



Integrated approach for addressing assisted population migration programs in forest management to climate change: Out-planting Performance, Genotype by Environment Interactions, Physiological and Molecular Response.

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Abstract

Forest ecosystems are likely to shift faster in response to climate change than their maximum natural rate at which they can migrate and establish. This thesis introduces an interdisciplinary approach to develop a proactive management strategy towards climate change through assisted populations' migration for two pine species; Aleppo pine (*Pinus halepensis* Mill.) and Black pine (*Pinus nigra* ssp. *salsmannii*).

The main objectives of this study were to (1) evaluate plantation performance and phenotypic plasticity in the broad context of genotype by environment interaction (GEI) of these pine seed sources out-planted in contrasting trial sites to test a hypothetical northwards migration for further selection under specific conditions, (2) compare the efficiency of joint regression and Additive Main effect and Multiplicative Interaction (AMMI) models in elucidating seed sources adaptation patterns in each site then, (3) explain the basis of the differential response of seed sources to induced drought and cold stresses through the physiological, metabolomic and proteomic analyses. This study reproduced real conditions of reforestation in potential future climatic conditions either in field or under phytotron controlled conditions.

The selective use of the intraspecific variability was demonstrated to have a potential contribution to alleviate adverse climate change impacts on forest ecosystems. For both species, certain seed sources were able to cope better with specific climate perturbations than others in response to the northwards shifts; seedlings not belonging to the target site could be selected for facing current climate irregularities in different environments. Here,

provenances moved from slightly different transfer distance metrics were the best performers. The main problem is the high expected seedlings mortality due to freezing events and drought stress mainly for seedlings belonging to warmer provenances. Seed sources phenotypic plasticity was low to moderate for height and diameter growth and the environmental effect had a great influence on their performance variation. The AMMI models demonstrated higher adequacy to analyse complex GEI than the joint regression analysis. An important finding is that specific adaptation to adverse environmental conditions was coupled with low phenotypic plasticity responses.

Differences among Aleppo pine seed sources subjected to induced drought conditions were significant for chlorophyll fluorescence, pigments and soluble sugars contents. However, induced cold stress changes transpiration rate, stomatal conductance, pigments and glucose contents. The decrease in photosynthesis under drought, unlike to cold stress, was due to stomatal closure. At the considered metabolomic level, drought tolerance was related to the decrease of glucose and fructose and the increase of sucrose contents in needles. However, the cold tolerance was associated to the decrease of glucose and the increase of sucrose and fructose contents. At the proteomic level, most of the identified proteins were related to the transcriptional machinery and sugar metabolism. The presence of enzyme related to the sulphur amino-acids metabolism could be the limiting factor for drought stress in Aleppo pine.

Keywords

Assisted population migration, Reforestation, *Pinus halepensis*, *Pinus nigra* ssp. *salzmannii*, Seed Sources, Out-planting performance, Survival, Growth, Phenotypic Plasticity, Genotype by Environment Interaction, Adaptation, Physiology, Metabolomic, Proteomic.

Resumen

En respuesta al cambio climático se prevé que los ecosistemas forestales se vean forzados a migrar a una tasa más rápida que sus posibilidades naturales. En esta tesis doctoral se presenta un enfoque interdisciplinario para desarrollar una estrategia de gestión proactiva hacia el cambio climático a través de la migración asistida de las poblaciones de dos especies de pino; *Pinus halepensis* Mill. y *Pinus nigra* ssp. *salzmannii*.

Los objetivos principales de este estudio son (1) evaluar el rendimiento y plasticidad fenotípica en un contexto amplio de interacción ambiente-genotipo (GEI) de estas diferentes materiales de base en sitios de ensayo representan diferentes condiciones climáticas, de forma que nos permitan ensayar una hipotética migración de estas especies hacia el norte, para selección posterior en condiciones específicas, (2) comparar la eficacia de los modelos de la regresión conjunta y lo de la interacción por efecto principal aditivo e interacción multiplicativa (AMMI) para elucidar los patrones de adaptación de las diferentes materiales de base en cada sitio, (3) explicar la base de la respuestas diferencial de cada material de base al estrés por sequía o frío a través de análisis fisiológicos, metabolómicos y proteómicos. Este estudio se ha hecho reproduciendo condiciones reales de repoblación en hipotéticas futuras condiciones climáticas ya sea en campo o en condiciones controladas en fitotrón.

El uso selectivo de la variabilidad intraespecífica se ha demostrado que puede contribuir a paliar los efectos del cambio climático en los ecosistemas forestales. Para ambas especies ciertos orígenes de semillas eran capaces de enfrentarse mejor a las perturbaciones climáticas específicas que otras en

respuesta a los cambios de localización hacia el norte. Semillas foráneas al campo experimental podrían ser mejor elección para afrontar las irregularidades climáticas en diferentes entornos. Semillas provenientes de diferentes distancias de transferencia fueron más eficientes. El principal problema fue la alta mortalidad debida a episodios de congelación y sequía por parte de semillas provenientes de entornos más cálidos. La plasticidad fenotípica para los diferentes orígenes fue entre baja y moderada para la altura y el diámetro y el efecto ambiental tuvo una gran influencia en la variación de rendimiento. El modelo AMMI demostró ser más adecuados para analizar el complejo GEI que el modelo de regresión conjunta. Un hallazgo importante es que la adaptación específica a las condiciones ambientales adversas correlacionaba con una baja plasticidad fenotípica.

Las diferencias entre las semillas del pino Carrasco sometidas al estrés por sequía fueron significativas para la eficiencia fotosintética, la acumulación de pigmentos y de azúcares solubles. Sin embargo, el estrés por frío cambia la tasa de transpiración, la conductancia estomática, la acumulación de pigmentos y de glucosa. La disminución en la fotosíntesis bajo sequía, al revés al estrés por frío, se debió al cierre estomático. Al nivel metabolómico, la tolerancia a la sequía se relaciona con una disminución en los niveles de sacarosa y fructosa. Al nivel proteómico, la mayoría de proteínas identificadas estaban relacionadas con la maquinaria transcripcional y el metabolismo de azúcares. La presencia de enzima relacionada con el metabolismo de aminoácidos con azufre podría ser un factor limitante para la tolerancia del pino Carrasco al estrés por sequía.

Palabras clave

Migración asistida de poblaciones, Reforestación, *Pinus halepensis*, *Pinus nigra* ssp. *salsmannii*, Supervivencia, Crecimiento, Plasticidad fenotípica, Interacción genotipo-ambiente, Adaptación, Fisiología, Metabolómica, Proteómica.

Resum

En resposta al canvi climàtic es preveu que els ecosistemes forestals es vegin forçats a migrar a una taxa més ràpida que les seves possibilitats naturals. En aquesta tesi doctoral es presenta un enfocament interdisciplinari per desenvolupar una estratègia de gestió proactiva al canvi climàtic a través de la migració assistida de poblacions per dos espècies; el pi aleppo (*Pinus halepensis* Mill) i el pi negre (*Pinus nigra* ssp. *Salzmannii*).

Els objectius principals d'aquest estudi són (1) avaluar el rendiment i plasticitat fenotípica en un context ampli d'interacció ambient-genotip (GEI) d'aquestes diferents varietats sembrades en camps experimentals que representen diferents condicions climàtiques, de manera que ens permetin assajar una hipotètica migració d'aquestes espècies cap al nord, per seleccionar les varietats millor adaptades, (2) comparar l'eficàcia de models de regressió conjunta i la interacció per efecte principal additiu i interacció multiplicativa (AMMI) per elucidar els patrons d'adaptació de les diferents varietats en cada emplaçament, (3) explicar la base de les diferents respostes de cada varietat a l'estrès per sequera o fred a través d'anàlisis fisiològics, proteòmics o metabolòmics. Aquest estudi s'ha fet reproduint condicions reals de repoblació en hipotètiques condicions climàtiques futures ja sigui en camp o en condicions controlades en fitotrò.

L'ús selectiu de la variabilitat intraespecífica s'ha demostrat que pot contribuir a pal·liar els efectes del canvi climàtic en els ecosistemes forestals. Per a ambdues espècies, certs orígens de llavors eren capaços d'afrontar millor les pertorbacions climàtiques específiques que altres en resposta als canvis de localització cap al nord; llavors foranes al camp experimental

podrien ser millor elecció per afrontar les irregularitats climàtiques en diferents entorns. Ja que en els nostres estudis llavors provinents de diferents distàncies de transferència van ser més eficients. El principal problema va ser l'alta mortalitat deguda a episodis de congelació i sequera per part de llavors provinents d'entorns més càlids. La plasticitat fenotípica pels diferents orígens va ser entre baixa i moderada per l'alçada i el diàmetre i l'efecte ambiental va tenir una gran influència en la variació de rendiment. El model AMMI demostrà ser més adequats per analitzar complexos GEI que el model de regressió conjunta. Una troballa important és que l'adaptació específica a les condicions ambientals adverses correlacionava amb una baixa plasticitat fenotípica.

Les diferències entre les diferents llavors de pi Aleppo sotmeses a estrès per sequera van ser significatives per a l'eficiència fotosintètica, l'acumulació de pigments i de sucres solubles. En canvi en condicions d'estrès per fred es pareieron canvis en la taxa de transpiració, la conductància estomatal, l'acumulació de pigments i el contingut en glucosa. La disminució en la fotosíntesi sota sequera, a l'inrevés que en estrès per fred, es va deure al tancament estomatal. A nivell metabòlic, la tolerància a sequera es va poder relacionar amb una disminució en els nivells de sacarosa i fructosa. A nivell proteòmic, la majoria de proteïnes identificades estaven relacionades amb la maquinària transcripcional i el metabolisme de sucres. La presència d'enzims relacionats amb el metabolisme d'aminoàcids amb sofre indica un paper en aquesta ruta metabòlica com a factor limitant per a la tolerància a estrès del pi aleppo.

Paraules clau

Migració assistida de poblacions, Reforestació, *Pinus halepensis*, *Pinus nigra* ssp. *salzmannii*, Supervivència, Creixement, Plasticitat fenotípica, Interacció ambient-genotip, Adaptació, Fisiologia, Metabòlica, Proteòmica.

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I dedicate this thesis

to the soul of my father may God mery be upon him,

to my family for their constant support and unconditional love,

to Leila, my strength, my illusion.

I love you all...

Khaled

Student: Dr. Einstein, Aren't these the same questions as last year's [physics]
final exam?

Dr. Einstein: Yes; But this year the answers are different.

— Albert Einstein

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Background of the Thesis

1. Background of the Thesis

1.1. Global changes in climate

Climate varies naturally on all timescales from decades to millennia due to changes in atmospheric and ocean circulation, solar output and volcanic activity. However, future climate change will be dominated by human influences unless and until the composition of the atmosphere is stabilized (IPCC 2012). The global average surface temperature has increased, especially since about 1950, by $0.74^{\circ}\text{C} \pm 0.18^{\circ}\text{C}$ with a rate of temperature increase ($0.13^{\circ}\text{C} \pm 0.03^{\circ}\text{C}$ per decade) that almost double the rate of the last 100 years (Solomon et al. 2007). Many changes are occurring in the amount, intensity, frequency and type of precipitation which tend to be significantly wetter in eastern North and South America, northern Europe and northern and central Asia, but drier in the Sahel, southern Africa, the Mediterranean and southern Asia (IPCC 2012).

