Genetic effect on physiological behavior of *Pinus nigra* in response to water deficit :case study of provenances trials in North Africa

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Abstract: The variability of gas exchange and hydraulic traits has been studied in four subspecies black pine species of which 19 provenances planted in common garden in Northwestern Tunisia in order to compare and select the best adapted provenances to draught conditions. The aim of this paper is to illustrate the ability of black pine species to cope with draught conditions. Therefore, gas exchanges, leaf water potential, hydraulic conductivity and anatomical characteristics were measured. Results showed significant differences between provenances to cope with draught conditions. Although, some provenances such as Kustendil (*nigra*ssp) tends to avoid water stress by an earlier stomata control. However, by reducing its stomatal conductance it allowed to reduce their ability to assimilate carbon. Other provenances, such as Olette (*salazmanni*ssp) and Crimée (*pallasiana*ssp) showed a very late control of transpiration. It seems that net photosynthesis followed the same trend as stomatal conductance and transpiration. In the other hand, Marghese, Cazorla, Tavola and Olette provenances showed an increase in sapwood and leaf specific hydraulic conductivity which makes it possible to decreases the tension in the xylem. In conclusion, black pine species revealed different drought response mechanisms among provenances which may be explained by a possible genetic variability.

Keywords: Black pine; Drought; Photosynthesis; Stomata control; Subspecies

1. Introduction

In the Mediterranean region, vegetation is most sensitive to climate and the impact of climate change should be important. Water availability is, with heat, one of the factors limiting the species distribution (Maherali *et al.* 2004; Haddou *et al.* 2023). So, there is a growing demand for plant material adapted to extreme environmental conditions. The Mediterranean climate is characterized by hot, dry summers; the different stages of vegetation are related to the duration and intensity of summer drought. Regional climate simulations predict a decrease in annual mean precipitation for the Mediterranean Basin (Sumner *et al.* 2003; Laita *et al.* 2024; IPCC 2007). Precipitation variability

could be particularly important because extreme events, such as extended droughts, could have much more drastic consequences on tree growth and survival than gradual changes in the average climate conditions (Granier*et al.* 2007, Zhang *et al.* 2007). Thus, if the frequency of extreme droughts in Mediterranean areas increases in the future, it could lead to a reduction in forest productivity and changes in species composition (Allen *et al.,* 2009; Allen *et al.* 2010). Forest species are affected by both climatic and edaphic droughts (Loustau *et al.,* 2001; Barba *et al.,* 2013; Barba *et al.,* 2016). In Europe, Scots pine populations has been affected by high mortality rates associated with recent drought episodes (Bigler *et al.*2006). In addition, several studies showed that Pinus nigra has been found to be a drought sensitive species which makes it susceptible to water deficit during spring and summer (Martin-Benito *et al.* 2010). In fact, it showed that black pine is sensitive to high summer temperatures and to water deficit during spring and summer (Martin-Benito *et al.,* 2010, Martin-Benito, 2011, Fkiri *et al.* 2018). Macias *et al.,* (2006) showed that increasing water stress seems to be the main cause of growth decline. In the same context, several investigations indicated that water availability restricts productivity, particularly if precipitation decreases during the growing period (Loustau *et al.,* 2005, Martínez-Vilalta *et al.,* 2008). In addition, several studies showed that rainfall deficits during the growing season might induce black pine forest growth (Martín-Benito *et al.,* 2010).

Black pine (*Pinus nigra* Arn.) is a long-lived frugal species able to adapt ecologically and physiologically to a large environment condition. The species has colonized various ecological niches and played a leading role in different stages within forest succession. It is one of the most extended pines, from the Iberian Peninsula to Turkey and from Northern Africa to Austria (Barbéro *et al.* 1998). Numerous subspecies, varieties, and forms have been named following that diversity; six subspecies are currently recognized: *nigra, salzmannii, dalmatica, pallasiana, laricio and mauretanica* (Medail 2003).

How water stress interacts with site conditions to affect tree growth could be essential to understand the response of many key forest ecosystems to climate change (Vaganov *et al.* 2006; El Abdouni *et al.* 2021). The response of leaf gas exchange to water stress in Mediterranean species has been extensively studied (Faria *et al.* 1996, Faria *et al.*1998, Goulden 1996). Stomatal closure is a common response to drought stress in these species, but there is an important interspecific variability in the sensitivity of the response (Duhmeand Hinckley 1992, Salmon *et al.* 2015).

In order to characterize physiological mechanisms developed by black pine to resist against drought, the main objective of our investigation was to evaluate the variability in leaf water potential, gas exchange responses and hydraulic traits across nineteen provenances of black pine growing in common garden in Northwest of Tunisia.

