalents (EC) per g dry weight (mgEC.g$^{-1}$DW). All the experiments were carried out in triplicates and the results were reported as means in box plot distribution.

### 2.6. Antioxidants activities of oak leaves methanolic extract

DPPH radical scavenging assay (DPPH) was realized according to Tammar, Salem [22], it was expressed as IC50 (µg ml$^{-1}$), the concentration required to reduce 50% DPPH free radical, butylhydroxytoluene (BHT) was used as the positive control. The ABTS radical cation (ABTS) was conducted according to the method reported by Yeddes, Chalghoum [19]. The ABTS scavenging capacity was expressed as IC50 (µg ml$^{-1}$), and BHT was used as the positive control. Total antioxidant capacity (TAC) was performed according to Rguez, Essid [23], and was expressed as mg gallic acid equivalent per gram dry weight (mg GAE.g$^{-1}$DW). The reducing power (RP) of oak leaves methanolic extract was determined according to Ouarghemi, Harbeoui [24]. Ascorbic acid was used as the positive control. The results are expressed as IC50 values (µg ml$^{-1}$). All the experiments were carried out in triplicates and the results were reported as means in box plot distribution.

### 2.7. Statistical analysis

One-way and multivariate analysis of variance (ANOVA) followed by Tukey’s test was performed by SPSS 15 (SPSS Inc. Chicago, IL, USA). Principal Component Analysis (PCA) was performed by XLSTAT (XLSTAT)
Matrix correlation was visualized by the R software package (Version 4.0.2 R Foundation for Statistical Computing, Vienna, Austria). Scatter plots were realized by Orange Software (version 3.4.5, University of Ljubljana, Slovene).

3. Results

3.1. Morphometric leaves variation in Cork oak and Zeen oak

The oak leaves morphology studied was represented in a box plot graphic representing the distribution of the measurements recorded for all the oak populations. For petiole length (17.7 mm), veins per leaf (13.1 Veins), lobs per leaf (10.2 lobs), biomass (3.12 g per 5 leaves), and foliar surface (4586.36 mm²), the highest values were recorded for the population of pure Zeen oak from the Bni Mtir region (BM). The leaves from the habitat of Hammam Bourguiba (HB) are characterized by the highest leaves length and width with a value of 113.7 and 65.6 mm, respectively (figure 2). These leaves belong to the specie Oak Zeen for a pure population. The student’s t-test carried out the interspecific variation of the leaf morphology. It was performed between the mean of the distribution of the averages of the measurements for each species (table 3). This analysis lets us know if a morphological trait is affected by the Specie factor.

Table 3. Variance analysis of morphological traits distribution by comparison their means.

| Trait                        | Cork          | Zeen          | Student’s t-test | P-value | Signification |
|------------------------------|---------------|---------------|-----------------|---------|---------------|
| Petiole length (mm)          | 12.35 ± 1.31  | 16.66 ± 1.11  | 5.03            | 0.003   | **            |
| Blade thickness (mm)         | 0.2 ± 0.12    | 0.23 ± 0.02   | 2.97            | 0.035   | **            |
| Veins (Veins per leaf)       | 6.11 ± 0.25   | 12.29 ± 0.84  | 14.12           | 0.000   | ***           |
| Blade width (mm)             | 26.37 ± 0.06  | 59.81 ± 3.56  | 18.51           | 0.000   | ***           |
| Blade length (mm)            | 51.16 ± 1.84  | 107.86 ± 4.74 | 22.3            | 0.000   | ***           |
| Ratio Width/Length           | 1.82 ± 0.06   | 1.95 ± 0.05   | 3.09            | 0.022   | *             |
| Lobs (Lobs per leaf)         | 5.04 ± 0.02   | 9.8 ± 0.03    | 26.97           | 0.000   | ***           |
| Biomass of 5 leaves (g)      | 0.75 ± 0.06   | 2.89 ± 0.23   | 17.98           | 0.000   | ***           |
| Foliar surface (mm²)         | 997.7 ± 101.6 | 4293.73 ± 230.54 | 26.17       | 0.000   | ***           |
| Leaf area mass (gm⁻²)        | 69.94 ± 7.84  | 76.87 ± 7.03  | 1.32            | 0.237   | NS            |

The data are expressed as the means of boxplot distribution (± SD). The differences between species were assessed at a p-value < 0.05 (*), p < 0.01 (**), p < 0.001 (***) which mean significant, very significant and strongly significant, respectively.
3.2. Nitrogen, carbon and $^{13}$C isotopic discrimination variation in Cork oak and Zeen oak