Other climatic factors are expected to change also along with changes in frequency and magnitude of extreme weather events with probable impacts on ecosystems and society (Hegerl et al. 2011). An increasing of heat waves occurrence probability and duration accompanied with a decrease in frequency but not magnitude of cold extremes temperatures are expected (Kodra et al. 2011). The expected changes in precipitation extremes (including droughts) seem to be more unclear, even if some general trends towards prolonged drought periods over the Mediterranean and more heavy rain events in Northern high latitudes are emphasized (Orlowsky and Seneviratne 2012).

1.2. Forests in a changing climate

Understanding the effects of a changing climate on forest ecosystems requires investigating responses to the simultaneous changes in several climatic variables such as temperature, precipitation and ambient CO₂ concentrations. At the ecophysiological level, trees respond almost instantaneously to their environment, which mainly affects forest functions, such as net primary productivity. Some structural aspects may respond over years, whereas others, such as species composition, may take centuries to respond to altered conditions (Zhang et al. 2007). In general, each species is affected differently by climatic change; some species will remain unaffected, some will become more and others less competitive (Pauls et al. 2013). Landscape-scale processes such as the lateral interactions between neighbouring patches through migration or fire may induce many changes in the local to regional forest ecosystems pattern (IPCC 2012).

1.2.1. Ecophysiological responses

- **To temperature and water availability**

Forest trees of most temperate and boreal species are supposed to increase growth in response to increased temperature and precipitation, and their higher growth rates could be observed near the warmest boundaries of their geographic ranges (Parmesan 2006). However, under limited water availability, increasing temperature increases evaporative demand leading to more severe water stress, which will adversely affect growth and may increase the risk of drought and fire. In warmer areas, photoperiodic limitations are also expected along with increasing the range of insect pests, which could cause considerable damage (Adams et al. 2008). The distribution of species is strongly determined by any changes in the total and seasonal availability of water (McCarthy et al. 2009).

- **To increased CO₂ concentration**

Atmospheric carbon dioxide (CO₂) is a substrate for plant photosynthesis. Increased atmospheric CO₂ concentration is believed to, therefore, increase photosynthesis rate by increasing CO₂ intake directly (Norby et al. 1999). Increased atmospheric CO₂ might induce a partial closure of stomata, reducing water loss by transpiration, which results in an increase in the ratio of carbon gain to water loss, i.e., water use efficiency. Consequently, this effect can extend the period of growing seasons in seasonally dry ecosystems, thus probably stimulating biomass accumulation, and enhancing ecosystem net primary productivity. This CO₂-induced enhancement in primary productivity and water use efficiency is commonly referred to the direct 'fertilization effect'. When plants have access to limiting amounts of water, growth is limited by the amount of CO₂ that can be obtained in the diffusive gas exchange during photosynthesis. Once the available water has been used up, tissue water potentials fall below threshold levels and growth ceases (Beedlow et al. 2004).

- **To carbon storage and nutrient availability**

Net ecosystem productivity may not increase, and may even become negative, even if increasing temperature may lead to higher net primary productivity because warmer temperatures also greatly stimulate soil organic matter decomposition which could release large amounts of CO₂ to the atmosphere (Kirschbaum 1995). However, the direct effect of increasing CO₂ concentrations may partly offset or in some cases even reverse this effect and make net primary productivity positive. Enhanced soil organic matter decomposition increases mineralizing nutrients processes mainly for nitrogen and phosphorus that are held in soil organic matter which make them available for plant growth (Solomon et al 2007).

1.2.2. Species distributions

Species have responded individually to past environmental changes. The fundamental ecological niche of a given forest species is the set of all possible environmental conditions in which it survives and reproduces. These conditions may become unsuitable to complete one or more stages of the life cycle, especially if some climate variables were to change significantly more than other variables with rapid climatic changes. Forest trees whose seedlings can now survive at a particular site will grow into adults in climates that may be unsuitable in 50-100 years; conversely, adults that could grow in an area in 50-100 years time must grow from seedlings that may be unable to survive current climatic conditions at those sites. The ability to reach reproductive maturity in a short time favours early successional species that grow in full sun, whereas slower-growing species that begin their life cycles as understory species under closed canopies may be lost (Solomon et al. 2007).

1.2.3. Transient responses in species compositions

Besides the doubt about the geographic distribution of areas potentially suitable for forests and the performance of trees under different environmental conditions, the effect of climate change on the functioning and structure of forest ecosystems during the transient phase constitutes a big concern. Changing environmental conditions can reduce growth in forest ecosystems while their magnitude will subject many species assemblages, within a life cycle of their main species and in most of their distribution area, to climates that now occur outside their current ecological range (Solomon et al. 2007).

1.2.4. Potential biome distributions

Climate and vegetation interact bi-directionally on many time and spatial scales. One clear manifestation of such interaction is the global pattern of vegetative land cover and climate (Cox et al. 2004). Climate may be regarded as the single factor that exerts the largest influence on vegetation distribution and its characteristics on a global context (Prentice 1992). Climate change has affected the global distribution of vegetation from the distant past and will likely affect it into the future (Solomon et al. 2007). On the other hand, changes in the distribution and structure of the vegetation may influence climate (Nobre et al. 2006).

1.2.5. Biodiversity

Forests ecosystems are of paramount importance for biodiversity because they harbour about two-thirds of all species on Earth. About 20% of the estimated total of 250,000 plant species are woody or tree species. Species diversity generally increases strongly as one moves from colder to warmer sites (Groombridge 1992), although the reasons for that trend are not well understood. When the size of an ecosystem is reduced to about 10% of its former size, about 50% of the species originally present generally will become extinct (Solomon et al. 2007).

Biodiversity can be affected by climatic changes either directly through altering the physiological responses of species or indirectly by altering inter-specific relationships or habitat degradation. In addition, biodiversity is affected also by deforestation and other land-use changes that cause further habitat degradation and fragmentation. Disturbance also may create opportunities for opportunistic or pioneer species, which will become more abundant and, over time, replace many species that are slower growing and require more stable conditions (Myers et al. 2000).

Species may extinct when local extinction cannot be reversed by re-immigration from neighbouring areas mainly if the climate changes from being favourable to being completely unsuitable rendering “sanctuaries” into “traps” (Aitken et al. 2008).

Genetic diversity could be affected by two fundamental but not mutually exclusive processes through (i) change in the geographical location and extent of the range and (ii) changes in the local selection regime.

- **Effects at the edge of the range of distribution**

Range shifts are usually the combined effect of the colonization of newly emerging suitable habitat coinciding with the migration and then extinction in areas that have become unsuitable. Arenas et al. (2012) show that the leading edge of a shifting range under climate change has low levels of neutral genetic diversity which is caused by recurring founder effects, allele surfing and because only a part of the original genetic variation moves to a newly colonized habitat.

Range contractions should lead to decreases in effective population size and, in extreme cases, to population bottlenecks that may reduce populations’ genetic diversity (Arenas et al. 2012). Range expansion may lead to complex patterns of population genetic structure, with regions of low genetic diversity separated by sharp gradients in allele frequency of individual loci and that these patterns can arise without the influence of rare long-distance dispersal events. However, this structure is likely to erode over time due to gene flow between contact zones of different adjacent regions. The rear-edge populations play here an important role in maintaining long-term genetic diversity (Hampe and Petit 2005).

- **Effects within the range of distribution**

Populations' neutral genetic diversity decreases with increased short-term regional climate variation and with increasing speed of the range shift. Besides reductions in overall genetic diversity, range expansions, range contractions and range shifts indeed alter the distribution of genetic diversity across the range (Arenas et al. 2012). Sork et al. (2010) showed that the importance of adaptation, migration and tolerance as climate change responses will vary in different parts of the species range. Climate change and range shifts may enhance levels of genetic diversity; apart from increasing overall mutation rate, is the modification of reproductive isolation among lineages and species (Hoffmann and Sgrò 2011). Postglacial climate shifts triggered expansions and migrations that brought previously isolated lineages into secondary contact, thereby mutually increasing local genetic diversity (Arenas et al. 2012).

- **Effects on small ranges of distribution**

Small-range species are considered more strongly threatened by climate change (Hering et al. 2009). Small-range species with low effective population size are more likely to have low genetic diversity and thus reduced adaptation potential, and small-range endemics of colder climates (high altitude or high latitude) may be locally adapted to colder climatic conditions. This makes them primary targets for conservation efforts, but the degrees to which most of these species are really climate sensitive remains unclear, because little is known about the ecological and phenotypic plasticity of most species (Morueta-Holme et al. 2010).

1.3. Climate change in the Mediterranean region

Scientists are confident that global warming due to current trends in emissions will be accompanied by significant changes in local climate. The Mediterranean region lies in a transition zone between the dry-hot arid climate of North Africa and the wetter-cooler and rainy climate of central Europe and it is affected by interactions between mid-latitude and tropical processes (Giorgi and Lionello 2008). Given the potentially significant climate changes in Africa and Europe under a warming climate, the regional climate in the Mediterranean is expected to be very sensitive to future increase in greenhouse gases (Cattiaux et al. 2013).

1.3.1. Temperature

Since 1970, South-Western Europe (Iberian Peninsula and southern France) has reported a temperature rise of about 2°C (IPCC 2007). This warming is also felt in North Africa, though it is more difficult to quantify due to an incomplete observation network. The majority of climate models suggest that during the 21st century the Mediterranean region will experience a warming period, with an average air temperature increase of 3.2°C, which is higher than the global average (2.6°C) noting that warming is expected to be greater in summer than in winter (Bartolini et al. 2012).

Climate predictions also indicate an increase in the number of extreme warm events across the region; summer heat waves are occurring more frequently, especially since the 1970s, and less frequent cold extremes. The number of warming events is likely to increase threefold by 2025, with three warming events expected every four years, compared to the current rate of one warm event every four years. Moreover, both frequency and intensity of these events will increase with heat waves maintaining higher temperatures for longer periods (IPCC 2012). A lower number of frost days and higher

maximum temperatures in winter in the second half of the 20th Century have been identified (Kuerbis et al. 2009; Orłowsky and Seneviratne 2012). The expansion of the frost-free season and the decreasing of cold extremes frequency have been reported (IPCC 2012).

Despite the general warming trend, cold extremes in winter or late frosts in spring are still likely to occur in the future, even if being less frequent. Rigby and Porporato (2008) showed that the late frost risk in a future climate is as sensitive to changes in variance of temperature, as to changes in mean, which means that the frost risk will stay more or less constant. Even though there will be fewer frost days in the future, severe frost events are still expected to occur (IPCC 2012).

