



Universitat de Lleida

## Evaluation of autumn frost resistance in *Juglans regia* L.

Mercè Guàrdia Bel

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# **EVALUATION OF AUTUMN FROST RESISTANCE IN *JUGLANS REGIA* L.**

Memòria presentada per Mercè Guàrdia Bel per optar al grau de Doctora per la Universitat de Lleida.

Realitzada sota la direcció de la Dra. Neus Aletà Soler i el Dr. Robert Savé Monserrat i sota la tutela del Dr. Jordi Voltas Velasco.

Maig de 2013

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## RESUM

Les diferents tasques exposades en aquesta tesi busquen aprofundir en els coneixements sobre la resistència de *Juglans regia* L. a les gelades de tardor, en les vessants metodològica, genètica i ecofisiològica.

El primer objectiu del treball va ser el determinar una metodologia adequada per avaluar el caràcter "resistència a les gelades de tardor" en *J. regia*, aspecte recollit en el Capítol I. Es va testar la utilitat del paràmetre Fv/Fm obtingut a partir de la mesura de la fluorescència de les clorofil·les del clorènquima de l'escorça en brots de l'any. Els resultats van ser comparables amb els dels mètodes habitualment utilitzats per avaluar els danys de gelades en brots llenyosos, com són l'anàlisi visual de danys i el càlcul d'alliberació relativa d'electròlits. Aquest primer estudi va permetre establir la temperatura de -8.5°C com la que ja provoca danys visibles sobre *J. regia* a la tardor. Tot i ser l'avaluació dels danys per fred mitjançant la Fv/Fm el sistema més fàcil i ràpid, a partir del II capítol, per la naturalesa dels objectius dels diferents assaigs, els mètodes emprats van ser els dos mètodes "clàssics" esmentats.

L'aprofundiment en els coneixements de la genètica d'aquest caràcter adaptatiu es va abordar als capítols II i III. En primer lloc, es va analitzar la relació entre el genotip i l'ambient i el pes relatiu de cada un dels factors pel que fa a la resistència a les gelades de tardor. L'estudi es va realitzar en quatre procedències de *Juglans regia* L. de la Península Ibèrica, plantades a dues zones ecològicament diferents també dins de la Península Ibèrica (una amb clima atlàntic i l'altra amb clima mediterrani). Es van detectar diferències temporals en l'aclimatació al fred entre els dos llocs d'assaig; no obstant, l'ordre que ocupava cada procedència en relació als danys observats a cada assaig fou el mateix. Així doncs, es va poder constatar la importància de la component genètica en l'expressió d'aquest caràcter. Dins d'aquest assaig es va observar un vincle altitudinal, en el que les procedències de les zones de menor altitud eren les més danyades. Posteriorment l'estudi es va centrar en estimar l'heretabilitat familiar del caràcter, a partir d'un test de progènies, format per 22 famílies de mitjos germans de *J. regia* d'orígens diversos, obtenint un valor de  $h_F^2=0.63$ . S'avaluà també el vincle del caràcter amb altres caràcters fenològics i vegetatius i s'observà una correlació genètica significativa i positiva tant amb la durada del període vegetatiu com amb el creixement anual en diàmetre. El valor de l'heretabilitat en sentit estricte calculat a partir de la correlació progenitor-progènie va ser de  $h^2 = 0,34$ .

L'últim objectiu, abordat al capítol IV, consistí en l'avaluació de la influència de l'estrès hídric estival (típic del Clima Mediterrani) sobre la resistència dels *Juglans* a les gelades de tardor. Aquest estudi es va realitzar sobre una progènie de *J. regia* i una d' híbrida de *J. ×intermedia*. Es varen aplicar diferents tractaments de reg tant en arbres adults com en juvenils. S'analitzaren diferents paràmetres relacionats amb l'aclimatació: la caiguda de fulla, el contingut hídric de les varetes i l'acumulació de sucres solubles i midó als teixits. Els resultats de la major part de les variables estudiades van mostrar un possible avançament de l'aclimatació en els individus sotmesos a tractaments hídrics restringits. Ara bé, aquesta aparent avantatge compromet negativament el creixement i per tant aquests resultats mereixen una avaluació acurada en les noves aforestacions de l'àrea mediterrània.

## RESUMEN

Las diferentes tareas que se exponen en esta tesis buscan profundizar en los conocimientos sobre la resistencia de *Juglans regia* L. a las heladas de otoño, en las vertientes metodológica, genética y ecofisiológica.

El primer objetivo del trabajo fue determinar una metodología adecuada para evaluar el carácter "resistencia a las heladas de otoño" en *J. regia*, recogido en el Capítulo I. Se testó la utilidad del parámetro Fv/Fm obtenido a partir de la fluorescencia de las clorofilas del clorénquima cortical de la corteza en brotes del año. Los resultados fueron comparables con los métodos utilizados habitualmente para evaluar daños producidos por heladas en brotes lignificados, como son el análisis visual de daños y el cálculo de la liberación relativa de electrolitos. Este primer estudio permitió establecer la temperatura de -8.5°C como la que ya provoca daños visibles sobre *J. regia* en otoño. Aunque la evaluación de los daños mediante la Fv/Fm fue el sistema más rápido y fácil, a partir del Capítulo II, debido a la naturaleza de los objetivos de los diferentes ensayos, los métodos utilizados fueron los "clásicos", mencionados anteriormente.

La profundización en los conocimientos de la genética de este carácter adaptativo se abordó en los capítulos II y III. En primer lugar, se analizó la relación entre el genotipo y el ambiente y el peso relativo de cada uno de los factores en relación a la resistencia a las heladas de otoño. El estudio se realizó en cuatro procedencias de *Juglans regia* L. de la Península Ibérica, plantadas en dos zonas ecológicamente diferentes también dentro de la Península Ibérica (una de clima atlántico y otra de clima mediterráneo). Se detectaron diferencias temporales en la aclimatación al frío entre los dos sitios de ensayo; no obstante, el orden que ocupaba cada procedencia en relación a los daños observados en cada ensayo fue el mismo. De esta manera se pudo constatar la importancia de la componente genética en la expresión de este carácter. En este ensayo se observó un vínculo altitudinal, en el que las procedencias de las zonas de menor altitud eran las más dañadas. Posteriormente el estudio se centró en estimar la heredabilidad familiar del carácter, a partir de un test de progenies, integrado por 22 familias de medios de hermanos de *J. regia* de distintos orígenes, obteniéndose un valor de  $h_F^2=0.63$ . Se evaluó también el vínculo del carácter con otros caracteres fenológicos y vegetativos como el crecimiento anual en diámetro. El valor de la

heredabilidad en sentido estricto calculado a partir de la correlación progenitor-progenies fue de  $h^2 = 0,34$ .

El último objetivo, abordado en el capítulo IV, consistió en la evaluación de la influencia del estrés hídrico estival (típico del clima Mediterráneo) sobre la resistencia de los *Juglans* a las heladas de otoño. Este estudio se realizó sobre una progenie de *J. regia* y una progenie híbrida de *J. ×intermedia*. Se aplicaron diferentes tratamientos de riego en árboles adultos y juveniles. Se analizaron diferentes parámetros relacionados con la aclimatación: caída de hoja, contenido hídrico de varetas y acumulación de azúcares solubles y almidón en los tejidos. Los resultados de la mayor parte de las variables estudiadas mostraron un posible avance en la aclimatación en los individuos sometidos a tratamientos hídricos restringidos. Esta aparente ventaja compromete negativamente el crecimiento, y por lo tanto, estos resultados merecen una evaluación precisa en las nuevas aforestaciones del área mediterránea



## SUMMARY

The work described in this thesis aims to deepen knowledge of autumn frost resistance of *Juglans regia* L. in methodological, genetic and ecophysiological terms.

The first objective of the study was to determine an appropriate methodology for evaluating the trait "autumn frost resistance" in *J. regia*, included in Chapter I. An assessment was made of the usefulness of the Fv/Fm parameter obtained from measurement of chlorophyll fluorescence in cortical bark chlorenchyma of annual budsticks. The results were comparable with those of methods commonly used to assess freezing damage to woody tissues, namely visual analysis and calculation of relative electrolyte leakage. This first study allowed establishing -8.5°C as the temperature which causes the first visible autumn freezing damage on *J. regia*. Although the assessment of cold damage by Fv/Fm was faster and easier, from Chapter II onwards the nature of the objectives of each assay obliged the use of the two aforementioned "classical" methods to estimate freezing damage.

In-depth knowledge of the genetics of this adaptive trait was addressed in Chapters II and III. First, the relationship between genotype and environment was examined as well as the relative weight of each factor on autumn frost resistance. The study was conducted in four provenances of *Juglans regia* L. from the Iberian Peninsula, planted in two ecologically different areas, the Spanish Atlantic and Mediterranean coast. Temporary differences were detected in cold acclimation entrance between the two trial areas; however, the order of each provenance in relation to freezing damage was the same in both areas. Thus, the importance of the genetic component in the expression of this trait was confirmed. The existence of an altitudinal link between provenances was also observed, with those from lower areas of elevation always the most damaged. The study then focused on estimating family heritability of the trait from a progeny test, integrated by 22 half-sib progenies of *J. regia* from different origins, obtaining a coefficient of  $h_F^2=0.63$ . The relationship of the trait with other vegetative and phenological features was also evaluated, and a significant positive genetic correlation was observed with both the length of the growing season and the annual growth in diameter. The value of the narrow-sense heritability obtained from the progenitor-progeny regression was  $h^2 = 0.34$ .

The final task, addressed in Chapter IV, consisted of evaluating the influence of summer drought stress (typical in the Mediterranean climate) on autumn frost resistance of *Juglans*. This study was conducted on a progeny of *J. regia* and a hybrid progeny of *J. ×intermedia*. Different irrigation treatments were applied to both adult and young trees. Several parameters related to cold acclimation were analysed: leaf fall, budstick water content and the accumulation of soluble sugars and starch in the tissues. The results of most of the variables showed a possible advance of acclimation in individuals subjected to water restricted treatment. However, this apparent advantage is offset by a negative effect on growth and therefore these results deserve careful evaluation in new afforestation in the Mediterranean area.



# General introduction |

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## GENERAL INTRODUCTION

In recent decades there has been increasing interest in establishing plantations of walnut in order to produce timber as an alternative to growing conventional Mediterranean crops. As a result of observing the damage that autumn frosts caused to these plantations, and especially to *Juglans regia*, steps were taken to evaluate “resistance to autumn frosts” in this species. The present work is based on the trials that were carried out in associated with this research.

All the activities were developed within the context of two lines of research conducted at the *Institut de Recerca i Tecnologia Agroalimentàries* (IRTA). The first formed part of a programme of adaptive and productive evaluation of different woody species for agroforestry exploitation. The second focused on the ecophysiological characterisation of natural and cultivated species subjected to biotic and abiotic stresses.

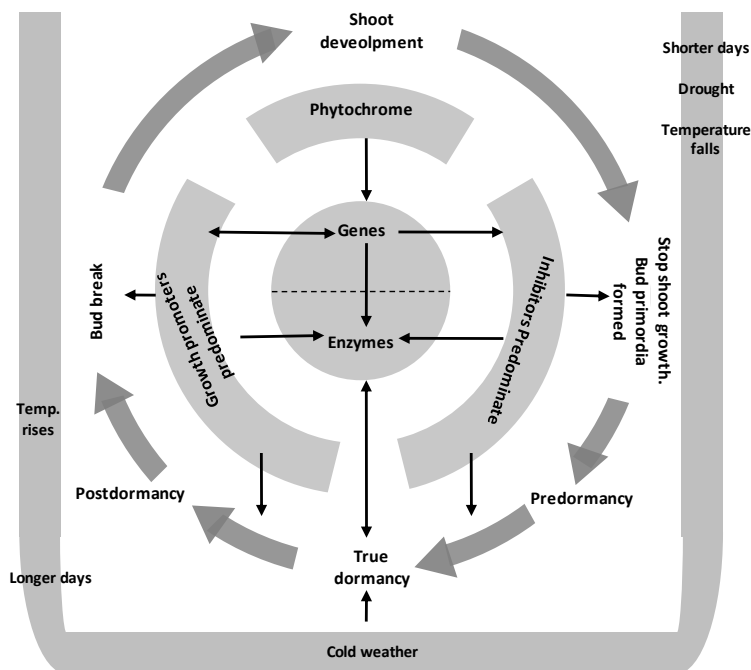
### Seasonal acclimation of species

Low temperatures are one of the most important environmental factors responsible for limiting the production and distribution of plants on Earth. Development throughout the growing period, the acclimation process and finally resistance to low temperatures all form part of an annual cycle in which plants establish a close relationship with the environment in which they develop through processes of adaptation (Sakai and Larcher 1987). However, when there is an unexpected event during the process of acclimation, with autumn temperatures suddenly falling, there is an increased risk of frost damage, or even the death of individual plants (Physiological Plant Ecology I. Responses to the physical environment 1981).

Under natural conditions, woody species that vegetate in a seasonal climate, like that of the Mediterranean, adapt to seasonal change through a number of different responses to environmental stimuli; this is what allows them to develop and to survive (Figure 1). Acclimation to winter frosts has two different stages (Weiser 1970). During the first stage, growth is halted and bud dormancy and leaf fall are induced (Sakai and Larcher 1987). To induce the process of growth arrest and hardening, the majority of woody species depend on their photoperiod; in other words, they are sensitive to exposure to short days. It has also been shown that the photoperiod can interact with various environmental factors, such as temperature and other sources of stress, to induce

acclimation (Lagercrantz 2009). During this period, woody species accumulate abundant quantities of organic substances which help them to survive low winter temperatures; these include carbohydrates, lipids and proteins (Levitt 1980; Sauter et al. 1996; Welling et al. 2004; Welling and Palva 2006). There is also a withdrawal of water from the xylem in order to prevent breakages due to the expansion of water when it freezes (Levitt 1980). In this state, plant cells can survive at temperatures around 0°C, but they are still not totally acclimated to the cold.

The second stage of acclimation is associated with direct exposure to temperatures of around 0°C or lower. In this stage, there are physiological and structural changes caused, amongst other things, by biochemical alterations to plasma membranes. These alterations may be due to qualitative changes in concomitant proteins, due to an increase in the fluidity of the membrane and to the enrichment of phospholipids and unsaturated fatty acids (Yoshida and Uemura 1990). All of these changes produce the highest level of tissue hardening and allow plants to survive freezing winter temperatures. At the end of this hardening process, plants enter a process of dormancy; this means that they cannot resume growth until they have overcome a long enough period of low temperatures to satisfy their “cold” requirements (Sakai and Larcher 1987; Taiz and Zeiger 2006).



**Figure 1.** Environmental influences (shaded U) and different interactions that affect the seasonal alternation between vegetative activity and dormancy in woody plants (Adapted from Sakai and Larcher 1987).

Once acclimation is complete, woody plants can survive freezing winter temperatures. In contrast, autumn freezes may cause serious damage to annual growth, above all during the first years of tree structure formation, when this occurs before these processes have been completed.

The capacity to tolerate freezing temperatures under natural conditions varies according to the tissue in question. Seeds, spores and other partially dehydrated tissues can remain almost indefinitely at temperatures near to absolute zero (Sakai and Larcher 1987). In unacclimated tissues, however, a sudden fall in temperature can cause the freezing of water and of both intracellular and extracellular aqueous solutions. Intercellular nucleation does not appear spontaneously unless cell temperatures fall to below  $-10^{\circ}\text{C}$  (Mazur 1977). At the moment of intracellular freezing, it is normal for cell death to occur, probably due to the mechanical destruction of biomembranes as a result of the growth of ice crystals inside the protoplast. The formation of extracellular ice occurs on the cell surface or between the protoplasts and the cell wall. The pressure of cellular water vapour is greater than that of extracellular ice, even when they are at the same temperature. As a result, the cell water flows through the plasmatic membrane and towards the extracellular ice. The cell then contracts due to the loss of water. When the functional temperatures for each specific plant material and physiological and phenological state are re-established, if they have not been damaged, the cells can again absorb water and regain their turgor. If they have been damaged, they remain compressed and cell death occurs (Physiological Plant Ecology I. Responses to the physical environment 1981; Taiz and Zeiger 2006).

Environmental stimuli are necessary for acclimation to occur, but when it begins, the extent to which it is expressed and how long it lasts depend on the species in question and its provenance (Sakai and Larcher 1987). The distribution of species in populations is determined by the response of individuals to different environmental factors associated with the specific territory. Thus, both the photoperiod, which is linked to latitude, and the temperature, which is associated with altitude, produce variations in plant senescence in certain species which depend on their provenance (Vitasse et al. 2011; Li et al. 2003; Pagter et al. 2008). Cases of the interaction between both of these factors have also been observed in studies of some genotypes of willow species, in which both the temperature and photoperiod are crucial for the development of resistance to low temperatures (those with more continental and more northerly provenances acclimatised before others) (Ogren

1999). The genetic diversity present within a given species provides an important anchor for surviving unexpected events.

Understanding the genetic variability of the “resistance to autumn frosts” characteristic would enable us to take more successful decisions to prevent early frost damage to plantations and reforestation areas. With this information, it should also be possible to adapt the management of new plantations to the environmental conditions foreseen as a result of climatic change. It is necessary to remember that such changes could turn out to be positive or negative for the territory, depending on many local environmental factors, on the crop chosen and on the agronomic management policy (ESCACC 2012).

In the Mediterranean region, a drier and warmer climate is expected (IPCC 2007a, b; ACCUA 2011) with a considerable increase in the concentration of atmospheric CO<sub>2</sub> (Aranjuelo et al. 2009). The predictions produced by different models for generating climatic change scenarios suggest that the Mediterranean region could be affected by medium-to-long and long periods of drought (lasting from 4 to 6 months and for more than 12 months, respectively) and by their increased frequency, which could rise to between 3 and 8 times the present levels (Sheffield and Wood 2008). An increase in the occurrence of extreme weather events (such as heat waves, frosts and storms) is also foreseen, with a high degree of regional variability (IPCC 2007a, b). With this new panorama, the phenology of the different species would be markedly affected by the foreseen changes (Way 2011), with growth periods becoming extended or shortened according to the region in question and with this affecting the final production of many crops. Under these conditions, plants could be exposed to sudden autumn frosts with very different degrees of hardening and acclimation and the resulting damage to plants.

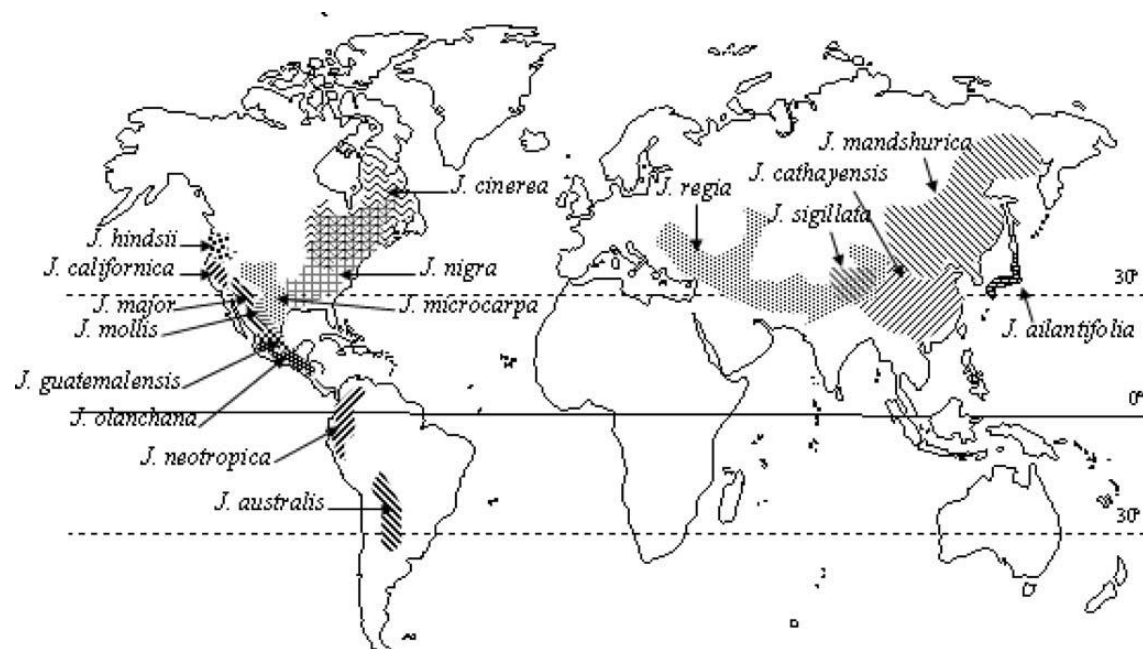
Within the *Juglans* genus, there is considerable uncertainty concerning the magnitude of the possible effects of climate change, which could affect factors ranging from the growth and development of individual trees to its distribution patterns and even the survival of the genus itself (Gauthier and Jacobs 2011). It is therefore necessary to work towards maintaining or improving the genetic capacity of populations, which is important for facilitating the adaptation of the species to climatic change. It is crucial to understand how these adaptation mechanisms work, as it may be



possible to improve the capacity for adaptation already inherent to the species through the application of planned measures for adaptation (Lindner et al. 2010; FAO 2013).

### The *Juglans* genus and its exploitation for timber

The genus *Juglans* belongs to the Juglandaceae family and forms part of the group of species which are commonly known as walnuts. At present, walnuts are classified into 21 species which are distributed throughout the world: 17 are native to the American continent and the rest have their origins in Central Asia. The genus is divided into four botanical sections: *Juglans* (previous known as *Dioscaryon*), which only includes the species *J. regia* L.; *Rhycocaryon*, which groups together the black walnut species, which originate on the American continent; *Cardiocaryon*, which includes the Central Asian walnuts; and *Trachycaryon* which includes the species *J. cinerea* L., which is found in the north-east of the USA and Canada (Figure 2). All of these species are diploids ( $2n=32$ ) and are characterised by the ease with which they hybridise amongst themselves, which gives rise to abundant natural hybrids (Leslie and McGranahan 1995).



**Figure 2.** Current distribution of natural populations of *Juglans* (The distribution of *J. regia* cultivation extends far beyond its natural area) (Map based on Mallikarjuna et al 2006).

The growing demand for fine leafy timber, and walnut in particular, and the fact that the natural populations of these species have been exhausted by overexploitation throughout the world, have contributed to the exploitation of *Juglans* in plantations exclusively managed for timber production (Hemery and Popov 1998). The high economic value of this wood combined with its production of quality timber to meet the demands of the transforming sector for top quality material, has triggered the expansion of these types of plantation (Ducci et al. 2010). They offer an economic alternative which helps to diversify the production of an agricultural exploitation and to provide medium to long term returns. The possibility of simultaneously planting timber producing species with agricultural crops, which is known as agroforestry, offers an advantage to these plantations at both the environmental level (landscape, increased diversity, better use of resources) and in terms of sustainable production and as a source of deferred revenue. The main species of *Juglans* currently exploited for their timber are *J. nigra*, which is mainly grown in the USA, and *Juglans regia* and *Juglans xintermedia*, in Europe (Montero et al. 2003; Aletà and Vilanova 2012; Woeste and Michler 2011).