The variation of Nitrogen, carbon, and $^{13}$C isotopic discrimination as illustrated in figure 3, the nitrogen percentage varies from 2.1% for the population of HBQzm to 1.5% for BMQcp. The difference in the distribution according to two oak species ($Q. canariensis$ and $Q. suber$) was weakly significant, with a p-value of 0.081 (table 4). The carbon content experienced a more categorical distribution by the habitat factor. The highest values were recorded for the $Q. suber$ specie, with levels of 49.4% for the BMQcp population. This observation was consolidated by the p-value of the inter-species variation (0.003). Contrariwise, the distribution did not affect the isotopic discrimination $^{13}$C variation according to the spece (p-value: 0.198). The value of $\delta^{13}$C varies from $-28.3\%$ for BMQzp to $-29.9\%$ for HBQcp and $\delta^{13}$C from 20.3 to 21.9 for BMQzp and HBQcp, respectively.

3.3. Phenolic composition and antioxidant variation in Cork oak and Zeen oak leaves extract

According to figure 4, the approximate phenolic composition was carried out with the dosage of total polyphenols content (TPC), total flavonoids content (TFC), and condensed tannin content (CTC). The colorimetric assay of total polyphenols showed that the content varied in a very significant way. The highest levels are recorded for the HBQcp and HBQcm populations with 241.4 and 267.4 mgEAG g$^{-1}$DW, respectively. The lowest values are found in BMQzm and BMQzp with 53.00 and 61.40 mgEAG g$^{-1}$DW,
respectively. This tendency is also observed in the dosage of total flavonoids and condensed tannin content. Therefore, according to these observations, it was evident that this composition represents a determining factor for the interspecific differentiation between the two sampling sites, likewise for the inter-species discrimination. These results were consolidated by the student’s t-test applied to the middle of the box plots of the different dosages with p-values of 0.009, 0.011, and 0.013 for TPC, TFC, and CTC, respectively (table 5).

Antioxidant activities were evaluated by the determination of total antioxidant capacity (TAC), DPPH radical scavenging assay (DPPH), ABTS radical cation scavenging assay (ABTS), and reducing power (RP). According to figure 5, the distribution of antioxidant activity measures was represented by IC50 values. Therefore, lower values represent higher activity. In fact, for the total antioxidant activity, the minimal results were recorded for the specie Zeen from the region of Hammam Bourguiba (HB) (9.2 and 8.6 mg EAG g⁻¹ DW for HBQzm and HBQzp, respectively). On the other hand, the DPPH activity, ABTS activity, and Reducing power tests are associated with the Cork species from the same region. HBQzm had the highest DPPH scavenging activity with an IC50 6.5 µg ml⁻¹. The lowest DPPH activity was recorded for HBQzp with an IC50 14 µg ml⁻¹.

### 3.4. Intra and interspecific variability effects on intrinsic water-use efficiency

According to the correlation matrix (figure 6), the intrinsic water-use efficiency can be a function of several morphological and physiological parameters of the oaks. In our study, we will focus on the correlation of the efficiency with the morphological properties that influence the oak’s biomass content. In fact, iWUE was positively correlated with petiole length (Pearson correlation coefficient: 0.560), leaves thickness (0.130), veins per leaf (0.570), blade width (0.370), blade length (0.400), lobs per leaf (0.530), foliar surface (0.520) and finally with biomass (0.500). On the other hand, iWUE was negatively correlated with the contents of phenolic compounds. Consequently, the efficiency was positively correlated with the IC50 of DPPH activity, ABTS activity, and reducing power. To better understand the effect of oak purity, each correlation between efficiency and morphological traits was visualized by correlation scatter plots grouped by sites and purity criteria with iWUE (figure 7). Recorded values for vein per leaf, blade width, blade length, lobs per leaf, foliar surface, and biomass were more correlated with the iWUE calculated for leaves from trees in mixed populations (Pearson correlation coefficient: 0.770, 0.600, 0.590, 0.680, 0.690, and 0.570, respectively). On the other hand, petiole length and leaf thickness were more correlated with the iWUE of the leaves from trees located in pure populations (0.790 and 0.60, respectively).