1.3.2. Precipitation

Elevation of temperatures induces higher evaporation rates and a higher water holding capacity of the atmosphere, which will intensify the hydrological cycle and, as consequence, increase of extreme heavy rain events. Increasing precipitation over Northern mid- and high latitudes and decreasing rainfall over Southern Europe and the Mediterranean have been observed (Orłowsky and Seneviratne 2012). Summer precipitation has become more intense, nevertheless rain events are less frequent in the north Mediterranean (Kysely et al. 2011). In winter, precipitation has experienced a small increase in the northern part of the Mediterranean. In contrast, major reductions in rainfall are expected in the southern Mediterranean. Precipitation, rainfall and snow, are expected to decrease by 30% in summer and 10% in winter by 2100. The largest decrease in summer precipitation (over 60%) by the end of this century may occur in the areas affected by the greatest warming in the southern Europe and the Mediterranean region (Orłowsky and Seneviratne 2012).

1.4. Climate change impacts on Mediterranean forests

The impacts of climate change on forest ecosystems are strongly species dependent according to the dominant key tree species and can even differ in severity within the distribution range of one single species as site conditions, phenotypes, and regional climatic changes may differ (Lindner et al. 2010).

In the Mediterranean region, rising temperatures and decreasing rainfall will lead to increased occurrence of drought periods and usually, as consequence, to the fire risk (Matesanz and Valladares 2013). Even drought-adapted ecosystems are influenced by drought, as shown for coniferous and evergreen species, since increased drought is likely to lead to reduced plant growth and primary productivity, reduced nutrient turnover and nutrient availability and altered plant recruitment (Sardans et al. 2006). Scots pine (*Pinus sylvestris*) has increased stem growth during the 20th century in Catalonia, but increasing temperatures had already negative impacts on drier sites (Martínez-Vilalta et al. 2008).

Even though forest stands show some plasticity, growth and survival are expected to suffer with changes in timing and duration of drought. Resco De Dios et al. (2007) noted that the changes in the rainfall distribution pattern may have a stronger effect on forest growth than the decreased precipitation because trees are adapted to grow within the constraints of a given climate and water regime. The negative effect of the increase in temperature may become stronger and not compensated by the positive effects promoted by increasing atmospheric CO₂ concentration. Elevated CO₂ increases water use efficiency of trees and this can counteract or offset potential negative effects of changes in climate. However, there is a limit on this effect and growth reductions are predicted for most species if rainfall does not increase.

Fire risk is expected to increase significantly in the Mediterranean region. Total burned area in Spain has increased six-fold between 1960 and 1990 and fire-return frequency has decreased during the same period. Climate warming is thought to have contributed greatly, because daytime temperatures increased and relative moisture decreased, affecting vegetation growth, fuel structure and combustibility (Resco De Dios et al. 2007). Moriondo et al. (2006) indicated that, under climate change, there is an increase in the number of years with fire risk, especially in the northern parts of the Mediterranean region (North Spain, North Italy and Southern France) where the number of years at risk increases by up to 50%.

The synergy created by increasing fire frequency and high intensity storms could, in turn, increase soil erosion. De Luís et al. (2001) showed that high intensity rainfalls after fire could adversely affect establishment of new plants, because of soil and nitrogen losses. In addition, drought and altered fire regimes may lead to more shrub-dominated landscapes (Resco De Dios et al. 2007).

The distribution range of a number of typical tree species is likely to decrease in the Mediterranean; drought periods will shift also species composition and the proportion of drought-sensitive species would increase in more moist localities due to a higher likelihood of co-occurrence of species that share moist climatic requirements (Lloret et al. 2007).

The forecasted global warming and fire increase may trigger irrecoverable biodiversity losses and shifts in vegetational composition within a few decades or centuries at most. Fire and drought-sensitive vegetation types seem particularly threatened by large-scale displacement. Extinction risk is likely to increase by approximately 20 to 30% for plant species known to science, if the 2°C target will not be met, and adverse impacts on unique or threatened ecosystems will amplify (Smith et al. 2009).

Forest stands will be weakened by the unfavourable environment and the higher incidence of extreme weather events which will increase biotic risks. As an example, water stress of eucalypt trees plays a crucial role in the colonization success, larval survival and growth of the phloem-boring beetle *Phoracantha semipunctata*. Pest species are likely to profit from climate change in case of altitudinal expansion or dislocation of the distribution range, especially when coupled with host switching as in the case of *Thaumetopoea pityocampa*. In case temperatures raise above optimal development conditions, certain species, such as *Lymantria dispar* and *Lymantria monacha*, may also face southern restrictions of distribution (Vanhanen et al. 2007).

Highly thermophilic pathogen species, already typical components of the micro-flora of Mediterranean tree species, are likely to become more serious in South Europe. Typical components of the endophytic microflora inhabiting Mediterranean tree species, such as *Biscogniauxia mediterranea* on *Quercus* spp. and *Diplodia pinea* on *Pinus* spp., may develop rapidly in case of water stressed host trees and cause sudden dieback (Resco De Dios et al. 2007). Temperature increase and summer droughts will promote outbreaks of various pathogenic fungi, such as the Dutch elm disease, *Ophiostoma ulmi*, poplar rusts, or the chestnut blight (Resco De Dios et al. 2007). Decline and death of oaks were recently associated with *Phytophthora cinnamomi* (Resco De Dios et al. 2007).

Furthermore species composition of ecosystems are expected to shift due to extinction, invasion, changing dominance patterns, range size reductions or expansions, poleward or altitudinal range shifts, as species do not respond synchronously to climatic stressors (Walther et al. 2009; Kardol et al. 2010).

1.5. Forest-species responses towards climate change

Individual forest species responses to such changes will depend on ecological and evolutionary factors such as niche breadth, competitive ability, phenotypic plasticity and adaptability (Soto et al. 2010). The responses of long-lived plant species to changes in environmental conditions are determined by the capacity of individuals to alter their structure and function to novel environments (i.e. phenotypic plasticity), adapt through natural selection or migrate (Matesanz and Valladares 2013). In this sense, migration and adaptation to climate change is of paramount importance in current and future restoration plans (Vallejo et al. 2012).

Chen et al. (2011) and Hoffmann and Sgrò (2011) resumed that species can react to climate changes by persisting in new conditions through (a) ecological plasticity or (b) adaptation or they can (c) avoid new conditions by shifting their habitat or their range. Failure to succeed in either of these mechanisms will likely lead to population extirpation or extinction of entire species.

1.5.1. Phenotypic plasticity

Phenotypic plasticity, or the capacity of a given genotype to render different phenotypes under different environmental conditions, is a means to cope with environmental heterogeneity that is particularly adequate for sessile organisms (Valladares et al. 2007). Plasticity is frequently invoked in studies exploring the impact of global change on key plant species and communities since it is a source of ample phenotypic variation that may promote adaptive divergence and, thus, evolution and speciation. The importance of phenotypic plasticity as a buffer against extinction has not been widely appreciated. In fact, the extent of species' losses may have been overestimated in many simulations of distribution shifts induced by global

change because the plasticity of individuals in populations of threatened species is not considered (Thuiller et al. 2005). In an environment rapidly changing on local and global scales, narrowly adapted populations with low plasticity in selectively important characters might be at a higher risk of extinction (Valladares et al. 2007). Plastic responses of plants to contrasting environments have been frequently reported as adaptive, but this is not always the case (van Kleunen and Fischer 2007), and examples of maladaptive plasticity do exist (Sánchez-Gómez et al. 2006).

Differences among species and populations in their plasticity may reflect differential selective pressures on plasticity, differential limitations acting upon the maximization of plasticity, or a combination of both (Valladares et al. 2007). Various pieces of evidence suggest that global change should in principle favour high levels of phenotypic plasticity in plants (Parmesan 2006). However, global change often involves simultaneous changes in two or more abiotic and biotic factors, which may impose restrictions on plastic responses to the environment. Consequently, we observe in nature a wide range of imperfect solutions to the conflicting situations faced by plants in changing and complex environments (Valladares et al. 2007).

1.5.2. Adaptation through natural selection

Since the rate and magnitude of projected climate change continue to increase and exert selective pressure on populations, natural selection will favour genes that increase trees survival in new environments, and may lead to the decline of genes that were dominant under previous conditions (Hoffmann and Sgró 2011). Evolutionary responses to climate change are less likely when genetic diversity is absent or beneficial alleles occur at a low frequency within a population (Bürger and Lynch 1995; Hoffmann and Sgró

2011). The extent to which populations will adapt will depend upon phenotypic variation, strength of selection, fecundity, inter-specific competition, and biotic interactions. Forests may acclimate or adapt to new climatic conditions to some extent, as evidenced by the ability of some species to thrive outside their natural ranges (Kawecki and Ebert 2004). Populations of temperate and boreal trees show moderate to strong clines in phenology and growth along temperature gradients, indicating substantial local adaptation. Traits involved in local adaptation appear to be the product of small effects of many genes, and the resulting genotypic redundancy combined with high fecundity may facilitate rapid local adaptation despite high gene flow (Aitken et al. 2008). Gene flow with pre-adapted alleles from warmer climates may promote adaptation and migration at the leading edge, while populations at the rear will likely face extirpation (Savolainen et al. 2007). Widespread species with large populations and high fecundity are likely to persist and adapt, but will likely suffer adaptational lag for a few generations.

As does genetic variation, adaptation potential varies across a species' populations and its range. It is thus likely that individual populations within a species will react differently to climate change, especially if there are stronger selective pressures in different parts of the range or changes in selection pressure (Hoffmann and Sgrò 2011), i.e. to stronger temperature changes in certain parts of a range, to accelerated range expansion in leading edge populations, or to stronger competition through climate amelioration in the centre of the range of Arctic species (Hill et al. 1999).

Under strong selection pressure such populations risk going extinct before beneficial genes have a chance to increase population fitness. However, there is increasing evidence that rates of evolution can be rapid when genetic variation for differing environmental tolerances already exists

in populations. Genetic diversity prevents unintended losses of traits that can enhance population survival, and increase the potential for adaptive evolution (Schwartz et al. 2012).

Phenotype changes in response to environmental changes may be due to evolutionary adaptation, phenotypic plasticity or a combination of both. The rate and magnitude of climate change may exceed the limits of phenotypic plasticity, at which point adaptive evolution or gene flow from other populations provide the only mechanisms for population persistence. Physiological responses are mediated by mechanisms involving biochemical adaptations, changes in membrane properties, and molecular adaptations such as shifts in gene expression in response to environmental fluctuations. However, physiological responses are limited in some populations, leading to sub-lethal impacts on fitness by increasing energetic demands on individuals (Williams et al. 2012).

1.5.3. Natural migration

The past glacial and postglacial migrations of forest trees, inferred from fossil pollen records and genetic data, suggest a robust capacity for range shifts (Hamrick 2004). Anecdotal evidence of the capacity of forest trees to adapt rapidly to new environments includes the development of steep genetic clines during post-glacial migration (Davis and Shaw 2001), and the existence of locally adapted land races of introduced tree species within one or two generations of introduction (Saxe et al. 2001). Shifts in the distribution of forest zones take place with significant time lags. The area occupied by different forest types has been quite plastic in the past 100,000 years, responding to changing environments with changes in species associations, structural properties of stands, and areas occupied. The change in annual mean temperature that occurs when one moves 100 km poleward

may be as high as 0.7°C in mid- and high latitudes, but less at low latitudes. For summer temperatures and toward the interior of the continents, this value may be higher. With altitude, temperature changes of about $0.5\text{--}0.7^{\circ}\text{C}$ per 100 m are also common. With an expected warming between 0.1 and 0.35°C per decade, this means that species would have to migrate $1.5\text{--}5.5$ km toward the poles per year or increase elevation by $1.5\text{--}5.5$ m per year in order to remain within similar climatic conditions (IPCC 2012).

