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## Characterization of Chestnut Behavior with Photosynthetic Traits

José Gomes-Laranjo et al.\*

*Centre for the Research and Technology of Agro-Environmental and Biological Sciences (CITAB), Universidade de Trás-os-Montes e Alto Douro (UTAD), Vila Real Portugal*

### 1. Introduction

European chestnut (*Castanea sativa* Mill.) covers in total 2.53 million hectares, of which 2.2 million hectares are forests and the remaining parts, are orchards. In Europe it is growing in an area comprised by 27° N and 53° N latitude, from sea level in seaside regions to 2000 m above sea level (a.s.l.) in the south of Europe. According to Fernández-López et al. (2005), chestnut species is characterized by the existence of some differentiation among extreme populations, which can be supposed due to its long-range distribution across the Mediterranean region, through varying climatic conditions. As reported by Heiniger (1992), chestnut is a good indicator of warm regions with oceanic climate.

In spite of *C. sativa* Mill. be characterized as a mesophilic species, nowadays the chestnut shows many growth limitations, which partially can be ascribed to the climate changes, since they influence abiotic and biotic factors and consequently photosynthetic productivity. From abiotic factors, water and heat stress have been the most important limitations, inducing less growth, less vigor increasing susceptibility to the biotic factors such as ink and blight diseases.

When compared with other hardwood species, the trees can absorb the same amount of carbon, but due to the fast-growing chestnut can store more carbon in less time. So, this agro forest system has been identified as an important way to slow climate change.

Photosynthesis, according to (Givnish, 1988), provides green plants with almost all of their chemical energy, being central to their activity to compete and reproduce. So understanding

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\* Lia-T. Dinis<sup>1</sup>, Luís Martins<sup>2</sup>, Ester Portela<sup>1</sup>, Teresa Pinto<sup>1</sup>, Marta Ciordia Ara<sup>3</sup>, Isabel Feito Díaz<sup>3</sup>, Juan Majada<sup>3</sup>, Francisco Peixoto<sup>4</sup>, S. Pereira Lorenzo<sup>5</sup>, A.M. Ramos Cabrer<sup>5</sup>, Changhe Zhang<sup>1</sup>, Afonso Martins<sup>1</sup> and Rita Costa<sup>6</sup>

<sup>1</sup>Centre for the Research and Technology of Agro-Environmental and Biological Sciences (CITAB), Universidade de Trás-os-Montes e Alto Douro (UTAD), Vila Real, Portugal

<sup>2</sup>Department of Forest & Landscape, Universidade de Trás-os-Montes e Alto Douro (UTAD) Apartado, Vila Real, Portugal

<sup>3</sup>(SERIDA), Consejería de Medio Rural y Pesca, Villaviciosa, Asturias, Spain

<sup>4</sup>CECAV, University of Trás-os-Montes e Alto Douro, Vila Real, Portugal

<sup>5</sup>University of Santiago de Compostela, Department of Crop Production, Campus de Lugo, Spain

<sup>6</sup>INRB, I.P. L-INIA Quinta do Marquês, Oeiras, Portugal

photosynthetic performances of each species, its varieties or even its cultivars is crucial to understand and advise the future.

This chapter will present an overview about photosynthetic studies done in chestnut species emphasizing the abiotic stresses (drought and heat stress) and biotic stresses (ink disease) issues, contributing to understand the impact of climate change in chestnut.

## 2. Ecological chestnut behaviour according to its European distribution

European chestnut is the only species of genus *Castanea* in Europe showing an outstanding evolutionary history from its likely origin in North-east Turkey and Caucasus region in respect to other European forest tree species during last 9,000 years before present (YBP) (Mattioni et al., 2008). Palynological studies support also two main fast expansion periods, about 5,000 YBP due to glacial Pleistocene Epoch and 2,000 YBP, during the Roman Empire. Actually, in Europe, *C. sativa* Mill. is commonly found between the Canary Islands, the most southern point (27° N latitude) and the most northern point defined by a line passing in the south of the United Kingdom, northern Germany, Poland and Ukraine (52° N latitude). In terms of altitude, chestnut is quite widespread, since it grows at sea level in coast regions until 400 m and 2000 m a.s.l. in the inner regions of the European continent. The lowest elevations are recommended for the highest latitudes and vice versa (Bounous, 2002).

The wide distribution of European chestnut, and the higher phenotypic plasticity observed in populations coming from arid regions in comparison to those that are from more wet areas, suggest the substantial adaptive variation existing among populations (Fernández-López et al., 2005). In a study comparing progenies from several European climatic contrasting locals growing under the same climatic conditions (EU funded Cascade project), populations from Greece started growth earlier followed by southern latitude progenies (south Italy and south Spain) while the plants from north Spain and North Italy initiated later. Height growth of the northern populations was higher than growth of the southern plants. The southern ones also showed an earlier growth cessation, budbreak, and a longer juvenile period than those from more north latitudes (Fernández-López et al., 2005). The importance of budbreak is due to the sensibility of the young leaves to latest frost during spring times, besides other factors such as drought tolerance.

The European chestnut presents a fair effective number of alleles, decreasing diversity from northern to the most southern populations (Eriksson et al., 2005b). Nevertheless, in Iberian Peninsula there were detected more than 350 genotypes in 574 accessions (Pereira-Lorenzo et al., 2010). According to these authors, in Iberian Peninsula the two main variability origins are located in North and Centre, being the most southern ecotypes (Andalusia and Canary Islands) assigned to both of these zones, which might suggest a colonization process. This colonization by varieties from the north part of Iberian Peninsula, since there are quite different edaphoclimatic regions, suggests that this species has potential to adapt themselves to new climatic conditions and by this way to the new context of climate changes. This ability of long-term species to respond and to adapt to environmental changes though natural selection is due mainly to their high intrapopulation genetic diversity (Martin et al., 2010), demonstrating genomic SSRs significantly higher levels of diversity in terms of number of alleles, expected heterozygosity and level of polymorphism among

populations. While previous studies using isoenzymes showed limited diversity, the new methodologies using microsatellites have demonstrated high number of alleles and gene diversity, even in European cultivated stands. More than a hundred of chestnut ecotypes from Portugal and Spain were characterized (Costa et al., 2009; Dinis et al., 2011a; Martín et al., 2007, Pimentel- Pereira, et al., 2007; Pereira-Lorenzo et al., 2006), showing significant adaptive differences to the different edaphoclimatic conditions: (Dinis et al., 2011a). In the same way, in Spain almost 50 varieties spreading from the northern to the southern and Canary Islands were studied, showing the chemical data of fruits low correlations with environmental parameters indicating that these differences could be mainly ascribed to genetic differences (Pereira-Lorenzo et al., 2006). In Spain this diversity will be certainly higher, since only in Andalusia (located in south of Spain), there were detected 43 ecotypes, showing them quite different morphological traits, namely at the level of leaves, spines on the burs, catkins, fruit shape and size (Martín et al., 2007). So, Man has also influenced the structural and genetic characteristics of chestnut populations through cultural practices (propagation, grafted plants, silvicultural or agronomical practices, etc), leading to widely heterogeneous stand typologies all over Europe: a) naturalized forests with coppices exploited for timber, b) managed coppice, an agro-forest system cultivated for fruit and timber production and c) orchards, a crop for fruit production (Mattioni et al., 2008), perfectly demonstrating the multipurpose character of this species. In spite of human influence, three main gene pools across Europe were identified (Villani et al., 2010): a) the north-eastern Turkish pool, b) the Greek gene pool and c) the Mediterranean Turkish gene pool, from which the European seems to be originated. In all these situations, they have been considered the natural hybridations and introgressions. These are due to the introduction in Europe during first half of the 20<sup>th</sup> Century, of Asiatic species a cause of the resistant ink disease breeding programs. Although climate and the grazing regime are influential, the degree of silvicultural management appears to be the most important factor determining floristic composition of forests and their long-term sustainability (Konstantinidis et al., 2008).

Because of its different domestication levels, chestnut has undergone natural and artificial selection pressure, which have shaped the actual genetic and phenotypic traits, and so expected capacity of adaptation to environmental stress factors. Particularly evident until now, there is the capacity to drought tolerance adaptation, which underlines a higher phenotypic plasticity in populations from arid regions as compared to those from humid areas (Villani et al., 2010). Concerning rainfall, the minimum for chestnut is 800 mm, but it can growth well in rainy areas (2000 mm) like Galicia, located in northwestern Spain (Pereira-Lorenzo et al., 2006).

Plants from this species are moderately thermophilic and well adapted to ecosystems with a annual mean temperature ranging between 8 and 15 °C and monthly mean temperatures between May and October over 10 °C. Temperature is also important for a good pollination (July), with an adequate range between 25 °C and 30 °C (Bounous, 2002).

The capability to take in water and nutrients and to make an efficient use of these resources is an important feature of species adapted to the Mediterranean environment (Zhangh et al., 2011). C<sub>3</sub> plants discriminate during photosynthesis against <sup>13</sup>C (1.1% of total carbon in atmosphere), the heavy stable isotope of C (Lauteri et al., 2004), being its depletion very affected by environmental conditions. So, the carbon isotope discrimination ( $\Delta$ ), a complex physiological trait involved in acclimation and adaptive

processes, estimates the amount of  $^{13}\text{C}$  in plants, which by its way is dependent on the plant response. Climatic conditions from each local, temperature and precipitation, can be conveniently characterized by the xerothermic index, which takes in account each growth season (Eriksson et al., 2005a), the biggest values meaning driest and warmest conditions. Across European chestnut populations displays significant  $\Delta$  variability (Lauteri et al., 2009). In general, chestnut growth decreases with the increase in xerothermic index, in opposition to the  $\Delta$  values. Then highest  $\Delta$  values were measured in progenies from the warmest locals (e.g. Greece and south of Spain). On the other way, the highest variability on  $\Delta$  values presented by ecotypes from Mediterranean locals in response to increase in temperature indicates a higher adaptation potential against climate changes than that of those from wet locals (Lauteri et al., 2004). The same is also true for drought tolerance, meaning that Mediterranean ecotypes might have more tolerance to drought than other ones from contrasting locals. The highest  $\Delta$  values correspond to the lowest water use efficiency (WUE) (Lauteri et al., 2004). These authors demonstrated that under the same climatic conditions, progenies from ecotypes originating from xeric locals (e.g. Mediterranean locals) can present higher  $A$  and  $g_s$  rates but surprisingly lower WUE than those from mesic (wet) locals.

Under field conditions, on Trás-os-Montes (located in the Northeast of Portugal) chestnut trees have maximal photosynthesis rate ( $A$ ),  $7.6 \mu\text{molCO}_2\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , in September (Figure 1), when temperature reaches  $25^\circ\text{C}$ . This month corresponds to an important phase of bur growth. In relation to maximal leaf transpiration ( $E$ ), it was measured during July ( $29^\circ\text{C}$ ,  $4.8 \text{mmolH}_2\text{O}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ), which can be positive in terms of leaf cooling and consequently in  $A$ . So, the worst  $A$  measured in July comparatively to September, might be due to heat stress, relieving the importance of leaf water content in this month, and indirectly the importance of xylem growth to allow leaf water uptake.