2. Methodology

2.1 Studied area and plant material

The experimental site (Souiniet arboreta) is located in the northwest of Tunisia (36°48'N, 8°48 E, 255 m, 492 m). The site was established in 1966. It is characterized by a cold and humid climate. The average annual precipitation is approximately 1534 mm/yr. The mean annual temperature is 15.6°C, the hottest month is July (31°C) and the coldest one is January (4.4°C). The geological formations are dominated by Oligocene. Soil is poor in organic matter and chemical material The work was conducted on nineteen provenances of *Pinus nigra* (black pine) from different geographic origins (Table 1), which have been planted since 1966 in provenances trials experimental site (Souiniet arboreta).

2.2 Soil water content (SWC%)

The TDR moisture meter was used to measure the moisture content of the soil (n = 42). It is based on the dielectric properties of the soil which mainly depend on its water content. The TDR probe sends an electromagnetic signal which is transmitted along the waveguides (15 to 20 cm deep) placed in the ground. The speed of propagation of the signal is a function of the water content of the soil. The measures were taken at the beginning of the dry season (before sunrise).

2.3 Leaf Water potential

Leaf water potential (LWP) was measured using the pressure chamber technique. Small (5-7 cm long) twigs were cut from the same trees at similar height and side and put in a pressure chamber fed by a Nitrogen gas cylinder and equipped with a lamp-carrying magnifying glass.

Table 1. Provenances List and geographic origin of Black pine

Code	Subspecies	Provenance Name	Origin	Altitude	Latitude	Longitude
P1	*Pinus nigra salzmanni*	Brouzet-lès-Alès	France	-	44°07'35.4N	4°05'09''E
P2	*Pinus nigra calabrica*	TrentaCoste (SilaGreca)	Italy	1050	39°25N	16°35E
P3	*Pinus nigra calabrica*	Les Barres	France	150	47°50N	2°45E
P4	*Pinus nigra calabrica*	Cosenza	Italy	1300	39°15N	16°17E
P5	*Pinus nigra corsicana*	Boisfrerot (Ardennes)	France	100	-	-
P6	*Pinus nigra austriaca*	Puget-Théniers	France	-	43°57'24.4N	6'53'53.0''E
P8	*Pinus nigra nigricana*	Kustendil	Bulgaria	900-1100	42°16N	22°41'49E
P9	*Pinus nigra pallasiana*	Alaçam	Turkey	800-1000	39°35N	28°35E
P10	*pinus nigra calabrica*	Catanzaro	Italy	-	38°54N	16°34E
P11	*Pinus nigra corsicana*	les Barres (leint)	France	150	47°50N	2°45E
P12	*Pinus nigra salzmanni*	Saint-Guilhem-le-Désert	France	350-400	43°41 N	3°35E
P13	*Pinus nigra pallasiana*	Crimée	Russia	500	44°33N	34°17E
P14	*Pinus nigra calabrica*	Grancia (SilaGreca)	Italy	850	39°41N	16°58E
P15	*Pinus nigra calabrica*	Aspromonto	Italy	1300	38°05N	16°00E
P16	*Pinus nigra salzmanni*	Cazorla	Spain	1500	37°50N	3°00O
P17	*Pinus nigra calabrica*	Tavola (SilaGreca)	Italy	950	39°25N	16°35E
P18	*Pinus nigra salzmonni*	Olette (Pyrénéesorientales)	France	-	42°36'01.0''N	2'14'25.8''E
P19	*Pinus nigra calabrica*	les Barres	France	150	47°50N	2°45E
P20	*Pinus nigra corsicana*	Marghese	Corse	1100	41°39N	9°12E

2.4 Gas exchange Measurements

Gas exchange measurements were carried during the beginning of dry season, carbon assimilation was measured on pine needles of 19 provenances of black pine were recorded using a portable gas-exchange system (LI-6400, Li-CorInc, Lincoln, NE, USA) equipped with 2x3 cm light-source chamber (6400-02B LED, Li-CorInc). Ambient conditions were recorded, the temperature was maintained at 25°C, the humidity of the incoming air was kept at 60% and radiation was fixed at 1500 ppm. The CO_2 are reserved at 400 ppm. After steady-state, reading was taken. Gas exchange parameters were calculated according to von Caemmerer and Farquhar (1981). Carbon dioxide and

water vapour concentration differing between intel and outlet gaz circulating through the leaf chamber, as well as leaf temperatures obtained were used to calculate An, stomatal conductance (gs) and transpiration (T). WUEi was computed as the ratio between A and gs.

2.5 Xylem hydraulic conductivity

Branch sections of the nineteen provenances were sampled in the begin of dry season. Then, it was cut in the field and resin secretion from the cuts was eliminated by placing both ends in a water bath at 95 °C for 10 min (adapted from Waring and Silvester 1994). The exposed xylem of the shoot segment was wrapped tightly in parafilm and fitted with a ruber tubing gasket.