### ***Juglans regia* L.: origins, uses and genetic variability**

This species has its origins in the mountain chains of Central Asia, which extend from Turkey and Iran, through the Caucasus area, and to western China and the eastern Himalayas. Over thousands of years, transportation - associated with trading of the walnut fruit - led to *J. regia* spreading throughout Europe and as far as the Mediterranean basin, until it finally became established as a naturalised species (Leslie and McGranahan 1995). The presence of the walnut on the Iberian Peninsula dates back to the last glaciation, with the oldest fossil remains belonging to the Upper Palaeolithic period (Rivera et al. 1997).

*J. regia* species have the capacity to adapt to different climatic conditions as a result of their ancestral naturalisation to wide areas of Eurasia. They have gradually differentiated materials adapted to both continental climates, such as those of Central Europe, and to other warmer zones, such as the examples that grow around the Mediterranean (Fady et al. 2003). The species can therefore stand winter frosts of -30°C in parts of Central Europe, but also grow in much more temperate areas, such as the south of Europe and even the north of Africa, where the accumulation of winter cold is minimal (Germain 2004). It is typical to find it as an isolated tree, especially in the warmer zones where this species only grows spontaneously, when there are sufficient water

resources at hand. It requires average monthly temperatures of over 10°C for at least six months of the year and a minimum annual precipitation of 700 mm; this should preferably be well-distributed throughout the year or, if this is not the case, it should be grown in soils with a great capacity for water retention (Garavel 1959; Becquey 1997). On the other hand, it cannot stand waterlogging. Its main thermal vulnerabilities for cultivation in the Mediterranean zone are its sensitivity to intense winter cold (particularly when temperatures fall below -20°C) and particularly to sudden out of season frosts, whether in autumn or in spring (Masson 2005).

Since time immemorial, the main use for *J. regia* has been for fruit production and its edible nut. It was not until the 14<sup>th</sup> century that its timber began to be appreciated in Europe for the manufacturing of quality furniture (Aletà 2005). During the 18<sup>th</sup> century, the species was mainly threatened by mass felling due to great demand from furniture makers, particularly in England. In the 20<sup>th</sup> century the large-scale felling of walnut trees continued in countries such as Spain and Greece and the quality of the surviving natural populations fell notably, mainly as a result of the absence of any form of reforestation. The present demand for common walnut timber within the EU is largely met by countries such as Iran, Turkey and Kirgizstan, where it is still possible to find numerous examples of *J. regia* forming part of the natural landscape (Hemery and Popov 1998).

Within the timber market, walnut is mainly exploited to extract sliced walnut veneer. The standardisation of the type of wood used has been imposed by furniture makers, who give priority to homogeneity, regular growth and the absence of significant defects (Jay-Allemand 1999). The logs used to make this type of veneered wood must be straight, at least 2.5 m long, and have diameters of around 35 to 40 cm (Giannini and Mercurio 1997). The final quality of the timber, which includes the previously mentioned traits, will depend on the good management of the plantation during its growth, but also on whether the productive and adaptive characteristics of the plant material used are suitable for the conditions of the exploitation. There have been few studies of adaptive and productive behaviour in *J. regia* or of the technological capacity of the timber produced by the great majority of the materials planted. A number of *J. regia* materials have been selected in Italy, France and Spain and the first data relating to some half-sib progenies or clones are now becoming available (Aletà et al. 2003; Fady et al. 2003; Bono and Aletà 2009; Ducci et al. 2010). EUFORGEN is a collaborative programme, involving various European countries, whose main aim is to promote the conservation and sustainable use of genetic forestry resources, including

those of the species *J. regia*. At present, the use of this species as a timber producer is limited in Europe by the scarcity of appropriate genotypes with respect to the existing level of demand. A basic material of *J. regia* was first registered in Europe in 2011 as the result of selection work carried out by the IRTA in collaboration with the public company Forestal Catalana. The UK and Italy are other countries which are actively working on the characterisation and selection of walnut materials for use as timber (Ducci et al. 2010; Hemery and Popov 1998). Within the framework of the selection of basic walnut materials for the production of timber, numerous ecophysiological studies have been carried out in recent years to broaden our knowledge of adaptive traits. The objectives of these studies often centred on how to control the possible negative effects that climatic change could have on the species (Gauthier and Jacobs 2011). Studies have, for example, examined the capacity of the walnut to resist and prevent damage associated with winter frosts when the tree is completely dormant. The contraction of living bark due to the formation of extracellular ice during a severe frost is therefore an example of how the adverse effects of freezing can be mitigated (Améglio et al. 2001). Susceptibility to embolisms, whether produced by drought or by a cycle of freezing and thawing, and the subsequent process of recovery also some of the adaptive processes that have been studied in this species (Améglio et al. 2002; Breda et al. 2006). Complementing this line of study, Fady et al. (2003) reported a high level of genetic variability in a number of adaptive features, such as the date of bud break and susceptibility to unseasonable frost events, in examples of *J. regia* from different provenances growing under different ecological conditions. Another study by Aletà et al (2009) showed genetic variability in the efficiency of water use in this species and the high level of heritability of this adaptive characteristic; this was clearly related to the origins of the provenances studied.

Within the same species there are fruit producing varieties which have been selected on the basis of both their productive and adaptive characteristics. In the latter case, a range of genetic variation has been observed in such traits as the ability to survive adverse climatic and soil conditions, phenology and flowering, which have been summarised by McGranahan and Leslie (1990).

The species of *J. regia* selected for their timber production should also share productive and adaptive traits, incorporating a certain degree of diversity in order to obtain forestry reproductive materials appropriated to areas which exhibit different climatic conditions.

## Methods for evaluating and selecting the type of study conducted

The adaptive trait on which the present work focuses is “resistance to autumn frosts” in *J. regia*. Various methodologies can be used to evaluate resistance to freezing temperatures that affect plant tissues and organs, some of which have been successfully applied to various woody species for several decades now. One of the most commonly used systems is that of the visual evaluation of damage. This can be conducted in the field or in the laboratory through the dissection of tissues and the direct observation of damage; this can be recognised by the necrotic colouring caused by frost damage (Díaz et al. 2009; Liu et al. 1998; Savé et al. 1992; Timmis 1976). Another very widely used method involves the relative quantification of the electrolyte leakage from damaged cells after the plant material to be evaluated has been subjected to freezing conditions (Jacobs et al. 2008; Morin et al. 2007; Murray et al. 1989; Savé et al. 1992; Save and Adillon 1990). Amongst other methodologies applied, it is possible to find a study of the damage caused to the photosynthetic apparatus of the organs subjected to freezing, such as the leaves (Savé et al. 1992), and the more recent study of chlorenchyma of stem bark (Peguero-Pina et al. 2008; Wilson and Jacobs 2012).

Many of the traits related to adaptation, such as resistance to autumn frosts, are not controlled by a single gene but involve a series of genes with minor, additive effects that interact with the environment at the moment of their expression. These types of traits are studied in quantitative genetics where specific models have been developed to analyse phenotypic expression when it is not possible to analyse the genotypes themselves. In this way, it should be possible to describe the dynamics of populations subject to natural or artificial selection in specific environments.

The individuals chosen to form part of breeding programme with the aim of producing genetic improvement are usually selected based on the quality of their phenotypes. It is then necessary to test their genetic value by studying their progeny. The progeny test allows us to evaluate the genetic value of the progenitors selected; their descendants can then be raised based on an estimation of the parental values that will be obtained from the cross. This makes it possible to distinguish between progenitors whose phenotype superiority was due to an environmental/local factor from those associated with a good genotype. Estimations are also made of the components of variance and heritability associated with the trait in question. Particularly in the case of an adaptive trait, it is important to know whether the environment has had a decisive influence on its expression; it is therefore necessary to conduct studies of the same plant materials subjected to

different environmental conditions (planned trials for Genotype x Environment). The heritability value will show us how easy it is to improve a particular trait, though in many cases this value will vary according to the age of the trees in question. This will therefore need to be treated according to the corresponding age-age correlation for the same trait. The main objective of the whole process is to obtain populations that possess better traits than those that have not been improved (Alía et al. 2005; Zobel and Talbert 1988). The final step could be the installation of fields with basic material with the capacity to reproduce those improved populations (Aletà and Vilanova 2011).

### **Structure and objectives of the work**

The different activities carried out by the IRTA between 2009 and 2012 focused on evaluating the capacity of the *Juglans regia* L. species to respond to autumn frosts. The different trials organised during this period sought to answer questions related to the potential impact that a sudden autumn freezing event could have on this species. Each of the four chapters that make up this work has its own objectives, but they all aim to help further our knowledge relating to this response.

#### **Chapter 1. Autumn frost resistance in several walnut species: methods, comparisons and the impact of leaf fall.**

This chapter includes the results of different trials carried out in order to respond to three questions:

- a) Which of the methodologies applied to other woody plants is the most appropriate for evaluating the damage caused by autumn freezing events to annual stems on walnut trees?
- b) From what temperature does the walnut exhibit frost damage to its annual wood production compared to other species of *Juglans* that are also used for timber production?
- c) How does the extent to which tree growth is arrested affect the amount of damage caused?

The results of this work have been accepted for publication in the journal "Forest Science" (a paper is scheduled for publication in October 2013; it has been available on-line since April 2013). The chapter corresponds to the article as accepted for publication.

## Chapter 2. **Genotype and environment: two factors related to autumn cold hardiness in Persian walnut.**

The activities included in this chapter focussed on determining the influence of Genotype and Environment on the extent of damage caused by autumn frost to the species *J. regia* and whether the interaction between the two factors is relevant. A comparison was made between two identical “field tests”, one of which was run on the Mediterranean coast (at Reus, Tarragona) while the other was conducted on the Atlantic coast (at O’Pino, A Coruña).

In February 2013, the results of this work (which are currently subject to second revision) were sent to the journal “Annals of Forest Science”. The chapter corresponds to the article as originally sent, which includes all the changes suggested by the reviewers of the journal.

## Chapter 3. **Genetics of autumn frost resistance in Persian walnut (*Juglans regia* L.).**

Taking advantage of a test carried out on progenies of *J. regia* at the IRTA (Constantí, Tarragona), which involved 22 x 9 year-old half-sib progenies, we estimated the genetic variability and heritability of the “resistance to autumn frost” trait and studied its genetic correlations with various phenological and growth traits. Within this framework, we also analysed the relationship between the female progenitor and its descendants in terms of the expression of this trait.

## Chapter 4. **Is summer drought influencing the autumn frost resistance of *Juglans* under Mediterranean conditions?**

In this fourth chapter, the aim was to evaluate the influence of one of the most common sources of abiotic stress on autumn frost resistance under Mediterranean conditions: summer drought. We compared frost damage to walnuts caused by different hydric conditions during the growing season. The responses of *J. regia* and *J. xintermedia* hybrids were analysed both at the juvenile phase, corresponding to one and two year-old seedlings (trial at IRTA Torre Marimon), and in 10 year-old adult trees under field conditions (trial at IRTA Reus, Tarragona).

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## **Chapter I.**

Autumn frost  
resistance on several  
walnut species:  
methods comparison  
and impact of leaf fall

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## AUTUMN FROST RESISTANCE ON SEVERAL WALNUT SPECIES: METHODS COMPARISON AND IMPACT OF LEAF FALL

### ABSTRACT

Four walnut tree species, of value for their wood production, were evaluated for autumn cold resistance: *Juglans regia* L., *Juglans nigra* L., *Juglans major* Torr. A. Heller and *Juglans ×intermedia*. Early frost events damage and reduce wood quality and lengthen timber harvesting rotation. The frost vulnerability of these species could be an even more important problem in the future, depending on to which Mediterranean climatic scenario develops.

The work is based on the use of the chlorophyll fluorescence parameter Fv/Fm -measured in cortical bark chlorenchyma- as a reliable estimator of freezing injuries in *Juglans*. Two methods traditionally used to evaluate the effects of frost on deciduous trees were also analyzed: visual damage and relative electrolyte leakage. The study was carried out using annual woody shoots collected in November of 2009, which were subjected to low temperatures (from 5°C to -16.5°C) in a controlled cold chamber. The relationship between leaf fall stages and autumn frost damage has also been discussed.

The results obtained using the different methods studied were comparable. No injuries were detected in any species until the temperature dropped below -8.5°C. In the study, black walnut clones were the most resistant to sub-zero temperatures, *J. regia* were the least, and *J. ×intermedia* showed an intermediate behavior with respect to its genitors. The assessment of cold damage using the Fv/Fm parameter was easy and quick. It seems that this would be particularly useful for screening a large number of samples.

**KEY WORDS:** *Juglans*, frost hardiness, low temperatures, Fv/Fm, leaf fall.

## INTRODUCTION

Frost resistance is one of the key factors that limits the survival and distribution of plants in many ecosystems (Grace 1988). The ability to increase frost resistance is a fundamental difference across species and this is the result of an evolutionary adaptation to cold stress via the mechanism of natural selection (Sakai and Larcher 1987). However, under global climate change-induced, non-negligible increases in autumn temperatures could lengthen the period of natural vegetative growth of species. Consequently, leaf senescence will delay and the risk of frost injury increase (Christensen and Christensen 2007; IPCC 2007; Menzel et al. 2006). Until now, the frequency of sudden frost events in the Mediterranean area remains quite stable but under this new scenario associated with climate change frost damage is expected that could be more severe. There is also evidence that some tree species growing under high CO<sub>2</sub> concentrations - e.g. *Eucalyptus pauciflora* - are more vulnerable to sub-zero temperatures, due the delay of the cold acclimation produced by this growing conditions (Barker et al. 2005; Woldendorp et al. 2008).

The *Juglans* genus is widely spread worldwide and several species play important roles in new afforestation programs due to the high quality of their wood and also their fast rate of growth (Aletà et al. 2003; Becquey 1997). High vigor is a very much appreciated trait in tree species used for timber production, as it reduces the time between planting and felling trees. Over the last 15 years, *Juglans* trees planted in southern Europe have mainly been selected for their growth characteristics, without considering their autumn frost adaptation (Aletà et al. 2004; Fady et al. 2003). Possible consequences of autumn damage include: the loss of leader dominance and tree shape; wood color alterations; and even mortality in young trees. Frost damage defects in the inner bark are unacceptable for first timber quality standards.

It is known, low temperature-acclimated walnut trees can resist winter temperatures ranging from -20 to -40 °C, although this depends on the species (Becquey 1997) and cultivars of walnut (Davarynejad et al. 2009). However, the real danger of frost damage appears during the process of cold acclimation, when plants are particularly vulnerable to fast decreases in temperature (Becquey 1997; Fady et al. 2003). The Northern black walnut (*Juglans nigra* L.), which has a short growth cycle, is also vulnerable to autumn sudden frost. Thomas and Reid (2006) reported that 41% of young trees were damaged by temperatures dropping to -6 °C when many of them were still fully foliated.

The market requirements of *Juglans* wood clearly points to the need to evaluate the frost autumn resistance of newly planted materials. This adaptive trait is the first aim to select *Juglans* progenies/clones. In other genera, like *Picea*, it has been demonstrated that rapid growth in summer and efficient frost hardening in autumn, are not mutually exclusive characteristics (Ogren 1997). In Douglas-fir, Aitken et al. (1996) observed that intraspecific genetic variation in frost resistance is greater in autumn than in winter. No conclusive data exist on genetics of this character in walnut species, therefore similar studies of those cited should be carried out in *Juglans*.

Several methodologies have been used to assess freezing damage in different species. Direct visual scoring of damaged tissues has been, and is currently used in many species (Fernández-López et al. 2005; Luoranen et al. 2004; Timmis 1976). However, the most common indirect evaluation is the analysis of relative electrolyte leakage (REL), which has been studied in a wide range species, including some Persian walnut cultivars (*Juglans regia* L.) (Aslamarz and Vahdati 2010) and a progeny of *Juglans ×intermedia* (Poirier et al. 2006). The Chlorophyll Fluorescence measurement in photosynthetic tissues, focusing on the Fv/Fm parameter determination, has been recently used in some species to provide information on maximum potential photosystem II (PS II) efficiency, related to the photosynthetic activity of the analyzed tissue (Genty et al. 1989). This is an easy and fast methodology to know the existing damage on the tissues compared to those cited above. Peguero-Pina et al. (2008) applied successfully this analysis on the chlorenchyma of stem bark in *Pinus sylvestris* L.

This study aimed to determine the effectiveness of measuring maximum potential PS II efficiency in the stem bark chlorenchyma in order to evaluate frost damage in *Juglans* species. Frost susceptibility was studied in different tissues of woody shoot -internal layers of bark, apical and lateral buds- and in four *Juglans* species. The fluorescence results (rate Fv/Fm) were validated by the information generated from the other two more conventional methodologies: the visual scoring and the analysis of the relative electrolyte leakage.

## MATERIAL AND METHODS

### Plant material and test site

Four walnut species, currently used for timber production, were chosen trying to obtain a wide potential range of susceptibility to sub-zero winter temperatures in the *Juglans* genus (Becquey 1997). The Northern black walnut (*Juglans nigra* L) is highly appreciated and economically important in North America, its area of origin, (Williams 1990). The Persian walnut (*Juglans regia* L.), the only walnut naturally spread in Europe, is a naturalized species which is valued for both its fruit and timber; currently it is included in the European Forest Genetic Resources Program (EUFORGEN) network for noble hardwoods (Fernández-López et al. 2002). The Arizona's black walnut (*Juglans major* (Torr. A. Heller)) grows in this arid zone of America. The fourth one is the natural hybrid *J. nigra* x *J. regia*, commercially known as Ng23xRa, one of the most common *Juglans* species planted for wood production in the recent South Europe afforestation programs, (Aletà et al. 2004; Fady et al. 2003). All of them were grown in the same plot at spacing of 5 m x 5 m, under Mediterranean conditions, in Caldes de Montbui (northeastern Spain). The annual average rainfall of this area is around 670 mm (Pm); the average annual temperature is 14 °C (Tm); and the mean annual max/min temperatures are 21 and 8 °C, respectively. At this site, during the period 2005-2010, the first autumn freeze was registered in mid-November. The field was drip irrigated from May to August, with the amount of water applied in 2009 being 1200 l/tree. A total of six clones were evaluated: two of *J. regia* (JR1 and JR2), two of *J. nigra* (JN1 and JN2), one of *J. major* (JM) and one of *J. ×intermedia* (JX). The *J. regia* clones came from different climatic areas: JR1 was from northwestern Spain (Atlantic coast) and JR2 was from Tarragona, in northeastern Spain (Mediterranean coast). The *J. nigra* and *J. major* trees came from the USA and they are originally seedlings. The *J. ×intermedia* was a natural cross between *J. nigra* and *J. regia* and came from the female genitor called 'Ng23' selected by INRA (La Grande Ferrade, Bordeaux- France). All of these trees were vegetatively propagated in 2006 and planted in the field in March 2007.

Annual woody shoots (budsticks) of each clone, 20 cm long and from apical position, were removed during the second week of November 2009 (this date belongs to the period in which sudden freezing events may occur at Caldes de Montbui). Immediately afterwards, they were sent to the

Forest Research Centre of Lourizán, where the controlled cold chamber was used, and stored in a refrigerator for four days before starting the test.

### **Leaf fall assessment**

In deciduous trees, leaf fall is a response to many of the internal and external factors involved in cold acclimation and dormancy. In 2009, the leaf fall period was controlled in order to compare the evolution of cold acclimation of each of the clones studied. Scores were made following the scale proposed by Díaz and Fernández-López (2005): 1 = tree without leaves; 2 = tree with less than 25% of its leaves; 3 = tree with 25-50% of its leaves; 4 = tree with 50-75% of its leaves; 5 = tree with more than 75% of its leaves. Data were recorded once per week from the beginning of October to the end of November, until the trees reached stage 3. The Julian day corresponding to each stage was noted in order to control the evolution of leaf fall. The day when samples were taken from the field plot, leaf fall stage of each of clones was recorded.

### **Artificial freezing test**

Budsticks collected from the six *Juglans* clones were placed in the freezing chamber at the Forest Research Centre of Lourizán (Pontevedra, Spain). They were then fixed by their bases in a tray full of vermiculite and covered with a metal box in order to prevent sample desiccation. The budsticks were distributed inside the freezing chamber in a randomized block design, with 6-7 replications of each clone per temperature. The initial temperature inside the chamber was 5°C and was reduced at a rate of 2.5°C/hour until it reached the temperature chosen for each treatment. Samples were kept for two hours at each sub-zero temperature and then again stored at 5°C for a progressive thaw. During these freeze/thaw cycles, the temperatures were registered and stored in data loggers. The real sub-zero temperatures applied were: -3.5°C (T1), -8.5°C (T2) and -16.5°C (T3).

### **Evaluation of frost hardiness**

Three different tests were applied to evaluate clone frost hardiness: visual scoring and relative electrolyte leakage, which are the most commonly applied methods, and chlorophyll fluorescence of cortical bark chlorenchyma.

### Visual scoring

After freezing treatments, budsticks were placed in a greenhouse at 20°C under high relative humidity which was maintained, by a fog-system, for two weeks. Damage was observed to the internal layer of the bark and to the apical and lateral buds of each budstick. Internal lesions of tissues were quantified following the methodology described by Díaz et al. (2009) in *Castanea sativa*. Five color scale levels were considered to evaluate internal stem damage: 0 = green, no damage, 1 = green with some light brown spots, 2 = light brown, 3 = dark brown, 4 = black, necrotic. Three scale points were also established for bud analysis (apical and lateral): 0 = completely green, living bud, 1 = partially brown bud, 2 = dead bud.

The visual damages of each sample were standardized. The final values of damage were from 0 (without damage) to 1 (completely damaged). The process was done with the division of the level of affection, following the above mentioned color scale, by the total number of levels in the scale: four and two levels on bark and bud damages, respectively.

### Relative electrolyte leakage (REL)

Before placing the budsticks in the greenhouse, a couple of slices of their distal parts; were removed and then introduced into test tubes containing 20 ml of distilled H<sub>2</sub>O. Wood samples were left overnight at room temperature. Following standard methodology (Murray et al. 1989), the samples were then shaken for 30 minutes and the conductivity of the solution was measured (C<sub>1</sub>). To ensure complete electrolyte leakage, sample tubes were heated in an oven for 90 minutes at 80°C and then again stored overnight at room temperature. After shaking for a further 30 minutes, solution conductivity (C<sub>2</sub>) was again measured. Relative electrolyte leakage (REL %) was calculated as:  $REL = C_1/C_2 * 100$ . Considering the REL value of unfrozen samples (REL<sub>0</sub>) according to Flint et al. (1967), we calculated the index of sample freezing injury at each temperature (t):  $I_t = (REL_t - REL_0) / (100 - REL_0)$ . The index ranges from 0, indicating no damage, to 1, indicating a completely damaged sample.