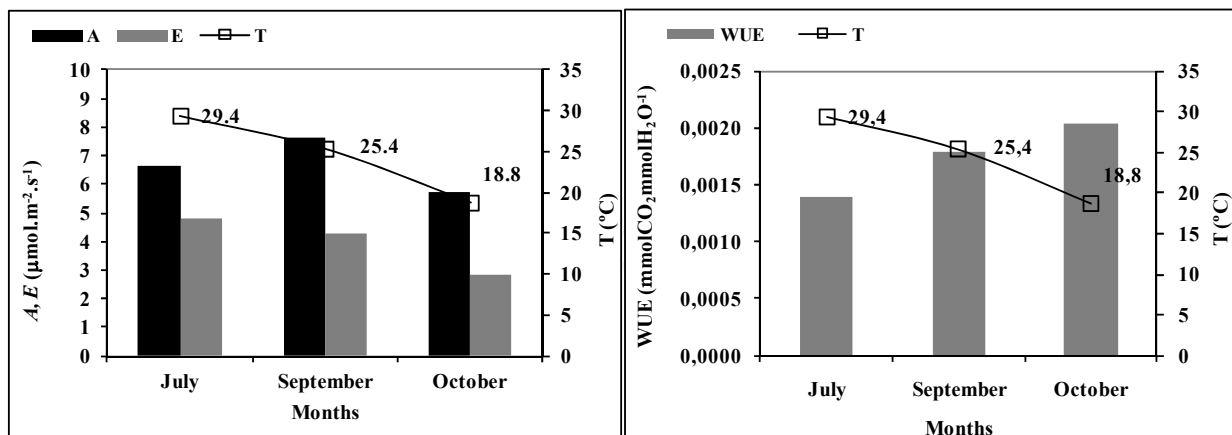


Fig. 1. Variation of photosynthesis ( $A$ ), leaf transpiration ( $E$ ), water use efficiency (WUE) and environmental temperature ( $T$ ) during July, September and October, in adult chestnut trees ( $n=8852$ ) with near 40 years old. Measurements were done in Trás-os-Montes region, located in the Northeast of Portugal, during 6 successive years.

The highest production of xylem cells is observed between mid-May and mid-June and phloem ones from mid-June until mid-July. Wood and phloem production mainly terminated in the middle of August while differentiation of xylem cells lasted until mid-



October (Cufar et al., 2011). The highest production in relation to water use efficiency (WUE) increases from July ( $0.0014 \text{ mmolCO}_2.\text{mmolH}_2\text{O}^{-1}$ ) to October (Gomes-Laranjo et al., 2007), following the standard curve above referred.

In conclusion, populations from wet regions seem to have less phenotypic plasticity than those from dry and warm regions, indicating that the first ones might also be more vulnerable to possible climate changes (Lauteri et al., 2009; Villani et al., 2010).

### 3. Ecophysiological characterization of chestnut

#### 3.1 Characterization of Portuguese varieties by gas exchange traits, as a function of temperature and radiation

Near 85% of Portuguese chestnut area (around 35,000 ha) is located in Trás-os-Montes, a region located in the Northeast of Portugal ( $41^{\circ}30'N$ ,  $6^{\circ}59'E$ ),  $9-13^{\circ}C$  being the mean annual temperature and 600-1200 mm the amount of annual rainfall (Figure 2).

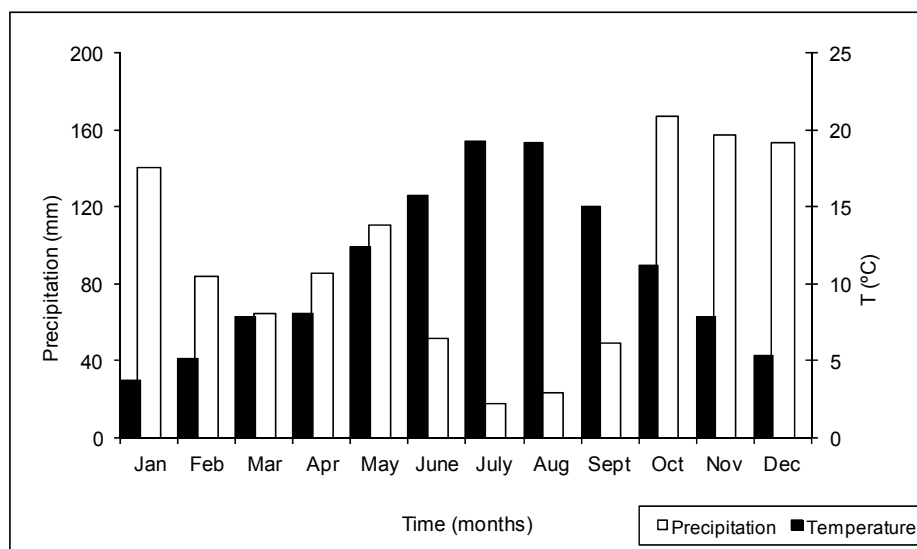


Fig. 2. Month values of precipitation amount and daily mean temperature in Trás-os-Montes region. Values represent the mean between 1988 and 1994.

Chestnut is a dim-light species. In a study done in Trás-os-Montes Region, including a sample constituted by 13 varieties (see Figure 6), half of the maximal  $A$  ( $A_{100}$ ) was obtained when PPFD level reached near  $400 \mu\text{mol}.\text{m}^{-2}.\text{s}^{-1}$ , instead 75% of  $A_{100}$  was measured under PPFD of  $850 \mu\text{mol}.\text{m}^{-2}.\text{s}^{-1}$ , corresponding this radiation to half of maximal PPFD. These results indicate that trees might have better adaptation to the shade north-facing slopes, rather than south-facing ones (Gomes-Laranjo et al., 2008a). South-facing orchards have higher mean temperatures than north-facing ones. In the former orchards, there is higher evapotranspiration but lower water availability, which induces less growing trees, and so less vigour, predisposing them to biotic stresses such ink disease (induced by the soil born oomycete *Phytophthora cinnamomi*). Maximal  $\text{CO}_2$  assimilation ( $A_{100}$ ), was obtained at about  $24^{\circ}C$  (Figure 3), while half of maximal  $A$  was measured around  $11-12^{\circ}C$  and  $37-38^{\circ}C$  of ambient temperatures.

This relatively low tolerance to hot temperatures was attributed to the level of thylakoid fatty acid saturation, so that the most tolerant cultivars (e.g. Aveleira) have a higher level of saturation than the worst ones (e.g. Judia) (Gomes-Laranjo et al., 2006).

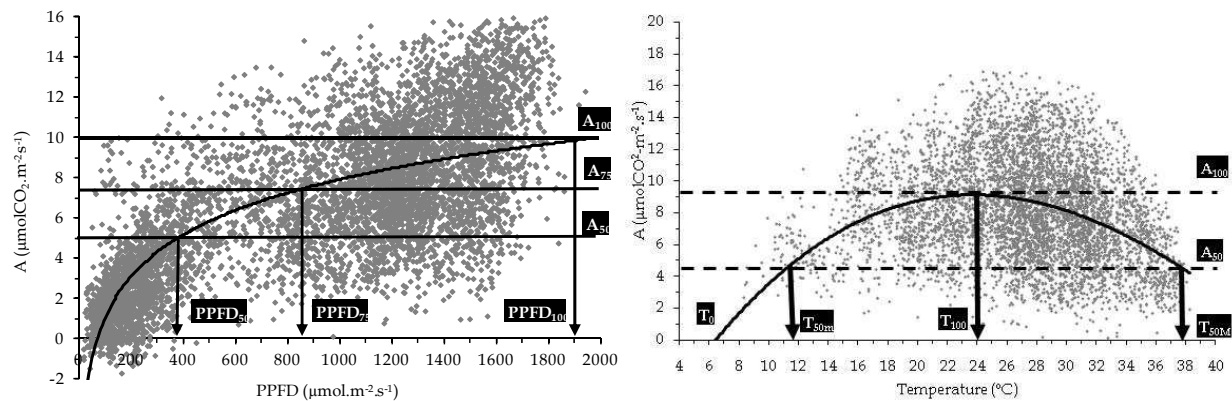


Fig. 3. Study of photosynthesis rate ( $A$ ) as a function of radiation (PPFD, left) and temperature ( $T$ , right). For PPFD,  $A$  was modulated by logarithmic curve  $A = 3.0108 \ln(\text{PPFD}) - 12.87$  ( $R^2 = 0.541$ ), instead of temperature, there was selected a second polynomial curve  $A = -0.0002T^3 - 0.0432T^2 + 1.6543T - 8.9259$  ( $R^2 = 0.121$ ). Measurements ( $n=8852$ ) were taken in adult trees (40 years old) from 13 Portuguese varieties, during 4 years, between July and October, with an infra-red gas analyser (model LCA-2, Analytical Development Co., Hoddesdon, UK). For PPFD curve, there was selected the measurements done between 16 °C and 32°C, the temperature curve was determined under plenty PPFD ( $>900 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ).

Leaf transpiration is a physical process of evaporation that causes the water content of the leaves to drop. This produces a gradient of water potential from the root to the leaves. In consequence a flow of water from the soil to the roots, stems, leaves and evaporation from here to the atmosphere through stomata, happens. This water movement has two main functions: a) promote the mineral upward to the plant, and b) promote the regulation of leaf temperature due to high specific heat value of water (Epstein & Bloom, 2005). So, a higher  $E$  is better for leaf temperature regulation. Contrarily to  $A$ , dim-light did not significantly

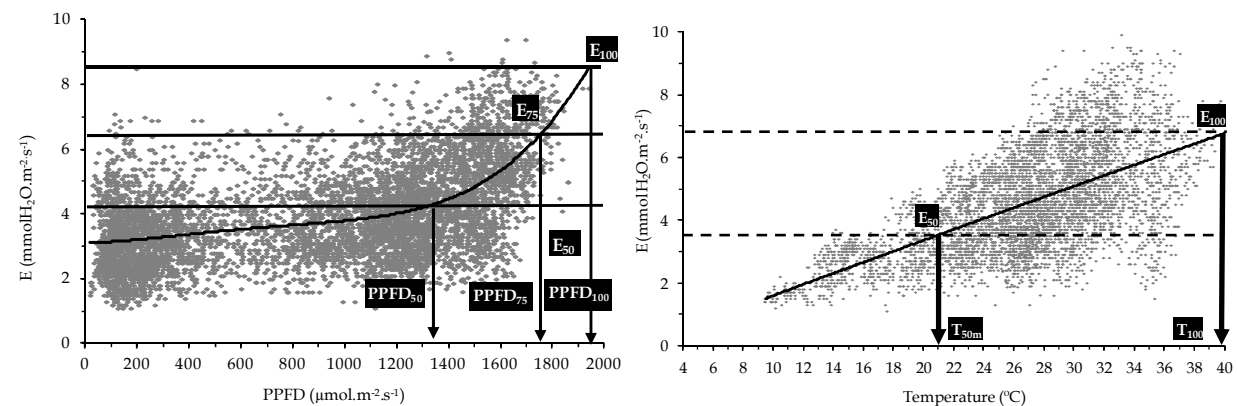


Fig. 4. Study of transpiration ( $E$ ) rates in European chestnut trees as a function of radiation (PPFD, left) and temperature ( $T$ , right). For PPFD the polynomial equation is  $E = 1E^{-12}\text{PPFD}^4 - 3E^{-9}\text{PPFD}^3 - 3E^{-6}\text{PPFD}^2 + 8E^{-5}\text{PPFD} + 3.109$  ( $R^2 = 0.308$ ) and for temperature,  $E$  can be represented by  $E = -0,0002T^2 + 0.1813T - 0.1967$  ( $R^2 = 0.360$ ). For other conditions see Figure 3.

affect  $E$ , half rate of it being around  $1300 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  (Figure 4). Although, above this PPFD level, this strongly influences  $E$ . On the other way, there is visible a strong increase in  $E$  as a response to the temperature rise (Figure 4), from 1.5 to 3.5 and to  $7 \text{ mmolH}_2\text{O}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  when temperature increases from 10 to 21 and to  $40^\circ\text{C}$ , respectively. On the other way it must be believed that a higher diversity of  $E$  was observed under highest temperatures than under lowest ones. This might indicate that significant differences among varieties in this parameter could be ascribed.

The water use efficiency (WUE) firstly considered as water used per unit plant material generated (Briggs & Shantz, 1914), varying between 0.001 and 0.005 or less, clearly indicates that transpiration is a wasteful process. This is the result between two conflicting requirements: the need for exposure of moist, green cells to light and that for open pathways to allow  $\text{CO}_2$  to diffuse to these cells. In what concerns the influence of PPFD, the strongest increase in WUE was obtained under dim-light conditions ( $\text{WUE}_{75} \sim 600 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , Figure 5). These findings are partially consistent with the influence of  $T$  on WUE, where the highest efficiency,  $0.003 \mu\text{mol CO}_2\cdot\text{mmol H}_2\text{O}^{-1}$ , was found under lowest temperatures ( $14^\circ\text{C}$ ). The half value of WUE appears when  $T$  reaches  $30^\circ\text{C}$ , a typical temperature measured during summer times in the South of Europe.