A hydrostatic pressure of 0.02 MPa was applied by placing the reservoir exactly 2 m above the stem section. For two hours, water drenched from the distal end of the branch section. Specific hydraulic conductivity (Kh) was measured following Sperry *et al.* (1988) with XYL'EM (Embolism Meter, Bronkhorst, Montigney-les-cormeilles, France). Kh was measured at low pressure. All segments were perfused with ultrapure, deionized, degassed and filtered (0,2 µm) water with 10 mM KCL (to minimize the displacement of air bubbles in open vessels). Kh was calculated as a ratio between the flow through each segment and the corresponding hydrostatic pressure gradient. After, Kh was divided by the xylem cross-sectional area which was converted to specific hydraulic conductivity Ks (kg m-1 MPa-1 s-1). The segment was perfused at 0.5 MPa for 5 min and then a second measurement of Ks was made to get the maximum specific conductivity, Ks max. Measurements of Ks and Ks max were further used to identify loss of conductivity, which can be attributed to xylem cavitation according to (eqn. 1):

PLC=100*(Kmax−Ki)/Kmax) where PLC is the percent loss of conductivity (%) due to cavitation.

Hydraulic conductivity Kl (Kl, kg s-1 m-2 MPa-1) can be define as the rate of water flow through the sheet per unit leaf area divided by the pressure exerted by this flow (Tyree *et al.* 2005, Sack and Holbrook, 2006). The Huber value (HV) is defined as the ratio of sapwood cross sectional area to leaf area.

2.6 LMA and Xylem density

Leaf mass per area (LMA g.m-2) is a morphological trait widely used as a good indicator of plant functioning which is the ratio between leaf dry mass and leaf area (Poorter *et al.* 2009). Wood density (WD) was calculated from mass in the oven dry state related either to volume (dry mass per fresh volume in kg m−3). Segments of 0.05 to 0.1 m long and 0.01 to 0.02 m diameter were cut from each branch after harvesting and immediately placed in plastic bags to avoid desiccation. In laboratory, the stem was cut into a small segment (0.02 to 0.05 m long) and its volume subtracted from the volume of the stem without bark and calculated by measuring stem length and the average diameter of the two perpendicular diameters the average diameter. All stems were then dried at 70–90 °C for three to four days and weighed. Xylem density, ρx, was then calculated as the dry mass divided by the green volume of the sample (Patino *et al.* 2009).

2.7 Data statistical analysis

The analysis of the variance was carried out using the Generalized Linear Model (GLM) of the SAS statistical program. The multiple comparison of means was performed by using the Newman–Keuls

test at a threshold of 5% (means with the same letters are not significantly different). Principal component analysis was conducted with Statistica 6.0 program.

3. Results

3.1 Soil water content

The mean soil water content (SWC, m^3 / m^3) measured at 20 cm deep using a TDR was estimated by 8.3%. The minimum value recorded was 7.3% and the maximum value was 10.9%.

3.2 Leaf Water potential

Statistical analysis revealed a highly significant difference between provenances ($p < 0.0001$). At the onset of the drought season, the water potential of *Pinus nigra* ranged from -0.3 MPa to -1.43 MPa. The highest water potential was observed in the Les Barres (P19) provenance of *P. nigra* subsp. *calabrica*, while the Crimée (P13) provenance of *P. nigra* subsp. *pallasiana* and the Olette (P18) provenance of *P. nigra* subsp. *salzmanni* subsp exhibited the lowest water potential values (Figure 1). These results suggest significant variation in drought resistance strategies among provenances, with certain provenances maintaining higher water retention under drought stress conditions.
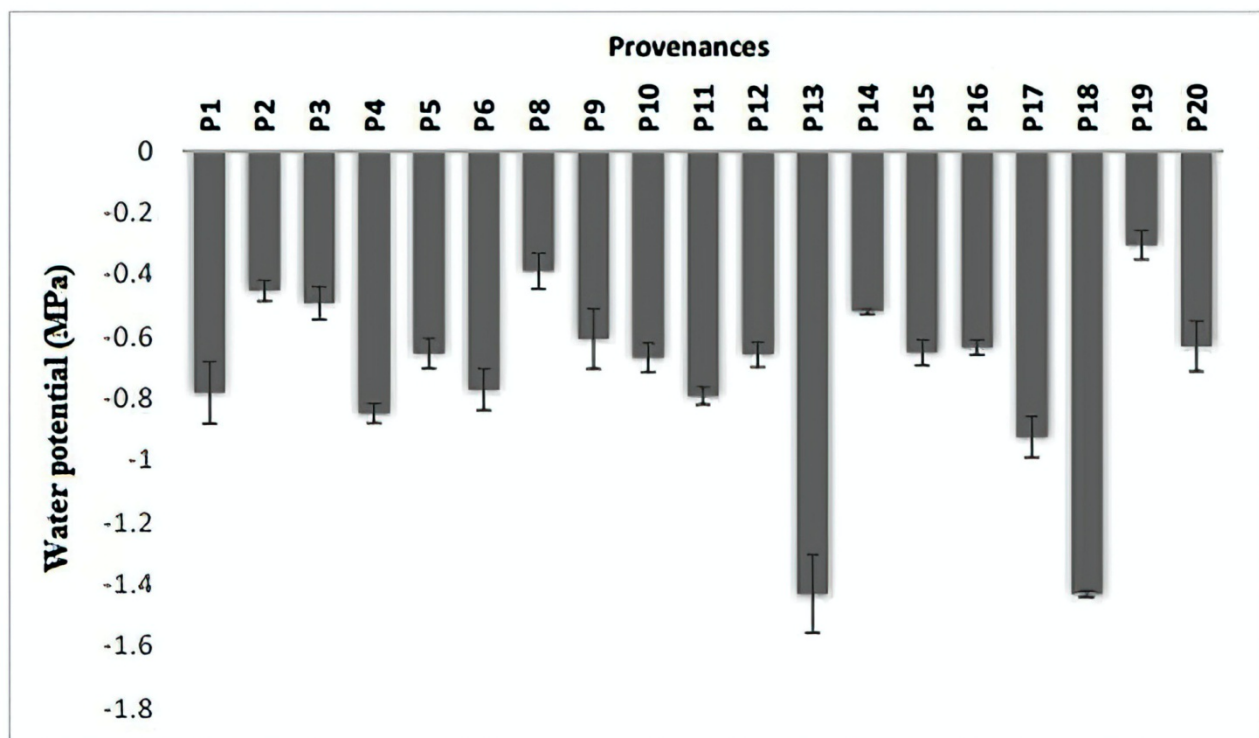