### Chlorophyll Fluorescence

After two weeks in the greenhouse and just before visual scoring, the budsticks were kept in darkness for 30 minutes and a strip of bark running from the top to the bottom was removed and the chlorophyll fluorescence was measured on the inner part of the excised bark. Minimum (F<sub>0</sub>) and

maximum ( $F_M$ ) fluorescence values were estimated for each sample using a Photosynthesis Yield Analyzer (Walz, MINI-PAM), as described by Peguero-Pina et al. (2008). The  $F_v$  parameter was calculated as proposed by Genty et al. (1989):  $F_v = F_M - F_0$ . The maximum potential PSII efficiency was represented by the ratio  $F_v/F_M$ . High values of  $F_v/F_M$  indicated an undamaged photosynthesis system of the plant (0.7-0.8).

### Statistical analysis

Statistical damage evaluations were independently conducted for each test considering the parameters  $F_v/F_M$ ,  $I_t$ , and the visual scoring, respectively, of each stem. An ANOVA analysis was performed under a General Linear Model, in which temperature and clone were considered fixed effects. On the visual test, differences between tissues (damage on bark and buds) were analyzed considering the tissue as a fixed effect too.

A Spearman correlation was made between  $F_v/F_M$  fluorescence parameter and the two other  $I_t$  and visual damage score.

## RESULTS

The JR1, JR2, JX and JM trees respectively reached "stage 3" of leaf fall in the field, 29, 24, 22 and 5 days after removing wood samples. In contrast, the two JN clones, JN1 and JN2, passed "stage 3" of leaf fall in the orchard four and five days, respectively, before the samples were collected. All the samples (160 woody shoots/budsticks) were evaluated, by visual scoring and REL methodology. However, readings for chlorophyll fluorescence activity on the inner bark ( $F_v/F_M$ ) were only obtained for two sub-zero temperatures ( $T_2 = -8.5^\circ\text{C}$  and  $T_3 = -16.5^\circ\text{C}$ )

The results of the ANOVA test, which are summarized in table 1, pointed to a significant interaction between clones and temperature (CxT). Data analyzed separately, and based on temperature, did not show any differences between the clones subjected to a temperature of  $-3.5^\circ\text{C}$  (results not shown).

**Table 1.** Temperature (T) and clone (C) effects on the susceptibility of *Juglans* woody shoots to autumn frost. The Anova corresponds to the General Linear Model for the index of freezing injury ( $I_t$ ), maximum potential photosystem II efficiency (Fv/Fm) and visual damage to the inner bark ( $VD_{IB}$ ).

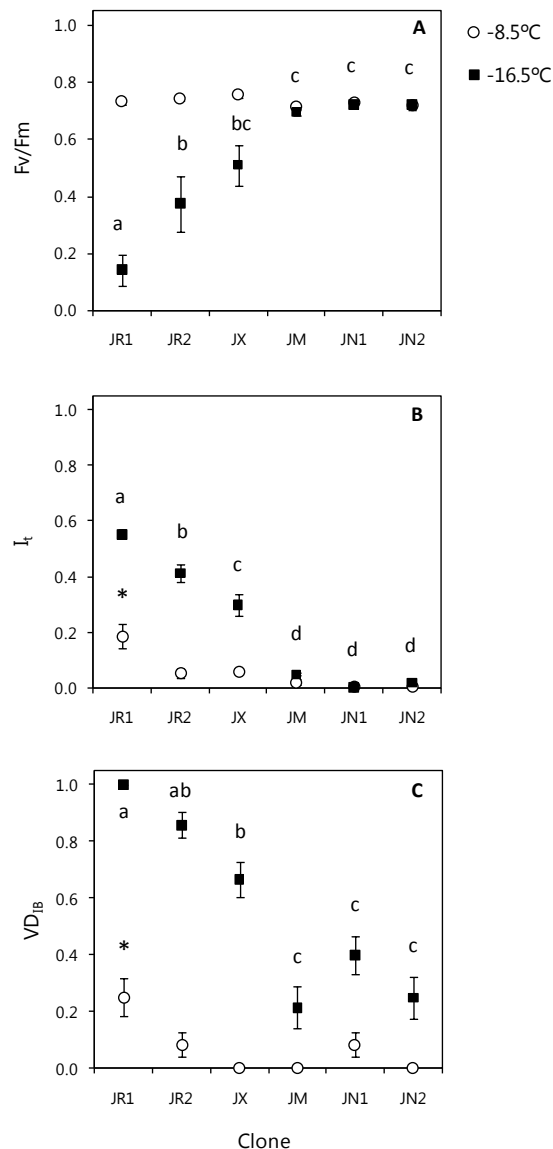
	Fv/Fm			$I_t$			$VD_{IB}$		
	Df	F	Prob<F	Df	F	Prob<F	Df	F	Prob<F
<b>Clone</b>	5	15.23	<0.0001	5	79.21	<0.0001	5	20.07	<0.0001
<b>Temperature</b>	1	74.22	<0.0001	2	196.12	<0.0001	2	217.85	<0.0001
<b>C × T</b>	5	17.3	<0.0001	10	42.5	<0.0001	10	11.22	<0.0001

Temperatures: Fv/Fm: T2=-8.5°C and T3=-16.5°C.  $I_t$  and Visual damage: T1=-3.5 °C, T2 and T3. Clones: JR1 and JR2 (*J. regia*); JN1 and JN2 (*J. nigra*); JX: (*J. ×intermedia*) and JM (*Juglans major*).

In Fig. 1, damage caused to the stem, in all of tests conducted, is graphed at -8.5°C and -16.5°C. Both the VD and  $I_t$  showed significant damage to JR1 at -8.5°C, while the Fv/Fm parameter revealed no differences between the clones observed. However, at -16.5°C, significant differences were observed between clones when applying the three methodologies. JR1 was always the most damaged material and it showed significant differences with respect to JR2 when data for the Fv/Fm parameter and the  $I_t$  were considered. The *J. ×intermedia* (JX) exhibited behavior that lay between those of the *J. regia* and the black walnut clones. While, no significant differences were found between the black walnut species (JN1, JN2 and JM) in which only minor VD damage was registered.

The Spearman correlation between  $I_t$  and Fv/Fm, considering all the temperatures applied, was consistent and significant at  $p < 0.001$ , with  $r_k = -0.447$ . Fv/Fm was negatively correlated with  $I_t$ , as expected. The correlation was narrower when analysis was only carried out with data of the lowest temperature (-16.5°C):  $r_k = -0.885$  ( $p > 0.001$ ). The VD of the stem and the Fv/Fm also correlated significantly ( $p < 0.001$ ) and inversely:  $r_k = -0.655$ . Again, the correlation improved when only the lowest sub-zero temperature applied in the study was considered:  $r_k = -0.789$ .

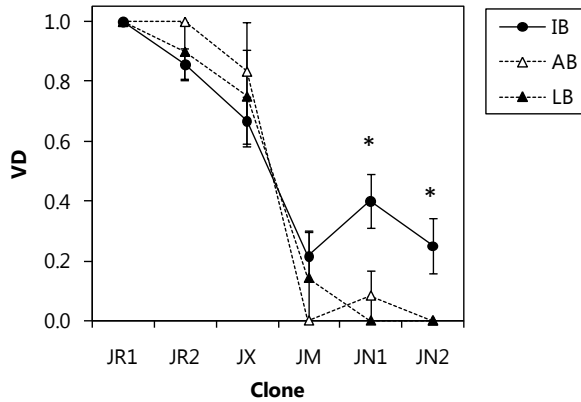




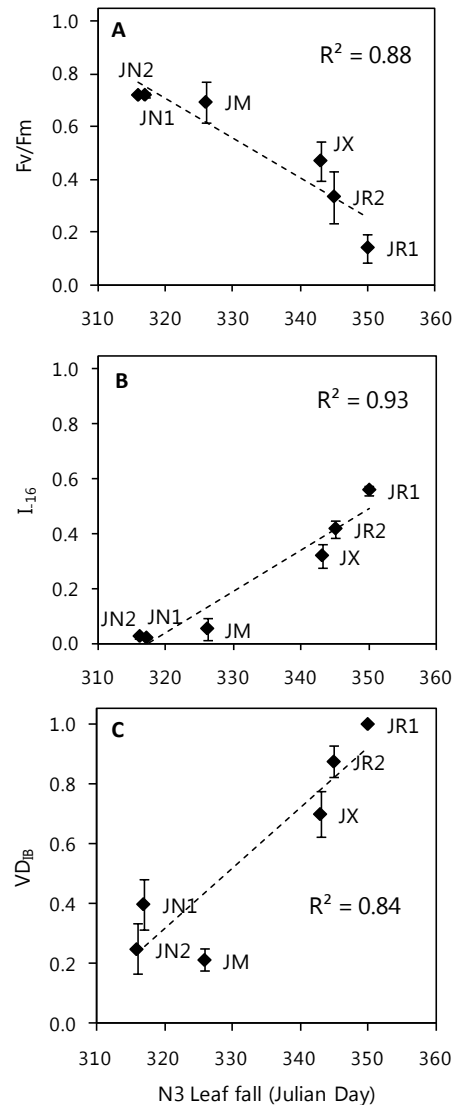
**Figure 1.** Mean and standard error of damage to budsticks submitted to - 8.5°C and -16.5°C measured on 6-7 replicates per clone by A) Chlorophyll fluorescence in the inner bark (Fv/Fm); B) Relative electrolyte leakage ( $I_t$ ) and C) Visual scoring of the inner bark (VD<sub>IB</sub>). In treatment T3: Post-hoc SNK mean test. The same letter represents no significant differences between clones ( $p < 0.05$ ). In treatment T2: Post-hoc SNK mean test. Clones with \*differ significantly from all the others ( $p < 0.05$ ).

Analyzing only data relating to the visual damage observed in the three tissues taken from each budstick and clone: inner bark, apical buds and lateral buds; no difference between tissues was detected at -8°C (data not show). However, at -16.5°C, JN1 and JN2 showed damage to the inner-bark which was also significantly greater than that to their buds (fig.2)

The linear regression between damage to each material, registered at  $-16.5^{\circ}\text{C}$ , and the Julian day on which each *Juglans* clone reached stage 3 of leaf fall, is plotted in figure 3. The data showed a high coefficient of determination ( $R^2$ ) in all the tests applied.



**Figure 2.** Mean and standard error of visual scoring on damaged budsticks (VD) at  $-16.5^{\circ}\text{C}$  on 6-7 replicates per clone, and separately considering the three tissues: inner part of the bark (IB), apical bud (AB) and lateral buds (LB). Clones with \* differ significantly in their response, which depends on the tissue



**Figure 3.** Linear regressions between data at stage 3 of leaf fall, expressed by the corresponding Julian day, and the mean damage at  $-16.5^{\circ}\text{C}$  ( $N=6-7$ ) for each clone, Measurements made by A) Chlorophyll fluorescence of the inner bark B) Index of freezing injury ( $I_{-16}$ ) and C) Visual scoring of inner part of the bark ( $VD_{IB}$ ).

## DISCUSSION

The ranking of the susceptibility of *Juglans* species to sub-zero temperatures studied here was essentially the same for the three tests conducted. The Fv/Fm parameter, which analyzes the inner bark photosynthesis system II efficiency status, was significantly correlated with  $I_t$  and VD, particularly when the lowest temperature was considered. Jiang et al. (1999) had already used the Fv/Fm parameter to evaluate photosynthetic efficiency and assess the viability of freeze stressed woody grape tissue during cold acclimation and also obtained a positive correlation between frost damage and the temperatures they applied. Peguero-Pina et al. (2008) studied frost damage in *Pinus sylvestris*, they examined the cortical chlorophyll fluorescence of stems and they obtained results which were also comparable with those obtained using traditional methodologies. Despite the good parallelism obtained in the present study between the response of the photosynthetic efficiency parameter and VD and  $I_t$ , it showed that Fv/Fm did not detect damage at the same level as the other two methods assessed. The rate Fv/Fm should not be used to estimate damage of autumn frost on the *Juglans* tissues, but it could be useful for screening frost resistance of a large number of samples as it is easy and quick to measure fluorescence with a Photosynthesis Yield Analyzer.

As expected, differences in sub-zero temperature susceptibility were observed between species in the three compared tests. At field conditions where the studied materials were grown, no frost damage was observed in analyzed samples until the temperature dropped to  $-8.5^{\circ}\text{C}$ . Black walnuts were the least susceptible and *J. regia* clones the most susceptible to frost damage. An intermediate level of damage was observed in the case of *J. ×intermedia*. This result provided more reliable information about the possibility of extending the use of hybrids in afforestation programs for wood production. The tree ranking from the highest to the lowest susceptible to sub-zero autumn temperatures was: JR1>JR2>JX>JN1=JN2=JM. These results can be explained by the different patterns of cold hardiness acclimation of each *Juglans* species. Under natural conditions, dormancy, cold hardiness and the onset of leaf fall are all related and connected to the annual cycle of deciduous woody plants (Li et al. 2004). According Charrier and Ameglio to (2011), leaf fall is a genetic trait linked to cold acclimation. In this study, the linear regression between leaf fall (Julian day corresponding to stage 3) and observed damage at  $-16.5^{\circ}\text{C}$  (fig. 3) indicated that the level of

cold acclimation could be established using leaf fall level as an indicator in *Juglans*. Stage 3 of leaf fall was revealed to be a useful parameter for comparing the status of cold acclimation between *Juglans* species and clones. In this assay, *Juglans nigra* clones (JN1 and JN2) reached cold acclimation before the other species studied. JN2, which was the least damaged species, reached stage 3 of leaf fall 27 days before JX and 29 and 34 days, respectively, before JR2 and JR1, the most damaged species. The relationship between date of leaf fall and frost damage imposes to take in consideration the compromise between growth and length of the vegetative period in selecting *Juglans* for timber production. In this study, *Juglans nigra* clones showed significant damage at  $-16.5^{\circ}\text{C}$  by visual observation comparing the inner bark with bud tissues; this result indicates that frost damage tends to be more severe on bark than on buds at this time. In this sense, frost injuries to *Juglans* used to be described as strips or patches of dried bark distributed throughout annual shoots and visible to the next vegetative period after the frost event (Aletà and Vilanova 2006; Fady et al. 2003; Germain et al. 1999). Southern *Juglans* species used in afforestation in the Mediterranean area have a later leaf fall than northern species. To avoid frost damage, only the latter should therefore be used in new plantations. However, a longer vegetative period is directly associated with greater annual growth (Aletà et al. 2004; Fady et al. 2003). Increasing timber production is an important objective in the Mediterranean area where forests tend to suffer low growth (S.E.C.F 2010). One way to overcome this would be to use species that are well-adapted to the local soils and climatic conditions but also species/clones that are able to make use of all the available growing period. The natural high vigor associated with *Juglans* hybrids (Aletà et al. 2003), together with their intermediate autumn frost resistance – shown in this experiment – increase the interest in using this material for afforestation in many parts of the Mediterranean area. In fact, commercial hybrid progenies tend to leaf fall only a few days before *J. regia* (Aletà et al. 2003).

In Europe, *J. regia* is one of the most appreciated broadleaves for high quality wood production; its wood is widely used in cabinet making. Even so, relatively few materials of this species have been specifically selected for timber production (Becquey 1997). The challenge lies in combining forest growth habit and vigor and adding resistance to drought and autumn frost (Aletà et al. 2009). Both of the *J. regia* clones evaluated in this experiment were from Spain, but their origins were different. JR1 came from the northwest coast of Spain, which is a rainy area belonging to what García del Barrio (2001) refers to as Spanish Provenance Region number 1; this region has a  $T_m=13.4^{\circ}\text{C}$ ,  $P_m=$

1508 mm and a period with the probability of freezing events (PFE) of 6.3 months per year. However JR2 came from the Spanish Provenance Region number 24, in the northeast of Spain, which corresponds to a dry, continental area, with  $T_m=16.8^{\circ}\text{C}$ ,  $P_m=612$  mm and a PFE= 5.2 months per year. The results of this experiment showed that both JR1 and JR2 were damaged by sudden frost in autumn but that JR2 seemed to tolerate these events better than JR1; differences were observed in the three methodologies assessed. In terms of leaf fall phenology, there was a difference of five days in their times to reach stage 3 (JR2 earlier than JR1) and the lengths of their growing periods differed by five days, with that of JR1 being longer than JR2. In a trial plot of *J. regia* clone evaluation for timber use, no significant differences between JR1 and JR2 were observed either in primary or in secondary growth at the fifth leaf (Bono and Aletà 2009). This work only provides preliminary data, the two *J. regia* clones should be interesting options for wood production. In order to know whether there was a significant intraspecific difference in autumn frost resistance in *J. regia* - as documented in other species such as Douglas-fir (Aitken et al. 1996), willow (Ogren 1999) and also in some cultivars of *J. regia* (Aslamarz et al. 2011) - a wider number of clones/provenances of *J. regia* should be evaluated.

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**Chapter II.**  
Genotype and  
environment: two  
factors related to  
autumn cold hardiness  
on Persian Walnut

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## GENOTYPE AND ENVIRONMENT: TWO FACTORS RELATED TO AUTUMN COLD HARDINESS ON PERSIAN WALNUT

### ABSTRACT

*J. regia* timber is appreciated for high-value wood products. In new plantations, biotic or abiotic events which could affect wood quality should be monitored. Autumn frosts could affect annual shoot development, with consequent loss of timber value or even tree death. In southern Europe, climate change forecasts include erratic and severe autumn frost events.

The relationship between genotype and environment regarding susceptibility to autumn frost damage was examined in four provenances of *Juglans regia* L., planted at two ecologically different sites, one subject to Mediterranean and the other Atlantic weather conditions.

Annual budsticks from eight trees per provenance were collected in November 2010 from each site. The samples were then submitted to freeze-thaw cycles down to -8, -13 and -18°C, plus a control treatment (keeping samples at 5°C). Damage to the stem was assessed using the Index of freezing injury calculated from relative electrolyte leakage at each temperature considered. Frost damage to stem, apical and lateral buds was recorded by visual scoring.

Differences in cold acclimation between sites were detected, with provenances exhibiting differences on senescence. A 'Provenance x Site' interaction was found in some of the analyses, but the same ranking of susceptibility was detected for all provenances at both sites and in all the tissues analysed. The differences between provenances could be related to their geographical origins, where an altitude gradient was observed.

**KEY WORDS:** *Juglans regia*, frost hardiness, autumn freezing events, genetic variation, environment influence.

## INTRODUCTION

Woody plants growing in areas of the world with highly seasonable climates must adjust their development and metabolic activity in order to survive seasonal changes. One of these processes is the cold acclimation which corresponds to a sequence of changes in the plant in which each stage opens the way for the next. This starts with growth cessation and is followed by bud set, senescence and dormancy. The last stage gives plants a high level of frost resistance (Sakai and Larcher 1987). Frost tolerance is not a constant property; it is a response to a number of environmental signals which usually precede the arrival of frost: shorter day length and decreases in temperature (Olsen 2010). In order to achieve the hardening required to avoid damage caused by freezing winter temperatures, a number of physiological processes take place. These include osmotic adjustment, a decrease in the water content of the stem and buds and/or the storage of starch and proteins (Sauter et al. 1996; Welling et al. 2004; Welling and Palva 2006). The sudden exposure of unhardened trees to sub-zero temperatures typically results in injury to their shoots, cambium and roots and often leads to the death of the plant. Recent studies have demonstrated that the warming produced by climate change can modify the timing of key phenological events (Way 2011; Savé et al. 2012). This can exacerbate the vulnerability of some species to the first freezing events of autumn. There is some evidence-based information regarding the effects of climate change on freezing periods which reveal a clear increase in climatic variability (IPCC 2007). To avoid frost damage, species have developed adaptive mechanisms during their evolution, resulting in variation in cold hardiness both between species and between genotypes (Sakai and Larcher 1987). Inter-specific variability in frost resistance has been studied in several species of pine (Climent et al. 2009) and Canary laurel (Rodríguez et al. 2005), showing that their ecological niches are related to frost tolerance. This variability also exists at intra-specific level. Some studies have analysed the distribution of populations in several species in order to avoid damage associated with winter freezes. The distributions can display a latitudinal or altitudinal cline, in which environmental factors play an important role, as observed in several species including *Betula pendula* (Li et al 2003), *Acer platanoides* L. (Pagter et al. 2010) and some *Salix* (Li et al. 2005; Ogren 1999). The use of local provenances in reforestation is a commonly accepted way of infer adequate autumn hardening in new plantations. Differences in cold resistance at the population level are

often found in tree species that grow in a wide range of environmental conditions, as *Juglans regia* L. (Fady et al. 2003). This is the only naturally distributed walnut species in Europe and it is highly appreciated for its valuable timber. The optimum growing environment for the Persian walnut is associated with well-watered and warm climatic conditions during the growing season. It requires at least 700 mm of water supply yearly, which need to be well distributed throughout the year as it cannot withstand summer droughts (Masson 2005). Six months with mean temperatures over 10°C will allow suitable growth (Garavel 1959; Masson 2005). The *Juglans* can display both freezing tolerance through deep supercooling (George et al. 1977) and freezing avoidance through stem shrinking, as observed by Ameglio et al. (2001). These processes can happen when trees are completely cold acclimated, but one of the main limitations, especially in *J. regia*, is sensitivity to out-of-season frost events in autumn or spring (Masson 2005).

The increasing interest in the use of *J. regia* in commercial afforestation in the western Mediterranean has enabled the study of traits specifically related to growth in conjunction with an analysis of adaptive traits (Fady et al. 2003). Variability in both the flushing date and water use efficiency of different progenies of *J. regia* showed that this genetic variation was related to the climate of origin (Aletà et al. 2009). Some differences in cold acclimation were also found in cultivars of *J. regia* that were selected for fruit production (Charrier et al. 2011). Knowledge of genetic differences in resistance to autumn frost in walnut trees from different origins could be of great relevance for future timber production. The damage that a sudden frost event may produce has a notable economic impact. One of the main consequences of this is a reduction in timber quality and even the death of young trees, directly affecting the profitability of the species. Choosing the right materials for each climatic area entails reducing risks of this kind in new plantations.

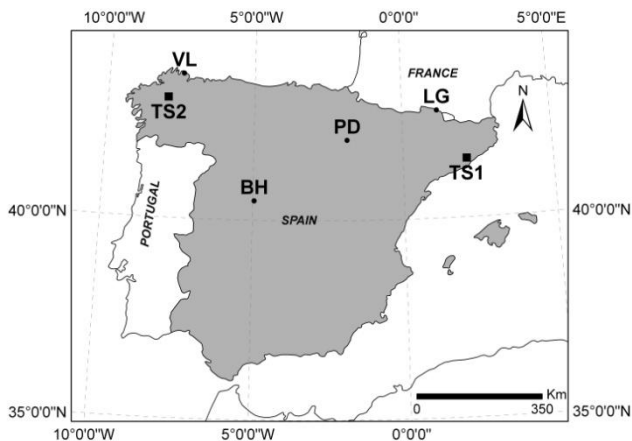
The main objectives of this work involving *J. regia* were: i) to compare the level of autumn frost hardiness of four Spanish provenances selected for their wood production and ii) to determine the importance of the provenance and the planting site for autumn frost resistance.