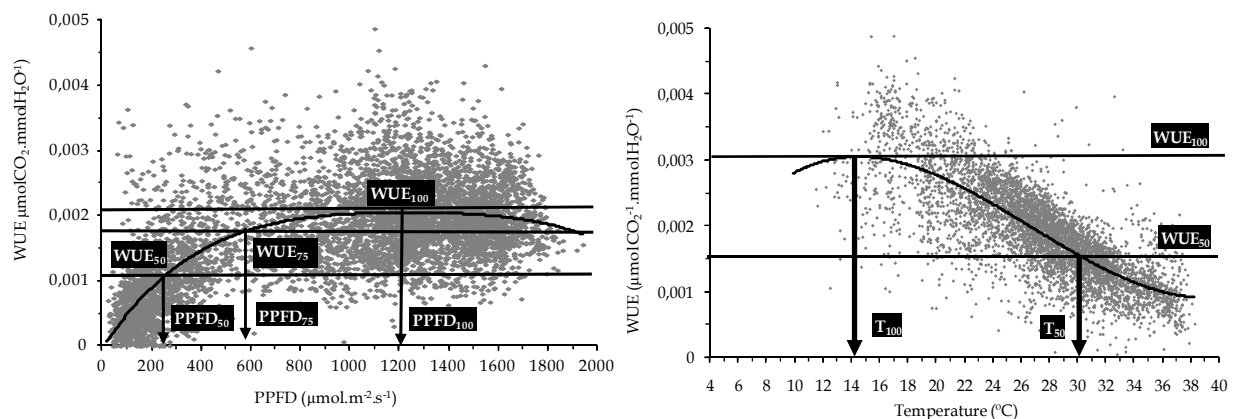


Fig. 5. Characterization of water use efficiency variation (WUE) as a function of the abiotic factors: radiation (PPFD, left) and temperature ( $T$ , right). Temperature influence can be modulated by the equation  $\text{WUE} = 0.0002T^3 - 0.0126T^2 + 0.2098T + 2.3388$  ( $R^2 = 0.627$ ), while for PPFD the respective equation is  $\text{WUE} = -6E-16\text{PPFD}^4 + 3E-12x^3 - 6E-09x^2 + 6E-06x - 5E-05$  ( $R^2 = 0.466$ ).

In relation to Portuguese varieties, optimal temperature ( $T_{100}$ ) changes between  $22^\circ\text{C}$  ('Lada') and  $29^\circ\text{C}$  ('Boaventura'),  $7^\circ\text{C}$  being the wide of the interval. Concerning the heat tolerance,  $T_{50}$  interval range is only  $3^\circ\text{C}$ , between  $35^\circ\text{C}$  ('Judia' and 'Negral') and  $38^\circ\text{C}$  ('Aveleira'). Results suggest that globally, the varieties from Valpaços (780 m a.s.l.) have lower  $T_{100}$  than those from Vinhais (780 m a.s.l.), while the varieties from Vila Real (730 m a.s.l.) presented high  $T_{50}$  (Figure 6). The infection of trees with *Phytophthora cinnamomi* Rands (the oomycete that causes ink disease) induces higher heat tolerance as the shift on  $T_{100}$  and  $T_{50}$ , presented by infected 'Judia' trees, indicates.

The state of the lipids plays an important function in the temperature response at the thylakoid membranes, since it might function as a buffer, in a determined range of temperatures. Fatty acids of the thylakoid glicerolipids must be sufficiently unsaturated in



order to allow a convenient mobility of the electron transfer carrier plastoquinone, but at the same time must preserve its integrity, namely in relation to the above cited impermeability of the protons (Blackwell et al. 1994). In chestnut chloroplasts the most significant fatty acids are  $\alpha$ -linolenic acid (18:3) (40-50%) and palmitic acid (16:0) (20-30%). Comparing the balance of the lipid composition in three varieties, Aveleira (Vinhais), Longal (Valpaços) and Judia (Valpaços), the ratio of unsaturated/saturated fatty acid was 1.86, 2.27 and 2.40, the former being also the variety with lowest  $T_{50}$ , followed by Longal, being Aveleira the most tolerant to heat.

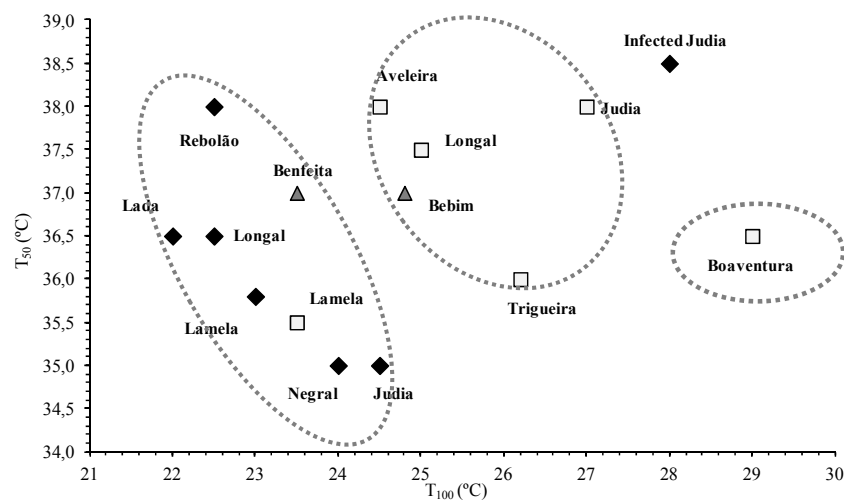


Fig. 6. Distribution of Portuguese varieties according to their optimal temperature ( $T_{100}$ ) and temperature for half ( $T_{50}$ ) of maximal rate for *A.* Varieties in different origins, Valpaços, Vila Real and Vinhais, are indicated by lozenge, triangle and square, respectively. The label 'Judia infected' correspond to infected trees with *Phytophthora cinnamomi* Rands.

### 3.2 Spanish varieties

In Spain, there are 124,053 hectares of pure *C. sativa* Mill. forests and orchards and 55,416 hectares of chestnut mixed with other broadleaves (mainly oak, holm oak, cork and beech) according to the Third National Forestry Inventory published in 2006 ([www.marm.es](http://www.marm.es)). However, this does not include the area formed by the Canary Islands, with 2000 hectares, or the estimated 5000 hectares in the provinces of Zamora, Salamanca, Ávila and Málaga (Pereira-Lorenzo et al., 2009). It is found from sea level in some parts of the northern coast of the Iberian Peninsula up to 1500 m in the Sierra Nevada (Granada, Southeastern Spain; 37° N, 03° W).

Four models of management for chestnut can be distinguished in Spain: i) orchards for nut production based on grafted trees with selected cultivars; ii) coppice stands for timber production; iii) high forests for timber production; iv) grafted orchards for nut and timber production with cultivars like 'Garrida', 'Loura' and 'Paredé' which, in humid areas, are pruned in a way which favours the development of long trunks that are sold at high prices (Pereira-Lorenzo et al., 2009). Chestnut coppice distribution is largely dependent on the silvicultural techniques applied historically in the region. As a result, coppice exploitation for timber is more common in Asturias than in the rest of Spain.

The identification and evaluation of the genetic resources of grafted chestnuts in Spain began by examining morphological characters and isoenzymes enabling the discrimination of these materials. Pereira-Lorenzo et al. (2006) evaluated 701 accessions corresponding to 168 cultivars; 31 from Andalusia (12 cultivars), 293 from Asturias (65), 25 from Castilla-Leon (9), 4 from Extremadura (2) and 348 from Galicia (80). The main morphological characters examined showed considerable variation in relation to geographic area. Furthermore, the isoenzyme data demonstrated greater variability in the North, a finding related to the historic importance of chestnut cultivation in this area. Examination of the F-statistics, however, showed that there is in fact only limited genetic differentiation, but high heterozygosity, linked to grafting, between areas. The same methodology was applied to the Canary Islands and a further 38 cultivars were characterized (Pereira-Lorenzo et al., 2007).

More recently, Pereira-Lorenzo et al. (2011) have used microsatellites to confirm this high degree of conservation of this genetic variability in the Iberian Peninsula, finding that diversity in chestnut orchards was mostly explained by hybridization (up to 77%), with mutation only accounting for 6%. Previous results (Pereira-Lorenzo et al., 2010) showed that there are two main origins of variability in the Iberian Peninsula, one in the North and the other in the Central Iberian Peninsula. This division roughly corresponds to contrasting climatic conditions in Spain; i.e., mesic areas (moderately humid environment) and xeric (drier environment) areas, respectively. The Northern group is characterized by a wetter and milder climate than the Southern, which has lower monthly rainfall in summer ( $P_s$ ) and both higher average annual T and higher average T of the hottest month (Allué-Andrade, 1990).

Water availability is known to be one of the most limiting factors in relation to photosynthesis and plant productivity (Boyer, 1996). Furthermore, predicted climate warming effects in the Iberian Peninsula (Ramírez-Valiente et al., 2009) suggest considerable changes in this respect. To address these issues, an ecological characterization of chestnut has been conducted: the first part of the investigation focused on water relations and growth traits (Ciordia et al., under review); the second concentrated on water relations and physiological traits (Ciordia, 2009) and the results are presented in the remainder of this section.

One and a half-year-old open pollinated seedlings of 10 half-sib progenies of the main Spanish fruit cultivars of *C. sativa* Mill. were chosen from each of the two main gene pools (Pereira-Lorenzo et al., 2010): Northern (specifically from Asturias and Galicia) and Central Iberian Peninsula (specifically from the Canary Islands and Andalusia in Southern Spain). Chestnut seedlings were either well-irrigated throughout the experiment, or were subjected to mild drought stress followed by suspension of irrigation, with the aim of determining the vulnerability to drought of each progeny and of assessing the effect of the treatment on the phenotypic variation of the plants. Significant variations in most adaptive traits studied, such as juvenile growth, biomass allocation, leaf morphology and water relations, were observed.

In terms of photosynthetic and water traits, our results show that, in general and irrespective of geographic provenance, water stress modified physiological response: significantly reducing water conductance (K), gas exchange measurements ( $CO_2$  assimilation rate, A; transpiration rate, E; stomatal conductance to  $CO_2$ ,  $g_s$ ) with the exception of internal  $CO_2$  concentration ( $C_i$ ), and the maximal apparent efficiency of Photosystem II, estimated as  $F_v/F_m$ , whilst intrinsic water use-efficiency ( $WUE_i$ ) and initial fluorescence ( $F_o$ ) increased and carbon isotope composition ( $\delta^{13}C$ ) became less negative.

As expected, given that *C. sativa* Mill. is considered to be an anisohydric species, lower values of water potential, both at predawn ( $\Psi_{\text{wpd}}$ ) and at midday ( $\Psi_{\text{wmd}}$ ), were obtained in plantlets subjected to water stress. However, the recovery of  $\Psi_{\text{wpd}}$  compared to that at midday was not a general strategy for all chestnut progenies under water stress. Adjustment in water potential, together with variation in  $E$ , led to variation in  $K$ , which was found to decrease significantly when the plants were subjected to water stress. This reduction was most marked in the materials originating from Andalusia and the Canary Islands.

$\text{CO}_2$  assimilation rate ( $A$ ) was also found to decrease when soil water availability is low and, in this sense, a negative lineal correlation between  $A$  and  $\Psi_{\text{wpd}}$  and  $\Psi_{\text{wmd}}$  occurs. Furthermore, in some genotypes where  $\Psi_{\text{wpd}}$  and  $\Psi_{\text{wmd}}$  became very low and were in fact very similar, indicating that the plants were at critical matrix potential ( $\Psi_{\text{mcrit}}$ ), the reduction in photosynthetic efficiency was drastic with values below  $6 \mu\text{mol CO}_2 \text{ m}^{-2} \cdot \text{s}^{-1}$ , a decrease mainly associated with a reduction in  $g_s$ . Stomatal closure is generally the first response to drought and it is the dominant limitation to  $A$  at mild and moderate drought (Flexas & Medrano, 2002). In the Spanish chestnut progenies studied, as in other plant species, an adaptive mechanism in response to water stress can be observed, which tends to increase  $\text{WUE}_i$ . This is because the relationship between reduction in carbon assimilation and  $g_s$  is not a linear one and results in photosynthetic efficiency being affected less, and less quickly, than water loss (Chaves et al., 2003).