| Table 5. Variance analysis of phenolic compounds and antioxidant activities distribution. |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Cork | Zeen | Student’s t-test | p-value | Signification |
|---|---|---|---|---|
| TPC (mg EAG g⁻¹ DW) | 223.63 ± 55.88 | 4.673 | 0.009 | ** |
| TFC (mg EQ g⁻¹ DW) | 195.99 ± 58.21 | 4.140 | 0.013 | ** |
| CTC (mg EC g⁻¹ DW) | 62.53 ± 13.39 | 2.454 | 0.056 | NS |
| DPPH IC50 (µg ml⁻¹) | 7.80 ± 0.79 | 7.007 | 0.001 | ** |
| ABTS IC50 (µg ml⁻¹) | 8.65 ± 0.86 | 5.753 | 0.001 | ** |
| RP IC50 (µg ml⁻¹) | 6.91 ± 0.66 | 6.474 | 0.001 | ** |

The data are expressed as the means of boxplot distribution (± SD). The differences between species were assessed at a p-value < 0.05(*), p < 0.01(**), p < 0.001 (***).
3.5. Biplot principal component analysis of oak populations according to physiological parameters

Principal Component Analysis (PCA) is one of the most widely used multivariate data analysis methods for visualizing statistical interactions. It makes it possible to explore multidimensional data sets of quantitative and qualitative variables \[25\]. One of the advantages of this method is that it can provide both optimal visualization of variables and data, and biplots mixing the two. Nevertheless, these models are reliable only if the sum of the percentages of variability associated with the axes of the space of representation, is sufficiently high. If this percentage is high (for example, 80%), the representation can be considered reliable. If the percentage is low, it is advisable to make representations on several pairs of axes to validate the interpretation made on the first two factorial axes \[26\]. In our case, the collection regions represent the individuals and the morphological and physiological parameters represent the variables. To improve the intra and interspecific variability effects, a principal component analysis (figure 8) with a Pearson correlation allowing the analysis of the interactions between morphological and physiological parameters was realized. The variability percentages associated with the axes of the representation space are presented by F1 (66.73%) and F2 (16.59%) with a sum of 83.32%, which shows that the representation is reliable. By focusing on the two key parameters of our study: water use efficiency and productivity, the distribution of populations was divided into two groups. The first group was characterized by high biomass and iWUE values and the second group essentially comprises the Cork specie with high content of phenolic compounds and increased antioxidant activity.

4. Discussion

In this study, the comparison of eight oak populations from two different regions was determined through the evaluation of the interactions between the physiological (the isotopic composition of carbon 13 and intrinsic water-use efficiency), morphological and biochemical parameters of Q. canariensis (Zeen oak) and Q. suber (Cork oak) to choose the appropriate species for reforestation.

The oak leaf morphology studied was represented in a box plot graphic representing the distribution of the measurements recorded for all the oak populations. According to the student’s t-test applied to morphological oak traits (table 3). Vein per leaf, blade width, blade length, number of lobs, biomass and foliar surface, were the most distinguished traits with a p-value < 0.001. Moreover, petiole length, blade thickness and ratio Width/Length were less sensitive to specie variation with p-values of 0.003, 0.035, and 0.022, respectively. However, LMA did not show any significant distribution according to specie. In a similar study, Mhamdi, Yahia \[27\]
showed that the multivariate analysis of morphological traits revealed an important interspecific diversity for most studied traits and separation of leaf shape and size characters into three taxa, each corresponding to one of the three species, *Q. suber*, *Q. canariensis* and *Q. afares*. Consequently, the results obtained clearly show that the morphological traits are factors of interspecific differentiation between *Q. canariensis* and *Q. suber*.

Elemental analysis of different leaves revealed that the Nitrogen variation does not seem to be affected by inter-species variability. The carbon content varies considerably among the species studied. The isotopic discrimination $\Delta^{13}C$ variation was not affected by the distribution according to the specie ($p$-value: 0.198). The value of $\delta^{13}C$ varies from $-28.3\%$ for BMQzp to $-29.9\%$ for HBQcp and $\Delta^{13}C$ from 20.3 to 21.9 $\%$ for BMQzp and HBQcp, respectively. For the oak, a C3 tree [28], the fixation of CO2 on ribulose 1,5-biphosphate is carried out by the enzyme rubisco. This enzyme primarily uses carbon dioxide containing only light isotopes (recognition linked to the shape of the substrate) [29]. This induces a very unfavourable fractionation to heavy isotopes for the organic carbon produced compared to the initial carbon dioxide ($\text{atmospheric }^{13}C$: $\delta^{13}C \sim -8\%$). Therefore, plants in C3 have a meagre $^{13}C/^{12}C$ ratio, hence very negative $\delta^{13}C$ values ($-25$ to $-30\%$) [30]. In a previous study and according to Damesin, Rambal [31], the broad range of $^{13}C$ values for *Q. pubescens* and *Q. ilex* was 4.4 and 3.1, respectively. The intra-site variability was approximately 3 per cent. The total mean for each species was the same. Although there were substantial disparities between sites, the means of $^{13}C$ at each location were not significantly different between species.