## MATERIAL AND METHODS

### Plant material and trial sites

The study was based on Persian walnut seedling trees of four Spanish provenances. These came from different environments on the Iberian Peninsula in which this species grows naturally (Fig. 1). The provenances studied were all from the north of Spain: Valadouro (VL), from the Atlantic coast (northwest of Spain), which is an area with a high number of widely dispersed Persian walnut trees; Pedroso (PD), near the Sierra de la Demanda, on Spain's northern plateau, which corresponds to a traditional walnut production area; Burgohondo (BH), where the walnut population grows on the banks of the River Alberche, in the northern part of the Sierra de Gredos; and La Guingueta (LG), which is located at medium altitude in the Aneu valley of the Pyrenees (Table 1).

Trees were planted in 2001 in two trial sites: one at Reus, near the Mediterranean coast, in the northeast of Spain (TS1), and the other at O'Pino, in the northwest of Spain, which has an Atlantic climate (TS2) (Fig. 1). Both trial sites were planted following identical experimental designs: the four different provenances were distributed in 25 randomized blocks with 8 to 12 trees per origin. Trees were spaced at 3 x 1.5 m at TS1, as this was a short-term test, and 3 x 3 m at TS2. TS1 was drip irrigated during summer in order to minimise summer drought stress in the Mediterranean area. The extra water applied was approximately 2500 m<sup>3</sup>·ha<sup>-1</sup>·year<sup>-1</sup>. In contrast, TS2 was located in a rainy area (Table 1) where summer irrigation was not necessary.



**Figure 1.** Map of Spanish Regions for the Identification and Utilization of Forest Reproductive material (RIUs) (Garcia del Barrio et al. 2001). Origins of the four *J. regia* provenances studied (BH: Burgohondo, LG: La Guingueta, PD: Pedroso and VL: Valadouro) and location of the two trial sites (TS1 on the Mediterranean coast and TS2 under Atlantic influence).



**Table 1.** Geographic references and climatic characteristics of the areas of origin of the four studied provenances and the two trial sites.

<b>Provenance</b>	<b>Latitude</b>	<b>Longitude</b>	<b>Alt.</b> (m)	<b>T M</b> (°C)	<b>TMINa</b> (°C)	<b>P</b> (mm)	<b>RIU</b>	<b>Fp</b> (month)	<b>Fs</b> (month)	<b>Tmin<sub>15</sub></b> (°C)
BH	40°27'12,3"N	4°28'25"W	888	14.1	7.2	584	19	7.1	0.5	-
LG	42°35'51.42"N	1°8'0.91"E	954	8.4	4.1	763	8	6.3	4.1	-
PD	42°18'17.82"N	2°43'42.09"W	780	9.5	6.8	558	15	7.1	3.4	-
VL	43°33'08"N	7°26'20"W	100	13.2	8.7	1004	3	6.1	0	-
<b>Trial Site</b>										
TS1	41°08'59"N	1°10'44"E	71	16.8	12.5	550	24	5.2	0	8.2
TS2	42°53'16"N	8°24'39"W	370	13.4	9.3	1508	1	6.3	0	7.6

Alt: altitude; T M: mean annual temperature; T MIN autumn: mean minimum temperatures during September, October and November; P: mean annual accumulated precipitation. RIU: Regions for the identification and utilization of reproductive forest material; Fp: number of months with a probable freezing event; Fs: number of months with freezing events. Tmin<sub>15</sub>: average minimum temperatures for the 15 days before sample collection (García del Barrio et al. 2001; Ninyerola et al. 2005).

### Leaf fall assessment, water content and diameter

The leaf fall stage of each tree was individually scored during the sample collection (2<sup>nd</sup> week of November 2010), both in TS1 and TS2. The date was chosen as a probable day of a first freezing event. The scoring system proposed by Díaz and Fernández-López (2005) was used: 5 - if the tree had over 75% of its leaves; 4 - if the tree had between 50 and 75% of its leaves; 3 - if the tree had between 25 and 50% of its leaves; 2 - if the tree still had some leaves but fewer than 25%; and 1 - when it had no leaves.

At TS1, two other parameters were registered to obtain detailed information of the provenances: the water content of stems (WC) and tree diameter. To obtain the water content, the fresh weights (FW) of the samples (a piece of 2 cm of annual budstick) were measured. After a desiccation it was obtained the dry weights (DW). The water content was calculated as  $(FW - DW) / DW$ .

The WC evolution was chosen to relate the leaf fall period to a physiological parameter closely linked to the winter acclimation process. This parameter was studied in two distinct provenances (VL and LG) in terms of the ecological conditions of their origin (Table 1). One annual apical budstick was removed from 10 trees of each provenance once per week in order to analyse its WC; the leaf fall level was also recorded. The diameter of the tree was measured at breast height (DBH) and this measurement was registered for each of the trees involved in an assay performed at TS1 in the year before the freezing study (on nine year-old trees).

### **Freezing treatments and frost damage assessment**

Four apical budsticks (of 20 cm long) from 8 randomly-selected trees of each provenance were taken to perform frost resistance assays. The samples were collected during the second week of November 2010 at the two trial sites. The budsticks were initially stored at 5°C and then immediately sent to the Forest Research Centre of Lourizán in Pontevedra (Galicia). The excised budsticks were moistened with distilled water and then wrapped in cheese cloth and aluminium foil, in groups of 8, in order to prevent desiccation. These packets, containing a sample of each provenance from the two trial sites, were then placed in the freezing chamber. From an initial 5°C, the temperature inside the chamber was reduced by 2.5°C/hour to final temperatures of -8°C, -13°C and -18°C, respectively, in each cycle. Once the chosen temperature was reached, it was maintained for two hours. To obtain a progressive thaw, the temperature inside the chamber was then increased at a rate of 3°C/hour until it returned to 5°C. In the control treatment, the packets of samples were kept at 5°C. In each freeze/thaw cycle the temperature was registered and stored in a data logger.

After the thaw, a couple of 1-cm-long pieces were cut from each budstick and the rest of the stick was taken to a greenhouse. The small off-cuts of wood were put into test tubes containing 20 ml of distilled H<sub>2</sub>O. These were then stored overnight at room temperature and shaken for 30 minutes before the first measurement of the conductivity of the solution (C<sub>1</sub>). To ensure complete electrolyte leakage, the tube-samples were kept in an oven for 90 minutes at 80°C and then stored overnight at room temperature. After a further 30 minutes of shaking, the conductivity of the solution was measured for a second time (C<sub>2</sub>). Relative electrolyte leakage (REL %) was calculated as:  $REL = C_1/C_2 * 100$ ; this parameter is widely accepted as a way of measuring frost damage in a

number of woody species (Jacobs et al. 2008; Climent et al. 2009; Davarynejad et al. 2009). Considering the REL value of unfrozen samples ( $REL_0$ ) according to Flint et al (1967), the Index of sample freezing injury at each temperature (t) was calculated as:  $I_t = (REL_t - REL_0) / (100 - REL_0)$ . This index ranged from 0 to 1, from no damage to a completely damaged sample, and has been used efficiently on *Juglans* spp. (Guàrdia et al. 2013). The sticks which were transferred to the greenhouse were kept at 20°C under high relative humidity for two weeks. The buds and the internal layer of the bark were then dissected and frost damage was recorded by visual scoring. Bud scoring was made according to the three-point scale described by Díaz et al. (2009) in *Castanea sativa* Mill.: 0 - if the internal bud is completely green and living; 1 - if the internal tissues are partially brown; and 2 - if the tissues are completely brown and the bud is dead. On the other hand, when evaluating internal stem damage, the scoring was established as: 0 - if the tissue was green and there was no damage; 1 - if tissue was green with some light-brown spots; 2 - if tissue was light brown; 3 - if tissue was dark brown; and 4 - if tissue was black and necrotic.

### Chlorophyll fluorescence

Measurements of chlorophyll fluorescence were taken to evaluate the initial state of the samples. These were taken from the inner bark of removed budsticks which were used as a control (and stored at 5°C). The sticks were kept in darkness for 30 minutes and a piece of stem bark was excised, cutting from the bottom to the top of each budstick. The minimum ( $F_0$ ) and maximum ( $F_M$ ) chlorophyll fluorescence scores were estimated with a Photosynthesis Yield Analyzer (Walz, MINI-PAM), as described in Guàrdia et al. (2013). The Fv parameter was calculated as proposed by Genty et al. (1989):  $F_v = F_m - F_0$ . The maximum potential PSII efficiency was represented by  $F_v/F_m$ , where values of around 0.7 indicated the correct functioning of the photosynthesis system of the plant.

### Statistical analysis

A variance analysis (a split-plot assimilated model, in which site was the primary factor and provenance the secondary factor) was applied to the data obtained from the index of freezing injury and from the visual scoring. The analysis was independently made for each sub- zero temperature, following the model:

$$Y_{jk(i)} = \mu + P_j + T_k + PT_{jk} + T(R)_{k(i)} + \varepsilon_{ij(i)}$$

where  $Y_{jk(i)}$  is the value of the response variable measured on the  $j^{\text{th}}$  provenance on the  $i^{\text{th}}$  replicate within the  $k^{\text{th}}$  trial site,  $\mu$  is the overall mean,  $P_j$ ,  $T_k$  and  $PT_{jk}$  are the fixed effects of the provenance, the trial site and the interaction between the two,  $T(R)_{k(i)}$  is the random effect of the replicate ( $n=8$ ) within the trial site, and  $\varepsilon_{ij(i)}$  is the residue that affects the analysis. Prior to variance analysis, the data relating to visually appreciated damage to the different tissues were transformed by temperature using a normal score transformation, as the traits were categorical and restoration of normality and homogeneity of the variances was required.

When the interaction between trial site and provenance was significant in any of the variables, the analysis was done again separately by trial site only with the fixed effect of the provenance. A Student-Newman-Keules test was performed to know the differences between provenances at each trial site.

The damage ( $I_{18}$ ) was related with the altitude and with the autumn minimum temperature of the provenance source, using a Pearson correlation.

The whole analysis was performed with the SAS/STAT software in SAS 9.2.

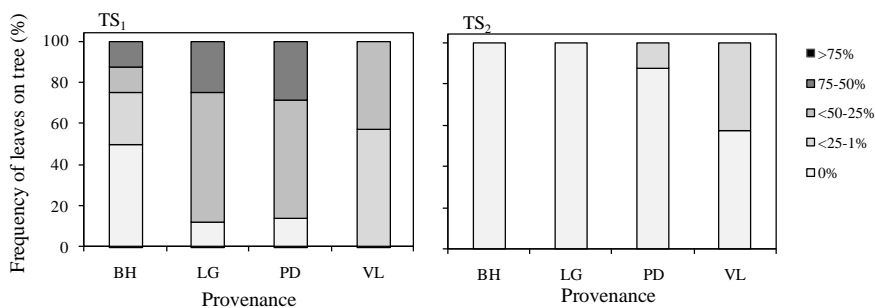
## RESULTS

### Evaluation of sample acclimation

There was a close relationship between the leaf fall stage of the tree and the water content of its annual budsticks; this was quantified by a significant linear regression whose coefficients were 0.89 ( $p<0.0001$ ) and 0.77 ( $p<0.0017$ ) in VL and LG, respectively (tested only for TS1).

Leaf senescence at TS1 was not as advanced as at TS2 (Fig. 2). In the case of the Atlantic site, at the time of sampling (15<sup>th</sup> November), all the BH and LG trees and nearly all of the PD trees were already at stage 1 (0% of leaves on the tree) and over 50% of the VL trees were at this same stage, with the rest being at stage 2 (between 1 and 25% of leaves on tree). However, at TS1, only the BH provenance had 50% of trees without leaves.

The photosynthetic efficiency data, expressed by the Fv/Fm parameter and obtained from the inner bark of the control treatment budsticks, also showed significant differences ( $p<0.001$ ) between the two trial sites. The values were lower for samples from TS2 ( $Fv/Fm=0.542\pm 0.01$ ) than for those from TS1 ( $Fv/Fm=0.675\pm 0.02$ ) (statistical analysis not shown).



**Figure 2.** Current leaf fall tree stage on the day of sampling. Frequency of trees at each stage and at each site by provenance (BH: Burgohondo, LG: La Guingueta, PD: Pedroso and VL: Valadouro). The frequency goes to 100% of leaves on tree to 0%.

### Frost damage to the stem assessed by the Index of freezing injury

Significant differences were found between the two trial sites and also between different provenances at the sub-zero temperatures analysed (Table 2). As expected, the samples from TS<sub>1</sub> were significantly more damaged than those from TS<sub>2</sub>. The origin of the genotypes seemed to play an increasingly important role as the sub-zero temperature decreased. Observing the results by provenance (Figs. 3a-3b), the most damaged trees were those belonging to VL at both of the TSs. Even so, the Provenance × Site interaction was significant at -13°C ( $p < 0.05$ ) and an analysis of provenance separated by trial site at this temperature revealed that at TS<sub>1</sub> the most damaged provenance was VL, while at TS<sub>2</sub> the extent of damage was similar for VL and PD.

### Visual score of damage to buds and stem

Visual scoring of stem showed higher damage than the index of freezing injury (Fig. 3). At all the sub-zero temperatures applied, greater damage was observed at TS<sub>1</sub> than at TS<sub>2</sub> ( $p < 0.0001$ ) (Figs. 3c-3d). There were significant differences among provenances and between sites at -8 and -13°C (Table 3). The Provenance × Site interaction was also significant at both temperatures. At -18°C neither provenance nor Provenance × Site interaction was significant. As the interaction was significant at -8 and -13°C, analyses of variance were done separately for each site. The results obtained showed that at TS<sub>1</sub>, the budsticks from VL were significantly more damaged than those from other provenances at both of the temperatures tested. At TS<sub>2</sub>, no differences were observed between provenances.

At all the sub-zero temperatures studied, the damages observed on the buds of the trees growing at TS<sub>1</sub> were greater, and significantly different, than the effect on the buds of trees at TS<sub>2</sub> (Figs. 3e-3f, 3g-3h). No differences were observed between different types of bud (apical and lateral) at any

of the sub-zero temperatures studied (Table 4). The Provenance x Site interaction was only significant at -18°C, when no differences between provenances were detected at TS1. Only VL was significantly more damaged than the other provenances at TS2 (Figs. 3e-3f, 3g-3h). At the temperature of -8°C, there were only significant differences relating to the site factor.

**Table 2.** Results of ANOVA analysis of damage expressed by the Index of Freezing Injury ( $I_i$ ) under the three sub-zero temperatures studied.

Fixed effect	$I_{-8}$		$I_{-13}$		$I_{-18}$	
	F	P-value	F	P-value	F	P-value
<b>Provenance (P)</b>	3.85	<b>0.0164</b>	5.95	<b>0.0019</b>	10.54	<b>&lt;0.0001</b>
<b>Trial Site (T)</b>	15.98	<b>0.0003</b>	39.01	<b>&lt;0.0001</b>	81.12	<b>&lt;0.0001</b>
<b>P×T</b>	2.56	0.0687	2.90	<b>0.046</b>	2.19	0.1044

*Bold P-values showed significant differences*

**Table 3.** Results of ANOVA analysis of damage to the stem, evaluated by visual scoring (VD), under the three sub-zero temperatures studied.

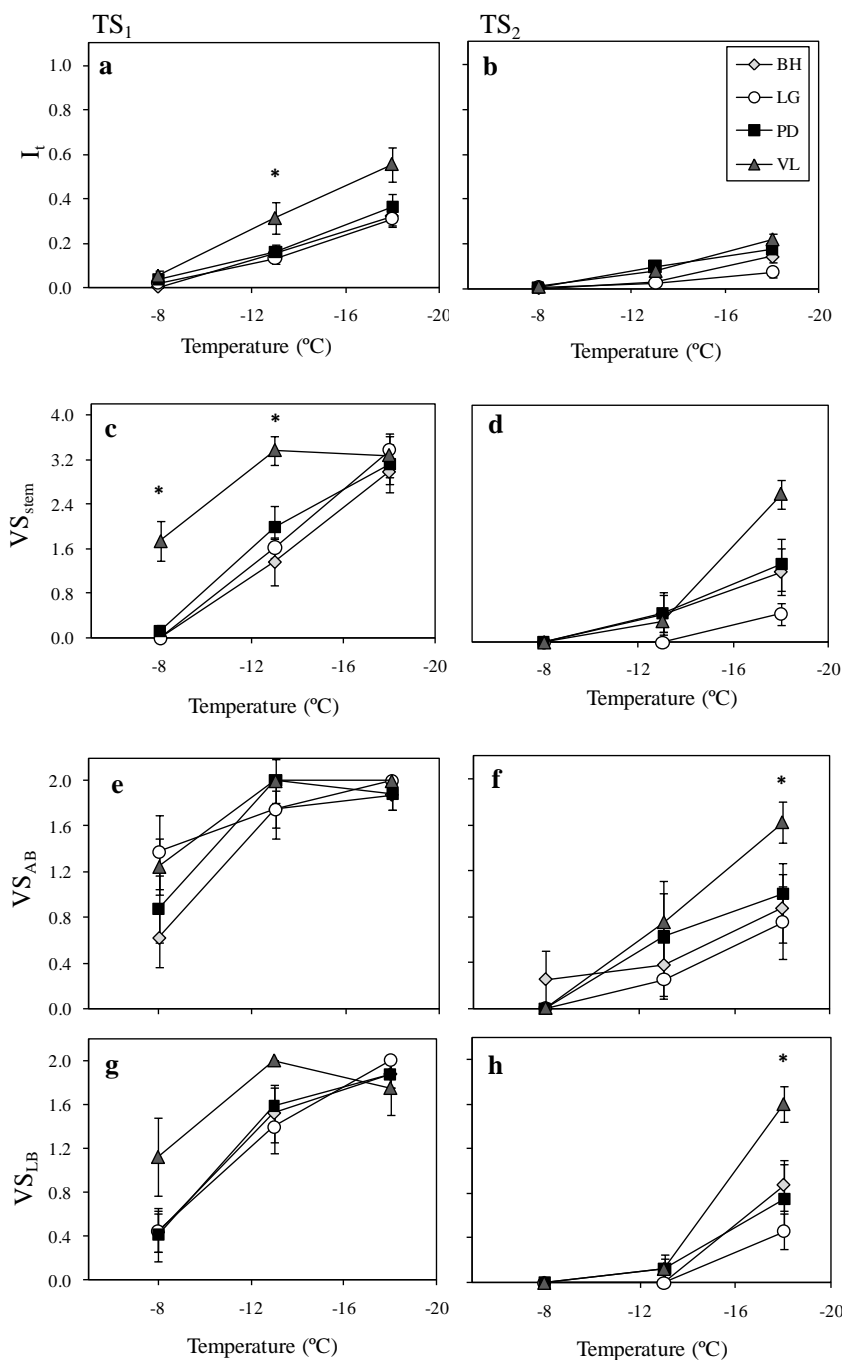
Fixed effect	$VS_{stem-8}$		$VS_{stem-13}$		$VS_{stem-18}$	
	F	P-value	F	P-value	F	P-value
<b>Provenance (P)</b>	19.05	<b>&lt;0.0001</b>	4.65	<b>0.0068</b>	2.57	<b>0.0672</b>
<b>Trial Site (T)</b>	204.47	<b>&lt;0.0001</b>	99.51	<b>&lt;0.0001</b>	39.24	<b>&lt;0.0001</b>
<b>P×T</b>	19.05	<b>&lt;0.0001</b>	3.31	<b>0.0289</b>	2.62	0.0635

*Bold P-values showed significant differences*

**Table 4.** Results of ANOVA analysis of damage to buds, evaluated by visual scoring (VD), under the three sub-zero temperatures studied.

Fixed effect	$VS_{buds-8}$		$VS_{buds-13}$		$VS_{buds-18}$	
	F	P-value	F	P-value	F	P-value
<b>Provenance (P)</b>	1.15	0.3327	2.93	<b>0.0374</b>	4.18	<b>0.0079</b>
<b>Trial Site (T)</b>	57.25	<b>&lt;0.0001</b>	123.18	<b>&lt;0.0001</b>	61.38	<b>&lt;0.0001</b>
<b>P×T</b>	2.08	0.1081	0.07	0.9775	5.68	<b>0.0012</b>
<b>Type of Bud</b>	0.01	0.9297	0.00	1.000	0.00	1.000

*Bold P-values showed significant differences*



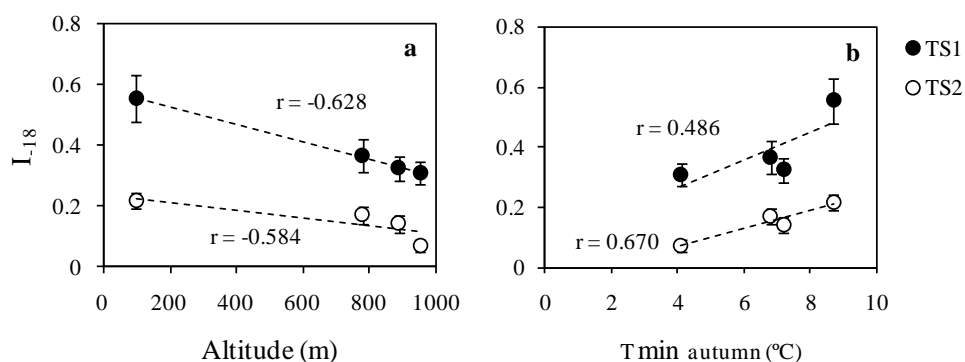
**Figure 3.** Mean and standard error (n=8) of damage for the four provenances (BH: Burgohondo, LG: La Guingueta, PD: Pedroso and VL: Valadouro) at each freezing temperature on trial sites 1 and 2. Fig. a-b: damage to the stem expressed by the Index of freezing injury; Fig. c-d: damage to the cambium by visual scoring; Fig. e-f: damage to terminal buds by visual scoring; Fig. g-h: damage to lateral buds by visual scoring. The \* show the significant differences between provenances, analysed separately by trial site when the interaction trial site × genotype was significant.

### Relationship between provenance origin and frost damage

A relevant relationship was found between frost damage at  $-18^{\circ}\text{C}$ , measured by  $I_{-18}$ , and some ecophysiological data relating to the environment of the place of provenance (Fig. 4).  $I_{-18}$  for the trees was negatively correlated with the altitude of their original growing area:  $r = -0.58$  ( $P < 0.001$ ) and  $-0.63$  ( $P < 0.001$ ), respectively, for TS1 and TS2 (fig. 4a). A significant, and in this case positive, correlation was also observed between the  $I_{-18}$  and the minimum autumn temperature of their original area, with  $r = 0.49$  ( $P < 0.05$ ) and  $r = 0.67$  ( $P < 0.001$ ), respectively, for TS1 and TS2 (fig. 4b).