Provided that materials collected from different habitats are sampled under uniform garden conditions, variation in  $\delta^{13}\text{C}$  among geographic origins can be employed to infer genetic diversity in  $\text{WUE}_i$ . In general, studies on genetic diversity in  $\delta^{13}\text{C}$  for forest tree species from both mesic and xeric environments suggest the existence of significant, and often considerable, variation in  $\text{WUE}_i$  (e.g. Cregg et al., 2000; Li & Wang, 2003). In our study  $\text{WUE}_i$  was seen to increase under water limitation as demonstrated not only by the doubling of the  $A/E$  coefficient in the experimental group, but also by changes in  $\delta^{13}\text{C}$ . This parameter was found to be more negative under control than experimental conditions. Family genetic variation ranged from 2.1‰ under drought conditions to 1.6‰ under control conditions. This is in line with findings from a common garden trial with 6 provenances of *Castanea* where the range of variation of carbon isotope discrimination ( $\Delta$ ) in leaf dry matter was also about 1.6‰ (Lauteri et al., 1997), which suggests a maximum between population differences in  $\text{WUE}_i$  of over 16% (Ferrio et al., 2005, as cited in Farquhar & Richards, 1984). Understanding genetic variation in  $\text{WUE}_i$  through  $\delta^{13}\text{C}$  in relation to geographic and/or climatic gradients is essential in evaluating adaptation patterns in drought-prone areas. However, in our study, we have not found there to be significant differences between the geographic areas of Northern Spain (humid area) compared to Andalusia and the Canary Islands (dry area). In fact, progenies from the latter cultivated under water stress conditions reached more negative values than those from the Northern group. This apparent contradiction can be explained if we take into account that stomatal opening and closing not only regulates transpiration but also plays an important role in thermal regulation.

The shape of the leaf also contributes to thermal regulation. According to Nicotra et al. (2008) more lobated leaves have a finer boundary layer leading to more efficient heat exchange and reducing differences in  $T$  between leaf and air. They also suggest that there is a relationship between degree of leaf lobation (ILB), carbon fixing and water loss and speculate that this is probably the effect of evolutionary convergence towards adaptation to drought and heat as indicated by the fact that more lobated leaves have a lower specific leaf

area (SLA) due to their higher proportion of veins and thus have an  $A$  per unit of mass equivalent to less lobated leaves.

The results from our experiment on the whole support the findings of Nicotra et al. (2008). We found that ILB is a plastic parameter which varies in relation to water availability, increasing under stress conditions, and that SLA decreases as water availability drops. According to Fischer & Turner (1978), lower values of SLA may be associated with higher resistance to drought. Contrary to the findings of Abrams (1994) who found that xeric genotypes generally produced leaves with lower SLA than those from the mesic region, in our study, SLA did not vary significantly between the two geographic groups. ILB could be considered a favourable selection characteristic in water limited environments, obviously to a large degree linked to summer rainfall which in turn is one of the environmental factors which distinguishes the two groups in this study.

However, we did not find increase in ILB to be associated with increases in carbon fixation: there was a trend, stronger for the Northern group, for  $C_i$  to increase under water stress simultaneous with reduction in carbon assimilation. This result may be explained by alterations in the photosystem II (PSII), which may also be the cause of reductions in the rate of  $A$  and  $E$ . In the chestnut progenies, the maximal apparent efficiency of PSII is maintained within a fixed range for healthy, well watered plants (Björkman & Demming, 1987). Any stress, such as drought, may be reflected by a drop in this relation (Öquist, 1987), and this was indeed seen in our trial, principally in relation to material from the Northern pool. We also noted a significant increase in initial fluorescence ( $F_0$ ), providing support for the idea that PSII reaction centres are damaged rather than a protective temporary inhibition in response to excess luminosity. Under water limitations, the proportion of absorbed light used by the plant in the course of photosynthesis and photorespiration is less than it would be in a turgid plant. In this situation, plants can reduce light absorption or divert the light through alternative processes such as thermic dissipation which can account for up to 75% of absorbed light (Niyogi, 1999). In such conditions, leaves experience a transitory reduction in PSII photochemical efficiency or suffer photoinhibition, and, in a worst case scenario, photooxidative destruction. Thus photosynthesis regulation can be seen to be a dynamic process regulated by dissipation of thermal energy (Chaves et al., 2003).

In summary, we found water stress to have a great effect on growth as well as most of the morphological and physiological traits studied in *C. sativa* Mill. juvenile progenies of the main fruit Spanish cultivars, all of which demonstrated its capacity to respond to drought. In spite of the large phenotypic variation observed between progenies, when analysing the data, significant differences were also found between the two gene pools (Northern and Southern) for most morphological and growth traits, as well as for  $K$ . This corresponds to the genetic differentiation between these groups found by Pereira-Lorenzo et al. (2010) and Martin et al. (2010) using molecular markers and detected previously by Mattioni et al. (2008). The increased vigour of progenies from the Northern Iberian Peninsula can therefore be attributed to differences in the gene pool of this group compared to that of plants from the rest of Spain.

Moreover, a significant geographical trend related to monthly rainfall in summer ( $P_s$ ) was found under both experimental and control treatments for juvenile growth and certain leaf morphology traits. According to previous studies (Villani et al., 1994; Lauteri et al., 1997, 2004), genetic, morphological and physiological differentiation of wild chestnut populations spread along a Turkish transect stretching from the Black Sea coast to the Mediterranean area were associated with macroclimatic variables, particularly with precipitation (Pigliucci



et al., 1990). However, Conedera et al. (2009) suggested studying the ecological plasticity of the chestnut in more depth in view of possible climate change, as a combination of increased temperatures and a significant reduction in summer precipitation, correlated to the geomorphologic site conditions, has been associated with damage in chestnut stands in the Southern Alps, thus leading the same authors to hypothesize that this species does not have an effective mechanism to protect against over-transpiration in extremely hot and dry weather.

The variability demonstrated in our study may be the result of domestication processes at the local level, i.e., the materials used in different localities are usually the ones which are best adapted to the local conditions even though they may not be the most productive. That said, both origins of chestnut genetic variability offer valuable experimental material for further studies of characteristics and processes involved in divergence and speciation as well as for the safeguarding and development of genetic resources for use in breeding and afforestation programs.

### 3.3 Hybrid clones

In an attempt to reduce the impact of the ink disease, many strategies have been taken into consideration, from characterizing soil suppressive features to examining orchard management related factors (Martins et al., 2010; Martins et al., 2005; Portela & Pinto, 2005) as well as assessing the impact of oomycete attack on the water relations of chestnut (Gomes-Laranjo et al., 2004; Maurela et al., 2001). In their studies, Robin et al., (2006) observed near 20% of tolerant trees in several populations of *C. sativa* Mill., suggesting that resistant genes could already exist within the species. In the work on genetic resources of *C. sativa* Mill. species, Aravanopoulos (2005) and Eriksson et al. (2005a) also found significant differences in tolerance against *Phytophthora cambivora* (Petri) Buisman infection in England, where the populations were the most sensitive, as opposed to those from Galicia.

Nonetheless, tree breeding in Europe has also focused on adding resistant genes to the gene pool of chestnut. This has been achieved through hybridization with the more resistant Asiatic *C. crenata* Sieb. and Zucc. species. These plants (*C. sativa* Mill. × *C. crenata* Sieb. and Zucc. or the reciprocals), known to be highly tolerant, are largely used as a clonal rootstock in European orchards (Cortizo et al., 1996; Fernandes, 1966; Gomes, 1982; Salesses et al., 1993). Portuguese production of hybrid clones started in the early 1950s by Fernandes (1952), and led to an important collection of clones, COLUTAD (Abreu et al., 1999), being one of the most popular and promising discoveries. Some of these clones showed good features for wood production while others are more suitable as rootstock for *C. sativa* Mill. varieties, thus making these clones first class material for new plantations, as proposed by the National Centre of Forest Seeds (CENASEF).

Japanese chestnut (*C. crenata* Sieb. and Zucc.) and Chinese chestnut (*C. mollissima* Blume) and *C. Sativa* Mill. species are thought to share the same origin, located in eastern Asia (Lang et al., 2007). According to these authors, *C. crenata* Sieb. and Zucc. is the most basal; in relation to Chinese species, it is positioned in a monophyletic clade; the North American and European species are its sister group. *C. crenata* Sieb. and Zucc. also presents the most divergent haplotype in relation to the other *Castanea* species, as a result of the unique climate of the island (Lang et al., 2007). According to Bounous & Beccaro (2002), *C. crenata* Sieb. and Zucc. grows in volcanic soils on the South of Japan, where summers register high levels of precipitation. Due to its origin, Japanese chestnut grows vigorously in humid and



warm climates. The earlier bud burst of these hybrids makes them more vulnerable to frost damage than *C. sativa* Mill., as well as less tolerant to drought.

In a study done with the Euro-Japanese clones growing in CENASEF, they showed 1.5 to 2 times significantly higher in  $g_s$ ,  $E$  and  $A$  values than the values measured in Portuguese variety 'Judia'. In fact the former's maximal photosynthetic rate was obtained between 32.5 and 35 °C, whereas for the latter the optimal  $T$  was 26.5 °C, explaining by this way the differences between both genotypes.

In terms of leaf water potential, the  $A_{100}$  occur between -0.5 and -1.1 MPa for all plants, whereas the values inducing a reduction of 50% in  $A_{100}$  are considerably different within hybrid clones, ranging between -2.8 MPa and -1.8 MPa. These values are higher than the -1.2 MPa determined for *C. sativa* Mill. plants.

On the other study to compare the effect of wild (*C. sativa* Mill.) and hybrid (*C. sativa* Mill. x *C. crenata* Sieb. and Zucc.- Ca90) rootstock on Judia, there was observed an increase on heat tolerance when there is used Ca90 rootstock.

### 3.4 Effect of the air temperature on photosynthetic capacity

Temperature controls the developmental rate of many organisms. The total amount of heat required, between the lower and upper thresholds, for an organism to develop from one point to another in its life cycle is calculated in units called degree-days (°D). This represents the physiological time that provides a common reference for the development of organisms (Cesaraccio et al., 2001; Zalom et al., 1983).

Analysis of Figure 7, shows a strict relation between daily mean  $A$ , mean  $T$  and their dependence on place's altitude in Trás-os-Montes Region (Portugal). In fact, as the altitude increases,  $T$  decreases but  $A$  also increases. The maximal value (11.5  $\mu\text{mol CO}_2\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) was measured at 23-25 °C, corresponding to the daily mean values observed at 850 and 1050 m a.s.l. places. The minimal  $A$  value (65% of the maximal rate) was measured in the warmest local, 450 m a.s.l. being 29.15 °C of daily mean  $T$  (Figure 7). In the lowest altitude local the amount of heat was 2587 °D and in the highest altitude place that was 1879°D (in the period between March and October in 2007). Other important difference observed in tree response to altitude, and consequently to the  $T$  is the daily variation of  $A$  (Figure 7). In the highest altitudes, it stays in the maximal range until the middle of the afternoon, while in the lowest altitudes it starts to decrease in the morning, indicating loss on productivity. On the other side,  $A$  is much higher in the highest altitude than in the lowest one.