After analysing the phenolic composition, it was proved that those traits represent a determining factor for the interspecific differentiation between the two sampling sites and the inter-species discrimination. The student’s t-test consolidated these results applied to the box plots means of the different measures with $p$-values of 0.009, 0.011 and 0.013 for TPC, TFC and CTC, respectively (table 5). Our results are similar to those reported by [32] for *Q. suber* specie. The methanolic extract is characterised by a total phenolic content of 210 mg GAE g$^{-1}$ DW. Likewise, the phenolic content coincided with those previously reported for *Q. suber* methanol extracts from Cork with 0.35 g GAE g$^{-1}$ of the extract [33]. Condensed tannin content reached the highest amount of
89 mgEAG·g⁻¹DW in the HBQcm population. Furthermore, other studies on the phenolic composition of leaves from different Quercus species, namely Q. coccifera and Q. suber, also reported high levels of tannins, in particular ellagitannins [34]. According to a prior study. Similar extracts obtained from Cork oak collected from a different geographic zone in Portugal had lower levels of phenolic and tannins but higher quantities of flavonoids. This is consistent with the impact of environmental factors on phenolic content [35, 36]. Antioxidant activities were evaluated by the determination of total antioxidant capacity (TAC), DPPH radical scavenging assay (DPPH), ABTS radical cation scavenging assay (ABTS) and reducing power (RP). The interspecific study between the responses of antioxidant activities provided by the student’s t-test showed that DPPH, ABTS, and RP assays are discriminating factors between the two species with p-values of 0.001. The IC50 of the DPPH assay of the Cork oak methanolic extract is very close to that found by Custódio, Patarra [32], Santos, Pinto [33]. According to Smirnoff [37], higher concentrations of reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide, hydroxyl radical, and singlet oxygen are commonly related to limited water availability.

Figure 7. Interspecific Scatter plots comparing iWUE and Petiole length, leaves thickness, vein number, blade width, blade length, lobs, foliar surface and biomass.
In addition to the ability to mobilise soil water, water use efficiency has emerged in recent decades as an interesting feature in agronomy, forestry and ecology [38]. Water use efficiency is often considered an essential characteristic for productivity under water deficit conditions [39]. It is used to establish a relation between biomass production and water management in forestry. Water use efficiency can be defined at the ecosystem level; it corresponds to the ratio between the primary production of the ecosystem and the actual evapotranspiration [40]. The revolution in this field has demonstrated a negative linear correlation between carbon isotope discrimination ($\delta^{13}C$) during photosynthesis and intrinsic water-use efficiency (iWUE) [15].

These different approaches to water use efficiency ($\delta^{13}C$, $\Delta^{13}C$ and iWUE) let us characterise this trait’s inter and interspecific diversity for many tree species of economic and ecological interest, such as oaks. By investigating the relationships between water-use efficiency and carbon isotope composition and specific leaf area of maize ($Zea mays$ L.) under water stress, Zhang, Zhang [41] revealed that the hydric stress restricted foliar growth and translocation of foliar assimilates, resulting in less $^{13}C$ being transported to the stems and more $^{13}C$ being enriched in the leaves. The $^{13}C$ of the leaves has increased, whereas the $\Delta^{13}C$ has decreased. Furthermore, the leaves have thickened, and the specific foliar surface has shrunk. As a result, the specific foliar surface was negatively correlated with iWUE since the specific foliar surface was negatively correlated with the leaf $\delta^{13}C$.