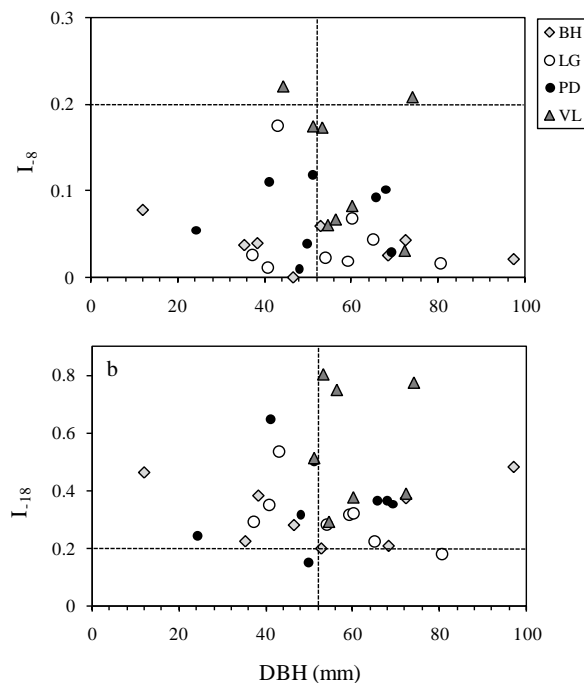
### Relationship between frost damage and growth (DBH)

Figure 5a and b shows the damage observed at temperatures of  $-8^{\circ}\text{C}$  ( $I_{-8}$ ) and  $-18^{\circ}\text{C}$  ( $I_{-18}$ ), respectively, and its relationship with breast height diameter (DBH) at TS1. Fig 5a shows that most of the trees exhibited an  $I_{-8}$  below than 0.2 and several of them showed no damage at all (values around 0). The lower right part of the Fig. 5a shows that this kind of tree can have high DBH, where 17 trees had values above the average level of growth for the assay. In fig 5b, considering the extreme sub-zero temperature assessed ( $-18^{\circ}\text{C}$ ), there are still trees with low damage values (around 0.2) and high DBHs, most of them being from LG.



**Figure 4.** Correlation between the Index of freezing injury at  $-18^{\circ}\text{C}$  and a) the altitude of the origin of the populations, b) the mean minimum temperatures during September, October and November of the original locations of the populations. Each point corresponds to 8 trees of the same provenance. In each figure, a correlation was made for the two trial sites (TS1 and TS2).





**Figure 5.** Relationship between diameters at breast height (DBH) with the Index of freezing injury calculated at a)  $-8^{\circ}\text{C}$  ( $I_{-8}$ ) and b)  $-18^{\circ}\text{C}$  ( $I_{-18}$ ), for each analysed individual from TS1. The vertical dashed lines show the average of the DBH for all samples.

## DISCUSSION

In the present study it has been observed that both factors: planting site and provenance (genotype), have influence on the acquisition of the autumn frost resistance.

### Importance of the planting site on autumn frost resistance

Significant differences in the level of damage by freezing temperatures were found between the two trial sites for all the tissues analysed, with the damage at Trial Site 1 (TS1) always being greater than at Trial Site 2 (TS2). These differences could be related to the level of cold hardiness that had already been developed by the time of sample taking at each trial site. The average minimum temperature during the 15 days before the samples were collected was lower at TS2 than at TS1 (Table 1). This temperature was highly related to the acclimation process as demonstrated for *J. regia* (Charrier and Ameglio 2011). The senescence process was more advanced at TS2 where most of trees had no leaves by 15<sup>th</sup> November 2010.

The Fv/Fm data obtained from the samples used as a control showed significant differences between the two trial sites (TS1>TS2). A winter drop in PS<sub>II</sub> efficiency was described by Levizou and Manetas (2008) in *Prunus cerasus* L. and suggested as a possible seasonal adaptive adjustment. More recently, a decrease in PS<sub>II</sub> efficiency has been reported in seedlings of *Juglans nigra* L. during a simulation of the hardening process (Wilson and Jacobs 2012). The differences between the two trial sites with respect to the Fv/Fm parameter could be related to the level of cold hardiness of the trees from TS2. This development of cold hardiness was more advanced at TS2 compared to the trees planted at TS1 at the time when the samples were collected.

Studies of the ecology of *J. regia* have established that this species has a high demand for water and heat during the growing period if it is to exhibit optimal growth (Masson 2005). The difference in annual rainfall was clearly evident in the results from the two assays; there was three times more rain at TS2 than at TS1, though this was offset by the use of drip irrigation at TS1. However, the optimum average annual temperature at this trial site was above 15°C and, from this point of view, TS1 could therefore be a better place for planting walnut trees than TS2 (Garavel 1959) (Table 1). Previous studies at these trial sites showed that, at the fourth growing season, dendrometric data for the two trial sites were very different for the average diameter at breast height (DBH), with the higher values being registered at TS1 (Díaz et al. 2006). Moreover, a warm autumn, with minimum temperatures of 12.5 °C, delayed the onset of the senescence period, which was later at TS1 than at TS2 (Díaz et al. 2006). The risk of frost was similar at the two sites (Table 1), but the expected severity of damage to plant tissue was higher on the plantation with a Mediterranean climate than that with an Atlantic influence.

In terms of walnut timber production, it is not known whether Climate Change could impact on the process of hardiness. In the Mediterranean region, summer drought periods could increase in frequency and length (Sheffield and Wood 2008). Walnut is well-known for exhibiting leaf abscission in periods of extreme drought (Ni and Pallardy 1991). If the period of active photosynthesis is too short, as a result of leaf abscission the amount of C that can be accumulated in autumn may be reduced, which could decrease its winter frost resistance (Gauthier and Jacobs 2011). Despite this fact, some relationship has been found in other species such as Holm oak, where seedlings collected from different areas of the Mediterranean region, showed that the most heat

tolerant were also the most freeze tolerant (Gimeno et al. 2009). An in-depth study relating the two stresses (drought and frost) needs to be performed taking into account the provenance regions.

### **Importance of the provenances on autumn frost resistance and its relationship with their origin**

Observing differences between the four provenances, the “Valadouro” provenance (VL) was always the most affected by freezing temperatures for each of the tissues tested and at both trial sites. Origin could have an influence on this behaviour: the observed correlations between frost damage and altitude and between frost damage and minimum autumn temperature, which were respectively negative and positive, support this view. Altitude and minimum autumn temperature could be important factors in the development of the hardening process of the chosen provenances, at their site of origin. The observed variability in frost damage followed an elevation gradient. The “La Guingueta” provenance (LG) from the 954 m high Pyrenean valley with the lowest autumn temperature displayed the least frost damage. In addition, VL - which was located only 100 m above sea level and had the highest autumn temperature and zero probability of an autumn frost-event - was the provenance that exhibited most frost damage. PD and BH were more similar to LG than to VL, as expected. This is common behaviour in other species such as *Pinus sylvestris* L. (Persson et al. 2010), *Picea abies* L. Karst (Kathke and Bruelheide 2011), and even in some deciduous species of the genus *Fagus*, *Quercus* (Vitasse et al. 2011) and *Castanea sativa* Mill., in which autumn bud damage to different populations has been related to low temperatures of their places of origin (Díaz et al. 2009). This would confer great importance to provenance (genotype) in the process of cold acclimation and resistance to autumn frost damage. However, the adaptive character of frost resistance should also be considered with respect to other specific traits related to timber production, such as growth rate and forest growth habit. In this sense, the results of previous studies involving dendrometric characterization of the first four growing seasons of provenances at the two trial sites (Díaz et al. 2006) reveal genetic differences, with VL being the most vigorous provenance. Focusing on TS1, where the highest growth was observed, the two characters (growth and frost resistance) seemed not to be related. Figure 5a shows the damage registered at -8°C, which represented a possible freezing-event in the Mediterranean area. The lower-right quadrant of this figure refers to material that was either less or not affected by sub-zero temperatures and

which had high diameter at breast height (DBH) values. These trees belong to different provenances; this suggests that breeding selection should be directed to individuals (family genitors). When applying extreme freezing temperatures (Fig. 5b), provenances such as VL showed frost sensitiveness and high DBH. However, for other provenances such as LG some of the trees showed DBH around the average of all the samples and they were also tolerant to autumn frost. This shows that autumn frost resistance and a good growth ratio are not mutually exclusive. This observed behaviour supports that extreme climatic events can influence on long-term survival. The main objective of breeding programmes in broadleaf species is to obtain a high growth ratio and thus optimize the use of the natural resources available at each planting site. Adaptive characters are becoming increasingly important in this respect. In *J. regia*, genetic differences have been found in traits such as water use efficiency (Fady et al. 2003; Aletà et al. 2009).

## CONCLUSIONS

The findings of this work point to differences in behaviour with respect to autumn frost resistance within *J. regia* origins.

At Trial Site 1, localized in the north east of Spain, the first freezing damage was observed by visual scoring at  $-8^{\circ}\text{C}$ . This situation should be carefully considered in afforestation with *J. regia* for timber production in the Mediterranean area.

Reforestation with local provenances or with trees from places with comparable environments is the usual choice of farmers/foresters looking to minimize damage from autumn frosts. Specific material selected for adaptive traits do not exist in the market. Farmers require trees selected for both adaptive and productive features in order to optimize their revenue, in each environment. Seedlings should therefore come from tested clonal seed orchards, or even better from selected family genitors; this should guarantee homogeneity and adaptability of reproductive forest material to the target environment of new afforestations (Aletà and Vilanova 2011).

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**Chapter III.**  
Genetics of autumn  
frost resistance in  
Persian walnut  
(*Juglans regia* L.)

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## GENETICS OF AUTUMN FROST RESISTANCE IN PERSIAN WALNUT (*JUGLANS REGIA* L.)

### ABSTRACT

The growing interest in broadleaf timber plantations in the Mediterranean area has promoted several studies focusing on the identification and characterization of sources of variability in main timber-producing tree species. *J. regia* is one of these species and is considered to be well-adapted to this area, but it is frost-susceptible. Breeding focused on productive traits should include knowledge of adaptations required to obtain a good selection capable of producing a suitable turnover from timber plantations. In this study, the main trait evaluated was autumn frost resistance and it was evaluated on 22 half-sib walnut progenies by submitting budsticks to sub-zero temperatures in a controlled chamber and then using measurements of relative electrolyte content (REL %) to calculate the resulting  $LT_{50}$  values ( $^{\circ}C$ ). The study was carried out on seven-year-old progenies and on their mother trees. The familiar heritability of autumn frost resistance in *J. regia* was  $h_F^2=0.63$ , and the narrow-sense heritability was  $h^2=0.64$ . This feature was genetically correlated with the length of the growing season ( $0.785\pm 0.219$ ) and inversely with secondary annual growth measured at breast height ( $-0.604\pm 0.233$ ). The relationship between autumn frost damage to progeny and their mother was heterogeneous and the narrow-sense heritability calculated from the regression progenitor-progeny was  $h^2= 0.34$ .

**KEY WORDS:** inheritance, cold hardiness, female progenitor, growth

### INTRODUCTION

The Persian walnut (*Juglans regia* L) is a species that has become naturalised in Europe and is considered a noble hardwood with valuable timber. It is native to central Asia (Leslie and McGranahan 1995) where it has been cultivated for thousands of years. Some authors claim to have established its presence in the Mediterranean region before the last ice age (Manchester 1989), with human activity enabling it to spread from different niches to all the temperate regions of

Europe, including the Iberian Peninsula (Huntley and Birks 1983). In ancient times, the main use of *J. regia* by humans was the production of its edible nut; the interest for other uses dates from the 14<sup>th</sup> century when fine wood started to be used for making furniture. It has been reported that the *J. regia* species was threatened in the 18<sup>th</sup> century due to a massive harvest; the high demand for this fine wood in England, for use in cabinet and other furniture making, led to the prohibition of its importation from France (Aletà 2008). Like other scattered broadleaf trees, poor silviculture, which failed to promote natural regeneration, together with the genetic introgression of local populations through the introduction of genotypes for nut production, contributed to the reduction of the size of wild populations in Europe (Demesure (1996). However, despite all of this, *J. regia* did not appear to be genetically impoverished (Fornari et al. 2001). The species is naturally present throughout Central and Southern of Europe in a wide variety of environments (Fady et al. 2003; Germain 2004).

Following the recent rise in interest in timber plantations in the Mediterranean region, several studies have focused on identifying sources of variability for several important tree traits linked to wood production (Germain 2004; Díaz and Fernández-López 2005; Aletà et al. 2004). These works also surveyed the diversity present in local populations and sought to identify the most representative tree species. Trees selected for timber production have to exhibit both suitable growth and good vigour; they must also show good adaptation to the environment where they grow. Adaptive traits are commonly associated with the ability of individual trees to survive biotic or abiotic stress. Several studies on different species have been conducted to increase our knowledge about the best combination of adaptive characteristics and economic traits. Some of the successful cases of resistance observed in different species could include resistance to Valsa canker in apple (Abe et al. 2011), frost hardiness in *Tsuga mertensiana* (Benowicz et al. 2001), and improved efficiency of water use in *Juglans regia* (Aletà et al. 2009b). All of these adaptive features were present in some individuals together with suitable productivity traits. These results allowed opening promising breeding ways.

Knowing more about the genetics of adaptive traits could also help to offer better responses/solutions in the face of potential climate change related problems that could be encountered in the future (IPCC 2007). In this regard, it is expected that the most suitable habitats of several species currently growing in the south of Europe, such as *Fraxinus angustifolia* Vahl (Temunovic et al. 2013), could be lost. Also pests and diseases, such as *Phytophthora cinnamomi* in

oak (Brasier 1996), could increase. These effects are more and more evident in the Mediterranean area. The selection of adaptive traits must therefore be made in wild populations before too much biodiversity is lost.

The present study focused on the adaptive trait of autumn frost resistance as the *J. regia* species is particularly sensitive to early frosts (Whoeste and Michler 2011; Guàrdia et al. 2013a). In winter, this species can survive freezing events with temperatures even falling below  $-20^{\circ}\text{C}$  (Becquey 1997), but in autumn, the same trees can be badly damaged before hardening is completed (Sakai and Larcher 1987). Autumn frost damage can produce losses of productivity by extending the number of training years required and by reducing tree vigour or by promoting undesirable levels of curvature of the central leader; these problems could even result in the death of young trees (Fady et al. 2003).

The aims of this study were: i) to assess the genetic variability of the “autumn frost resistance” trait in a set of 22 half sib progenies of common walnut from different origins installed in a progeny test; ii) to report variance components and to estimate the heritability nature of the trait and link it to phenological and growth traits through genetic correlations; iii) and to look for the relationship between the frost resistance in the mother tree and in its offspring (narrow-sense heritability).

## **MATERIAL AND METHODS**

### **Field test and plant material**

Progenies of outstanding *J. regia* trees, selected from different surveys made between 1988 and 1998 involving wild Spanish populations (Germain et al. 1997), were planted, according to the structure of a progeny test, in the northeast of Spain (Constantí-Tarragona). In the present study, 19 progenies were evaluated together with three references, selected for their supposed winter frost resistance. The references were two cultivars from the USA: ‘Chase D-9’ from Oregon and ‘Serr’ from California and a chance seedling of ‘Serr’ called ‘S-89’ (Germain 2004). A field trial was established in 2003. Seeds from the selected plus tree were harvested from the *J. regia* germplasm collection of Mas de Bover (Constantí-Tarragona) and then grown for one year at a nursery. Eighteen seedlings per progeny were planted at 5m x 5m under a randomized complete block design, with three replications and six trees per experimental unit. Table 1 summarises the main

ecological characteristics of the point of origin of the progenies studied and the characteristics of the study site. The orchard management was standard for *Juglans* grown for timber production in the Mediterranean area. From April to August supplementary irrigation was applied, with the total amount supplied being equivalent to 100 mm y<sup>-1</sup>. After the second growing season, corrective pruning was carried out every year, in late spring, in order to eliminate any multiple leaders and branches that were too great in diameter.

### Freezing treatments

The budsticks chosen for the assay were apical and 20 cm long. They were removed from annual shoots in the third week of November, a time when there was a probability of having freezing temperatures at the test site (García del Barrio et al. 2001). The progenies were evaluated in 2010 and the mother trees in both 2010 and 2011.

A total of 12 trees per progeny were analysed (four trees per block). Budsticks with similar characteristics were collected from each tree for each of the temperatures assessed in order to provide a homogenous group of four samples. Budsticks destined for the same treatment were wrapped in cheese cloth and aluminium foil to prevent their desiccation during freezing and placed in a testing chamber. The initial temperature inside the test chamber was 5°C. This was then reduced, by 2.5°C/hour, until the programmed sub-zero temperature had been reached. The chamber was then kept at the same temperature for two hours and then increased (also at rate of 2.5°C/hour) to a temperature of 5°C. The temperatures assessed in the control chamber were: -14°C and -18°C<sup>1</sup>. Another treatment consisted of freezing samples at -75°C for 24h to achieve complete cell destruction. Finally, in the control test, samples were kept at a temperature of 5°C in a fridge, without submitting them to any freeze/thaw cycle. These sub-zero temperatures were not chosen as potential natural autumn freezing temperatures, but to be sure to get frost damage in order to estimate the LT<sub>50</sub> parameter (explained on the following section).

Five budsticks were analysed per mother tree for each genotype and temperature (i.e. five replicates per treatment). The treatments applied were the same as those applied to the progenies. In 2011, however, mother tree samples were only submitted to one freeze/thaw cycle, down to -14°C.



**Table 1.** Geographic origin and climatic characteristics (°C) of 22 *Juglans regia* families and details of the trial site

Family	RIU	Spanish provenance region	Latitude	Longitude	Elevation (m)	T min an (°C)	P an (mm)	P gs (mm)
F1	1	Galician Coast	42°80' N	8°12' W	330	7.8	1427	490
F2	1	Galician Coast	42°82' N	9°03' W	380	10.6	1667	558
F3	1	Galician Coast	42°41' N	8°76' W	10	9.2	1557	502
F4	1	Galician Coast	42°42' N	8°23' W	455	7.4	151	523
F5	2	Inner Galicia	43°48' N	8°23' W	1	9.8	1350	459
F6	2	Inner Galicia	43°30' N	8°20' W	50	8.7	1080	378
F7	2	Inner Galicia	43°36' N	8°18' W	65	9.8	1238	426
F8	3	Asturias Coast	42°81' N	7°11' W	490	6.8	1389	497
F9	3	Asturias Coast	43°27' N	8°22' W	50	8.6	1001	355
F10	3	Asturias Coast	42°78' N	7°01' W	720	5.9	1431	514
F11	9	Pre-Pyrenees	41°88' N	2°52' W	630	5.8	854	448
F12	9	Pre-Pyrenees	41°87' N	0°88' W	322	7.6	506	276
F13	11	Northern Ebro	41°30' N	1°03' W	850	7.4	612	325
F14	11	Northern Ebro	41°27' N	1°00' W	830	5.9	555	306
F15	24	Levant	40°72' N	0°50' W	75	11.2	648	339
F16	24	Levant	40°90' N	0°05' W	40	11.2	491	277
F17	24	Levant	40°60' N	0°45' W	130	11.2	598	307
F18	24	Levant	40°72' N	0°50' W	75	11.2	648	339
F19	35	Cazorla-Segura	38°15' N	2°30' W	1600	5.6	432	194
<b>References</b>		Origin						
F20	S-89	-	-	-	-	-	-	-
F21	Chase D-9	USA	-	-	-	-	-	-
F22	Serr	USA	-	-	-	-	-	-
<b>Trial Site</b>	Constantí	-	41°09' N	1°12' E	90	10.9	537	-

RIU: Regions for the identification and utilization of reproductive forest material (García del Barrio et al. 2001). T min an: mean of the minimum annual temperatures; P an: (average of 30 years). P gs: mean of the accumulated precipitation during the growing season. (Data obtained from "Atlas Climático Digital" (Ninyerola et al. 2005))

### Quantification of frost damage by electrical conductivity (EC)

The damage caused by the freezing temperatures was evaluated after thawing out a couple of slices taken from each budstick. These pieces of wood were put into test tubes containing 20 ml of distilled H<sub>2</sub>O and were stored overnight at room temperature. The samples were shaken for 30 minutes before measuring the conductivity of the solution (C<sub>1</sub>). Then, to ensure complete electrolyte leakage, the sample tubes were heated in an oven for 90 minutes at 80°C and then stored overnight at room temperature. After a new 30-minute shaking, the conductivity (C<sub>2</sub>) was measured for a second time. The relative electrolyte leakage (EC) was calculated as:  $EC = C_1/C_2 * 100$ , for each sample (Jacobs et al. 2008). The parameter used to estimate the frost hardiness of each genotype was LT<sub>50</sub> (the sub-zero temperature at which one genotype exhibited damage to over 50% of its cells). To calculate LT<sub>50</sub>, we assumed the logistic sigmoid function proposed for walnut trees by Charrier and Ameglio (2011):

$$EC = A / (1 + e^{B(C-X)}) + D$$

In this equation, the  $X$  corresponds to the temperature (5, -14, -18 and -75°C),  $A$  and  $D$  define the asymptotes of the function and  $B$  is the slope at the inflection point:  $C$ . The parameter LT<sub>50</sub>, which indicates the level of frost hardiness, was estimated as the abscise temperature of the inflection point:  $C$  (Repo and Lappi 1989). This value was obtained using a nonlinear regression (JMP software, version 8.0) for each tree.

### Field traits

The diameter at breast height (DBH) was measured each year at the end of the growing season for all the replicates in the progeny test. The annual increment (DBHI) was calculated as the difference between two consecutive years. Phenological traits were also recorded for each tree. Two main traits were registered: flushing (BB, stage  $C_f$  following the nomenclature described by Germain et al (1999)) and leaf fall (LF, the stage registered when 50% of the leaves have fallen (Díaz and Fernández-López 2005)). All of the values were expressed in Julian days. The length of the growing season (GSD) was the number of days between BB and LF. All of these traits were monitored for at least four years between 2006 and 2010 for each tree.

### Statistical analysis

The values of  $LT_{50}$ , DBH and the phenology traits were analysed, using mixed model analysis, at the individual-tree level as a randomized complete block design (following the test progeny design):

$$Y_{ijk} = \mu + B_i + f_j + bf_{ij} + e_{ijk}$$

where  $Y_{ijk}$  is the observation of the  $k$ th individual of the  $j$ th family in the  $i$ th block,  $\mu$  is the overall mean,  $B_i$  is the fixed effect of the  $i$  block,  $f_j$  is the random effect of the  $j$ th family,  $bf_{ij}$  is the residual effect of the  $j$ th family in the  $i$ th block, and  $e_{ijk}$  is the random tree effect of the  $k$ th individual in the  $ij$ th plot.

Family heritability was calculated from restricted maximum likelihood (REML) estimates of variance components as:

$$h_F^2 = \frac{\sigma_F^2}{(\sigma_F^2 + \sigma_{BF}^2/B + \sigma_e^2/BK)}$$

Where  $\sigma_F^2$  is the family variance,  $\sigma_{BF}^2$  is the family by block interaction variance,  $\sigma_e^2$  is the between tree within plot variance,  $B$  is the number of blocks and  $K$  is the number of trees per plot. Standard errors of  $h_F^2$  were calculated according to Gilmour et al (2002). The Z score was used as an approximate statistic to test whether the family variance component differed from 0.

Genetic correlations and their standard errors were calculated from the variance-covariance component matrices obtained from bivariate analyses of variance involving the traits of interest (Gilmour et al. 2002).

$$r_{12} = \sigma_{12} / \sqrt{\sigma_1^2 \times \sigma_2^2}$$

where  $\sigma_{12}$  stands for the estimated family covariance component between traits 1 and 2;  $\sigma_1$  is the family variance component of trait 1 and  $\sigma_2$  is the family variance component of trait 2.