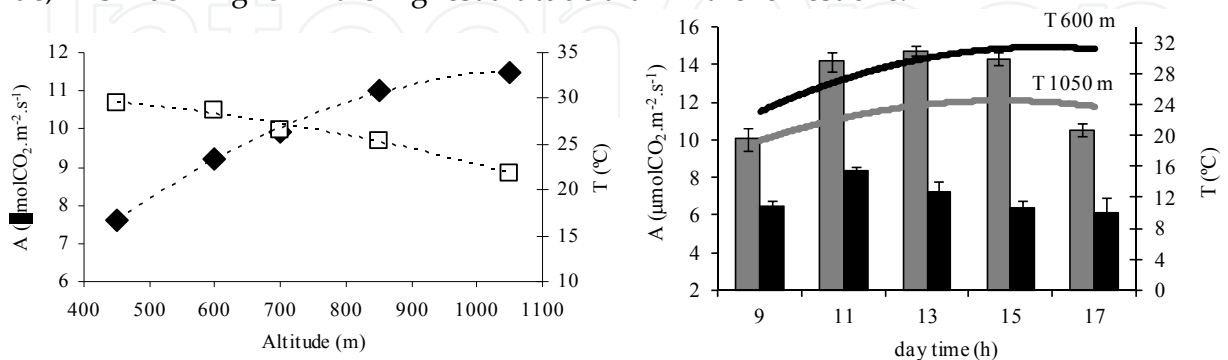


Fig. 7. Variation of mean daily photosynthesis rate ( $A$ , left, closed symbols) in function of altitude. As the altitude increases mean daily ambient temperature decreases (9, 11, 13, 15 and 17 h; open symbols). Daily variation of  $A$  (right) at 450 m a.s.l. (black bars) and 1050 m a.s.l. (gray bars).

Trees exhibit maximal  $A$  when  $\Psi_w$  is situated in the range of -0.8 to -1.3 MPa;  $A$  was inhibited by 10% at -1.6 MPa (Gomes-Laranjo et al., 2008a). Under dry soil conditions water potential can decrease to -2.0 MPa, which can induce a reduction in  $A$  of about 50%. The influence of a pair of factors, water stress and thermoinhibition, may constitute part of the explanation for the Fv/Fm diminution during morning in the warmest localities (450, 600 and 700 m a.s.l.), and the increase for the highest altitude orchards (1050 m a.s.l.). It is important to say that this parameter reflects the maximal efficiency of PSII, and consequently adequate internal conditions to have a high  $A$ . These conditions are strongly dependent on  $T$ , since physical state of thylakoid membranes (where photosynthetic electron chain takes place) is strongly  $T$  dependent. Adaptation of membranes to  $T$  has been reported by Burkey et al., (1997), since an augmentation in unsaturation level was detected as a function of the altitude increase (Table 1). Variations on Chla/b and Chl/Car ratios, which reflect sun and shade adaptation, also indicate adaptation of plants to the environmental conditions, because Chla/b ratio inversely increases in function of altitude increment and Chl/Car has a similar variation against altitude (Gomes-Laranjo et al., 2008b). These two variations are consistent with the known acquired tolerance to warm and sunny conditions, since Chla is the main pigment present in the photosystem I, which is located in exposed thylakoid membranes, and carotenoids have the chlorophyll protection function against photoinhibition.

Altitude (s.l.m)	Chltot mg.cm <sup>-2</sup>	Chla/b	Chl/Car	Total fatty acid		PI	UI	Malonic aldehyde x10 <sup>-4</sup> (mM)	
				Saturated (%)	insaturated (%)			Control	ADP-Fe
1050	121.5 b	3.12 c	4.8 a	27.0	73.0	111.5	171.3	1.35	3.88
900	145.9 a	3.10 c	5.0 a	32.4	67.6	97.5	154.2	1.67	4.57
700	99.1 c	3.30 b	4.4 b	38.2	61.8	119.2	156.3	2.00	4.91
600	143.9 a	3.40 b	4.6 b	33.1	66.9	47.5	146.1	1.90	4.53
450	80.9 d	3.60 a	3.9 c	43.9	56.1	79.6	121.2	3.12	5.36

Table 1. Variation of photosynthetic pigment content (n=10), fatty acid composition (n=3) and malonic aldehyde (n=3) in leaves from adult trees (var. 'Judia'). PI represents the peroxidation index and UI the unsaturation index.

Leaf pigment content was higher on trees growing in lower altitude than that in higher altitude regions, since thermoinhibition speeds light saturation of the photosynthetic process (Boardman, 1977). Moreover, increase in Chla/b suggests higher proportion of stacking thylakoid membranes, which in turn might induce higher  $A$ , if any stress factor imposes (Anderson et al., 1988). Additionally, thiobarbituric acid reactive substances presented as malonic aldehyde show a decrease in peroxidation activity as the altitude increases, which could indicate a better response to oxidative stress with the altitude.

### 3.5 Photosynthetic apparatus characterization of leaves from different sides of chestnut canopy

Chestnut (*C. sativa* Mill.) is a large deciduous tree, reaching a height of 40 m and 6 m to 7 m diameter of canopy (Bounous, 2002). In such canopies, it is possible to identify a deep heterogeneity in the light availability around the crown (north, east, south and west regions) besides an enormous internal canopy region (Figure 8).

According to the measurements, leaves located in the north side only have less than 20% of the south side level of PPFD, making the south part of the canopy hotter than the opposite part. Concerning east and west sides, they have between 74% and 67% of the south PPFD (Table 2). These facts induce a cascade of occurrences at many levels of biological organization (Boardman, 1977; Osmond & Chow, 1988). Leaf area of the south, east, west and north of tree top was different. The highest leaf area was in the northern canopy. The shadow leaves were larger, thinner and adapted to responding more effectively to the less light available and its diffused nature.

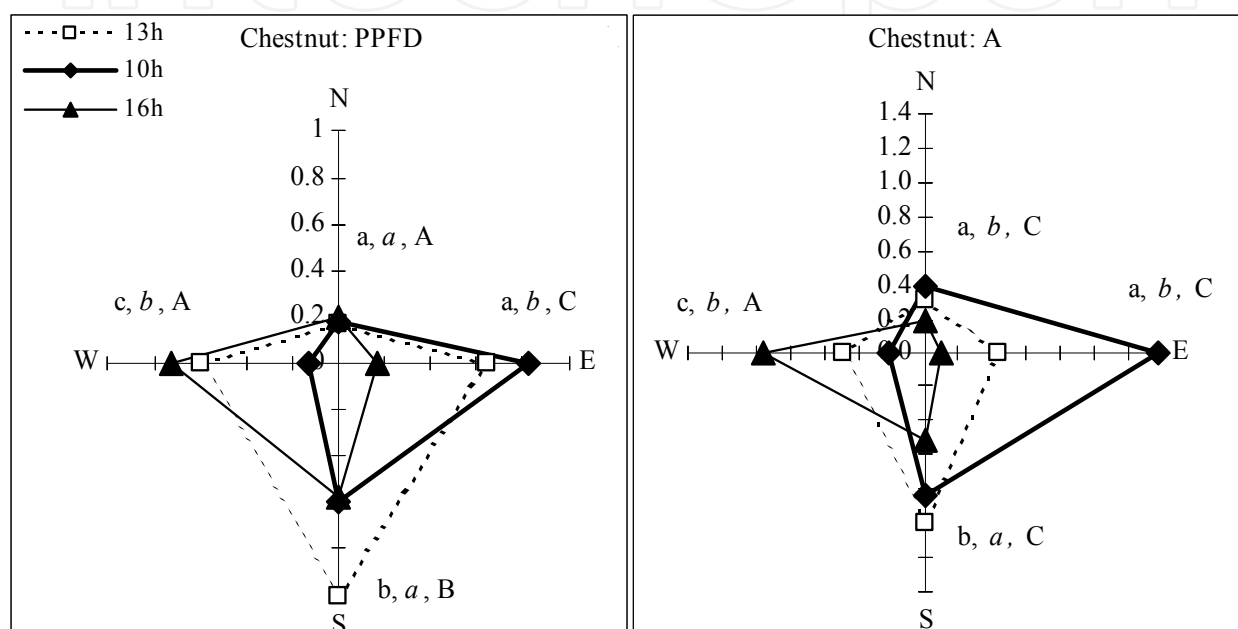


Fig. 8. Study of the radiation (PPFD) (left) and photosynthesis (A) (right) variation at 10, 13 and 16 h in north, east, south and west sides of 'Judia' crown. In each side, letters represent the result of the comparison between 10 h (normal letters) 13 h (italic letters) and 16 h (caps letters) hours. The values with common letters are not significantly different, according to Fisher test, 5%.

This species has typical mesomorphic leaves with a dorsiventrally flattened lamina. Concerning the lamina internal anatomy, in the upper epidermis there are two layers, and in the lower epidermis only a single layer (Figure 9). Both epidermises have cells with straight anticlinal walls. However, the cells were more regular in the upper than in the lower surface. In the glabrous adaxial surface there is a thicker cuticle layer than that in the abaxial surface, which is coated by a dense trichome layer (depending on variety, it can be almost absent in north leaves).

Differences in light saturation point between south and north leaves were found (Figure 10), showing the former leaves highest PPFD saturation point, as there is known for other sun and shade broadleaves (Lichtenthaler, 1985; Osmond & Chow, 1988). Additionally, north leaves presented higher A than south leaves below 400  $\mu\text{mol m}^{-2} \text{s}^{-1}$  radiation intensity. The former also presented lowest compensation point

Parameters	North	East	South	West
PPFD ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )	137 <i>d</i>	673 <i>b</i>	912 <i>a</i>	611 <i>c</i>
LT ( $^{\circ}\text{C}$ )	24.4 <i>b</i>	25.1 <i>a</i>	25.1 <i>a</i>	24.8 <i>ab</i>
<i>Histology</i>				
Thickness ( $\mu\text{m}$ )	188.7 <i>a</i>	194.6 <i>c</i>	239.2 <i>a</i>	208.7 <i>b</i>
Upper epidermis ( $\mu\text{m}$ )	22.0 <i>c</i>	23.8 <i>b,c</i>	29.1 <i>a</i>	24.4 <i>b</i>
Lower epidermis ( $\mu\text{m}$ )	15.2 <i>a</i>	15.0 <i>a</i>	16.2 <i>a</i>	15.4 <i>a</i>
Palisade mesophyll ( $\mu\text{m}$ )	82.4 <i>c</i>	87.8 <i>b</i>	110.8 <i>a</i>	86.8 <i>b</i>
Spongy mesophyll ( $\mu\text{m}$ )	73.4 <i>b</i>	66.6 <i>c</i>	82.2 <i>a</i>	83.6 <i>b</i>
Pal/Spongy	1.19 <i>b</i>	1.38 <i>a</i>	1.39 <i>a</i>	1.07 <i>c</i>
Transmittance (%)	5.44 <i>b</i>	5.25 <i>a</i>	5.24 <i>a</i>	5.36 <i>a</i>
<i>Photosynthetic pigments</i>				
Total Chlorophyll ( $\text{mg.cm}^{-2}$ )	63.3 <i>b</i>	63.9 <i>b</i>	67.6 <i>ab</i>	67.0 <i>a</i>
Chla /Chlb	2.91 <i>b</i>	3.09 <i>a</i>	3.14 <i>a</i>	3.08 <i>a</i>
Carotenoides ( $\text{mg.cm}^{-2}$ )	12.1 <i>a</i>	13.0 <i>a</i>	14.2 <i>a</i>	14.5 <i>a</i>
Chl/Car	5.23 <i>a</i>	4.92 <i>b</i>	4.76 <i>c</i>	4.62 <i>c</i>
<i>Gas Exchanges</i>				
$g_s$ ( $\text{mmol m}^{-2} \text{s}^{-1}$ )	169 <i>b</i>	190 <i>a</i>	188 <i>a</i>	183 <i>a</i>
A ( $\mu\text{mol}(\text{CO}_2) \text{m}^{-2} \text{s}^{-1}$ )	1.47 <i>c</i>	3.14 <i>b</i>	3.89 <i>a</i>	3.02 <i>b</i>
E ( $\text{mmol}(\text{H}_2\text{O}) \text{m}^{-2} \text{s}^{-1}$ )	3.26 <i>c</i>	3.54 <i>b</i>	3.74 <i>a</i>	3.56 <i>b</i>
WUE ( $\mu\text{mol}(\text{CO}_2) \text{mmol}(\text{H}_2\text{O})^{-1}$ )	0.49 <i>c</i>	0.84 <i>b</i>	1.05 <i>a</i>	0.75 <i>b</i>
A/PPFD ( $\mu\text{mol}(\text{CO}_2) \text{mmol}^{-1}$ )	0.0064 <i>c</i>	0.0037 <i>b</i>	0.0044 <i>a</i>	0.0048 <i>b</i>
Ci (ppm)	292 <i>a</i>	281 <i>b</i>	268 <i>d</i>	275 <i>c</i>

Means followed by the same letter are not significant different at  $P < 0.05$  (Fisher test).