Natural rates of trees migrations have been estimated to range from 40 to 500 m per year. Migration rates for Scots pine from several sites were only 40-80 meters per year (Gear and Huntley 1991) while much faster dispersal rates of up to 1-2 km per year have been reported for other species such as white spruce (Robledo-Arnuncio 2011). It is not always clear whether the observed past rates were maximal rates of migration or whether they were limited by the rate at which the climate changed (Prentice 1992).

Pollen may have a faster dispersion much than seeds, so the movement of genes of different ecotypes within a species might be able to match the speed of climatic change (Robledo-Arnuncio 2011). Migration of a tree species involves movement of propagules to new locations, establishment of seedlings, growth of individuals to reproductive maturity (which may take from years to decades), and production of new propagules. Seed production, in particular, often depends on a phased sequence of development over at least two years, including many steps from floral bud differentiation to seed ripening (Johnston et al. 2010). More flexible species, such as those with wider seed dispersal, or a more invasive growth habit, may be able to move to more favourable habitats, whereas less mobile species are likely to be left behind and would be at a disadvantage as their former habitat becomes climatically unsuitable (Gonzalez et al. 2010).

1.6. Selected forest pine species

Pine (*Pinus* spp.) has been extensively used for reforestation and land restoration in the Mediterranean regions and other parts of the world because it is stress tolerant, has pioneer features, and facilitates the long-term development of late-successional hardwoods (Pausas et al. 2004).

1.6.1. Aleppo pine (*Pinus halepensis*)

Aleppo pine (*Pinus halepensis* Mill) is the most widely distributed pine in the Mediterranean basin (Quézel and Médail 2003). It has been widely introduced throughout the world and is considered one of the most invasive pine species as well as a very successful colonizer (Maestre and Cortina 2004). Marked differences among Aleppo pine populations in terms of growth and survival have been reported (Bariteau 1992; Atzmon et al. 2004). Studies on the genetic variability of Aleppo pine, show high levels of polymorphism and of genetic differentiation among populations (Schiller et al. 1986; Teisseire et al. 1995; Agúndez et al. 1997). Common garden studies assessing Aleppo pine intraspecific variability revealed adaptive variation to climate (Voltas et al. 2008; Atzmon et al. 2004), which demonstrated the selective role of climate variables in determining populations fitness in this species (Climent et al. 2008). In Spain, Agúndez et al. (1997) reported that Aleppo pine selection for drought and frost tolerance has led to adaptation of provenances. The species is very drought resistant and grows especially well on dry rocky limestone soils (Schiller and Atzmon 2009).

1.6.2. Black pine (*Pinus nigra*)

Black pine (*Pinus nigra*) is one of the most important pines for high elevation sites in southern Europe and especially the Mediterranean basin (Isajev et al. 2004). *Pinus nigra*, with its (sub-)Mediterranean distribution is

considered very drought-tolerant (Isajev et al. 2004), and was therefore identified as a potential substitute for conifer species, like *Picea abies* and *Pinus sylvestris* that are threatened by the changing climate in Central Europe. Lebourgeois et al. (1998) showed that the drought tolerance of *P. nigra* saplings is attributed to their capacity to effectively counteract water stress by stomatal control of transpirational water loss. *P. nigra* exhibited a significantly lower mortality rate after two natural droughts than *P. sylvestris* in Eastern Spain (Martinez-Vilalta and Piñol 2002). Yet, a trade-off between this survival strategy under water limited conditions, and a reduction in growth becomes evident. Especially, late-wood formation was found to be sensitive to the precipitation regime during summer months (Biel et al. 2004; Martin-Benito et al. 2010).

The Salzmann pine (*Pinus nigra* ssp. *salzmannii*) is an endemic subspecies of black pine native to the Western Mediterranean basin and covers extensive areas of 350 000 ha in Spain (Isajev et al. 2004). Because of threats on their typical habitat Salzmann pine habitat is currently protected under the European Directive 92/43/ EEC (9530). In Spain, Salzmann pine exhibited a high genetic diversity among populations most probably due to the geographic isolation and the fragmented (sub-) Mediterranean distribution of populations (Rubio-Moraga et al. 2012). Differences among provenances and populations, most likely, display differences in their phenotypic plasticity and adaptive capacity and at this point, provenance trials could reveal a diverse performance of provenances from various geographic origins (Varelides et al. 2001; Seho et al. 2010). Martin-Benito et al. (2010) suggested that impacts of increasing temperature on the performance of *P. nigra* will differ between geographic regions; warming stimulates growth in the more temperate parts of its distribution, while it

increases water stress in the Mediterranean region and thus negatively effects the growth of this species.

1.7. Intraspecific variability and climate change

Biodiversity has an important effect on ecosystem functioning and reliability as the higher number of species, the more likely the function of a failing species can be adopted by other species in the system. The conversion into mixed forests through the increase of species diversity as insurance against adverse biotic and abiotic impacts has become popular over the last decades (Knoke et al. 2005). With respect to the described uncertainties and the potential positive effects of biodiversity on risk abatement, the mixing of provenances has been suggested by several authors (Kolström et al. 2011). The existence of genetic differentiation between populations of the same species is a key aspect of adaptation to different environments in forest trees (Schiller and Atzmon 2009).

Intraspecific genetic variation provides the basis for any evolutionary change and is thus the most fundamental level of biodiversity (Pauls et al. 2013). Climate change is expected to induce changes; (a) in the distribution of genetic variants in space and time as the ranges of populations and species vary, (b) in the levels of phenotypic plasticity of individuals and populations as they respond to new environmental conditions, and (c) in the evolutionary adaptation way to changing environmental conditions (Hoffmann and Sgrò 2011).

Provenance-trials experiments in forestry have revealed high among-population levels of genetic variation for quantitative traits related to adaptation, geographic structuring of that variation along climatic gradients, and genotype-by-environment interaction, providing strong evidence of local adaptation of populations to climate (Savolainen et al. 2007).

Evidence from these trials suggests that differences within species are distinct, at least in forest trees; differences in phenotypic expression could be highlighted on a genotypic level by molecular methods whereas the phenotypic and the genetic variation could be expressed in local adaptation to climate conditions or other abiotic factors (Savolainen et al. 2007). *Pinus halepensis* exhibits a high genetic differentiation among populations for neutral (Agúndez et al. 1999) and adaptive traits (Atzmon et al. 2004; Chambel et al. 2007; Voltas et al. 2008), suggesting that both non-selective genetic processes and directional selection have contributed significantly to its geographical genetic structure. *Pinus nigra*, with its spread distribution range, shows strong genetic differences between populations and subspecies (Soto et al. 2010) which are supposed to have been enhanced by geographic isolation during the Pleistocene (Aguinagalde et al. 1997). Besides, Varelides et al. (2001) and Seho et al. (2010) signalled differences in performance of provenances from various geographic origins. Considering the outlined potential impacts of changing climatic conditions on functions and services of forest ecosystems, the introduction of provenances or ecotypes from regions within the distribution range of the species with current climatic conditions similar to the expected conditions for the target area has therefore been suggested as one potential tool in climate change adaptation (Bolte and Degen 2010).

1.8. Neutral vs. adaptive genetic variation under climate change

Genetic variation and adaptive potential across a species' gene pool and adaptation can be important to help tolerate new conditions but also when selection acts on dispersal capabilities of species (Canale and Henry 2010). The term 'neutral' refers to a gene (or a locus) that has no (or almost no) effect on fitness, e.g. in terms of offspring produced. As natural

selection does not act upon these genes, they are of no direct adaptive value and are selectively neutral or, in short, neutral (Conner and Hartl 2004). Neutral genetic variation is the genetic variation estimated at such neutral genes. The terms ‘adaptive’ or ‘selective’ refer to a gene (or a quantitative trait) that has an effect on fitness. The genotypes are thus of adaptive or selective significance (Conner and Hartl 2004). Selected genes have a tendency to be monomorphic within populations, because selection removed all the unfit variants. Adaptive genetic variation is the genetic variation that is estimated at such adaptive genes.

Valladares et al. (2007) noted eventually that life history traits and resulting phenotypes are the factors that determine whether a species or population is able to cope with climate change and not the neutral genetic variation. Maximizing an individual’s ability to respond to changing environmental conditions through phenotypic plasticity comes at the cost of reducing energy inputs elsewhere, as reproduction (Canale and Henry 2010). Thus, there are limits to the potential phenotypic plasticity of individuals, populations and species; phenotypic plasticity should be highest in long-lived organisms as they cannot withstand high selective pressures, due to slow generation times, and need to invest energy in reproduction less frequently than species with short generation times (Pauls et al. 2013). Trees are an exception of long-lived organisms that can rapidly adapt to changing environmental conditions due to the immense effective population size, high genetic variation (even in individual trees) and great reproductive potential. Environmentally responsive plasticity (polyphenism) that is linked with genetic polymorphism between individuals may also be a first step in the evolutionary process. Thus, preserving (or losing) the varying degrees of plasticity within individual populations of a morphospecies has evolutionary implications as well.

1.9. Proactive vs. reactive adaptation strategies to climate change

From a forest management point of view, predicted climate change implies that current practices may need to be adapted for future conditions. By forest management it would be possible to respond to and adapt forests to the environmental changes and following changes in forest dynamics (Saxe et al. 2001). Climate change adaptation is an emerging approach that focuses on how to prepare for and respond to the impacts of current and future climate change. The Intergovernmental Panel on Climate Change (IPCC) Fourth Assessment, defined adaptation as “initiatives and measures to reduce the vulnerability of natural and human systems against actual or expected climate change effects” (IPCC 2007a) and as “adjustment in natural or human systems in response to actual or expected climatic stimuli or their effects, which moderates harm or exploits beneficial opportunities” (IPCC 2007b). The recent IPCC special report on extreme events (IPCC 2012) defines adaptation as “in human systems, the process of adjustment to actual or expected climate and its effects, in order to moderate harm or exploit beneficial opportunities. In natural systems, the process of adjustment to actual climate and its effects; human intervention may facilitate adjustment to expected climate”. However, maladaptation, defined as “any changes in natural or human systems that inadvertently increase vulnerability to climatic stimuli; an adaptation that does not succeed in reducing vulnerability but instead increases it”, is a concept that refers to responses to climate change that may actually be detrimental to the system of interest (IPCC 2007).

Because adaptation fundamentally is about managing change, it can best be thought of as a process, rather than a fixed outcome. Although specific adaptation goals and objectives may be set, ongoing environmental and climatic change, and the ecological and human responses to these

changes, will require continual reevaluation and adjustment of adaptation approaches (Fazey et al. 2010).

Vulnerability, as noted by the IPCC, refers to the degree to which an ecological system or individual species is likely to experience harm as a result of changes in climate (IPCC 2007b). Vulnerability to climate change is a function of exposure to climate change (i.e., the magnitude, intensity and duration of the climate changes), the sensitivity of the species or community to these changes, and the adaptive capacity of the system (Williams et al. 2008). Species and ecosystems that are more vulnerable are likely to experience greater impacts from climate change, whereas those that are less vulnerable may be more likely to persist or even benefit from changes in climate (Glick et al. 2011).