The phenotypic age-age correlation between the mother tree data for 2010 and 2011 was calculated to know the stability of the  $LT_{50}$  values within a genotype between different years.

The narrow-sense heritability ( $h^2$ ) was calculated as the ratio of genetic variance ( $\sigma_A^2$ ) respect to phenotypic variation ( $\sigma_P^2$ ):  $h^2 = \sigma_A^2 / \sigma_P^2$

The narrow-sense heritability ( $h^2$ ) was also calculated from the relationship between the progenitors and their progenies for the same parameter ( $LT_{50}$ ), which is equivalent to twice the progenitor-progeny regression slope (Zobel and Talbert 1984).

## RESULTS

### Trait variability

The coefficients of variation (CV) of  $LT_{50}$  for the evaluated half-sib progenies were from 3 to 13% (Table 2) and significant variability was found in the autumn frost resistance trait (the associated Wald Z-Score probability was 0.021) (Table 3). The reference progenies which were chosen by their winter frost resistance (F20, F21 and F22) all showed high resistance to autumn frost. In the field trial, the difference between extreme progenies by GSD was 23 days (F16 vs F21); by diameter of annual growth, it was 0.5 cm (F20 vs F21) (Table 2), by LF, it was 9 days (F9 vs F2), and by BB it was 23 days (F18 vs F21) (Table 2).

### Variance components and heritability of features

In the case of the variance family component ( $\sigma_F^2$ ), the highest value corresponded to BB, which represented 61% of the total variance, while that of autumn frost resistance was 16%. For vegetative traits like GSD and DBHI, the  $\sigma_F^2$  values were 18.3% and 18.4%, respectively, and for LF, it was just 10% of the total variance. As expected, the  $h_F^2$  for BB was very high ( $0.92 \pm 0.04$ ), while the autumn frost resistance and GSD traits exhibited similar levels of heritability ( $0.63 \pm 0.12$  and  $0.62 \pm 0.15$ , respectively) (Table 3). The narrow-sense heritability calculated from the variance components was  $h^2 = 0.64$ .

**Relationship between autumn frost resistance and vegetative traits**

Genetic correlations between  $LT_{50}$  values, which were obtained for each tree, and their corresponding dates of LF, BB and GSD, or DBHI, are summarized in Table 4. GSD was positively correlated with  $LT_{50}$  ( $r=0.785\pm 0.219$ ), while DBHI was negatively correlated ( $-0.604\pm 0.233$ ). The relationship between  $LT_{50}$  and these traits is graphed in Figure 1 and Figure 2, showing that good frost hardiness is compatible with good increases in diameter and with long vegetative periods. Genetic correlations were also calculated within phenological traits and growth, with the most significant finding being the negative correlation between GSD and BB ( $-0.792\pm 0.269$ ) and the lack of correlation observed between DBHI and LF and GSD.

**Table 2.** Mean ( $\pm$ SE) of  $LT_{50}$  and several traits of the families analysed: growing season duration (GSD), leaf fall (LF), diameter at breast height increment (DBHI) and bud break (BB). CV is the coefficient of variation of the  $LT_{50}$  parameter (n=7-12)

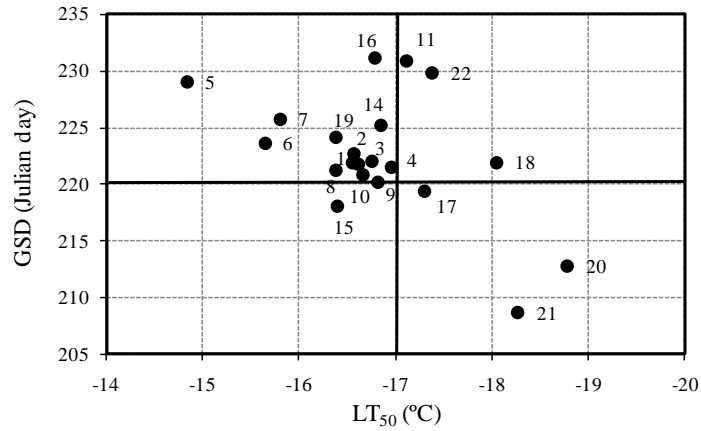
<b>Family</b>	<b><math>LT_{50}</math> (°C)</b>	<b><math>CV_{LT_{50}}</math> (%)</b>	<b>GSD (Julian Day)</b>	<b>LF (Julian Day)</b>	<b>DBHI (mm)</b>	<b>BB (Julian Day)</b>
F1	-16.62 $\pm$ 0.15	2.58	221.77 $\pm$ 2.97	317.93 $\pm$ 2.56	8.55 $\pm$ 0.99	95.71 $\pm$ 0.86
F2	-16.57 $\pm$ 0.45	9.15	222.65 $\pm$ 2.08	320.40 $\pm$ 1.73	9.38 $\pm$ 0.46	97.31 $\pm$ 1.01
F3	-16.75 $\pm$ 0.59	11.32	221.97 $\pm$ 2.30	315.72 $\pm$ 2.51	11.24 $\pm$ 1.39	93.70 $\pm$ 0.74
F4	-16.96 $\pm$ 0.34	6.49	221.43 $\pm$ 2.52	312.75 $\pm$ 1.81	9.91 $\pm$ 0.56	91.72 $\pm$ 1.33
F5	-14.84 $\pm$ 0.46	8.21	228.95 $\pm$ 5.05	315.25 $\pm$ 1.30	7.15 $\pm$ 0.97	92.93 $\pm$ 0.86
F6	-15.64 $\pm$ 0.48	8.80	223.61 $\pm$ 1.77	314.18 $\pm$ 2.66	7.45 $\pm$ 1.08	89.61 $\pm$ 0.81
F7	-15.80 $\pm$ 0.24	4.43	225.73 $\pm$ 3.97	319.16 $\pm$ 3.46	6.77 $\pm$ 0.53	93.12 $\pm$ 0.81
F8	-16.38 $\pm$ 0.47	10.07	221.23 $\pm$ 2.57	315.25 $\pm$ 1.32	10.10 $\pm$ 0.64	96.79 $\pm$ 1.11
F9	-16.82 $\pm$ 0.59	10.70	220.22 $\pm$ 1.35	311.20 $\pm$ 1.00	8.51 $\pm$ 0.66	90.43 $\pm$ 0.78
F10	-16.66 $\pm$ 0.29	6.12	220.89 $\pm$ 2.01	312.62 $\pm$ 1.60	7.35 $\pm$ 0.59	91.50 $\pm$ 0.68
F11	-17.10 $\pm$ 0.65	11.42	230.86 $\pm$ 2.64	317.77 $\pm$ 1.24	10.81 $\pm$ 0.57	91.03 $\pm$ 1.12
F12	-18.44 $\pm$ 0.37	5.82	-	311.25 $\pm$ 2.10	9.83 $\pm$ 0.74	-
F13	-16.54 $\pm$ 0.68	12.40	221.91 $\pm$ 1.41	315.50 $\pm$ 0.89	9.82 $\pm$ 0.62	94.81 $\pm$ 1.37
F14	-16.85 $\pm$ 0.50	8.96	225.16 $\pm$ 1.09	314.27 $\pm$ 1.12	9.58 $\pm$ 0.50	88.06 $\pm$ 0.39
F15	-16.39 $\pm$ 0.32	6.57	218.00 $\pm$ 2.80	308.31 $\pm$ 2.58	10.01 $\pm$ 0.71	89.43 $\pm$ 0.56
F16	-16.78 $\pm$ 0.60	11.32	231.10 $\pm$ 1.71	320.27 $\pm$ 0.94	10.08 $\pm$ 0.70	88.73 $\pm$ 1.36
F17	-17.30 $\pm$ 0.56	10.42	219.35 $\pm$ 2.35	312.37 $\pm$ 1.32	10.86 $\pm$ 0.80	93.89 $\pm$ 1.39
F18	-18.04 $\pm$ 0.44	8.20	221.93 $\pm$ 2.94	309.01 $\pm$ 2.33	9.96 $\pm$ 0.72	85.68 $\pm$ 0.75
F19	-16.38 $\pm$ 0.61	11.32	224.08 $\pm$ 4.87	313.41 $\pm$ 3.01	10.15 $\pm$ 0.36	96.08 $\pm$ 1.10
F20	-18.78 $\pm$ 0.68	12.63	212.82 $\pm$ 2.32	313.00 $\pm$ 2.10	11.75 $\pm$ 0.51	102.46 $\pm$ 2.05
F21	-18.27 $\pm$ 0.32	4.98	208.71 $\pm$ 4.31	316.90 $\pm$ 3.19	6.74 $\pm$ 0.49	108.50 $\pm$ 1.81
F22	-17.38 $\pm$ 0.67	12.81	229.82 $\pm$ 3.31	313.11 $\pm$ 1.76	-	87.27 $\pm$ 1.55

**Table 3.** Variance components and family heritability ( $h_F^2$ ) of frost hardiness ( $LT_{50}$ ), growing season duration (GSD), diameter at breast height increments (DBHI), date of leaf fall (LF) and date of bud break (BB).  $\sigma_F^2$  corresponds to the family variance component,  $\sigma_{FB}^2$  is the family by block interaction variance component,  $\sigma_e^2$  corresponds to the error component.

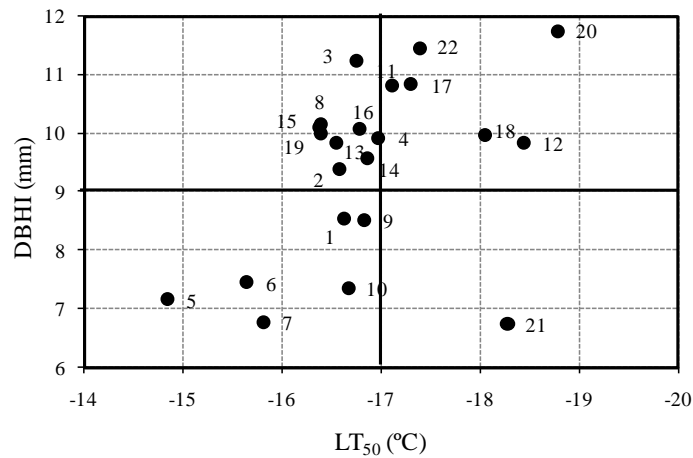
Variable	$\sigma_F^2$	$\sigma_{FB}^2$	$\sigma_e^2$	$h_F^2$
<b>LT<sub>50</sub></b> (2010)	16.0 (0.0211)	0	84.0	0.63±0.12
<b>GSD</b> (2006-08-09-10)	18.3 (0.0309)	10.4	71.3	0.62±0.15
<b>LF</b> (2006-08-09-10)	10.0 (0.0980)	17.1	73.0	0.42±0.21
<b>DBHI</b> (2006-08-09-10)	18.4 (0.0399)	21.4	60.1	0.57±0.16
<b>BB</b> (2006-08-09-10)	61.0 (0.0157)	4.4	34.7	0.92±0.04

**Table 4.** Genetic correlations ( $\pm$ SE) between several traits:  $LT_{50}$ , growing season duration (GSD), date of leaf fall (LF), diameter at breast height increment (DBHI) and date of bud break (BB). The phenological and the growth traits have been extracted from a four-year mean: 2006-08-09-10.

Variable	$LT_{50}$	GSD	LF	DBHI
<b>GSD</b>	0.785±0.219	-	-	-
<b>LF</b>	0.493±0.304	0.155±0.320	-	-
<b>DBHI</b>	-0.604±0.233	0.076±0.300	-0.431±0.377	-
<b>BB</b>	-0.434±0.337	-0.792±0.141	0.385±0.317	-0.548±0.269



**Figure 1.** Diagram of LT<sub>50</sub> (X axis) and the Growing Season Duration (GSD) (Y axis), using the mean for the 22 families. The GSD is the mean of the four-year increase (2006-08-09-10). The data for family 12 was not available.



**Figure 2.** Diagram of LT<sub>50</sub> (X axis) and the increase in diameter at breast height (DBHI) (Y axis), using the mean for the 22 families. The DBHI is the mean four-year increase (2006-08-09-10).

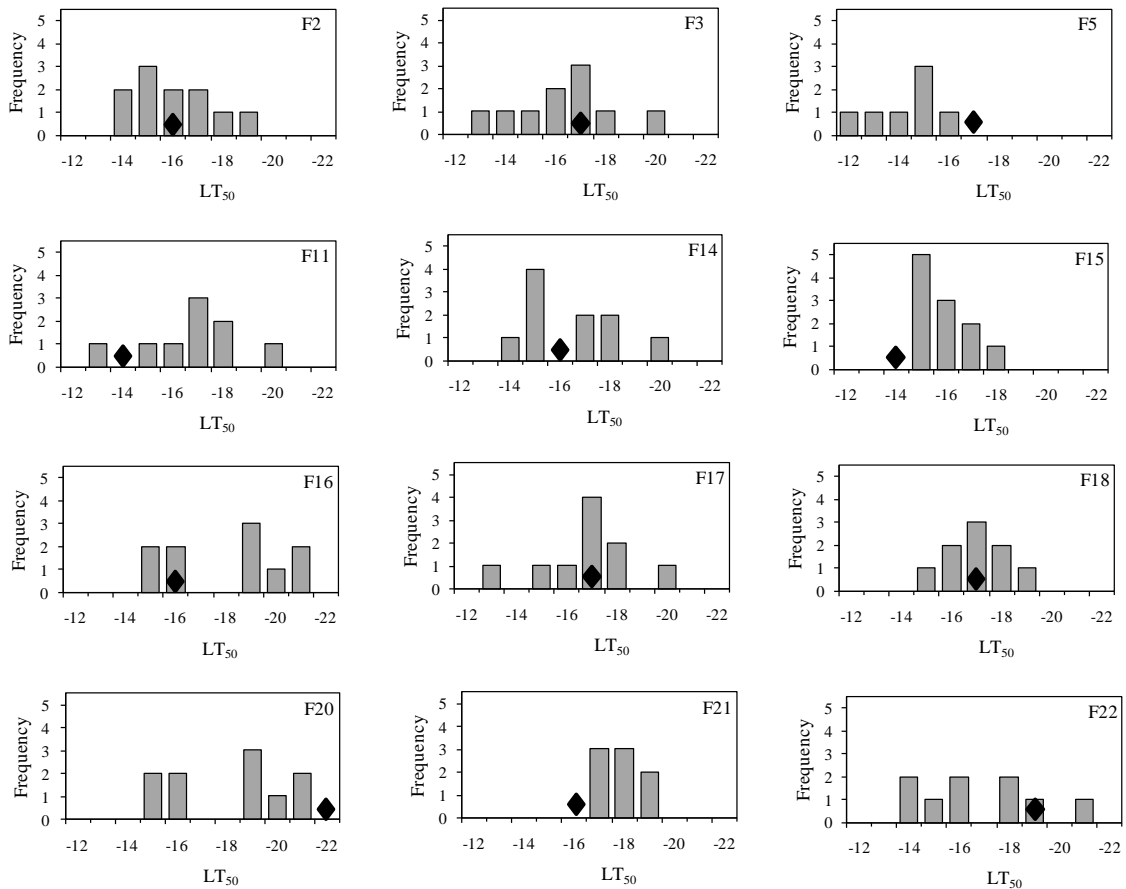
### Relationship between mother trees and progenies

In Figure 3, the ranges of LT<sub>50</sub> frequency by family are represented (n=7-12) together with the LT<sub>50</sub> of each corresponding mother tree (MT). All the families exhibited a normal distribution (Shapiro - Wilk W. test). Some of the families exhibited higher LT<sub>50</sub> values as their MT (f. 11, 15 and 21) and some other exhibited an inverse situation, where the offspring had lower values than their MT



(families 5, 20 and 22). Six of the twelve families studied had, on average, similar  $LT_{50}$  to MT values (families 2, 3, 14, 16, 17 and 18).

The age-age correlation for frost damage to the mother trees, which was evaluated at  $-14^{\circ}\text{C}$  in 2010 and 2011, was  $r = 0.712$  ( $p < 0.005$ ) (data not shown). The narrow-sense heritability calculated from the Progenitor-Progeny regression was  $h^2 = 0.34$ .



**Figure 3.** Distributions of the offspring of each family ( $n=7-12$ ) for the  $LT_{50}$  (frost hardiness in  $^{\circ}\text{C}$ ) and representation of the value for the mother tree of each family (rhombus).

## DISCUSSION

### Family heritability for autumn frost resistance and its relationship with growth and phenological traits

The significant family genetic component of the total phenotypic variance (16%) together with the high value for family inheritance of cold hardiness ( $0.63 \pm 0.129$ ) points to potential success in selecting for this trait in *J. regia*. Values of  $h^2_F$  of 0.37 have been reported for cold hardiness in needles of *Pinus sylvestris* (Abrahamsson et al. 2012) and of 0.30 in Douglas-fir full-sib families (Hawkins and Stoehr 2009). The family variance component and the heritability of GSD and DBHI obtained were as expected for this species (Aletà et al. 2009b).

In this study, a significant positive genetic correlation was found between GSD and  $LT_{50}$ . The length of the growing season seemed to be mainly influenced by the BB, with this exhibiting a strong negative correlation, while it was not dependent on LF (Table 4). The length of the growing season could be related to the climate of origin of each family due to the effect of natural selection on wild populations which synchronize environment and growth, studied in a wide range of species (Sakai and Larcher 1987). Despite the high genetic correlation ( $r=0.785 \pm 0.219$ ), some families (like F11 and F22) had long GSD and low  $LT_{50}$ ; this indicated that an advance or delay in cold acclimation was not the only factor which could explain autumn frost resistance (Figure 1). The relationship between DBHI and  $LT_{50}$ , showed families exhibiting high autumn frost resistance which had the highest DBHI values (Figure 2). These differences in increases in annual diameter could also have been related to the origin of the different families. In the case of *Tsuga Mertensiana* (Benowicz et al. 2001), it was found that the maximum growth rate (cm/week) was significantly and positively correlated with the number of annual frost days, showing that the seedlings from the coldest places had the greatest growth rates. In *J. regia* a positive relationship was observed between altitude and the acquisition of frost hardiness (Guàrdia et al. 2013b). Progenies from low-rise places (F5, F6 and F7) corresponded to the highest  $LT_{50}$  values and lowest levels of resistance. In the opposite situation, both F11 and F12, which came from the Pre-Pyrenees region, had low  $LT_{50}$  values, as expected. F17 and F18, from the Levant region, also exhibited low  $LT_{50}$  values; they came from a continental but not a mountainous region. Origin is obviously important, but not always it could be known which the

most influent factor is. In this study, the altitude could be important to confer autumn frost resistance in *Juglans*.

In the present work it should also be noted that growth did not show any direct relationship with GSD: families which differed significantly in terms of GSD were able to obtain similar DBHI values by the end of the season. DBHI was negatively correlated with BB, but no correlation was found with LF. The greatest growth rates consequently correspond to the first weeks of the growing season, as in some species that grow in the Mediterranean region. Along these lines, spring frost damage should also be analysed as *J. regia* is also highly susceptible to it (Masson 2005; Díaz et al. 2009).

### **Relationship between progenies and mother trees**

LT<sub>50</sub> progeny segregation was similar between families (the Bartlett test did not show any significant differences in the homogeneity of the variances – data not shown) and the highest coefficient of variation (CV) was 13%, which was supported by the high  $h^2_F$ .

However, the relationship between mothers and their offspring did not follow any well-defined pattern (figure 3). Perhaps the “father effect” could explain this: 17 days separated the earliest budbreak from the latest and flushing data were closely linked to female blooming in *J. regia*. This wide range of dates allowed many different female/male combinations in the germplasm collection from which the original seeds were collected. For several characteristics of *J. regia*, the male parent has an important influence on the acquisition of characteristics by the offspring; one such case was the inheritance of the leafing time (Germain 1990). The narrow-sense inheritance of the autumn frost resistance trait obtained from the progenitor-progeny regression was quite considerably smaller than the narrow-sense heritability calculated from the variance components. Perhaps age differences between mother trees and progenies could have affected this relationship. It has been observed that the inheritance of some traits varies with tree age in *J. regia*; it is known that this applies to dominance, ramification, straightness and some adaptive traits such as leaf fall (Aletà et al. 2009a)

The original site of the mother tree could also be a reason for the observed differences in LT<sub>50</sub>. The mother tree seedlings came from seeds which had developed in environmental conditions that were completely different from those of the germplasm collection where the mother trees that were evaluated produced their seeds. Several studies on *Picea abies* have pointed to the possibility

of the timing of cold acclimation being influenced by the temperatures during female sexual reproduction (Johnsen et al. 1996; Webber et al. 2005). Besnard et al (2008) who also worked on *Picea abies* found that the maternal environment influenced the performance of the progenies and that this could be affected by long-lasting epigenetic memory and regulated by the prevailing temperature and photoperiod during seed production. In the present study, most of the progenies which show similar  $LT_{50}$  values to their mother trees correspond to families for which the environmental characteristics of the places of origin of the mother trees were similar to those of the seed orchard where the progenies developed (F2 and F3 or F16, F17 and F18). This is a question that should be more extensively studied in *J. regia* before any basic procedures are undertaken to produce seeds for timber production.

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**Chapter IV.**  
Is summer drought  
influencing the  
autumn frost  
resistance of *Juglans*  
under Mediterranean  
conditions?

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## IS SUMMER DROUGHT INFLUENCING THE AUTUMN FROST RESISTANCE OF *JUGLANS* UNDER MEDITERRANEAN CONDITIONS?

### ABSTRACT

In Europe, the main species used for walnut timber plantations are the *Juglans regia* L. and some hybrid progenies between *J. regia* and *J. nigra*. The optimum growing conditions of these species include a minimum of 700 mm of water evenly distributed over the year, as they do not stand summer droughts very well. They are also sensitive to out-of-season frost events. In the Mediterranean area, summer drought is considered the main factors that can limit biomass production and species distribution. In view of climate change, summer droughts are expected to become more severe as a result of drier and warmer weather. The aim of this study is to investigate the influence of summer drought on autumn freezing injury. Different amount of water was applied during the growing season to young potted trees of *J. regia* and *J. ×intermedia* and to 10-year-old field-grown trees, all under Mediterranean climate conditions, over two consecutive years (2010 and 2011). Leaf fall process and final growth were recorded in each trial and year of the study. In autumn, annual budsticks were collected and starch, soluble sugars and water contents were analysed from different irrigation treatments. A freeze/thaw cycle down to -12°C was applied to the budsticks samples collected in autumn.

Data of leaf fall and soluble sugar content indicate that hardening processes commenced earlier in trees submitted to summer drought, though no differences between treatments were detected for either species in 2010 with respect to freezing injury. In 2011, the freeze/thaw cycle was performed on three occasions during the autumn period (2<sup>nd</sup> November, 20<sup>th</sup> November and 10<sup>th</sup> December) which allowed observing a progress in frost damage quicker in the water restricted treatments than in the control ones in both species.