Figure 1. Leaf water potential (MPa) of 19 provenances of black pine species growing in the Souiniet common garden in north-west Tunisia

3.3 Leaf-level Gaz exchange variables

The statistical analysis revealed highly significant differences for net assimilation rate (An), stomatal conductance (gs), transpiration rate (Tr), and intrinsic water use efficiency (WUE) ($P < 0.0001$). The net assimilation rate (An) in *Pinus nigra* varied between 11.6 µmol CO_2 $m^{-2}s^{-1}$ and 18.5 µmol CO_2 $m^{-2}s^{-1}$. The lowest An value was observed in the Kustendil (P8) provenance of *Pinus nigra* subsp. *nigricans* var., whereas the highest value was recorded in the Saint-Guilhem-le-Désert (P12) provenance of *Pinus nigra* subsp. *salzmanni* (Fig. 3). Stomatal conductance (gs) and transpiration

rate (Tr) exhibited similar trends, both positively correlating with An (Table 2). Likewise, WUEi followed the same pattern as Tr and gs (Table 2), with higher values observed in the Kustendil (P8) and Olette (P18) provenances (Figure 2). These results suggest a strong physiological link between photosynthetic activity and water use efficiency in these populations.
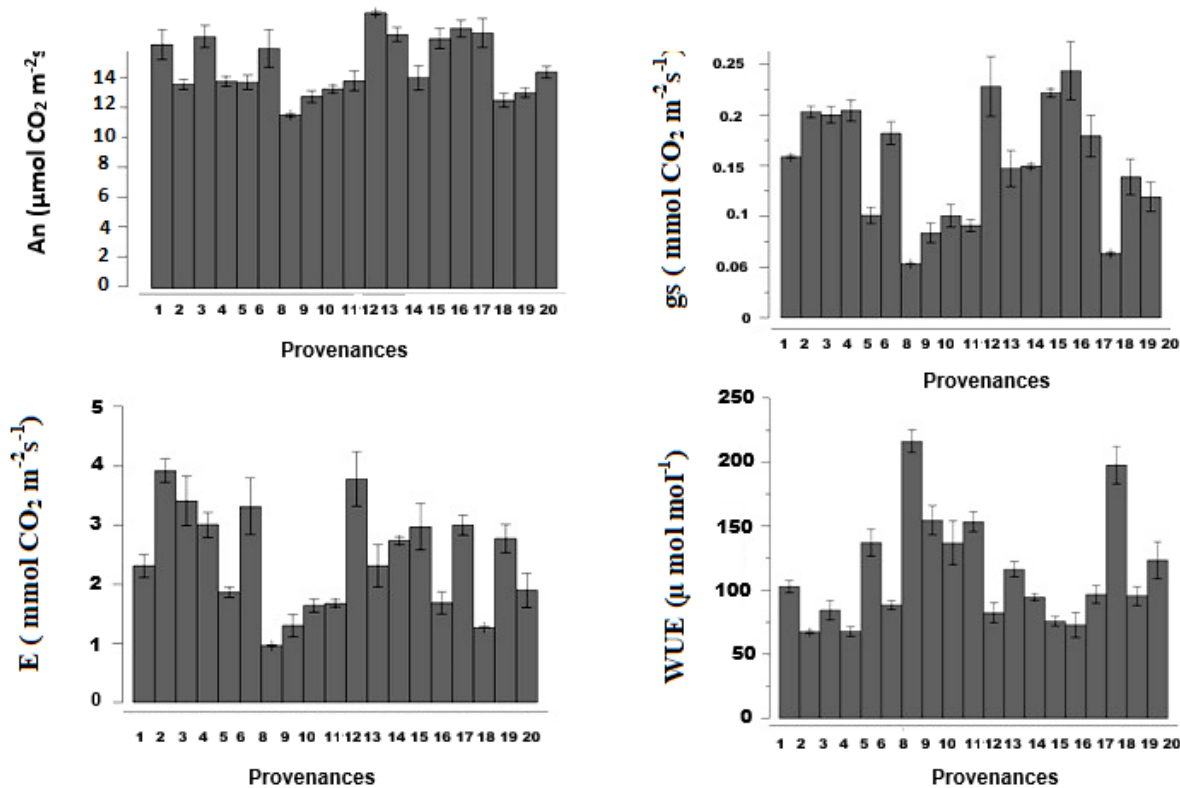