Table 2. Comparison of the mean values of photosynthetic active radiation (PPFD), leaf temperature (LT) and leaf histology, contents of photosynthetic pigments and gas exchanges among canopy sides ( $n = 225$ ).

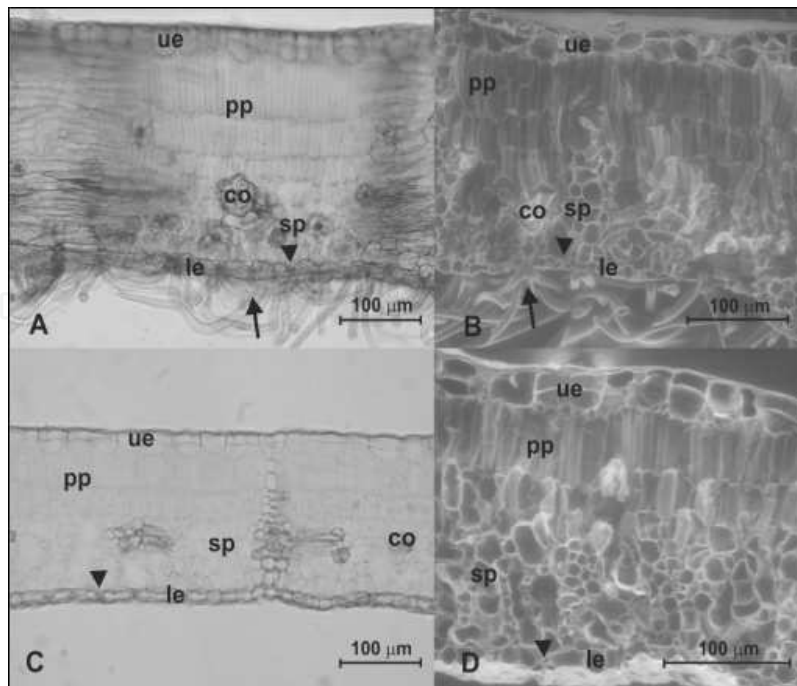


Fig. 9. Optical (A, C) and scanning electron microscopic (B, D) micrographs of the south (A, B) and north (C, D) of leaf cross-section from 'Martainha' variety. Legend: upper epidermis (ue), lower epidermis (le), palisade parenchyma cells (pp), spongy parenchyma (sp), calcium oxalate crystals (co), stomata (arrowheads), trichomes (arrows).

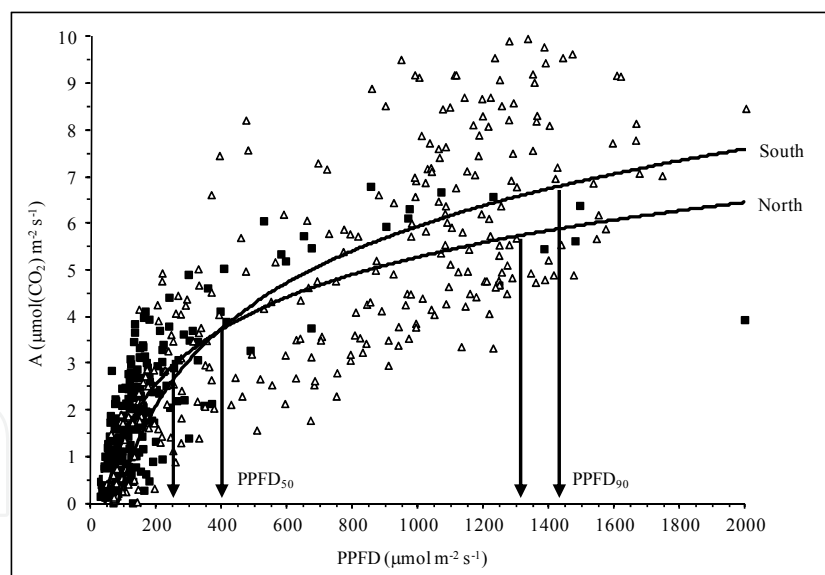


Fig. 10. Study of the correlation between PPFD intensity and photosynthesis rate in north and south sides. Data were obtained in July, August and September. Arrows represent the PPFD value for 90% and 50% of maximal  $A$  in south and north sides. Logarithmic equation analysis was used to determine the equation of the best-fitting line. The values of  $r^2$  were 0.64 (north) and 0.68 (south), respectively.

Changes in thylakoid membrane surface potential, induced by the electron transfer chain, were studied. South side chloroplast presented highest thylakoid membrane potential (Figure 11). This acclimation to high T is normally associated with a greater degree of



saturation of fatty acids in membrane lipids which turns membranes less fluid (Gomes-Laranjo et al., 2006). These authors also demonstrated that chloroplasts from north side showed approximately 5% more of surface thylakoids than those from south chloroplasts.

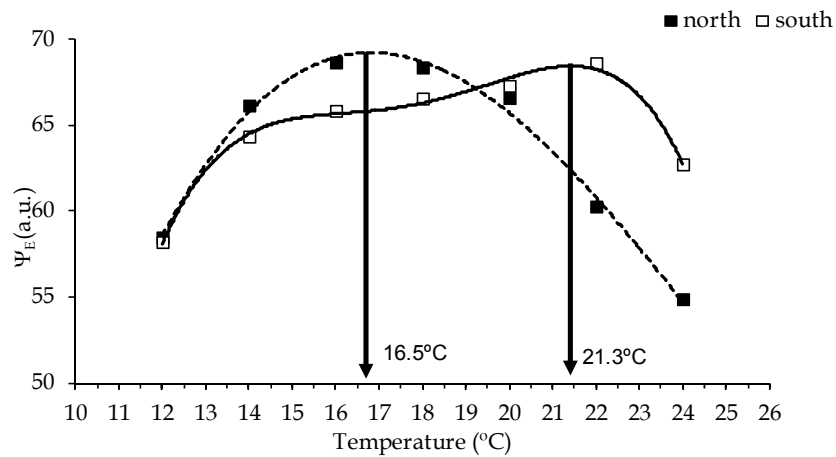


Fig. 11. Influence of temperature in thylakoid membrane potential of 'Judia' chloroplasts from leaves collected in south and north-side of canopy. For membrane potential measurements there was used the molecular probe 9-amino-6-chloro-methoxyacridine (Gomes-Laranjo et al., 2006).

#### 4. Impact of different orchard management strategies on gas exchange rates

##### 4.1 Soil management

Producers have submitted their orchards to intense management practices, such as soil tillage, fertilization and irrigation to increase productivity. In a study done between 2003 and 2007, in Trás-os-Montes region (Portugal), the effect of tillage and irrigation on the predawn leaf water potential ( $\Psi_{wpd}$ ) (Figure 12) and gas exchanges rates (Figure 13) were demonstrated.

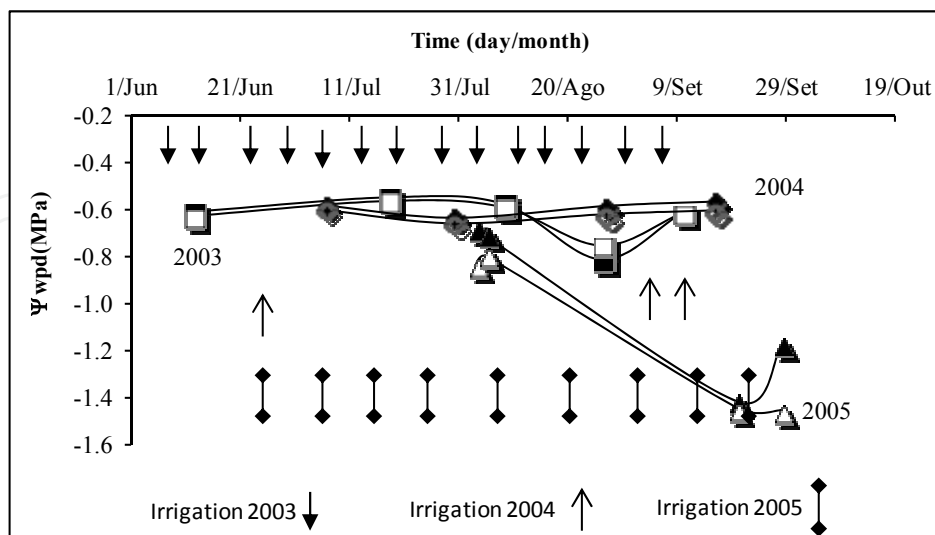


Fig. 12. Predawn leaf water potential values ( $\Psi_{wpd}$ ) during the studied period in three years, for TTC (▲, soil surface tillage with a tiny cultivator), USP (△, no tillage with rain feed seeded pasture under canopy) and ISP (■, no tillage with irrigated seeded pasture under canopy) treatments (n=12). The arrows display irrigation dates.

As shown in Figure 12, in 2003 and 2004, no significant differences in  $\Psi_{\text{wpd}}$  were found among treatments, with a level around -0.6 MPa. These results showed a complete recovery of the water level during the night, suggesting the availability of enough water for chestnut requirements in the soil, even without irrigation, and favourable conditions for chestnut growth. This observation provides an evidence that trees can absorb water according to its availability across the root zone, which is consistent with earlier observations above-mentioned. In relation to photosynthetic rate ( $A$ ) (Figure 13), no significant differences in cultural practices were detected in 2003 and 2004, following the same pattern as  $\Psi_{\text{wpd}}$ . Nevertheless, significant differences were detected between the measurements at different  $T$ . Independent on the measurement date, highest values of  $A$ , in the range of 9-9.5  $\mu\text{mol CO}_2\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , were measured when  $T$  reached 28-29 °C, decreasing to 8-8.5  $\mu\text{mol CO}_2\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  at 33 °C and to 4-5  $\mu\text{mol CO}_2\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , in August, when  $T$  rose up to 37.5 °C. These data are consistent with above lines and previous studies (Gomes-Laranjo et al., 2006) as also with the values obtained for August 2005, showing that photosynthetic rate of *C. sativa* Mill. seems more dependent on atmospheric  $T$  than on soil water, under the climatic conditions of the studied region.

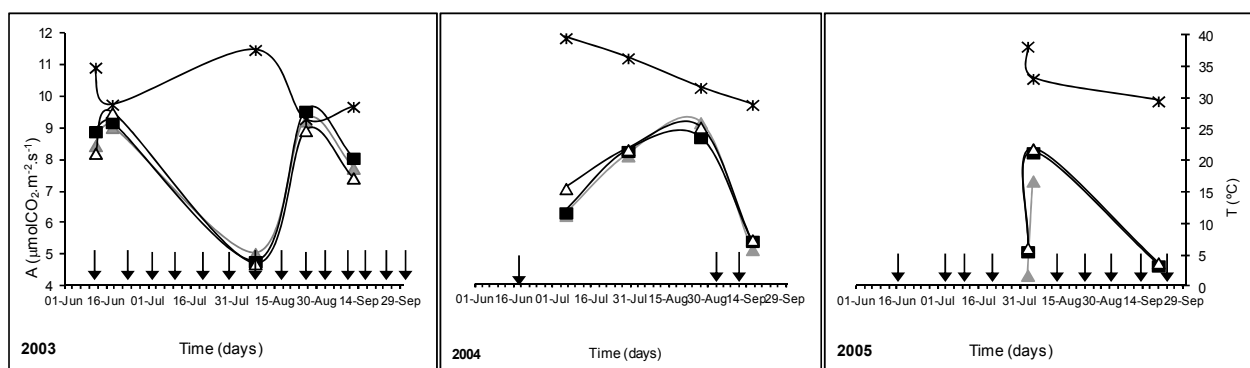


Fig. 13. Obtained results for photosynthesis rate ( $A$ ) and atmospheric temperature ( $T$ ) on TTC ( $\blacktriangle$ , soil surface tillage with a tine cultivator), USP ( $\triangle$ , no tillage with rain feed seeded pasture under canopy) and ISP ( $\blacksquare$ , no tillage with irrigated seeded pasture under canopy) treatments in 2003, 2004 and 2005 from June to September. When present, each arrow indicates irrigation in the ISP treatment.