Adaptive capacity is perhaps the most challenging to put into practice among the previously cited components of vulnerability. Adaptive capacity refers to the ability of a natural system to accommodate climate change impacts with minimal disruption (Williams et al. 2008). One aspect of adaptive capacity is the ‘intrinsic adaptation potential’ of a natural system to climate change (Glick et al. 2011), resulting from factors such as dispersal ability, genetic diversity, and plasticity at the population or species level, or factors such as functional redundancy and patch size at the ecosystem level. Nevertheless, the ability of a system to realize its intrinsic adaptive capacity is also influenced by extrinsic factors, such as barriers to dispersal and permeability of the landscape (Glick et al. 2011).

Resilience represents the potential responses of biological systems to the impacts of climate change; the concept of resilience is defined in the ecological literature as a measure of the persistence of systems and their ability to absorb change (Holling 1996). Resilience has been characterized as the capacity of a system to absorb disturbance and reorganize while still

maintaining the same relationships among its components (Walker et al. 2004). Gallopín (2006) limited resilience to the adaptive capacity component of vulnerability whereas Magness et al. (2011) treated it as an index of the non-exposure components of vulnerability. The concept of resilience is sometimes expanded to encompass properties of the system that promote resistance (Walker et al. 2004), facilitate the emergence of new system trajectories (Folke, 2006), or even allow systems to function "differently" (Lawler 2009).

Proactive or *anticipatory adaptation* refers to the actions undertaken to prepare for anticipated climate change impacts while actions in response to climate-driven impacts are often referred to as reactive adaptation (Adger et al. 2005).

Adaptive management is a concept that has been applied to resource management as a tool for helping resource managers makes decisions in response to climate change (Williams et al. 2008). Planning of adaptive forest management would require forestry modelling tools that are usable under the expected climate change. They should be at their best both responsive to changing environmental conditions and be able to use traditional forest inventory information as inputs to produce reliable predictions on forest growth and yield for application scales of practical forestry.

Increasing species diversity in forest management and assisted migration are two adaptation strategies to consider in managing forest stands and landscapes to buffer the negative impacts of climate change, and foster increased resilience to extreme weather events.

1.10. Assisted populations migration as an adaptation strategy for climate change

Assisted population migration, also called managed relocation, managed translocation, or assisted colonization, refers to objectives of protecting rare or endangered species or populations in the face of climate change and other disturbances. However, in the forestry context the objective is most often related to ensuring health and productivity of planted forests (Johnston et al. 2010). Forest tree species often ‘drive’ community structure and ecosystem processes (i.e., they are ‘foundation’ species) (Ellison et al. 2005); therefore, planting forests with trees that are adapted to the climate of their planted environment is fundamental to establishing resilient ecosystems. Consequently, we propose expanding the definition provided by Vitt et al. (2010) to: the purposeful movements of species to facilitate or mimic natural population or range expansion to help ensure forest plantations remain resilient in future climates.

Assisted migration is defined by Williams and Dumroese (2013) as the movement of species and populations to facilitate natural range expansion in direct management response to climate change and refers to the movement of seed sources beyond their contemporary climates have gained more interest during last decade. Generally, seed sources are moved northward and upward in elevation in a manner that mimics recent observed climate change and expected shifts over the next few decades.

Three distinct applications were used to achieve this purpose; (1) assisted population expansion through the movement of populations within a species’ range; (2) assisted range expansion throughout the movement of populations to locations adjacent to the species’ existing range and (3) the translocation of exotics, inter-regional, transcontinental, or intercontinental, species far outside its current geographic range (Johnston et al. 2010).

To explore assisted migration and the critical debate surrounding its application, it is helpful to break the discussion down into streams: the broader, conservation biology perspective, and the more specific case of applying assisted migration within forestry. In conservation biology, the emphasis is on rescuing species at risk of extinction or extirpation as a result of climate change or other disturbances. Consequently, much of the discussion involves translocation of exotics or assisted range expansion (Ricciardi and Simberloff 2009). In operational forestry, the emphasis is usually on maintaining adaptation of plantations. Consequently, required migration distances are shorter, potentially less risky and, for the most part, supported through extensive provenance testing. Migrations being considered within forest management involve movement between similar biogeoclimatic units and are usually within species' existing ranges. Climatic migration distances under discussion in forest operations translate to geographic distances of approximately 200-400 m up in elevation or up to 300 km northward (Ukrainetz et al. 2011). In the subsequent two sections, we describe the application of assisted migration to species conservation and to forestry more specifically.

Effective implementation of assisted migration can only occur if target transfer guidelines are developed because current seed transfer guidelines, established to guide the movement of plant materials, lack inherent spatial and temporal dynamics associated with climate change (Potter and Hargrove 2012). Planting seed sources and species that are adapted to current and future climates exploits finely tuned plant-climate adaptations wrought through millennia of natural selection to help maintain forest health and productivity.

1.10.1. Assisted populations migration for species conservation

Assisted populations migration is proposed for conservation biology as a means of avoiding predicted species extinction as a result of various forms of disturbance including climate change. Species or populations that are unable to migrate to new locations or adapt through natural selection would be intentionally moved to a region, often outside of their current or historic range, where stresses or threats are fewer. The approach is proposed as a conservation strategy for species with poor dispersal abilities in highly modified landscapes subject to the effects of climate change (Shirey and Lamberti 2010).

Conservation biologists and ecologists consider moving species beyond their current distribution without sufficient knowledge of their ecology and how they may interact with climates and other species and other biotic and abiotic factors on novel sites has to be too risky and may led to catastrophic invasions of ecosystems by species that are able to reproduce and out-compete native species (Clavero and Garcia-Berthou 2005).

However, some biologists are now suggesting the need to re-visit the prohibition of species movement in order to circumvent climate-driven extinction (Richardson et al. 2009). McLachlan et al. (2007) and Hoegh-Guldberg et al. (2008) argued that, by increasing understanding of the habitat requirements and species distributions, low-risk situations can be identified and thus, the benefits of assisted migration can be realized with less adverse outcomes.

1.10.2. Assisted populations migration for forest management

Assisted migration refers here to reforesting sites using neighbouring seed sources adapted to future climates to ensure that plantations remain healthy and productive in future climates. Natural selection has provided forest trees with a suite of adaptive traits best suited to its climate (e.g., cold hardiness, drought resistance) and local pests and disease. Identifying and maintaining these natural adaptations through assisted populations' migration to areas predicted to occupy their ancestral climates may offer an effective and practical alternative (Vitt et al. 2010).

Assisted migration in the form of assisted population expansion represents the movement of seed within a species current range to encourage the use of seed sources of native species from climates anticipated to exist at the target environment in the future. Depending on the magnitude of the migration distance and the size of the current seed zone, this form of assisted migration may involve little movement of seed beyond its current seed transfer limit. However, assisted range expansion refers to the movement of a species outside its current natural distribution which may pose greater risk of ecological disturbance because of the unknown interactions complexity of the introduced species with the native species (Leech et al. 2011).

The risk of ecological disturbance posed by exotic translocation differs from that of assisted range expansion because the suite of novel species encountered by the introduced species is much greater in exotic translocation than in assisted range expansion. The mechanism by which assisted migration may be implemented depends on the seed transfer system in use whether seed is procured within the fixed boundaries of a seed zone (fixed system where zones may stay fixed and seed would be procured from adjacent or climatically suitable zones) or is procured from within a defined

geographic, climate, or adaptive distance of the plantation (focal point zones where deployment ranges may be shifted to encourage use of seed from warmer locations and prohibit use of seed from colder locations) (Leech et al. 2011).

Aitken et al. (2008) and Ukrainetz and O'Neill (2009) proposed a migration distance equivalent to the climate change expected at one quarter to one third of the rotation, i.e., approximately 0.5°C mean annual temperature to encounter the greater sensitivity of trees to stress in early life. As climate change involves multiple aspects of climate (e.g., precipitation, extreme events), similar evaluations of multiple climate variables should be used in developing a multivariate migration distance (Ukrainetz et al. 2011).

1.11. Forest tree genomics

Genomics can be defined as the structural and functional analysis of the complete genome of an organism, including mapping, sequencing and gene expression profiling. The development of genome-wide experimental approaches, encompassed with new and potent bioinformatics tools, allows biologists to take on the study of adaptive genetic diversity and its association with phenotypic trait variation in non-model organisms such as forest trees (Abril et al. 2011).

Forest genomics finds a key challenge in the complexity and highly variable size of the forest trees genomes. The genome of most pines is more than 8-fold the human genome size (Tuskan et al. 2006) and contains about 30,000 genes, as estimated from known plant species genomes (White et al. 2007).

The study of natural genetic variation in forest trees has traditionally been investigated using either of two approaches; first, quantitative genetics

via common gardens and trial sites or second, population genetics by means of markers (Neale and Ingvarsson 2008).

The *quantitative genetics* through common garden approach estimate genetic variation based on the measurement of phenotypic variation where common gardens can be replicated over many different environments using either clonal or family-based testing. This allows estimation of genotype by environment interactions (GEI). Quantitative genetic parameters such as heritability and additive and dominance variance components are used to characterize genetic variation (White et al. 2007). This approach is extremely useful for characterizing broad patterns of adaptive genetic variation and has been used in a practical manner to define seed or breeding zones in reforestation programs. However, the individual genes underlying complex adaptive traits are not known, so single-locus population genetic theory cannot be applied (Neale and Ingvarsson 2008).

For *population genetics*, the use of genetic markers such as isozymes, RFLPs, RAPDs, AFLPs, SSRs, and ESTPs have all been used for the study of natural genetic variation as an alternative but for the most part all reveal neutral genetic variation. Such markers are useful for characterizing demographic patterns of variation (migration and drift) but are not instructive of adaptive patterns of genetic variation (Neale and Ingvarsson 2008). Complex trait dissection experiments are needed before applying population genetic theory to genes controlling complex adaptive traits. The QTL approach was first used, but because of low map resolution of QTLs, the underlying genes could not be determined (White et al. 2007). After the genes are identified that govern adaptive traits, then it is just one more step to discover the naturally occurring allelic variation in populations and test for the presence of selection using modern population genetic methods (Neale and Savolainen 2004).

The *comparative genomic* approaches can also be used to understand the evolution of adaptation. Forest trees, notably the pines and poplars, are well suited to comparative genomics. Forest geneticists have generally worked on the species of regional importance. This situation has been viewed as a limitation because of dispersed and lack of focused investigation. However, as the power of comparative genomics is realized, this situation may soon be viewed as an asset, rather than a liability. To that end, forest genomics researchers have taken early steps toward developing collaborative networks and a comparative genomic framework (Neale and Ingvarsson 2008). The Conifer Comparative Genomics Project (<http://dendrome.ucdavis.edu/ccgp>) was started several years ago and led to a series of low-density comparative maps (Pelgas et al. 2006). Comparative resequencing project (<http://dendrome.ucdavis.edu/crsp>) was begun to provide novel insight into within and between species genetic diversity and will lead to deeper understanding of speciation and the genetic basis of phenotypic differences among species (Neale and Ingvarsson 2008).