Figure 2. Variability of leaf-level gas exchange among 19 provenances of *P. nigra* species growing in Souinet common garden in north west of Tunisia: Net assimilation rate (An), Stomatal conductance (gs), transpiration rate (E) and instantaneous water use efficiency (WUEi) at 400 ppm CO_2 and 25 °C

Table 2. Linear correlations between hydraulic traits (Ks and Kl) and gas exchanges (An, gs, E and WUE) parameters

|	Gs	Tr	LWP	KS	KL
An	Y=39,591x+9,7526 **R² = 0,8202**	y =1,84x + 10,483 **R² = 0,6079**	y=0,388x+11,889 **R² = 0,3795**	y = 3,795x + 13,567 **R² = 0,4487**	y = 3143,3x + 13,658 **R² = 0,3112**
Gs	-	y=15,27x+ 0,2218 **R² = 0,8907**	y = -79,688x+7,3599 **R² = 0,9276**	y = 0,0485x + 0,1906 **R² = 0,6781**	y = 41,39x + 0,1935 **R² = 0,5526**
Tr	-	-	y = -1,6737x - 3,4138 **R² = 0,7797**	y = -0,6603x + 2,8984 **R² = 0,2732**	y = -740,93x + 2,9986 **R² = 0,3106**
WP	-	-	-	y = 0,046x + 0,1833 **R² = 0,161**	y = 5E-05x + 0,0002 **R² = 0,1948**

3.4 LMA and xylem density

The Puget Thenier (P6) provenance of *Pinus nigra* subsp. *nigracana* exhibited the highest leaf mass per area (LMA), reaching 2.47 g.cm², indicating a robust leaf structure (Figure 3). In comparison, the Trenta Coste provenance of *Pinus nigra* subsp. *calabrica* had a significantly lower LMA of 1. 87 g.cm². As for xylem density, Kustentil (P8) displayed the highest value at 639.58 Kg/m³, suggesting greater wood density and potentially higher resistance to water stress. In contrast, the Alaçam (P9) provenance recorded the lowest xylem density at 56.89 Kg/m³ (Figure 3). These variations in LMA and xylem density reflect the distinct physiological adaptations of each provenance to its environment, emphasizing their potential resilience or vulnerability to climatic conditions.
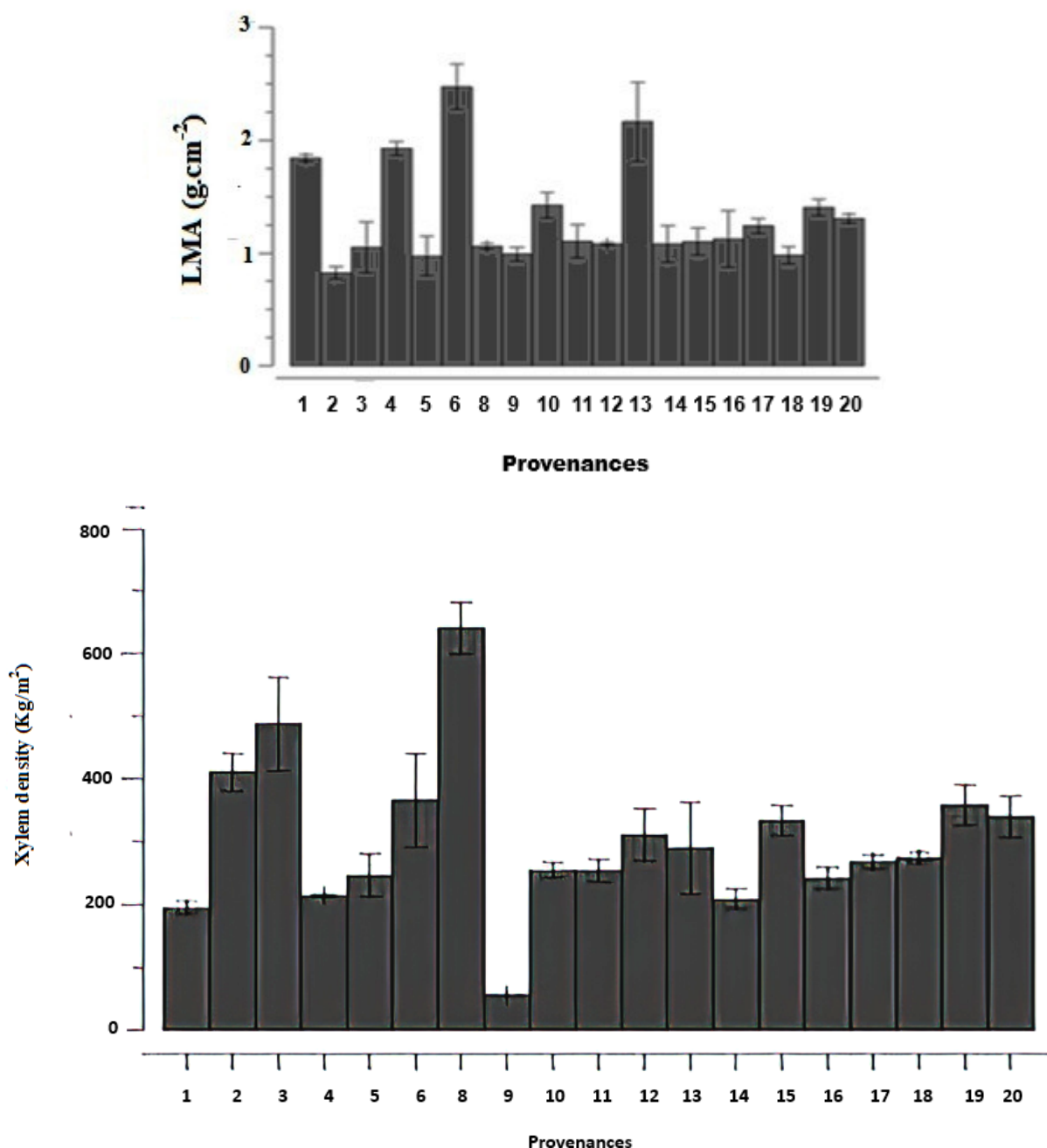
Figure 3. Leaf Mass Area (LMA) and Xylem density of nineteen provenances of black pine species growing in northwestern Tunisia in the Souiniet common garden.