Nevertheless, related to 2005, a decrease in  $A$  was observed from 5<sup>th</sup> August to 20<sup>th</sup> September, from 8.4 to 4.8  $\mu\text{mol CO}_2\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , in spite of the maintenance of atmospheric  $T$  at 30-32 °C, which might be due to the water stress as the very low  $\Psi_{\text{wpd}}$  (-1.6 MPa) suggested.

Fertilization with nitrogen, phosphorus and potassium is common practice among producers; however applications of magnesium (Mg) and boron (B) are hardly carried out in chestnuts. Both nutrients, Mg and B greatly affect vegetative growth, namely the size and shape of the leaves and obviously LAI and photosynthetic activity. Portela et al. (1999) showed that trees with pronounced yellowing due to Mg deficiency may reduce LAI to values less than 50%, and nut size and productivity were also greatly decreased (Portela et al, 2003). Although B deficiency in chestnuts has been identified and B fertilization was carried out in some orchards with unequivocal positive effects on nut production, only recently post-treatment evaluation was carried out (Portela et al., 2011).

#### 4.2 Boron applications

The trees suffering B deficiency show death of shoot tips and the terminal buds systematically fail; many branches are leafless; leaves are small and narrow, sometimes malformed and with irregular shape; the internodes are shortened and a rosetting arrangement of younger leaves can be observed (Portela et al., 2011). In 2008, fruit setting and productivity were increased fourfold with B applications. Epstein and Bloom (2005) emphasized the role of B in increasing the translocation rate of the sugars through the phloem from the photosynthetic tissues to the actively growing regions and also the developing fruits.

Soil B application in a non-irrigated orchard (around 15 years old) located at 600 m a.s.l in Trás-os-Montes (corresponding to an amount of 2500 °D, in the period of March-October), can increase the water potential in chestnut. This conclusion was drawn from 3 years of B experiment (2006-2009), where in October the leaf water potential ( $\Psi_w$ ) in fertilized trees with B was -2.14 MPa, while in control plants the value shifted to -2.24 MPa. As a consequence, a cascade of benefits was obtained. In the gas exchange rates (Table 3, unpublished data), an increase in  $g_s$ ,  $E$ ,  $A$  were observed as so in chlorophyll content. Photosynthesis rate increased by 52% in 2008 (under 27°C) and by 16% in 2009 (under 31°C). It is noteworthy that values from 2009 were obtained under heat stress (optimal T for chestnut is around 27 °C) inducing a reduction of about 50% in  $A$ .

Year	T	PPFD	$g_s$	$E$	$A$	WUE	A/Ci	A/ $g_s$	$\Psi_w$
Treatment	(°C)	( $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ )	( $\text{mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ )	( $\text{mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ )	( $\mu\text{molCO}_2\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ )	( $\mu\text{molCO}_2\cdot\text{mmolH}_2\text{O}$ )	( $\mu\text{molCO}_2\cdot\mu\text{bar}$ )	( $\mu\text{molCO}_2\cdot\text{mmol}$ )	(MPa)
2007									
B0	28,09	1180,00	127,79 a	2,57 b	11,77 a	5,24 a	0,08 a	0,11 a	-1,24 a
B1	29,58	1180,80	129,28 a	2,89 a	12,19 a	4,59 b	0,09 a	0,11 a	-1,26 a
2008									
B0	27,60	1305,00	84,00 b	2,27 b	8,29 b	3,66 b	0,04 b	0,10 a	
B1	27,38	1305,00	158,00 a	3,13 a	12,65 a	4,08 a	0,06 a	0,08 b	
2009									
B0	31,50	1392,00	33,02 b	1,18 b	6,73 b	7,15 a	0,38 a	0,25 a	-2,44 a
B1	31,65	1392,00	48,42 a	1,65 a	7,79 a	5,11 b	0,14 b	0,18 b	-2,14 b

Table 3. Effect of boron fertilization on gas exchange rates in chestnut trees.

Boron application to the soil might have created better conditions for development of the root system and therefore a better acquisition of water from the soil. The role of B in root development is well supported by Dell and Huang (1997).

#### 4.3 Silicon applications

Silicon (Si) is regarded as a beneficial element of higher plants (Epstein, 2001). It may increase plant resistance against biotic, e.g., diseases and pests (Hou & Han, 2010; Osunacanizalez et al., 1991) and abiotic stresses, e.g., water deficit (Gong et al., 2003), salt (Saqib et al., 2008) and heavy metals (Neumann & zur Nieden, 2001). Our recent work has shown that foliar application of Si significantly increased chestnut plant  $g_s$  and  $A$  (Figure 14, unpublished data).

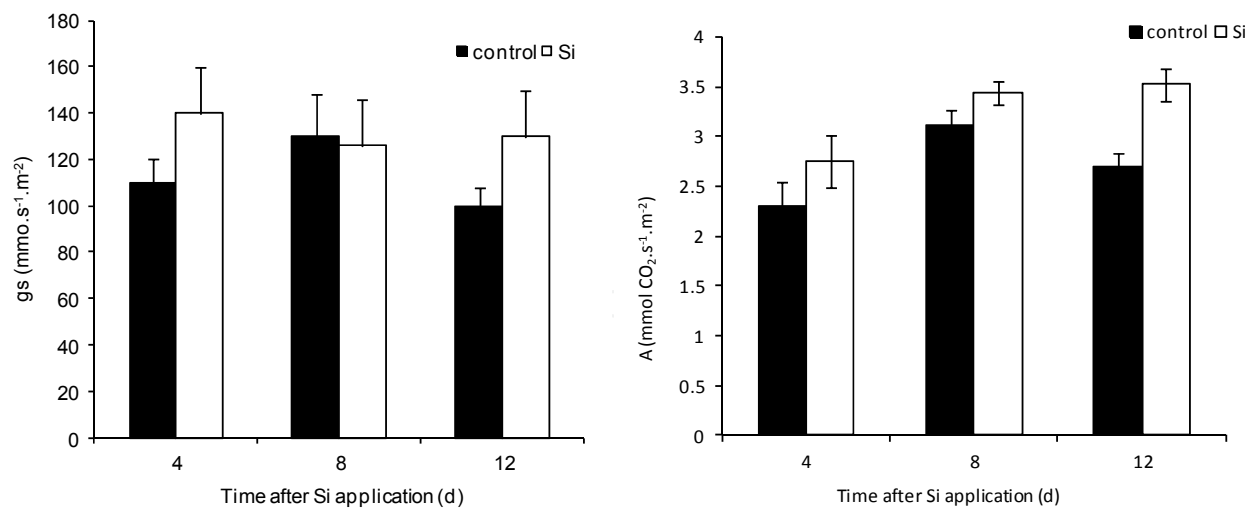


Fig. 14. The effect of silicon (Si) treatment on chestnut (*C. sativa* Mill. M.) plantlet  $g_s$  and  $A$ .

## 5. Understanding mechanisms of ink disease tolerance by analysing alterations in secondary metabolite synthesis

The oomycete pathogen *Phytophthora cinnamomi* Rands. is generally found in areas with acid to neutral soils containing low amounts of nutrients (mainly low content of phosphorus and calcium), low organic matter, and fewer micro-organisms and also presenting poor drainage, where average annual rainfall is greater than 500 mm. The severity of the symptoms is related to climatic and soil characteristics, which have been shown to be greater in south-facing stands (Martins et al., 2005). The metabolism seems to be directly affected by the water stress provoked by the presence of the oomycete. The most important cause could be the blockage of the xylem and the consequent lack of water in the leaves (Bryant et al., 1983). Infection frequently occurs as a response to several biochemical stimuli exuded from host plants, which are mainly near root injuries. These stimulating compounds are sugar enriched, which attract and promote zoospore germination in plant tissues. A recent study, where resistant and sensitive plantlets were infected, shows an increase of soluble sugars in resistant ones (Dinis et al., 2011b). It is known that the increase in water stress induces accumulation of soluble sugars in leaves (Quick et al., 1992), and consequently an increase in osmotic strength, which in most of times occurs at expenses of starch content diminution (Gomes-Laranjo et al., 2004a). On the other side, there is expected an inhibition in the phloem loading, due to the lack of water, increasing by this way, the starch synthesis in chloroplasts. It is well known that the water stress can also alter carbon assimilation and the partitioning between sucrose and starch (Quick et al., 1992), resulting in an increase in sucrose concentration (Chaves, 1991). Soluble sugar, such as sucrose and glucose, either acts as substrate for cellular respiration or as osmolyte to maintain cell homeostasis. Fructose is not related to osmoprotection but it is related to secondary metabolite synthesis, namely with the synthesis of erythrose-4-P, which acts as a substrate for lignin and phenolic compound syntheses (Rosa et al., 2009).

Dinis et al. (2011b), have observed a strong increase in phenols content (50%) in resistant plantlets with increase of soluble sugars, constituting a part of the defence mechanism against the hyphae invasion. Resistance to ink disease is attributed essentially to phenolic content, because most of them have fungicidal activity. Gallic acid is the most predominant

phenolic acid in chestnut (De Vasconcelos et al., 2009). Non-structural carbohydrates tend to accumulate and more phenolic compounds are produced (Bryant et al., 1983). Some of those secondary metabolites, like the polyphenols, function as plant antioxidants due to their free radical scavenging property.

Concerning the protein content opposing results were found. Gomes-Laranjo et al. (2004b) has shown an increase in infected plants with *P. cinnamomi* Rands., as many other authors also found in plants under water stress (Bacelar et al., 2006; Rosa et al., 2009; De Vasconcelos et al., 2009). However, Dinis et al., (2011b) obtained a decrease in soluble proteins content in the first answered period after infection. This could be a consequence of an increase in protein hydrolysis to provide an increase in amino acids under leaf drought conditions (Yadav et al., 1999). One of the most important responses of plants to drought is an overproduction of different types of compatible solutes (Ashraf & Harris, 2004; Serraj & Sinclair, 2002) namely total free amino acids, proline and soluble sugars. Among several biochemical indexes of water stress, proline accumulation and the decline in protein synthesis in plants have been widely reported. Other authors reported that the accumulation of certain cytosolutes, particularly proline and glycine betaine (a quaternary amine) could avoid the negative effects of the cell osmotic potential decrease, without interfering with protein synthesis (Raggi, 1994). Proline synthesis has been associated with protein hydrolysis induced by water deficit (Bacelar et al., 2006). In the second period, the soluble protein content increased (Dinis et al., 2011b), probably due to defense enzyme activity against the oomycete invasion (Ricardi et al., 1998; Tabaeizadeh, 1998).

In conclusion, leaf water deficit, caused by xylem blockage, due to the invasion of the oomycete hyphae, triggers phenol synthesis and phloem loading, resulting in the damage at the physiological and biochemical levels, which ultimately leading to plant death.

## **6. Genetic modifications targeting improved chestnut tolerance against abiotic stresses**

Abiotic stresses adversely affect growth and productivity and trigger a series of morphological, physiological, biochemical and molecular changes in plants. Drought, extreme T, and saline soils are the most common abiotic stresses that plants have to face. Globally, approximately 22% of the agricultural land is saline (FAO, 2004), and areas under drought have been already expanding and this is expected to increase further (Burke et al., 2006). Gene expression profiles of either drought- or salt-stressed barley plants indicated that, although various genes were differentially regulated in response to different stresses, they possibly induce a similar defense response (Ozturk et al., 2002).