1.12. Forest tree proteomics

Expression of different genes can be studied by using microarrays. The problem is that it has long been reported that there is a lack of correlation between mRNA and protein abundance (Gygi et al. 1999). When plants adapt to a particular environmental challenge, the gene expression pattern drastically changes, but most of the genes up- or down-regulated are irrelevant for the stress response, being most important the posttranscriptional regulation of these genes. For instance 5% of the model organism baker's yeast or the model plant *Arabidopsis thaliana* are changed after salt stress. That means that about 300 to 1200 genes change its expression. In yeast it has been shown by mutational analysis that most of

these genes are not relevant for the stress response. Therefore in order to obtain valuable data for assisted migration we must focus on proteomics or on the biochemical level.

Proteomics constitutes a priority research for any organism and configures a fundamental discipline in the post-genomic era. Proteomics is and will be in the frontier of such knowledge better than any other approach. Pioneer publications in forest tree proteomics come from maritime pine genetic and variability studies and data from the late 1980s (Bahrman and Damerval 1989). Starting by just doing 2-DE, protein patterns of 56 haploid megagametophytes from a single maritime pine were performed to analyze the genetic determinism, and pleiotropic effects of both qualitative and quantitative variation. Later on the 2-DE analysis was implemented for protein identification, first by micro-sequencing (Costa et al. 1999) and then by MS (Gion et al. 2005). In the 1990's a number of papers were published on this topic and species at the French INRA (Gerber et al. 1993; Bahrman and Petit 1995; Plomion et al. 1997; Thiellement et al. 1999 among others). Costa et al. (1998) identified drought stress-responsive proteins during a progressive water deprivation on two-year old plants raised on the ground. Plomion et al. (2000) identified proteins involved in the changes in cell-wall structure that can be associated with the formation of compression wood. Proteomics approaches involve various experimental techniques that produce large volumes of highly heterogeneous data, including protein amount using 2-DE gel image analysis and protein identification by MS. In order to manage this vast quantity of data, a number of forest tree databases have been established (Ferry-Dumazet et al. 2005).

1.13. Proteomics in the genus *Pinus*

Maritime pine (*Pinus pinaster* Ait.), the first species used for reforestation in South-western Europe, has been the main focus of high throughput proteomic analysis in pines, published in part from contributions on *Pinus radiata* (Valledor et al. 2010), *Pinus bungeana* (Wu et al. 2008) and *Pinus halepensis* (Ariza Mateos et al. 2009). 2-DE constitutes a valuable tool for providing informative markers for genetic diversity analysis, the establishment of linkage maps and QTL analysis. Coupled with micro-sequencing (Costa et al. 1999) or mass spectrometry (Gion et al. 2005), 2-DE made possible to evaluate protein regulation by genetic, environmental and developmental factors. Damerval et al. (1994) were the first to investigate the genetic determinism of quantitative variation of proteins separated by 2-DE using a QTL (Quantitative Trait Loci) detection strategy. Several studies have demonstrated that organ-specific proteins are more variable than organ-unspecific proteins and that the level of genetic variability depends on the organ or tissue considered (Bahrman and Petit 1995). Many cell functions are affected by drought and different types of proteins have been induced by this stress. The experiment conducted on eight maritime pine genotypes to identify drought stress-responsive proteins during a progressive water deprivation on two-years old plants raised on the ground (Costa et al. 1998) showed that some proteins were expressed de novo while others accumulated or were suppressed in drought stressed plants. The internal micro-sequencing of some water-deficit responsive polypeptides allowed the characterization of their function and their putative role in the stress. A modification of polypeptide abundance was observed for a diverse set of proteins, including those involved in photosynthesis (Rubisco activase), cytoskeleton (actin), enzymes with antioxidant activity (superoxide dismutase, glutathione peroxidase), and lignification (CoA-O-

methyltransferase). Based on this previous screening, Costa (1999) was set up to study 163 spots chosen among the previous set and likely to be drought-responsive in more genotypes (120). It was observed that 62.5% (75 spots) were down-regulated, whereas 37.5% (45) were up-regulated. Based on known function proteins, clusters were identified biologically-meaningful such as: (i) proteins corresponding to specific gene products (gene family), (ii) proteins characterized by a similar function (same metabolic pathway), (iii) protein located in the same cell compartment, and (iv) protein involved in different metabolic pathways but showing similar patterns of regulation in the two environmental conditions tested.

Pinus radiata needles were studied by combining a proteomic and transcriptomic approaches to describe the changes which occur during the transition from needle primordia to fully developed needle. A total of 280 protein species and 176 genes related to energy metabolism pathways, photosynthesis, and oxidative phosphorylation characterize mature needles, whereas biosynthetic and stress response processes were over-expressed in immature needles (Valledor et al. 2010).

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2. Synopsis of the Thesis

2.1. Problem statement

Climate change is triggering vegetation range-shifts in Europe where these changes are expected to result in the northward shift of Mediterranean ecosystems (IPCC 2012). Quézel and Médail (2003) pointed out an altitudinal shift from one vegetation stage to another over the Mediterranean basin in response to climate change and thus, the theoretical limit between species should quickly shift in elevation and in latitude (Venetier et al. 2007). Moreover, Metzger et al. (2008) reported that Mediterranean North and Mediterranean Mountain environments are predicted to shift, respectively, to South and North Mediterranean, indicating a potential expansion of deserts habitats, and an increased risk of forest fires and drought.

This bioclimatic envelopes shift northwards and higher up in elevation is expected to affect tree species distributions and thus, the range of Aleppo pine (*Pinus halepensis*) and Black pine (*Pinus nigra* ssp. *salzmannii*) widely distributed in the Mediterranean regions might retreat from the south to the north and upwards in the mountain regions. Here, trees establishment in new environments and mainly survival and growth could be significantly constrained.

2.2. General objectives and approach

Considering the challenges that forest ecosystems management and conservation will have to meet in spite of climate changes. The overall objective of this thesis is to implement an integrated approach for

addressing assisted population migration programs in forest management to encounter climate change by (a) assessing the potential of the selective use of within-species variability (provenances, seed sources, ecotypes...) as a tool for climate change adaptation through assisted population migration within the natural distribution range of the species and (b) identifying provenances or ecotypes in *Pinus halepensis* and *Pinus nigra* ssp. *Salzmannii* which are better adapted to future climate conditions in the Mediterranean regions, a transitional climatic zones between arid and humid regions of the world where global climate change expected to have the greatest impacts.

The first part of the study reproduced real conditions of reforestation in potential future climatic conditions in different trial sites; the first chapter (**Manuscript 1:** Testing Aleppo pine seed sources response to climate change by using trial sites reflecting future conditions) discusses the need to analyse seed sources (i.e. plant material from which forest reproductive material is derived) responses to environmental conditions in order to develop effective reforestation policies and to cope with the new environmental cues from climate change through Assisted Migration since reforestation guidelines that focus on local seed sources may become irrelevant, as these stocks will not be adapted to the new environmental conditions. The aim was to compare field performance (survival and growth) of eleven Aleppo pine seed sources, from a wide range of distribution of the species in Spain, outplanted in core and marginal habitats over three years and to assess their phenotypic plasticity for further screening under specific conditions in particular reforestation areas. Three sites of different and contrasting climatic conditions within the natural distribution of the species, representing the dry situation, the cold situation and the current core habitat for the species, were compared. We addressed the question of whether current core habitats for Aleppo pine in Spain can shift towards more

warmer and drier marginal habitats (shift from core to drier marginal habitat) where drier provenances could perform better than local provenances in such situations. At the same time, current marginal habitats for the species due to low temperature (high altitude belts) could be suitable in the near future (shift from cold marginal to core habitat), as they become core habitats (and thus, marginal for other species).

In a second step, the study was oriented to the understanding and the use of information about genotype by environment interaction (GEI) structure, phenotypic plasticity and adaptation of Aleppo pine vegetative traits under various growth conditions as a complementary selection strategy for identifying suitable seed sources for further introduction in specific areas in the context of assisted population migration towards predicted climate changes. Thus, the second chapter (**Manuscript 2: Addressing Genotype by Environment Interaction in *Pinus halepensis* towards assisted population migration programmes in response to climate change**) was addressed to (a) identify the existence of ecotypic differentiation among *P. halepensis* seed sources under core-to-dry and cold-to-core drifts, and (b) to determine whether different seed sources show divergence in their phenotypic plasticity for growth in response to abiotic stress. To achieve these goals, (1) seed sources variation in their phenotypic plasticity and the reaction norm in the broad context of GEI has been evaluated and (2) the efficiency of joint regression (Finlay and Wilkinson 1963) and Additive Main effect and Multiplication Interaction model (AMMI, Gauch 1992) has been compared in elucidating the pattern of the adaptation of the seed sources regarding height and diameter growth under both drift situations; taking into account both the transplant shock (one year after out-planting) and the final establishment of seedlings at early growth stage (after three years).

The same previous methodologies and approaches tested before on Aleppo pine were applied as well on six seed sources of Salzmann pine (*Pinus nigra* ssp. *salzmannii*) out-planting over three years in three trial sites in the distribution range of the species; two core sites of Mediterranean and Sub-Mediterranean climates and a marginal habitat were presented in the third chapter (**Manuscript 3:** On the assisted population migration in *Pinus nigra* ssp. *salzmannii*: early plantation performance and genotype by environment interaction of different seed sources in contrasting ecological sites).

In the fourth chapter, the last part of the study, 25 weeks old seedlings of the previous selected Aleppo pine seed sources grown under controlled conditions were exposed to temperature (low temperature vs. control) and drought (drought vs. control) treatments. Seed sources were tested under controlled conditions in order to verify, explain and complete field observations using deep ecophysiological and molecular studies (**Manuscript 4:** Physiological, biochemical and proteomic response of Aleppo pine seed sources to water and cold stress as a contribution for assisted population migration in the species). This part of work was devoted to the comparison of the intraspecific variability among nine Aleppo pine seed sources in response to induced drought and cold stresses through the analysis of several physiological and biochemical variables then by focusing on the study of stress responsive proteins to the identification of potential molecular markers of tolerance or of sensibility to such abiotic stressor by the proteomic approaches for further selection of candidate provenances or ecotypes that might replace or complement local ones.

2.3. References

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Chapter one:

Testing Aleppo pine seed sources response to climate change by using trial sites reflecting future conditions

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Abstract

Large-scale biogeographical shifts in forest tree distributions are predicted in response to the altered precipitation and temperature regimes associated with climate change. Adaptive forest management to climate change experienced in either stable or rapidly changing environments must consider this fact when carrying out reforestation programs or specifically assisted population migration for conservation purposes. The aim of this study was to compare field performance of eleven seed sources of Aleppo pine outplanted in core and marginal habitats and to assess their phenotypic plasticity for further screening under specific conditions in particular reforestation areas. We hypothesize that current marginal habitat due to low temperature is shifting toward conditions found on the core habitat and that current core habitat will shift toward warmer and drier marginal habitat. Our study reproduced real conditions of reforestation in potential future climatic conditions. Results suggest that it is difficult to predict Aleppo pine provenances' performance in different natural sites from their performance at a single location, even though 'Levante interior' and 'La Mancha' seed sources showed the best overall response among sites. On a site basis, provenances were matched in groups according to their survival and growth responses. Seedlings grown from local seed sources or seed orchards performed better on the core habitat. However, as conditions shifted to marginal habitats, seedlings from climatically similar regions performed better than local sources at least in the short term; in this sense, current plantations in areas already affected by global change could have better establishment.