3.5 Hydraulic Traits

The hydraulic conductivity parameters, Ks and Kr, followed similar trends. Ks ranged from 2.42 kg⁻¹m⁻¹MPa⁻¹ to 0.075 kg⁻¹m⁻¹MPa⁻¹, while Kr varied from 0.002 kg⁻¹m⁻¹MPa⁻¹ to 0.00014 kg⁻¹m⁻¹MPa⁻¹ ($P < 0.0001$) (Figure 4). These findings suggest a significant variation in hydraulic efficiency across provenances with notable differences in water transport capacities. The highest values of both Ks and Kr were observed in the Bois Frerot (P5) provenance of *Pinus nigra* subsp. *corsicana*, whereas the lowest values were recorded in the Corse (P20) provenance of *Pinus nigra* subsp. *calabrica*. Additionally, the Puget Thenier (P6) provenance of *Pinus nigra* subsp. *austriaca* exhibited a higher Huber index, coupled with the lowest Ks values.
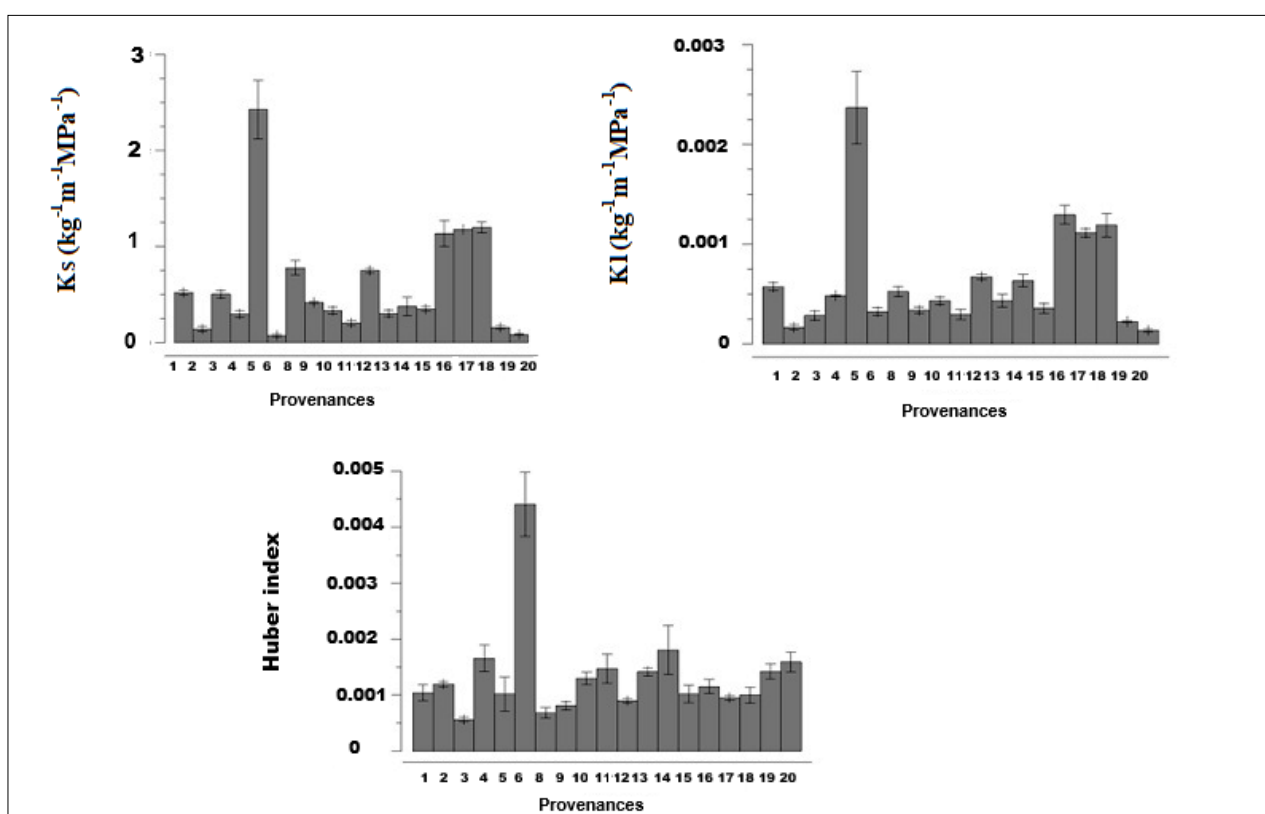