Progress in breeding for drought tolerance has consequently been limited. Molecular biology, however, provides new tools that promise better understanding of the mechanisms of drought stress and drought tolerance. Drought tolerance is a complex trait, and breeding for tolerance has been hampered by interactions between genotype and environment. From the conventional plant breeding point of view, several characteristics and processes have been considered important in drought tolerance improvement.

Similarly, many physiological and morphological (phenotypic) characters are considered important in adaptation to drought stress. Osmotic adjustment, in which the plant increases the concentration of organic molecules in the cell water solution to bind water, is one example of the mechanism that alleviates some of the detrimental effects of drought. A



thicker layer of waxy material at the plant surface and more extensive and deeper rooting are the others. Root development plays a major role in a plant's response to water availability. Root development is restricted in acid soils, because of aluminium toxicity. Phosphorus is also highly fixed in acid soils and this too adversely affects root development. Therefore, improving aluminium tolerance and phosphorus uptake indirectly improves drought tolerance. Similarly, physiological and biochemical traits that might enhance drought tolerance have been proposed but only a few of these mechanisms have been demonstrated to be casually related to the expression of tolerance under field conditions.

It has been reported that photosynthesis and several other related physiological traits differ significantly between drought-tolerant and susceptible genotypes. Some crops are naturally more drought tolerant than others, and are obviously better suited to drought environments (Gomes-Laranjo et al., 2006).

Gomes-Laranjo et al. (2006) studied the T effect in three chestnut (*C. sativa* Mill.) varieties' behaviour, 'Judia', 'Longal' and 'Aveleira' and they found differences among them indicating, the least adaptability of 'Judia' to high T (T100, 23.5 °C) and also 'Longal', showing a shift of one degree increase (T100, 24.5 °C) in the optimal T for photosynthesis, and 'Aveleira' which exhibits quite different behaviour (T100, 26 °C and T50, 38.5 °C). When different clones of the 'Judia' variety were studied in terms of heat tolerance, some intra-clonal variety was observed, clones with higher tolerance to heat were found and that seems to be associated with a "memory" heat, since they followed a pattern of behaviour similar to the climatic conditions of the place of origin (Dinis et al., 2011c, d). These genotypes may be interesting for selective breeding for heat resistance. Adaptive traits have been evaluated in progenies from local varieties (Ciordia et al., 2011) between the most genetically differentiated areas in Spain (Pereira-Lorenzo et al., 2010, 2011), the North and the Centre-South. Progenies showed significant differences in growth and morphological aspects, as a strategy for adaptation to water stress, quite common in central and southern Spain.

Identification of areas of the genome that have a major influence on drought tolerance, namely Quantitative Trait Loci (QTL), could allow marker assisted selection (MAS) to be used to identify those plants from a population that are likely to be better adapted to drought. These areas of the genome are invariably numerous and large, and it is a further step to identify the genes underlying the QTL and assess their contributions to drought tolerance. In addition to accounting for variation in drought tolerance directly, these QTL will also largely determine root morphology and development, and may well govern expression of a whole range of other associated genes. Once the major QTLs have been identified, they might be transferred among plants using linked molecular markers associated with them.

In the mapping project undertaken for the European chestnut (*C. sativa* Mill.) a family obtained from controlled crosses between two trees with contrasting phenotypes, with respect to the efficiency of water use was used: the female parent ('Bursa') belonging to type adapted to drought in the Western part of Turkey, and the male parent ('Hopa') adapted to flooding in Eastern Turkey. Two genetic linkage maps were constructed for the first time for European chestnut, based on this plant material and different markers: RAPD, ISSR and isozyme markers for identification of genomic regions (QTL) related to water use efficiency (Casasoli et al, 2004). QTLs were detected for bud flush, growth and carbon isotope discrimination using female and male parents originated from two Turkish chestnut populations adapted to a drought and humid environment, respectively (Casasoli et al.,

2004). Phenology showed the higher proportion of stable QTLs. Phenotypic correlations and co-localizations among QTLs for different adaptive traits was related with the genetic adaptation of the female parent to drought. Homeologous genomic regions identified between oak and chestnut allowed to compare QTL positions (Casasoli et al., 2006). Co-localization of the QTL controlling the timing of bud burst was significant between the two species.

To date, 517 markers were mapped in *C. sativa* Mill. linkage map, covering 80% of their genome, and 12 linkage groups that were aligned to obtain a consensus between the male and female parents. In order to obtain orthologous markers for comparative mapping, microsatellite markers developed for species of *Quercus* and *C. sativa* Mill. were tested in both species. Nineteen loci, 15 of which from *Quercus* and four from *C. sativa* Mill., were integrated into the two maps previously established, allowing the first comparative mapping between the linkage groups of the two species (Barreneche et al., 2004). The same loci were also used to perform the alignment of the maps obtained for the European chestnut with inter map x-specific *C. mollissima* Blume x *C. dentata* (Marsh.) Borkh. (Sisco et al., 2005).

The candidate-gene approach is a powerful and robust method. Compared to the genome wide mapping strategy, the chances of finding markers linked to putative QTL are maximized, since the selection of candidate-gene markers is based on known relationships between biochemistry, physiology and the agronomic character under study.

Genetic association between allelic variants and trait differences on a population scale is a powerful, and relatively recent approach to identifying genes or alleles that contribute to variation in adaptive traits (Long & Langley 1999; see Neale & Savolainen, 2004 for conifers). Regarding forest trees, progress on identification of drought-related genes and development of expressional studies are relatively recent (Chang et al. 1996; Dubos & Plomion, 2003; Watkinson et al., 2003). The molecular basis of dehydration tolerance in trees is extremely complex and a wide variety of expressional candidate genes have been suggested (González-Martínez et al., 2006).

Being multigenic as well as a quantitative trait, it is a challenge to understand the molecular basis of abiotic stress tolerance and to manipulate it as compared to biotic stresses (Amudha & Balasubramani, 2010). Stress-induced gene expression can be broadly categorized into three groups: (1) genes encoding proteins with known enzymatic or structural functions, (2) proteins with as yet unknown functions, and (3) regulatory proteins. Intuitively, genetic engineering would be a faster way to insert beneficial genes than through conventional or molecular breeding. Also, it would be the only option when genes of interest originate from cross barrier species, distant relatives, or from non-plant sources (Bhatnagar-Mathur et al., 2008).

Initial attempts to develop transgenics (mainly tobacco) for abiotic stress tolerance involved "single action genes" i.e., genes responsible for modification of a single metabolite. However, that approach has overlooked the fact that abiotic stress tolerance is likely to involve many genes at a time, and that single-gene tolerance is unlikely to be sustainable (Bhatnagar-Mathur et al., 2008).

Therefore, a second "wave" of transformation attempts to transform plants with the third category of stress-induced genes, namely, regulatory proteins has emerged. Through these proteins, many genes involved in stress response can be simultaneously regulated by a single gene encoding stress inducible transcription factor (Kasuga et al., 1999), thus offering

possibility of enhancing tolerance towards multiple stresses including drought, salinity, and freezing. Regarding the genetic transformation of chestnut, apart from the success obtained for American chestnut (Polin et al., 2006), where we can find reports of transgenic plants with resistance genes to *Cryphonectria parasitica* in field trials (Powel et al., 2005), the studies attempted for European chestnut didn't show significant results of stable transformation (Seabra & Pais, 1998, Corredoira et al., 2004).

## 7. Conclusion

A great deal of work has been done to assess the biodiversity in chestnut species, part of them with the aim to understand species' potentialities facing to climate changes.

Chestnut is quite spread in the world, mostly of those regions being located in the North Hemisphere. The cradle of chestnut, is attributed to a region in east of China, from where it spread for many regions, Europe being one of the most important areas. Nowadays, European chestnut occupies areas in the south part of Europe, mainly corresponding to the Mediterranean countries, ranging between 27° N and 53° N, where climate change impacts can be more significant. In fact, it is consensual that the main consequences of those, might be an increase in T and a decrease in the water availability. The large spread of chestnut in Europe during thousands of years has induced long-term adaptations and by this way a certain specialization of genotypes according to the local edaphoclimatic characteristics of each one. According to elegant studies performed in the Cascade project (Eriksson et al., 2005b), chestnut presents a substantial adaptive variation among populations. They demonstrated that Mediterranean ecotypes might have more tolerance to drought than other ones from wet locals. Additionally, Pereira-Lorenzo et al. (2010) verified that the south Iberian areas of chestnut were colonized by genotypes from the north Iberian region concluding by this way that this species has potential to adapt itself to new climatic conditions and eventually to the new context of climate changes. Anyway, chestnut is not very much heat tolerant as outlined in the present chapter. Air temperatures in the range of 22 to 30 °C are optimal for its growth. Thus for this species, the summer T is one of the most decisive factors.

Chestnut is a dim-light species presenting significant adaptive degree even inside same canopy, where shady and sunny leaves were also characterized. Chestnut is also an anisohydric species, with considerable buffer capacity during summer times on leaf water Potential, being -0.8 MPa is the typical value of predawn leaf water potential without water stress. This buffer capacity is attributed to deep root system which allows plant to absorb water from the deep soil layers. As this species is less heat tolerant, transpiration plays a decisive role in leaf cooling and by this way in promoting the best photosynthetic rates for such edaphoclimatic conditions. In consequence, chestnut needs great quantity of water, perfectly supporting the popular dictate for chestnut "Chestnut wants to boil in July and drink in August". So, increase of water absorption capacity, must be integrated into the new management strategies. The impacts of boron and silicon applications were studied with promising results. The above referred abiotic stresses, which induce loss of plant vigor, sensitize plant to pests and diseases. The most common, the root rot ink disease attacks the fragile chestnuts, blocking xylem and stopping water uptake, triggering phenol synthesis.

Besides agronomic characteristics, heat and drought stress are two important traits that must continue to be in account for future studies. Morphological and ecophysiological studies in strict connection with genetic studies aiming to identify tolerant genotypes, including the necessary variety breeding programs, must continue in future. The employ of

QTL's, which allows the identification of determined genome areas, could allow marker assisted selection (MAS) to be used to identify tolerant plants from a population that are likely to be better adapted to those stresses.

## 7. References

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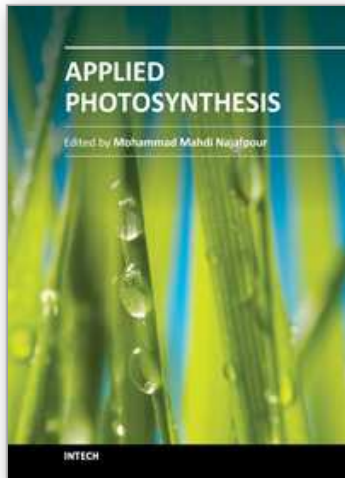
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Edited by Dr Mohammad Najafpour

ISBN 978-953-51-0061-4

Hard cover, 422 pages

**Publisher** InTech

**Published online** 02, March, 2012

**Published in print edition** March, 2012

Photosynthesis is one of the most important reactions on Earth, and it is a scientific field that is intrinsically interdisciplinary, with many research groups examining it. This book is aimed at providing applied aspects of photosynthesis. Different research groups have collected their valuable results from the study of this interesting process. In this book, there are two sections: Fundamental and Applied aspects. All sections have been written by experts in their fields. The book chapters present different and new subjects, from photosynthetic inhibitors, to interaction between flowering initiation and photosynthesis.

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José Gomes-Laranjo, Lia-T. Dinis, Luís Martins, Ester Portela, Teresa Pinto, Marta Ciordia Ara, Isabel Feito Díaz, Juan Majada, Francisco Peixoto, S. Pereira Lorenzo, A.M. Ramos Cabrer, Changhe Zhang, Afonso Martins and Rita Costa (2012). Characterization of Chestnut Behavior with Photosynthetic Traits, Applied Photosynthesis, Dr Mohammad Najafpour (Ed.), ISBN: 978-953-51-0061-4, InTech, Available from: <http://www.intechopen.com/books/applied-photosynthesis/characterization-of-chestnut-behavior-with-photosynthetic-traits>

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中国上海市延安西路65号上海国际贵都大饭店办公楼405单元  
Phone: +86-21-62489820  
Fax: +86-21-62489821

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