Keywords

Pinus halepensis, plantation performance, core and marginal habitats, growth, survival, assisted population migration.

3.1. Introduction

Mediterranean regions, transitional climatic zones between arid and humid regions of the world, are areas where global climate change is expected to have the greatest impacts. These areas are particularly prone to degradation because of the combination of climate change and human activities leading to increased habitat fragmentation, deforestation and land abandonment (Cortina et al. 2013). Climate change is triggering vegetation range-shifts in Europe where these changes are expected to result in the northward shift of Mediterranean ecosystems (IPCC 2001). Quézel and Médail (2003) pointed that global change should induce an altitudinal shift from one vegetation stage to another over the Mediterranean basin and thus, the theoretical limit between species should quickly shift in elevation and in latitude with climate changes (Venetier et al. 2007). Moreover, Metzger et al. (2008) reported that Mediterranean North and Mediterranean Mountain environments are predicted to shift, respectively, to South and North Mediterranean, indicating a potential expansion of deserts habitats, and an increased risk of forest fires and drought. Despite the uncertainties surrounding climate change projections over the Mediterranean region, the latest and most advanced sets of global and regional climate model simulations give a collective picture of a substantial drying and warming of the Southern and Eastern parts of Spain; precipitation decreases by 20 to 30% and warming increases by 4 to 5°C, depending on the scenario (Giorgi and Lionello 2008; Garrido et al. 2012).

Individual species responses to such changes will depend on ecological and evolutionary factors such as niche breadth, competitive ability, phenotypic plasticity and adaptability (Soto et al. 2010). The responses of long-lived plant species to changes in environmental conditions are determined by the capacity of individuals to alter their structure and function

to novel environments (i.e. phenotypic plasticity), adapt through natural selection or migrate (Matesanz and Valladares 2013). In this sense, mitigation and adaptation to climate change is of paramount importance in current and future restoration plans (Atzmon et al. 2004; Vallejo et al. 2012).

Climate change adaptation strategies such as assisted migration, defined as the movement of species and populations to facilitate natural range expansion in direct management response to climate change, have gained more interest during last decade (Williams and Dumroese 2013). Effective implementation of assisted migration can only occur if target transfer guidelines are developed because current seed transfer guidelines, established to guide the movement of plant materials, lack inherent spatial and temporal dynamics associated with climate change (Potter and Hargrove 2012).

Restoration of the Mediterranean regions is currently carried out using many native tree species taking into account other objectives than productivity, i.e. plant biodiversity and functionality, soil conservation, climate change mitigation, among others (Hernández et al. 2010; Vallejo et al. 2012). Aleppo pine (*Pinus halepensis* Mill.) is preferentially chosen for reforestation because of low-technical requirements for nursery production and high-resistance to drought and degraded environments (del Campo et al. 2007a). Although it is considered a pioneer species and favours establishment of late successional, Aleppo pine is also considered part of the climax community (Quézel and Médail 2003). However, forest restoration with Aleppo pine in the Mediterranean presents high variability in the establishment success, mainly due to variation in site quality and stock quality (del Campo et al. 2007b). In addition, the use of unsuitable seed sources for afforestation dramatically decreases the chances of success

(Zobel and Talbert 1984), especially on cold and drought-prone sites (Oliet et al. 2013).

In Spain, *P. halepensis* has a total of twenty regions of provenance (Alía et al. 2009), defined as territories subjected to almost uniform ecological conditions and consisting of populations which present analogous phenotypic or genotypic features (Esteban et al. 2010). Gandullo and Sánchez-Palomares (1994) reported climatic, physiographic and edaphic variables throughout the geographic area occupied by the species in 131 experimental sites. They defined marginal interval as that one encompassing the lowest or highest 10% of the values in a variable, whereas the core interval encompasses the 10-90% range. On a site, the higher the number of key ecological variables falling out of the central interval the higher the marginality of that habitat for the species.

The existence of genetic differentiation between populations is a key aspect of adaptation to different environments in forest trees (Schiller and Atzmon 2009). Marked differences among Aleppo pine populations in terms of growth and survival have been reported (Bariteau 1992; Atzmon et al. 2004). Common garden studies assessing Aleppo pine intraspecific variability revealed adaptive variation to climate (Voltas et al. 2008; Atzmon et al. 2004), which demonstrated the selective role of climate variables in determining populations fitness in this species (Climent et al. 2008). Agúndez et al. (1997) reported that Aleppo pine selection for drought and frost tolerance has led to adaptation of provenances. The contribution of local adaptation and plasticity across populations to the persistence of species under present and future climate change could be decisive (Benito-Garzón et al. 2011). High adaptive phenotypic plasticity would decrease the risk of species loss due to climate change by improving phenotypes suited to the new environments (Matesanz and Valladares 2013). Identifying how

populations grow and survive in response to abiotic factors constitutes a key element for a deeper understanding of ecosystems dynamics in a changing environment and should be considered in reforestation activities (Oliver et al. 2012; Potter and Hargrove 2012).

Reforestation guidelines that focus on local seed sources may become irrelevant, as these stocks will not be adapted to the new environmental conditions (Williams and Dumroese 2013). The need to analyse plant material responses to environmental conditions in order to develop effective reforestation policies and to cope with the new environmental cues from climate change has long been put into writing and is currently among the research priorities for most international organizations (EFI 2009; United Nations et al. 2011; FAO 2013). In this sense, investigation of how different seed sources (i.e. plant material from which forest reproductive material is derived) of Aleppo pine respond to extreme environmental conditions in strongly contrasting environments renders valuable information for the current afforestation programmes of degraded regions and to manage the prospect assisted migration of this species.

We address the question of whether current core habitats for Aleppo pine can shift towards more marginal habitats where drier provenances could perform better than local provenances in such situations. At the same time, current marginal habitats for the species (high altitude belts) could be suitable in the near future, as they become core habitats (and thus, marginal for other species). The aim of this study is to build and expand the knowledge of several Aleppo pine seed sources by assessing their performance (survival and growth) and their phenotypic plasticity under three different and contrasting climatic conditions within the natural distribution of the species, and to provide knowledge on seedling adaptive transfer in ecological restoration, tree breeding and conservation of genetic

resources. Three sites, representing the dry situation (shift from core to drier marginal habitat), the cold situation (shift from cold marginal to core habitat) and the current core habitat for the species, are compared. The predicted response of growth and survival of the different seed sources under the expected climate change in the core and marginal habitats was also addressed throughout this study. In this context, our results allow for further screening for specific conditions, such as drought and low temperature events, to help reforestations programs and provide knowledge to potential assisted migration efforts.

3.2. Materials and methods

Plant material

Eleven seed sources (basic materials) of *Pinus halepensis* were selected considering not only spatial distribution but also environmental heterogeneity, prioritizing those that represent contrasted environments (Table 1). This selection covers most of the climatic and ecologic regions of the natural range of this species in Spain with a wide spectre of molecular and phenotypic variation (Climent et al. 2008; Voltas et al. 2008; Grivet et al. 2009). These seed sources correspond to nine Spanish provenances defined for the species (Alía et al. 2009) and two seed orchards (Table 1).

Table 1. Site descriptions for the eleven seed sources of *Pinus halepensis* Mill. (from Alía et al. 2009). T_M : annual mean temperature; T_{MC} : mean minimum daily temperature of the coolest month; T_{MW} : mean maximum daily temperature of the warmest month; DM: drought months; SP: summer precipitation; AP: annual precipitation.

Seed source ^(class)	ID	Coordinates	Altitude (m)	T_M (°C)	DM	T_{MC} (°C)	T_{MW} (°C)	SP (mm)	AP (mm)
H.S. Alaquàs ³	HSA	39°27'N-0°28'W	43	17.2	4.0	5	31.1	58	444
Litoral levantino ¹	Lit	39°45'N-0°18'W	200	16.5	3.2	4.8	30.3	64	599
Cataluña ^{2*}	Cat	41°46'N-2°46'E	333	13.9	1.1	1.3	29.3	128	714
Los Monegros-Depresión Ebro ¹	Mgr	41°53'N-0°52'W	401	14.2	3.7	0.9	32.3	79	399
Ibérico Aragonés ¹	Arg	40°47'N-0°04'W	555	14.0	2.7	1.5	30.2	91	497
H.S. El Serranillo ³	HSS	40°40'N-3°10'W	635	13.3	3.0	-0.4	33.0	46	380
Levante Interior ¹	Lev	39°05'N-1°12'W	673	14.4	3.1	2.6	30.5	67	524
Maestrazgo-Los Serranos ¹	Mst	39°47'N-1°11'W	763	13.6	2.5	1.4	30.0	93	546
Bética septentrional/sur ¹	Bet	37°55'N-2°55'W	789	14.5	4.0	1.9	31.9	55	410
La Mancha ¹	Man	39°55'N-2°14'W	856	13.1	3.2	-0.1	32.0	68	496
Alcarria ¹	Alc	40°24'N-2°24'W	869	12.4	2.8	-1.1	32.2	82	591

Note: Class of seed source: 1, seed source (identified); 2, stand (selected); 3, seed orchard (qualified). *This is a composite seed source from Cataluña interior and Alta Cataluña seed sources.

Table 2. Characteristics of three sites in Spain used to test survival, growth and ecophysiological conditions of Aleppo pine seed sources. Climatic variables are measured or historical value followed by first and second year weather station estimates in parentheses. (AP: annual precipitation, SP: summer precipitation, T_M : annual mean temperature, T_{MW} : mean maximum daily temperature of the warmest month, T_{MC} : mean minimum daily temperature of the coolest month, PET: potential evapotranspiration).

Site, coordinates	Altitude (m)	AP (mm)	SP (mm)	T_M (°C)	T_{MW} (°C)	T_{MC} (°C)	PET (mm)	Sum of water deficits (mm)	Texture (USDA)	Slope (%)	Aspect	Soil depth (cm)	pH
Lower marginal/core/higher marginal ¹	70-350//970-1375	243-325//705-901	9-23//133-233	10.6-12//16.2-19.3	Not def.	Not def.	641-692//846-996	119-261//551-734	Not def.	0-8//60-75	Not def.	Not def.	6.5-7.7//8.5-8.9
La Hunde ² , Ayora-Valencia, 1°12'W; 39°05'N	800	477 (648;736)	80 (27;145)	13.5 (14;12.3)	31 (35.3;35.3)	0.9 (-2.7;-1.7)	765 (793;718)	-301 (-410;-256)	Silty loam	5	NE-SW	53	8.6
Granja d'Escarp ³ , Lleida, 0°20'E; 41°23'N	310	353 (392;310)	47.7 (97;24)	14.2 (13.3;14.7)	31.3 (32.7;31.9)	1.0 (0;0.1)	779 (755;794)	-426 (-289;-484)	Silty loam	17-44	S-NW	55	8.5
Tramacastiel ⁴ , Teruel, 0°40'W; 39°12'N	1150	531 (586;445)	119 (162;94.1)	11 (10.3;11.9)	28.5 (30;29.5)	-1.6 (-3.2;-1.8)	659 (639;686)	-177 (-133;-241)	Loam	9	SW	40	7.8

Note: 1, digits separated by “-” are the marginal ranges (low and high) and digits separated by “//” represent the core range. Type of habitat: 2, core habitat; 3, marginal dry habitat; 4, marginal cold habitat.