Figure 4. Sapwood and leaf-specific hydraulic conductivity (Ks and Kl) and leaf-to-sapwood area ratio (Huber index) of 19 provenances of black pine species growing in northwestern Tunisia in the Souiniet common garden

Correlation between hydraulic and gaz exchanges parameters

Results of principal component analysis showed that stomata conductance, transpiration, water potential, net photosynthesis (An), WUEi, hydraulics traits (Ks, Kr, Huber Index) and LMA, were the most significant variables for classification of the *P. nigra* provenances. These parameters were considerably loaded into the two major principal components (Dim1 and Dim 2) explaining more than 66% of the variance (**Figure 5**). According to our analysis, six different groups were revealed. The first group contained only Kutendil (P8) provenances which showed the lowest An, gs and Tr and the highest WUEi. The second group was represented by Olette (P18) which showed the highest water potential.
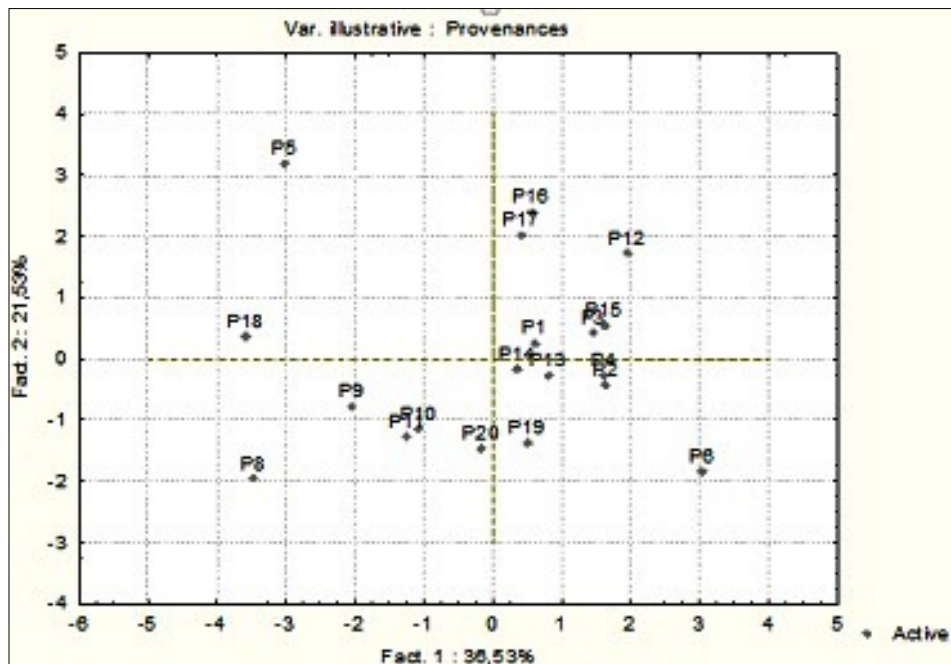
Figure 5. Factorial analysis and the distribution of the 19 provenances of black pine

The third group contained P17, P12, 16 which had the main An value. The fourth group was composed by P5 which had the highest Ks and Kl values. The fifth group was represented by P6 characterized by the highest LMA and Huber index values and the lowest values of Ks. Finally, the sixth group regrouped all the other provenances studied. An was strongly correlated with gs and WP respectively ($r2 = 0, 82$; $r2= 0, 90$). However, it was less correlated to Ks and Kr respectively ($r2 = 0,44$; $r2= 0,31$). Leaf water potential was significantly correlated with gs ($r2 = 0, 93$).