According to the correlation matrix (figure 6), the intrinsic water-use efficiency depends on several morphological and physiological parameters of the oaks. iWUE was positively correlated with petiole length, leaves thickness, veins per leaf, blade width, blade length, lobs per leaf, foliar surface and with biomass. Furthermore, iWUE was negatively correlated with the contents of phenolic compounds. Consequently, the efficiency was positively correlated with the I$_{50}$ of DPPH, ABTS, and reducing power. Consequently, the efficiency was positively correlated with the I$_{50}$ with DPPH, ABTS, and reducing power. Consequently, the iWUE was positively correlated with the IC$_{50}$ of DPPH, ABTS, and reducing power. Consequently, the efficiency was positively correlated with the IC$_{50}$ of DPPH, ABTS, and reducing power. Consequently, the efficiency was positively correlated with the IC$_{50}$ of DPPH, ABTS, and reducing power. Consequently, the efficiency was positively correlated with the IC$_{50}$ of DPPH, ABTS, and reducing power. Consequently, the efficiency was positively correlated with the IC$_{50}$ of DPPH, ABTS, and reducing power.
Regarding the inter-species discrimination, Zeen oak had the highest biomass markers than Cork oak. These results are related to iWUE means of $70.2 \mu$mol CO$_2$ mol$^{-1}$ H$_2$O for the pure population and $67.6 \mu$mol CO$_2$ mol$^{-1}$ H$_2$O for the Zeen oak mixed with Cork oak, in addition. These outcomes agree with the results of Abdessamad, Dhib [42], Abassi, Zouaoui [43], which showed that the Zeen oak from the Bni Metir region is distinguished by remarkable productivity even under water stress conditions. Similar results were reported by Gailing, Lind [44] by identifying patterns and morphological differentiation of two oak species ($Q. rubra$ L. and $Q. ellipsoidalis$ EJ Hill), which hybridised when co-existing and showed that the use of Shape leaf traits allowed clear separation of species. Based on morphological criteria also related to size and shape, results similar to ours on other oak species allowed the distinction between the leaves of $Q. pubescens$ (with relatively more minor leaves, with a petiole short and with few primary lobs) and $Q. petraea$, $Q. virgiliana$ and $Q. robur$ [45].

The Biplot principal component analysis of interactions between morphological and physiological parameters of 8 populations of $Q. suber$ and $Q. canariensis$ under different growth conditions revealed that the variables were divided into two groups (figure 8). The first group was characterized by high biomass and iWUE values. Similarly, this distribution also includes morphological traits, number of veins, petiole length, foliar surface, number of lobs, thickness of leaves, length and width of leaves. The most abundant species in this set was Zeen oak growing in the area of Bni Mtir at an average altitude of 734 m above mean sea level. Samples belonging to these populations were distinguished by the highest $^{13}$C isotopic discrimination, which is consistent with the position of the iWUE in this group. Plants with significant $^{13}$C discrimination are those that effectively manage water assimilation. Hence, we can conclude that these trees are not under water stress. Which may explain the low levels of phenolic compounds and low antioxidant activities. The second group essentially comprises the Cork specie with high content of phenolic compounds and increased antioxidant activity. Moreover, this group is associated with the Hammam Bourguiba region, a region of relatively low altitude compared to the other area (an average of 583 m above mean sea level). The criterion of purity did not show any association in this distribution, which shows that even if the two species are in a mixed habitat, the morphological and physiological response remains the same.

According to the Tunisian pedologic soil map [46], the soil nature of Hammam Bourghiba and Bni Mtir regions was reported as a calcimorphic lithosol, regosol, therefore, it does not play an important role for discrimination between trees populations. The relation between soil and $^{13}$C leaves was widely investigated. According to Yu, Wu [47], there was a positive correlation between litter $^{13}$C and leaves $^{13}$C, however, the $^{13}$C was negatively correlated with those determined in soil. which proves that the $^{13}$C isotopic composition content of the soil comes from the lillter (all dead leaves and decomposing plant debris).

5. Conclusion

In conclusion, following the evaluation of the quantitative and qualitative distribution of morphological and physiological parameters between 8 populations of $Q. suber$ and $Q. canariensis$ under different growing conditions. We found that the leaves morphology of these two species is a significant discriminating factor. On the other hand, the interspecific effect of each specie for the mixed and pure populations is not approved. Carbon 13 isotopic discrimination and intrinsic water-use efficiency are correlated with several morphological and physiological parameters of the oaks. The $Q. canariensis$ specie growing in the Bni Mtir region is characterised by a high intrinsic water-use efficiency and high biomass recovery. These results let us suppose that $Q. canariensis$ is the perfect specie for reforestation, given its ability to produce a large amount of biomass with less water.

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Data availability statement

No new data were created or analysed in this study.
Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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