**KEY WORDS:** water stress, growth, frost hardiness

## INTRODUCTION

In recent years, in the western Mediterranean there has been a growing interest in the cultivation of common walnut (*Juglans regia* L.) due to its high economic value as timber. However the scarcity of improved planting stock available for timber production of common walnut led landowners to use some hybrid progenies, outstanding for their vigour, which result from the natural hybridisation of some selected clons of black walnut species (mainly *J. nigra* L.) plus *J. regia* (Becquey 1997). Currently, in Europe, *J. regia* and these hybrid progenies (*Juglans* ×*intermedia*) are planted in new afforestations because of both materials are capable of producing good quality timber (Masson 2005; Aletà et al. 2003). These species need approximately 700 mm of water supply evenly distributed throughout the year, as they do not stand summer droughts very well. One of the main limitations is its sensitivity to out-of-season frost events in autumn of *J. ×intermedia* and in both autumn and spring of *J. regia* (Masson 2005; Garavel 1959).

In the Mediterranean area, summer drought is considered one of the main factors that can limit biomass production and plays an important role in the distribution of the species (Sanchez-Gomez et al. 2006). However, in Mediterranean continental areas, cold temperatures are also important in terms of limiting plant survival and growth (Gimeno et al. 2009; Terradas and Save 1992). Both these stresses have to be taken into consideration when planning walnut afforestation projects, due to their importance in relation to the growing results. Summer drought can affect annual growth and may have implications on the final tree-felling time. Out-of-season frost events can damage the timber and adversely modify tree shape, affecting stem straightness which is a very important condition to obtain high quality timber (Macdonald and Hubert 2001; Burton et al 2008). Young trees can even die as a result of these first-freeze events.

As a consequence of climate change, the weather is expected to become drier and warmer in the Mediterranean region (IPCC 2007b; ACCUA 2011). Predictions arising from different models for generating climate change scenarios show how the Mediterranean region could be affected by drought periods lasting between four and six months or even longer (over 1 year) and with a frequency of occurrence between 3 and 8 times higher than at present (Sheffield and Wood 2008). Moreover, an increasing frequency of extreme events (heat waves, frosts and storms) is forecast, with a high degree of regional variability (IPCC 2007b). Under these conditions, plants can be expected to be

subjected to more common sudden frost events with varying damage level in relation to the hardening process. Plant phenology can also be affected, with a shortening or lengthening of the growing season depending on the region that will influence final crop production (IPCC 2007a). In terms of walnut timber production, it is not known whether summer drought could impact on the process of hardening and on the resistance to first-freezing events. A relationship between summer drought and cold resistance was found in Holm oak when seedlings were collected from different areas of the Mediterranean region, with the most heat tolerant also being the most freeze tolerant (Gimeno et al. 2009). Previously, and also in Holm oak, a relationship between summer environmental conditions and low temperature stress has also been defined (Savé et al. 1999; Terradas and Save 1992). All these results concur with the pioneering work of Tranquillini (1982) on timberline trees. In the evergreen Azalea, water stress imposed at the end of summer and early autumn affected the cold hardiness of the plants which were dependent on their current water status (Anisko and Lindstrom 1996). Due to the commercial interest in developing woody plantations, which sometimes are not optimally sited (Guardia et al 2013), a better understanding is needed of how the relationship of these climatic events (summer drought and first-freeze events) could affect walnut timber plantations, focusing on productivity (growth) and on adaptation of these materials to the environmental conditions in the Mediterranean region.

The aims of the assays that were undertaken involved evaluating the effects of several levels of summer drought on annual growth, leaf fall and autumn frost hardiness in young and adult trees of two progenies of the *Juglans* genus usually planted for timber production.

## **MATERIAL AND METHODS**

### **Plant material, study site and drought treatments**

The assays were carried out on a progeny of *J. regia* L. and a hybrid progeny of *J. ×intermedia* (Ng23xRa). The experiment consisted of two assays related to tree age and growing conditions:

Assay 1: This comprised the application of two irrigation regimes on one-year-old potted trees of the two progenies mentioned above. The trees were planted in 25l pots (7:3 peat: perlite) and placed outdoors at IRTA research centre in Torre Marimon (Caldes de Montbui, Barcelona, Spain). The annual average rainfall of this area is around 670 mm and the annual mean temperature 14°C. The watering

regimes applied daily by drip pipe during the growing season (from bud break to the end of leaf fall) were: R1 irrigation with 100% of the water requirements, calculated from the ETo of Torre Marimon, and R2 irrigation with 50% of the ETo. The pots were covered with plastic bags to avoid substrate evaporation and rain. Eighteen seedlings of each progeny and irrigation treatment were randomly distributed in the experimental plot.

Assay 2. This experiment was carried out on ten-year old trees of the same two progenies of *J. regia* and *J. xintermedia* submitted to three irrigation regimes. The trial site was at IRTA's experimental facilities in Mas Valero (Tarragona, Spain). Average annual rainfall in this area is around 550 mm and annual mean temperature is 16.8°C. Each experimental plot unit had four individuals, that is 12-16 trees per irrigation treatment and progeny. Irrigation, applied by four drippers per tree, was initiated after bud break and was calculated weekly based on the water balance between ETo and precipitation on the shaded area of the canopy and considering the Kc (crop coefficient) of the common walnut. The three regimes were: Complete irrigation (C) with water supplied throughout the growing season until September 15; restricted irrigation (R) with water supplied only until July 15 and No irrigation (N) with no water supplied during the growing season. The C and R treatments had been applied to the orchard from planting that is for 10 years while N was applied from 2010 onwards on randomly chosen plots belonging to C or R on previous years. Both assays were carried out during two consecutive years (2010 and 2011).

### **Field traits: stomatal conductance, phenology and growth**

In both assays, stomatal conductance was recorded monthly throughout the 2011 growing period in four trees per treatment and species always at 12:00 h (solar time). Five measurements per tree were made on fully developed mature leaves.

The diameter evolution of each tree was recorded on 2010 and 2011. The diameter of young trees was measured at 5 cm from root collar every 15 days from June to September (Assay 1). In adult trees, the diameter was measured monthly at breast height (Assay 2)

Two stages of vegetative phenology were recorded per tree, in both assays and years: leaf flushing and leaf fall dates (Julian days). Leaf flushing was taken at Cf stage, following the description proposed by Germain et al. (1999) and leaf fall was registered when 50% of leaves have fallen (Díaz and Fernández-López 2005).

### **Autumn parameters**

In the second week of November, 2010, annual budsticks (6-8 samples per irrigation regime and species) were collected. First, a transversal piece of each budstick (1 cm thick) was cut to determine the starch, sugar and water contents of samples. The same budsticks were initially stored at 5°C and then submitted to a cycle of freezing/thaw down to -12°C.

Samples were collected on three dates of autumn 2011 (November 2, November 20 and December 10) in order to follow the evolution of cold hardiness. Each day samples removed from trees were directly submitted to a cycle of freeze/thaw to -12°C. Water content evolution was analysed only on adult trees (Assay 2), samples of Assay 1 were too small to get samples recurrently in each date.

### **Water, starch and soluble sugars content**

In 2010, the fresh weights (FW) of the samples (a piece of annual budstick) were measured to obtain the water content. These samples were subsequently frozen with liquid nitrogen and then lyophilized to obtain the dry weights (DW) and also starch and soluble sugars contents. Water content was obtained as  $(FW - DW) / DW$ .

Starch and sugar analysis were carried out at the UMR-PIAF Laboratory (INRA-Clermont Ferrand) following the methodology developed for walnut stem described by Charrier and Ameglio (2011). The contents were expressed in mg/g DW.

### **Freezing and characterization of damage according to the freezing injury index**

The samples of both 2010 and 2011 were moistened with distilled water and then wrapped in cheese cloth and aluminium foil, in groups of 8, in order to prevent desiccation. These packets were then placed in a freezing chamber. From an initial 5°C, the temperature inside the chamber was reduced by 2.5°C/hour to a final temperature of -12°C. Once the chosen temperature was reached, it was maintained for two hours. To obtain a progressive thaw, the temperature inside the chamber was then increased at a rate of 3°C/hour until it returned to 5°C. A control treatment was also employed in which the packets of samples were kept at 5°C. In each freeze/thaw cycle the temperature was recorded and stored in a data logger.

After thawing samples for REL analysis were prepared following standard methodology described specifically for *Juglans* in Guardia et al. (2013). Relative electrolyte leakage (REL %) was:  $REL = C_1 / C_2 * 100$ .

To compare sample frost damage the index of freezing injury was calculated as:  $I_t = (REL_t - REL_0) / (100 - REL_0)$  being  $REL_0$  the REL value of unfrozen samples (at 5°C) in accordance with Flint et al. (1967). This index ranged from 0 to 1, from zero damage to a completely damaged sample.

### Statistical analyses

The effect of irrigation treatment and species on the different variables studied was assessed using a general linear model (GLM):  $y_{ij} = \mu + T_i + S_j + TS_{ij} + \varepsilon_{ij}$

where  $y_{ij}$  is the value of the response variable measured on the  $i^{\text{th}}$  irrigation treatment and  $j^{\text{th}}$  species,  $\mu$  is the overall mean,  $T_i$ ,  $S_j$  and  $TS_{ij}$  are the fixed effects of the irrigation treatment, the species and the interaction between the two effects, respectively, and  $\varepsilon_{ij}$  is the residue that affects the analysis.

The freezing injury index of 2011, for both assays, was analysed as the other parameters, but with the addition of a new factor, sampling day, and its interactions with the other factors. The procedure was:

$y_{ijk} = \mu + T_i + S_j + D_k + TD_{ik} + SD_{jk} + TS_{ij} + \varepsilon_{ijk}$ , where  $D_k$  was the sampling day.

Significant differences between effects were assessed by Tukey's HSD test, with  $\alpha = 0.05$ .



## RESULTS

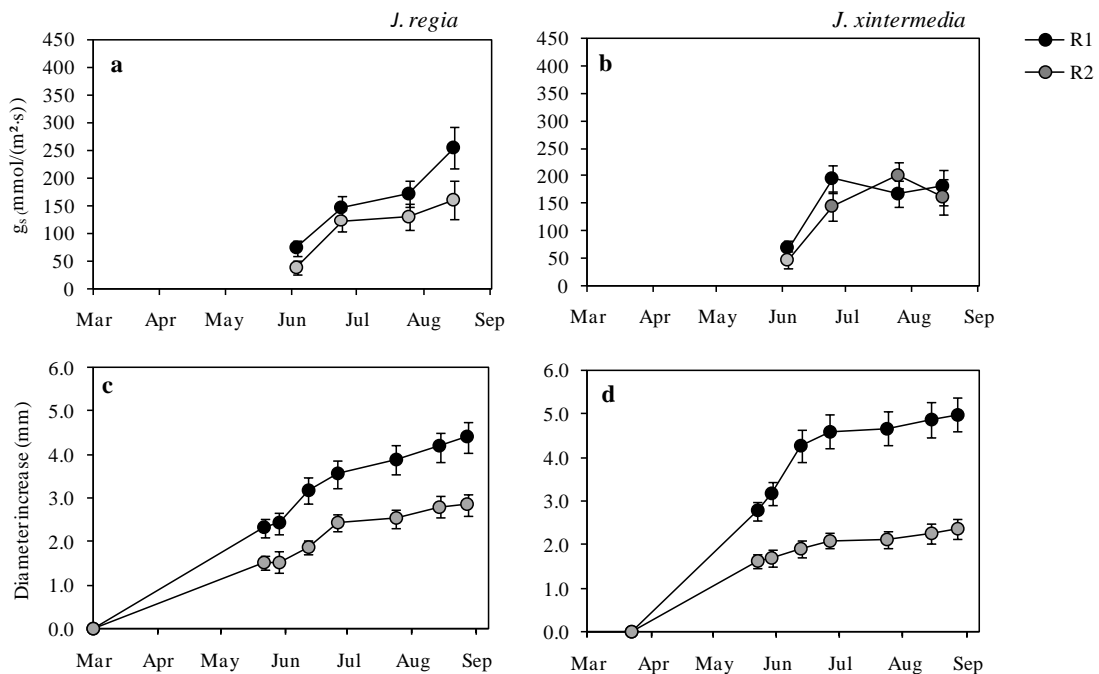
### Assay 1: young potted trees

#### Growing season: annual growth and stomatal conductance

In 2010, the final diameter showed differences between the two species, with the annual diameter increase of *J. regia* (JR) higher than that of *J. xintermedia* (JX). Differences between treatments were also found, with R1 showing a higher annual diameter increase than R2 in both species (Table 1 A-B).

During the 2011 growing period, the annual evolution of stomatal conductance and annual diameter increase, graphed on Fig. 1 a-b and c-d, showed both *Juglans* species had similar behaviour by the two hydric regimes. However, at the end of year growth significant differences on diameter increase existed between species (JR>JX) (Table 1B).

**Figure 1.** Mean and standard error of stomatal conductance (a-b) and diameter increase at root collar (c-d) of the trees of Assay 1 during the growing season of 2011. R1 is the irrigation treatment with 100% of total water requirements and R2 is the irrigation treatment with 50% of total water requirements, both calculated from the ETo.



### **Starch, soluble sugars and water content and leaf fall**

These parameters are summarized in Tables 1A-B. Analysis of starch content, made in budsticks removed at the second week of November 2010, showed the amount of starch depended as on the species as on the hydric regime, being the interaction  $I \times R$  significant. When results were studied by species, the total amount of starch in JR was significantly higher in R1 ( $101.21 \pm 3.3$ ) than in R2 ( $84.44 \pm 3.36$ ) while no difference existed in JX ( $R1=61.52 \pm 5.05$ ;  $R2=60.41 \pm 5.05$ ). These results were in accordance to those of leaf fall, less amount of starch corresponded to the earliest leaf fall and were opposite to the total SS content; SS content was significantly different between species,  $JX > JR$ , the earliest and latest fallen leaf species respectively. The water content parameter showed only significant differences between hydric regimes.

In 2011 (Table 1B), the leaf fall parameter not showed interaction  $I \times R$  but there was a significant differences between species ( $JR > JX$ ) and JR reached level 3 of leaf fall 13 days later than JX but no differences were found between irrigation treatments.

### **Index of freezing injury**

In 2010, the samples were collected in the second week of November and submitted to a freeze/thaw cycle down to  $-12^{\circ}\text{C}$ . The analysis revealed significant differences between the two species, with JR more damaged than JX samples. No differences were found between irrigation treatments being the interaction between irrigation and species not significant (Table 1A).

During autumn of 2011, the samples were collected on three different days. In this case, the interaction between species and sampling day ( $SD \times S$ ) was significant (Table 1B) and the analysis was performed separately for each species. Figure 2 shows the evolution of freezing injuries during autumn for the two species. On JR, no differences were found between the two irrigation regimes, but a progressive hardening of the samples could be observed over autumn. Considering JX, significant differences between irrigation regimes were observed, with the R2 samples being more frost resistant than the R1 samples on the first sampling day (November 2). Comparing the two species, JX was well hardened before JR (Figure 2).

**Table 1 A-B.** Results of ANOVA analysis and means ( $\pm$  SE) of several traits analysed on 2010 (A) and 2011 (B) in Assay 1 (young trees): damage expressed by the Index of Freezing Injury of samples submitted to  $-12^{\circ}\text{C}$  ( $I_{-12}$ ), annual diameter increase (Diam), starch accumulation (Starch), soluble sugars content (SS), water content (WC) and Level 3 of leaf fall (L3).

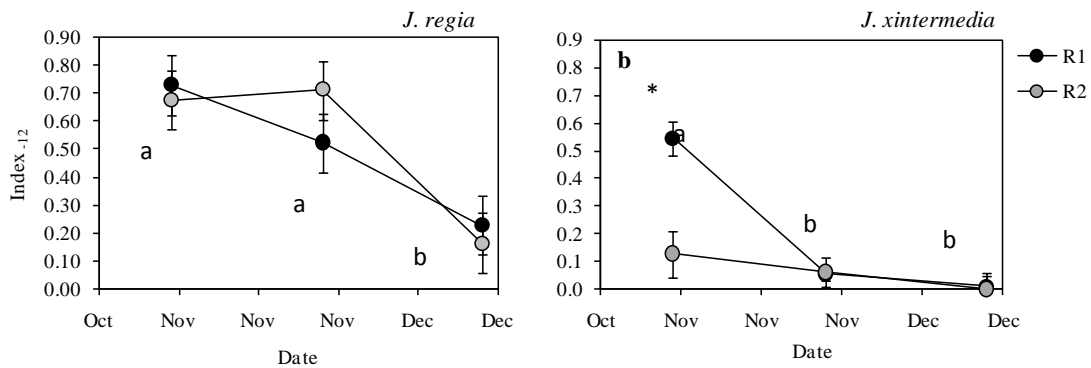
A) 2010	Index. <sub>12</sub>			Diam (mm)			Starch (mg/g DM)		
	df	Prob>F	Mean	df	Prob>F	Mean	df	Prob>F	Mean
<b>Irrigation (I)</b>	1	0.8694 <sup>ns</sup>		1	0.0001 <sup>***</sup>		1	0.0779 <sup>ns</sup>	
R1			0.04 $\pm$ 0.02			4.03 $\pm$ 0.10 a			72.98 $\pm$ 3.0
R2			0.04 $\pm$ 0.02			2.78 $\pm$ 0.22 b			80.81 $\pm$ 3.0
<b>Species (S)</b>	1	0.0472 <sup>*</sup>		1	0.0056 <sup>**</sup>		1	<0.0001 <sup>***</sup>	
JR			0.07 $\pm$ 0.02 a			3.85 $\pm$ 0.19 a			92.82 $\pm$ 3.0 a
JX			0.00 $\pm$ 0.02 b			2.97 $\pm$ 0.24 b			60.97 $\pm$ 3.0 b
<b>I <math>\times</math> S</b>	1	0.9225 <sup>ns</sup>			0.9696 <sup>ns</sup>		1	0.0455 <sup>*</sup>	

A) 2010	SS (mg/g DM)			WC (mg/g DM)			L3 (J. day)		
	df	Prob>F	Mean	df	Prob>F	Mean	df	Prob>F	Mean
<b>Irrigation(I)</b>	1	0.0649 <sup>ns</sup>		1	0.0260 <sup>*</sup>		1	0.0171 <sup>*</sup>	
R1			43.42 $\pm$ 1.4			58.59 $\pm$ 2.9 a			308.83 $\pm$ 1.8 a
R2			47.31 $\pm$ 1.4			49.11 $\pm$ 2.8 b			302.41 $\pm$ 1.8 b
<b>Species (S)</b>	1	0.0174 <sup>*</sup>		1	0.7199 <sup>ns</sup>		1	0.7264 <sup>ns</sup>	
JR			42.82 $\pm$ 1.4a			54.57 $\pm$ 2.9			305.16 $\pm$ 1.8
JX			47.91 $\pm$ 1.4b			53.11 $\pm$ 2.8			306.08 $\pm$ 1.8
<b>I <math>\times</math> S</b>	1	0.6783 <sup>ns</sup>		1	0.2493 <sup>ns</sup>		1	0.0181 <sup>*</sup>	

Different letters show significant differences. \*\*\*  $p < 0.001$ ; \*\*  $p < 0.01$ ; \*  $p < 0.05$ ; <sup>ns</sup>  $p > 0.05$

B) 2011	Index. <sub>12</sub>			Diam (mm)			L3 (J. day)		
	df	Prob>F	Mean	df	Prob>F	Mean	df	Prob>F	Mean
<b>Irrigation (I)</b>	1	0.2855 <sup>ns</sup>		1	0.2855 <sup>ns</sup>		1	0.2082 <sup>ns</sup>	
R1			0.34 $\pm$ 0.03			4.88 $\pm$ 0.5			322.58 $\pm$ 1.7
R2			0.29 $\pm$ 0.03			4.09 $\pm$ 0.5			319.40 $\pm$ 1.8
<b>Species (S)</b>	1	<0.0001 <sup>***</sup>		1	0.0302 <sup>*</sup>		1	<0.0001 <sup>***</sup>	
JR			0.50 $\pm$ 0.03a			5.30 $\pm$ 0.4a			327.27 $\pm$ 1.7a
JX			0.13 $\pm$ 0.03b			3.67 $\pm$ 0.6b			314.71 $\pm$ 1.7b
<b>I <math>\times</math> S</b>	1	0.1332 <sup>ns</sup>		1	0.0153 <sup>*</sup>		1	0.9913 <sup>ns</sup>	
<b>Sampling day (SD)</b>	2	<0.0001 <sup>***</sup>							
Day 1			0.51 $\pm$ 0.04a						
Day 2			0.33 $\pm$ 0.04b						
Day 3			0.10 $\pm$ 0.04c						
<b>I <math>\times</math> SD</b>	2	0.0579 <sup>ns</sup>							
<b>SD <math>\times</math> S</b>	2	0.0302 <sup>*</sup>							
<b>I <math>\times</math> S <math>\times</math> SD</b>	2	0.2802 <sup>ns</sup>							

Different letters show significant differences. \*\*\*  $p < 0.001$ ; \*\*  $p < 0.01$ ; \*  $p < 0.05$ ; <sup>ns</sup>  $p > 0.05$



**Figure 2.** Mean and standard error of the Index of Freezing Injury at  $-12^{\circ}\text{C}$  ( $I_{-12}$ ) of trees of Assay 1 in autumn of 2011. R1 is the irrigation treatment with 100% of total water requirements and R2 is the irrigation treatment with 50% of total water requirements, both calculated from the ETo. Different letters means significant differences between sampling days ( $p < 0.001$ ). \* means significant differences between irrigation treatments.