4. Discussion

The mean objective of this study was to evaluate physiological traits to classify black pine provenances according to its resistance to drought. Results showed significant differences between provenances. In fact, inter-provenances variability of basic potential water values in the same site suggests an influence of the tree genotype on this parameter (Ducrey 1988).

Leaf water potential of black pine varied between -0,3 MPa and -1,43 MPa which explains that the species was in condition of low and severe water deficit. Our study showed that the water economy and the resistance of the photosynthetic apparatus to water deficits, which resides in stomatal regulation, are one of the factors of resistance to drought for this species. During drought season, stomata control plays a major role in regulating the transpiratory flow of forest trees (Leo et al. 2014, Salmon et al. 2015). It is usual to observe a reduction in stomatal conductance (El Aouni 1976, Schulze et al. 1987, Leo et al. 2014, Salmon et al. 2015, Martínez-Sancho 2017) and a fall of the basic water potential (Aussenac and Granier1978).

Therefore, Stomatal closure appears to be the mechanism used by Kustendil provenances (*P. nigra* ssp. *nigricans*) to avoids lethal water stress, in this case the conductance declines at relatively high leaf water potential (-0,39 MPa). In addition, it showed a very early control of transpiration to keep their water situation in safety during draught season. However, reducing the stomatal conductance to

water loss led to reduce the ability of the species to assimilate carbon, which may further heat correlated to growth decline. Thus, it appears that regulation and blockage of transpiration at high potentials allows the tree to resist against drought, but also result in poor photosynthetic efficiency. Our result was confirmed by Aussenac (1980) which found that an earlier stomatal closure (-0,9 MPa) and a better hydric state (52% moisture content) at the end of closure of the stomata of *P. nigra* ssp. Var. *Nigricans* planted in Armance Arboreta in France.

Other provenances, such as Olette (*salzmanni*ssp) and Crimée (*Pallasiana*ssp) showed a beginning of closure of the stomata at low potentials (- 1,4 MPa). It shows a very late control of transpiration. Yurukov (2003) showed that *P. nigra* ssp. *pallasiana* seems well adapted to high summer temperatures. Then, *P. nigra* ssp. *salazmanni* had a very high efficiency use of water, it accepts a very marked and frequent drought (Debazac 1964, Aussenac 1980). In other studies, it was reported that Salzmann pine is sensitive to water deficit during spring (Amodei*et al.* 2012, Martin-Benito 2011, Fkiri *et al.* 2019). Aussenac 1980 showed a good adaptation to drought of two provenances of *Salzmanni* ssp. (Cazorla and St-Guilhem) planted in Armance Arboreta in France, which was confirmed by our results.

Pinus nigra ssp. *Lariciocorsicana* (Marghese, Boit Frerot and les Barres (leint)) followed the same trend for gas exchanges parameter at 0, 69 MPa, that had a good carbon assimilation and low transpiration with good water efficiency. In France, *P. nigra* ssp. *Laricio* showed that rainfall deficits during the growing season might induce forest growth decline of two varieties of laricio pine (corsicana and calabrica) (Lebourgeois 1998, Lebourgeois 2000).

The Intraspesific differences between the branch-level hydraulic traits of black pine are probably the result of the divergent ecophysiological and hydraulic strategies of provenances (Martinez Sanchot 2017). According to the results of our study, Marghese, Cazorla, Tavola and Olette provenances showed an increase in sapwood and leaf specific hydraulic conductivity (Ks and Kl) which makes it possible to decrease the tension in the xylem and total leaft area. For the other provenances under conditions of hydric stress, the xylem may be subjected to very low water potentials, which can lead to dysfunction and in particular to embolisms due to cavitation and the entry of air into the conductive tissues. These phenomena considerably reduce the conductivity of the xylem (Tyree and Sperry, 1988). We can conclude that the evading of low water potentials causing high losses of hydraulic conductivity can be achieved by a stronger stomatal regulation (Rambal 1993).

Under drought conditions, pine species adjust its leaf-to-sapwood area ratio, sapwood and leaf-specific hydraulic conductivity and total leaf area (Sterck*et al.* 2008, Martínez-Vilalta *et al.* 2009). It is the same result found as few black pine provenances in this study.

Conclusion

Black pine is one of several species in Europe with contrasting hydraulic architecture as well as different physiological strategies to contract with water limitations. In this study, we shown that, in the beginning of dry season, black pine species tends to avoid water stress by a stomata control. This strategy might affect carbon gain during prolonged drought periods however; conserve the statute of the hydraulic system by avoiding possible damages during extreme events. In fact, some provenances showed a very early control of transpiration (isohydric behavior). In contrast, other provenances maintained high transpiration and stomatal conductance (anisohydric behavior) under moderate

drought conditions. In addition, several provenances adjust sapwood and leaf-specific hydraulic conductivity. Differences occur among provenances which would attest interaction of environment and genetic controls of physiological response. In this context, the differences in hydraulic architecture and strategy to cope with drought may be decisive for the capacity of resistance and elasticity and therefore, in future provenances selection for reforestation.

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Compliance with Ethical Standards: This article does not contain any studies involving human or animal subjects.

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