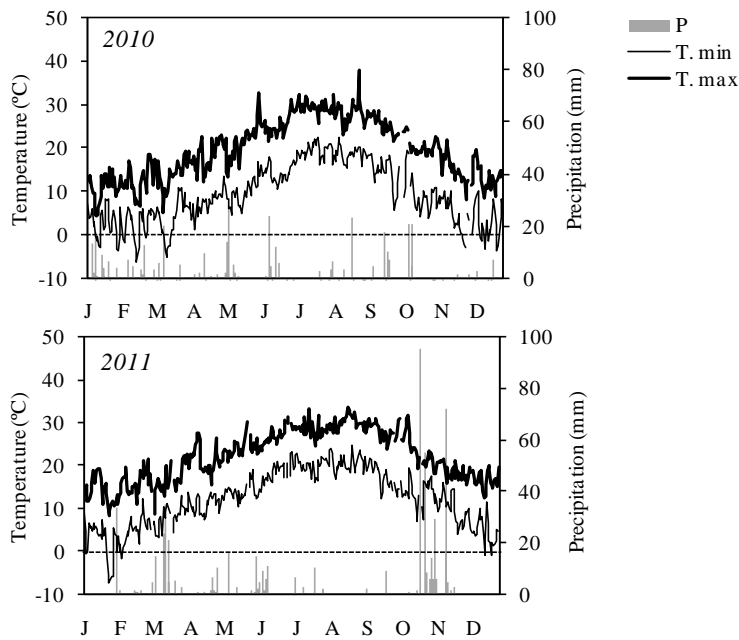
## Assay 2: field adult trees

### Growing season: secondary growth and stomatal conductance

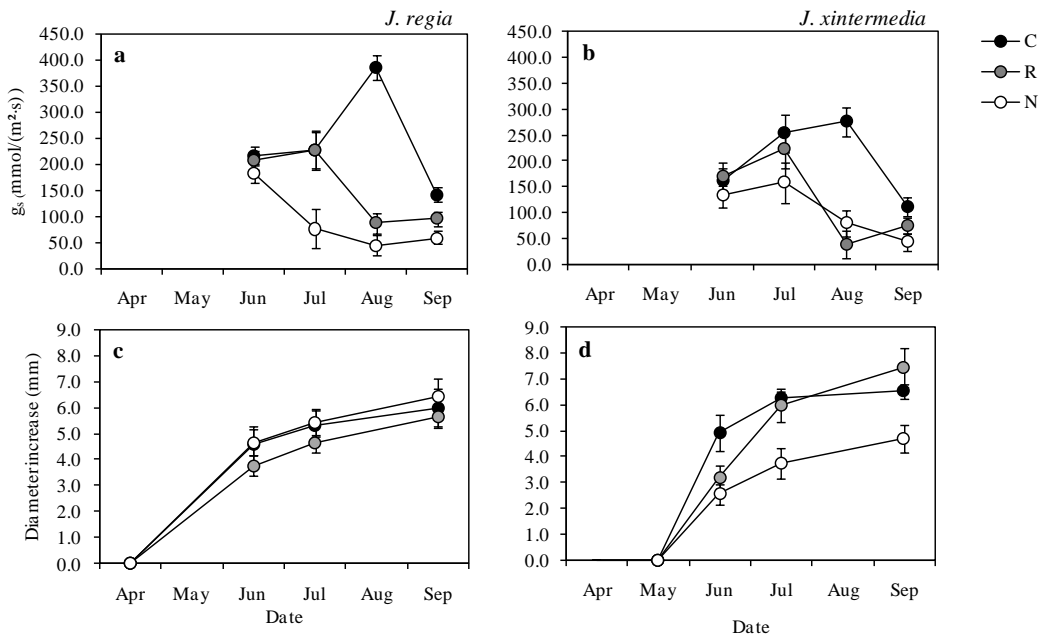
In 2010, the final diameter increase showed significant differences between species (*J. regia* grew more than *J. xintermedia*). Significant differences were also observed between irrigation treatments with trees subjected to complete irrigation showing a higher increase than those subjected to restricted irrigation and a higher increase still compared to the trees with no irrigation (Table 2A).

During 2011, the secondary growth, diameter at breast height, was depending on the species and the irrigation treatment (interaction  $I \times S$  was significant) and statistical analysis was made by species (Table 2B); both species showed similar growth without significant differences in JR between the three amount of water applied. However, the results graphed on Figure 4 c-d showed that no irrigation supposes less significant growth in JX.

Stomatal conductance ( $G_s$ ), which growing season evolution was measured in 2011, was clearly different between the three irrigation treatments in both species (Fig. 4 a-b). The “no irrigation” treatment showed lower  $G_s$  values throughout the vegetative period than the other treatments. Restricted irrigation had a more similar behaviour to the strict drought than to the complete irrigation treatment from July 15 onwards, as it was expected. However, the different photosynthetic activity which can be related to  $G_s$  observed, did not result on secondary growth in either species.



**Figure 3.** Daily minimum and maximum temperature and daily precipitation during 2010 and 2011. Data obtained from AEMET (Spanish National Meteorological Agency) meteorological station.



**Figure 4.** Mean and standard error of stomatal conductance (a-b) and the diameter at breast height increase (c-d) of the trees of Assay 2 during the growing season of 2011. The control treatment (C) was applied throughout the growing period. The restricted treatment (R) was applied until 15<sup>th</sup> July and the “no irrigation” treatment (N) involved no irrigation water.

**Starch, soluble sugars and water content and leaf fall**

In 2010 Starch content showed differences between species (JR>JX), and between irrigation treatments, with the "no irrigation" trees having the highest values, followed by the complete irrigation (Table 2A). SS content and the WC at collecting budsticks (15<sup>th</sup> of November) depended on the species and on the irrigation (for both parameters interaction I×S was significant). If the analysis is made by species, the watering regime N (41.18±1.08) had more SS content than R (31.18±1.53) and C (33.93±1.53) in JR, while in JX the C (43.19±1.08) and N (43.84±0.79) had the same content of SS which is significantly higher than R. By species WC not differed between irrigation treatments. Finally, no differences between species or between irrigation treatments were observed for leaf fall on reaching level 3 as in 2010 as in 2011 (Table 2 A-B).

**Index of freezing injury**

The freezing injury index of 2010 showed differences between species. The JR samples were more damaged than the JX samples. No differences between irrigation treatments were observed for both species and year (Table 2A). Same results were obtained in 2011 and significant differences between species were detected again (JR was more damaged than JX) as well as differences between the freezing injuries on sampling days. Curiously higher damage was detected on the first sampling day (November 2) than on the second.

**Table 2 A-B.** Results of ANOVA analysis and means ( $\pm$  SE) of several traits analysed on 2010 (A) and 2011 (B) in Assay 2 (adult trees): damage expressed by the Index of Freezing Injury of samples submitted to  $-12^{\circ}\text{C}$  (I<sub>-12</sub>), annual diameter increase (Diam), starch accumulation (Starch), soluble sugars content (SS), water content (WC) and Level 3 of leaf fall (L3).

A) 2010	Index <sub>-12</sub>			Diam (mm)			Starch (mg/g DM)		
	df	Prob>F	Mean	df	Prob>F	Mean	df	Prob>F	mean
<b>Irrigation (I)</b>	2	0.5911 <sup>ns</sup>		2	0.0001 <sup>***</sup>		2	0.0016 <sup>**</sup>	
C			0.152 $\pm$ 0.02			7.155 $\pm$ 0.35a			88.49 $\pm$ 3.85ab
R			0.181 $\pm$ 0.02			6.086 $\pm$ 0.35a			81.43 $\pm$ 3.85b
S			0.149 $\pm$ 0.01			4.166 $\pm$ 0.57b			98.74 $\pm$ 2.76a
<b>Species (S)</b>	1	<0.0001 <sup>***</sup>		1	0.0159 <sup>*</sup>		1	0.0057 <sup>**</sup>	
JR			0.273 $\pm$ 0.01a			6.43 $\pm$ 0.36a			95.38 $\pm$ 2.87a
JX			0.048 $\pm$ 0.01b			5.17 $\pm$ 0.35b			83.73 $\pm$ 2.88b
<b>I <math>\times</math> S</b>	2	0.9413 <sup>ns</sup>		2	0.2848 <sup>ns</sup>		2	0.7018 <sup>ns</sup>	

A) 2010	SS (mg/g DM)			WC (mg/g DM)			L3 (J. day)		
	df	Prob>F	mean	df	Prob>F	mean	df	Prob>F	mean
<b>Irrigation (I)</b>	2	<0.0001 <sup>***</sup>		2	0.9114 <sup>ns</sup>		2	0.0568 <sup>ns</sup>	
C			38.56 $\pm$ 0.93			0.46 $\pm$ 0.0			308.69 $\pm$ 1.44
R			34.02 $\pm$ 0.93			0.46 $\pm$ 0.0			311.65 $\pm$ 1.47
S			42.51 $\pm$ 0.67			0.46 $\pm$ 0.0			314.74 $\pm$ 2.08
<b>Species (S)</b>	1	<0.0001 <sup>***</sup>		1	<0.0001 <sup>***</sup>		1	0.2230 <sup>ns</sup>	
JR			35.42 $\pm$ 0.69			0.48 $\pm$ 0.0			310.49 $\pm$ 1.42
JX			41.30 $\pm$ 0.70			0.43 $\pm$ 0.0			312.89 $\pm$ 1.33
<b>I <math>\times</math> S</b>	2	0.0204 <sup>*</sup>		2	0.0011 <sup>**</sup>		2	0.1830 <sup>ns</sup>	

Different letters show significant differences. \*\*\*  $p < 0.001$ ; \*\*  $p < 0.01$ ; \*  $p < 0.05$ ; <sup>ns</sup>  $p > 0.05$

B) 2011	Index <sub>-12</sub>			Diam (mm)			L3 (J. day)		
	df	Prob>F	Mean	df	Prob>F	Mean	df	Prob>F	Mean
<b>Irrigation (I)</b>	2	0.4755 <sup>ns</sup>		2	0.1388 <sup>ns</sup>		2	0.9011 <sup>ns</sup>	
C			0.175 $\pm$ 0.02			6.16 $\pm$ 0.45			289.75 $\pm$ 2.36
R			0.155 $\pm$ 0.02			6.32 $\pm$ 0.44			291.12 $\pm$ 2.41
S			0.164 $\pm$ 0.02			5.28 $\pm$ 0.36			289.63 $\pm$ 3.41
<b>Species (S)</b>	1	<0.0001 <sup>***</sup>		1	0.8332 <sup>ns</sup>		1	0.2125 <sup>ns</sup>	
JR			0.27 $\pm$ 0.01a			5.87 $\pm$ 0.35			288.16 $\pm$ 2.32
JX			0.09 $\pm$ 0.01b			5.97 $\pm$ 0.33			292.18 $\pm$ 2.19
<b>I <math>\times</math> S</b>	2	0.5400 <sup>ns</sup>		1	0.0130 <sup>*</sup>		1	0.8247 <sup>ns</sup>	
<b>Sampling day (SD)</b>	2	0.0102 <sup>*</sup>							
Day 1			0.185 $\pm$ 0.02a						
Day 2			0.121 $\pm$ 0.02ab						
Day 3			0.183 $\pm$ 0.02b						
<b>I <math>\times</math> SD</b>	4	0.1721 <sup>ns</sup>							
<b>SD <math>\times</math> S</b>	2	0.1229 <sup>ns</sup>							
<b>I <math>\times</math> S <math>\times</math> SD</b>	4	0.7275 <sup>ns</sup>							

Different letters show significant differences. \*\*\*  $p < 0.001$ ; \*\*  $p < 0.01$ ; \*  $p < 0.05$ ; <sup>ns</sup>  $p > 0.05$

## DISCUSSION

### Differences between species

In 2010 the diameter increase in both assays, potted young trees and adult trees aged 10 in plantation was bigger on *Juglans regia* (JR) than on *J. ×intermedia* (JX) trees. In 2011, the young trees showed the same behaviour. However, on plantation the JX tree diameter increase was similar to that of JR, with the response subordinated to the significant interaction between species and irrigation treatment. It has been attributed in other studies that JX trees have a good apical dominance and rectitude but also vigour higher than JR trees (Aletà et al. 2003). The calcareous soil in which trees grew in the plantation could explain these results; it is referred the hybrid progeny used, Ng23xRa, does not adapt to poor and calcareous soils while *J. regia* naturally grows in many places of the whole of the Peninsula Ibérica having this edaphic limitations (Becquey 1997; Aletà and Vilanova 2012).

In autumn of 2010, for both adult and young trees, the freezing injuries measured during the second week of November after the freeze/thaw cycle were higher on JR than JX. This behaviour has been reported in a previous study comparing different *Juglans* species (Guàrdia et al. 2013). This difference could be due to the higher soluble sugars content in JX than JR (in both assays). Recently, a strong relationship has been observed between frost hardiness and soluble sugars content on walnut (Charrier and Ameglio 2011). The soluble sugars can protect against the freezing damages, as they can lower the intracellular freezing point by 1.86°C per mole of solute dissolved per kg of water (Hansen and Beck 1988). However, the concentrations of these sugars are not enough to explain the protection against very low temperatures but they must play an indirect role in cold hardiness development, depressing the effects associated with dehydration (Lenne et al. 2009), which is the primary cause of freezing injury in woody plants (Loris et al. 1999). On the other hand, the enzymes which hydrolyse the starch increase their activity in autumn from very low levels in late summer to maximum levels in winter, as demonstrated on poplar (Elle and Sauter 2000) and on walnut (Charrier and Ameglio 2011), and can generate starch hydrolysis. In the present study, it was observed that the JX (both young and adult) had less starch content than the JR trees, which could be related to the advanced hardening process of the hybrids. Moreover, on adult trees higher water content in JR than JX was observed which supported the significant difference between species in freezing injury. In autumn of 2011 the same response to freezing was observed between species as in 2010 (in both assays). The level of



hardening was significantly different considering the factor sampling day. In young trees the interaction between species and sampling day was significant and studying separately the response by species it was observed the process of hardening was longest in JR than JX (Figure 2). The results on plantation were quite erratic, having more freezing damages on the first day of sampling, less in the second and again the same as on the first on the last sampling day, and must be carefully considered. In this case, it is known that frost hardiness of JX trees displays a behaviour that can be described as mid-way between that of its progenitors (*J. regia* × *J. nigra*) and that hardening starts earlier than for JR trees (Fady et al. 2003; Guàrdia et al. 2013).

### **Differences between irrigation treatments**

In Assay 2 (adult trees), the different irrigation treatments only affected diameter growth of JR trees in 2010 while in Assay 1 differences existed each studied year (Table 1 A-B). The differences between years in plantation (Assay 2) could be due to the distribution of the precipitation. In 2010, precipitation was spread throughout the growing season in small amounts, so the effect of the irrigation treatments was evident with large differences observed in terms of diameter increase. In 2011, precipitation was high from flushing to the end of June when the highest growth increase probably occurs; the effect of the irrigation treatments was not visible. This is the probably reason why there were no differences between treatments in terms of diameter growth in 2011.

Starch content analysed on autumn 2010 showed differences between irrigation treatments in adult trees but the interpretation is not clear. In young trees a significant interaction detected between factors affected both starch content and leaf fall. Analysing by species, JR showed the hardening process more advanced in the restricted irrigation treatment, with less amount of starch and earliest leaf fall, at the time the trees were sampled. In autumn of 2011, significant interaction was again observed between sample day and species; in JR there was no difference between irrigation treatments along the sampling days while in JX from the first sampling day the treatment R2 was already hardened. The results of these trials suggested it exist a potential influence of the irrigation regime on autumn hardiness on the species of *Juglans* studied, as it is referred in others. On conifers, the short day together with no irrigation in autumn triggered the cessation of height growth (Calme et al. 1993). Also, several genotypes of *Eucalyptus globulus* subjected to drought hardening became freezing tolerant or increased their freezing tolerance, as explained by Coopman et al (2010).

Although the general trend with climate change is elongation of the growing season, locally at southern latitudes, as is the case of the Mediterranean zone, the trend is towards a shortening of the growing season (IPCC 2007a). This trend was observed in both assays, where the treatments with restricted water supply were likely to see initiation of the cold hardening process earlier than the well-irrigated treatments.

However, results observed in these preliminary assays must be taken in consideration to plan new research activities with this purpose. It should be noted that in most cases water stress caused a notable reduction in growth. It should therefore be evaluated whether this summer hardening may have some application at management level, taking into account the importance of the annual growth to shorten turnover in new afforestations.

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## General discussion |

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## GENERAL DISCUSSION

The main objective of this work was to provide greater in-depth knowledge of the adaptive trait “autumn frost resistance” for the species *J. regia*.

The first task of the present work was to find an appropriate methodology to evaluate the aforementioned adaptive trait. Several methods were used and compared: visual scoring of different tissues (VS), relative electrolyte leakage (REL) from the stems and measurement of chlorophyll fluorescence in cortical bark chlorenchyma of the stems (using the Fv/Fm parameter). The first two methods have been used widely on several species and on *J. regia* (Díaz et al. 2009; Jacobs et al. 2008). All of them gave good results and were able to show the freezing damage. Despite the good results obtained, the VS was the most time consuming, most subjective and the method which required the largest infrastructure. The REL method was also time consuming, though less so than VS, and the final data were easier to obtain. Although the Fv/Fm was the easiest and fastest method, the results were not always as good as with the other two methods. VS and REL showed damage to the most sensitive species analysed (*J. regia*) when its budsticks were submitted to -8.5°C, while Fv/Fm detected no damage at this level (Chap. I). However, this parameter had been used recently to estimate autumn hardening on *J. nigra* (Wilson and Jacobs 2012). One of the possible reasons of the differences between the methods could be the different kind of tissues analysed and their different frost susceptibilities. Even so, the Fv/Fm could be a good method for frost damage estimation, for example to perform a screening when a large number of samples have to be analysed. However, its lower sensitivity was the main reason why in the remaining chapters the fluorescence method was discarded as a means of evaluating frost hardiness on *J. regia*. In the third chapter, the VS method was also discarded as a result of the dimension of the assay, which was based on a frost hardiness analysis of a progeny test. To estimate the heritability and calculate the genetic correlations of the trait an integrative and highly detailed parameter was required to evaluate the “autumn frost resistance”. For this reason, it was decided to choose the LT<sub>50</sub> (the lethal temperature at 50% is the freezing temperature required to cause 50% of electrolyte leakage), obtained from the REL method.

Another problem involved defining the sub-zero temperature necessary to achieve measurable damage on the *J. regia* budsticks. The study made using different species of *Juglans* gave three



clear responses: a) a ranking of frost resistance in *Juglans* from highest, in this case black walnut species of North America, to lowest, common walnut; b) a temperature of at least  $-8.5^{\circ}\text{C}$  was needed before freezing damage occurred in *J. regia* and c) the autumn frost resistance of the walnut species *J. ×intermedia* - a hybrid obtained from *J. regia* and *J. nigra* species - displayed an intermediate behaviour to that of its progenitors. This last result indicated the existence of genetic inheritance in the acquisition of the trait, which was important to justify continuing in the present research line.

Besides the freezing injuries, the evaluation of phenological variables played an important role in the present work in terms of calculating the degree of frost hardiness of the trees. Leaf fall was recorded during the end of the growing period. The stage used is called level 3 of leaf fall (L3), defined as the stage when the tree still has 50% of its leaves (Díaz and Fernández-López 2005). A close relationship between freezing damage and L3 was observed when considering the different walnut species (Chap. I). L3 was again successfully used as a parameter to compare the frost hardiness level of *Juglans regia* growing in different trial sites and to understand the progeny response to early frost (Chap. II). However, there was a very small and non-significant variation when it was used as an indicator to classify different progenies of *J. regia* in terms of frost hardiness when growing in the same trial site (Chap. III).

To know the extent to which first-freezing events affected different tissues, several parts of the budsticks were analysed. The visual score of lateral and apical buds showed no significant differences between the two bud types. Comparing these results with visual score for stem damage, it could be observed that the internal layer of the bark showed less damage than the buds (this comparison was not statistically tested) (Chap. III).

The major part of the thesis centred on a genetic evaluation of "autumn frost resistance" in *J. regia*. The interaction between the genotype and the environment was studied in Chapter II, and the inheritance, together with the genetic correlations with phenological and growth traits, was analysed in Chapter III. Results indicated that both genotype and environment are influential factors in the development of autumn frost resistance. The evaluated *J. regia* provenances showed that genotype make-up in relation to autumn frost resistance was linked to minimum autumn temperature (Tanino et al. 2010; Olsen 2010) and altitude of the original site, observed in other

species (Li et al. 2005; Vitasse et al. 2011), while the environment of the planting site mainly contributed to advancing or delaying the trees' entrance into dormancy (Chap. II). The genetic variance and inheritance of the trait in *J. regia* species, estimated in a progeny test comprising a wide range of materials from varied origins, were high. Autumn frost resistance was genetically and positively correlated with the duration of the growing season - which mainly depended on bud break - and with the annual diameter increase (Chap. III). The observation that the "autumn frost resistance" trait and good annual growth are not mutually excluding features, together with the family heritability value ( $h_F^2 = 0.63$ ), suggest that this trait could be successfully included in a breeding program of *J. regia* for wood production.

The role of female parent trees in acquisition of the trait by the progeny was not clear, with the narrow-sense heritability ( $h^2$ ) calculated from the female progenitor-progeny regression being much lower than the  $h^2$  obtained from the variance components (Chap. III). The importance of epigenetic factors in this response should be evaluated, due its influence had been observed in other species (Besnard et al. 2008; Webber et al. 2005), and if the goal is to obtain well adapted Reproductive Forest Material (RFM) from basic materials selected for quality timber production.

During the last few years, our observations have recorded than the death of young *J. regia* trees, recently planted and frost-damaged occurred with far greater frequency than that of older trees, even installed on the same site. Similar results were also observed in black walnut (*J. nigra*) (Thomas and Reid 2006). In Chapter IV, in which the assays were carried out on both young and adult trees, it was observed that on the first sampling day, during the evaluation of injuries over autumn 2011, freezing damage was higher on young trees than on adult trees. Despite this, at the end of autumn, the level of hardiness was similar in both adult and young trees. Inheritance of the "autumn frost resistance" trait could vary with tree age and should be carefully evaluated. However, data of this study (Chap. III) correspond to 7-year-old trees, an age at which the inheritance of many other features related to productive characteristics has shown stability in many woody species, including walnuts (Aletà et al. 2009).

A study was undertaken in Chapter IV of the influence on the "autumn frost resistance" trait of summer drought, one of the main stresses in the Mediterranean area. This drought is forecast to become more severe as a result of Climate Change (IPCC 2007). The results indicate that mild

drought stress may be beneficial as it can advance cold hardening in autumn. This possibility was suggested after analysis of certain indicators (including starch and soluble sugar accumulation and the advance/delay of leaf fall). Nevertheless, annual growth in most cases was reduced by drought during the growing season. Management of a controlled summer drought on irrigated new afforestations to advance autumn hardening could be used to ensure the annual growth required to attain a final marketable product with the desired timber quality.

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## Conclusions |

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## CONCLUSIONS

- 1) The Fv/Fm parameter obtained from chlorophyll fluorescence in cortical bark chlorenchyma was fast and easy to obtain on *Juglans* annual budsticks. It could be useful for the evaluation of autumn frost damages when a previous screening is needed due to a large sample size.
- 2) An intermediate level of autumn freeze damage compared to that of its progenitors (*J. regia* and *J. nigra*) was clearly observed in the case of *J. ×intermedia*.
- 3) *J. regia* was the most damaged of the several *Juglans* species analysed, but its damages were not visible until the temperature dropped to  $-8.5^{\circ}\text{C}$ .
- 4) The scale used to score leaf fall during autumn was adequate to compare acclimation levels between *Juglans* species and even to know differences between planting sites. Not enough difference existed within *J. regia* trees planted in the same site to use this parameter to evaluate their frost hardiness.
- 5) Both the genotype and environment factors were relevant in the expression of the level of autumn frost resistance on *J. regia*.
- 6) An altitudinal gradient in autumn frost resistance expression was observed between provenances of *J. regia*.
- 7) The high family heritability of the studied adaptive trait suggests that it could be successfully included in a breeding program of the species *J. regia*.
- 8) Annual diameter growth and growing season duration were positively correlated with autumn frost hardiness on *J. regia*. This suggests that selection by autumn frost resistance is not incompatible with the attainment of suitable vegetative traits.
- 9) The difference between heritability in the narrow-sense calculated by the female parent-progeny regression and that obtained from the variance components indicates that the relationship between female parent tree and progeny is not only attributable to genetics.
- 10) Watering restrictions in summer seem to affect *J. regia* and *J. ×intermedia* by positively advancing autumn cold hardiness, but these restrictions also reduce annual growth. The use of such management in the Mediterranean environment must be evaluated more carefully.



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