Seed lots were collected as open-pollinated bulk populations either from seed sources or stands and grown as one-year-old container stock in a public nursery located in Centro Nacional de Mejora Genética Forestal de Alaquàs, Valencia (Spain), in growing years 2008 and 2009. Seedling lots were grown as one-year-old container stock under the standard conditions for the species (shade-house, 33 weeks, 200 cm³ container, sphagnum peat and a 15-4-7.5 slow release controlled fertilizer added at 7 g l⁻¹). During nursery culture, plant materials were arranged in a randomized block design with four replicates in order to avoid important differences in stock quality associated with microsite differences in the nursery benches. However, stock differed slightly among seed sources. Stock dimensions (average and standard deviation) in the 2008 stock were height: 35.4±8.0 cm, stem diameter at root collar: 4.4±0.59 mm, total dry biomass: 8.38±2.51 g, shoot/root: 3.18±1.18 g g⁻¹, N concentration (needles): 2.21±0.13 %, root growth potential: 43±24 mg of new roots in optimum conditions (del Campo et al. 2007a). The values for the 2009 stock were respectively: 18.2±2.1 cm, 2.97±0.32 mm, 2.88±0.63 g, 1.73±0.34 g g⁻¹, 1.91±0.08 %, 8.3±4.1 mg, much lower due to the cooler climate conditions during that year in the nursery.

Site selection and characterization

The study was performed in three climate-contrasting sites in Eastern Spain (table 2), encompassing zones in both core and more marginal habitats of this species in Spain (as defined by Gandullo and Sánchez-Palomares 1994). The three sites have similar soil characteristics (especially depth) and naturally regenerated Aleppo pine trees (Table 2).

The Granja d'Escarp site is located in the province of Lleida, NE Spain, and represents the driest site. It was chosen to represent the shift

from current core habitat to marginal dry habitat. This area has a dry continental Mediterranean climate with cold winters and hot dry summers.

The site is located in the provenance region of Aleppo pine ‘Cataluña Interior’ and borders ‘Ibérico Aragonés’ and ‘Monegros Depresión del Ebro’ provenances.

The Tramacastiel site is located in Monte UP 283, south of Teruel province (central-eastern Spain), and represents a current marginal habitat for the species (cold) that could become core habitat with climate change, and thus marginal habitat for species, such as *Pinus nigra* or *Juniperus thurifera*. The site is characterized by a continental mountain climate where frosts are common from November to April. Summer drought is mitigated by altitude and storms. Before a wildfire in 1994, this area was occupied by a natural stand of *Pinus nigra*, *P. pinaster* and *Juniperus thurifera*, whereas *P. halepensis* was minority. Since the fire, minimal natural regeneration of these species has occurred whereas Aleppo pine saplings are becoming present. This site is located in the Aleppo pine provenance region ‘Maestrazgo-Los Serranos’.

The third site acts as a control site (La Hunde, Ayora, Valencia, SE Spain) and belongs to a core habitat of the species according to Gandullo and Sanchez-Palomares (1994). Aleppo pine forest, either naturally regenerated or planted, is the most common vegetation type on the landscape. This site is located in the Aleppo pine provenance region ‘Levante Interior’. Climatic variables for the three sites were monitored from weather stations located in the vicinity of the experimental plots.

Site preparation, planting works and experimental layout

The trials Granja d’Escarpi and Tramacastiel were established in February 2010 whereas the trial site La Hunde was established in February 2009. The sites selected for trials were all considered suitable for forest field

experimentation. Seedlings were outplanted using standard reforestation techniques.

Before outplanting, sites were disked and each planting hole (0.5 x 0.5 x 0.4 m) was opened with a backhoe excavator. Experimental layout was a randomized complete block design with three blocks. Each seed source was represented by thirty-three trees per block for a total of ninety-nine trees per experimental site (1089 seedlings per site). Seedlings were outplanted in rows and spaced 2.5 m apart.

Plantation monitoring and measurements

We measured survival and growth expressed as the monthly relative growth rate in height (RGR-H) and stem diameter (RGR-D) three times during the first year: at outplanting (before spring growth), before summer (June-July, after shoot elongation and at the early summer drought period), and after summer (September-October, once the late growth period concluded). Survival and growth were also measured in the spring (May) of the third year for all sites. By this way, the first three years of growth and the first two summer periods were recorded; sufficient data to evaluate early plantation performance (Landis et al. 2010). Appending 1, 2, 3, or T to the RGR variables indicate the measurement period: 1 corresponds to growth from first to second assessment, 2 from second to third assessments, 3 from third to fourth assessments and T (total) from first to fourth assessments. Seedling height was measured to the nearest 0.5 cm from soil to the terminal. Seedling stem diameter was measured at 1.0 cm above the soil surface. Survival was assessed by determining the status (alive or dead) of each individual.

Transpiration and photosynthesis were measured on May 2012 under similar atmospheric conditions on the same tissues of five plants per seed

source per site (randomly selected in one block) using an LCpro+ Portable Photosynthesis System with leaf chamber (ADC Bioscientific Ltd. Hoddesdon, Hertz, EN11 0DB) with a light unit; ambient CO₂ was set at 360 ppm, air humidity at 60–70%, temperature at 25°C and photosynthetic photon flux density (PPFD) at 800 $\mu\text{mol m}^{-2} \text{s}^{-1}$. These conditions were selected because they yielded maximal photosynthetic rates. *In vivo* measurements were performed on intact needles with no visible symptoms of damage. After each measurement, needles were cut and put into plastic bags on ice until analysis of leaf area, which was performed with a numeric scanner connected to the software WinRhizo (Regent instruments Inc, Quebec, Canada). Net photosynthetic (PN, $\mu\text{mol}(\text{CO}_2) \text{ m}^{-2} \text{ s}^{-1}$) and transpiration (E, $\text{mmol m}^{-2} \text{ s}^{-1}$) rates were expressed on a needle area basis. The efficiency of photosynthetically active radiation utilization (PAR) was measured by quantifying photon flux density incident on needles surface area ($\mu\text{mol m}^{-2} \text{ s}^{-1}$). The efficiency of photochemical reactions driving photosynthesis was assessed by chlorophyll fluorescence measurements. Minimal fluorescence (F₀) yield was obtained upon excitation with a weak measuring beam from a pulse light-emitting diode, while maximal fluorescence yield (F_m) was determined after exposure to a 0.8 s saturating pulse [$>10.000 \mu\text{mol}(\text{photon}) \text{ m}^{-2}\text{s}^{-1}$] of white light. The maximal PSII photochemical efficiency (F_v/F_m) was then estimated as the ratio of variable (F_v=F_m–F₀) to maximal fluorescence.

Statistical analysis

Geographic and climatic variables with recognized influence on ecotypic variation of Mediterranean pines (Climent et al. 2008; Voltas et al. 2008) were selected (Tables 1,2) to calculate the transfer distance from the trial site to the corresponding seed source native site (Aitken et al. 2008;

Reich and Oleksyn 2008; Benito-Garzón et al. 2011). Correlations between measured (growth and survival) and seed sources (geographic coordinates, climatic variables and transfer distance) variables were determined based on the coefficient of *Pearson*. Seed sources from seed orchards (Alaquàs and El Serranillo) were excluded in these analyses (n=9) because they are grown from various sources. The Kaplan-Meier method (Kaplan and Meier 1958) was used to compare the survival functions of the different seed sources on a site basis. The statistics of LogRank, Breslow and Tarone-Ware were used to detect significant differences either among overall seed sources or for pairwise comparisons when significant differences appeared. The initial seedling height was not homogenous among seed sources (in spite of the nursery experimental design), which can affect field performance (del Campo et al. 2011). To control for this factor, initial height was introduced as a covariate in a Cox prediction hazard model together with the categorical predictor (seed source), resulting in this variable having no significant influence on the survival function. Regarding growth, differences among seed sources were tested on the RGR-H and the RGR-D, determined every two consecutive measurements, as well as the overall RGR value (last and first measurements). For this purpose, a multivariate analysis of variance (MANOVA) using the Wilks' Lambda statistic was performed on all RGR variables (height and diameter) as dependent variables and block and seed sources as factors. Follow-up analyses (ANOVA) were performed to assess whether differences among groups on the population means for each dependent variable were significant. ANOVA was also used to test for differences in physiological parameters. Post-hoc comparisons of means were made using Tukey's test. In all cases, data were examined for normality and homogeneity of variances and identified for any violations of assumptions. Variance components estimation was performed to partition

the phenotypic variance into its seed source, site and seed source by site components using the restricted maximum likelihood procedure for all the measured traits and was expressed as a percentage of variation explained by each fact.

Multivariate screening of seed sources was carried out to make additional comprehensive assessments of the overall performance. Canonical discriminant analysis was used to summarize the intra-site among seed sources variability on survival (mean survival time, first post-winter, first post-summer and final survival), growth (final height, final diameter and RGR for the first spring (1), first summer (2) and total period (T)) and physiological traits showing statistically significant differences between seed sources at the considered site according to ANOVA analyses. The multivariate generalized distances of Mahalanobis were computed later on the same variables used for the multivariate analysis and were used to cluster seed sources into groups with similar performance in terms of growth and survival (physiological traits not included, see the results section). The agglomerative hierarchical clustering procedure based on Ward's method was used. Data were analysed with SPSS© 17.0 at a significance level of p -value <0.05 for all analyses.

3.3. Results

3.3.1. Climate variation among sites

Climatic variables in the three experimental sites during the first and second years after planting showed some deviation from their historical means (Table 2), although the underlying hypothesis for this work was maintained that temperatures and precipitation in La Hunde were more suitable for the species than Granja d'Escarp (dry) and Tramacastiel (cold) (Gandullo and Sanchez-Palomares 1994) (Table 2). For instance, seedlings grown in the dry site of Granja d'Escarp received only 310 mm in 2011, which falls into the marginal value of precipitation for Aleppo pine (less than 10% of the Spanish populations of Aleppo pine grow in this threshold). Contrary, in the cold site of Tramacastiel, mean temperatures in the first year were considerably below the historical record, reaching a monthly average of lows of -3.2°C in January 2010 (0.16°C in March when plantation was finished), whereas summer drought was mitigated in this site.

3.3.2. Plantation performance

Decomposition of variance indicated that site was the most important factor affecting survival and growth traits (total variation attributed to site ranged from 84.6 to 99.8%). In contrast, seed-source and the seed sources by site interaction explained an extremely low proportion of the total variance (Table 3).

Table 3. Sources of variation and percentage of explained variation of the survival and the relative growth rate (RGR) (height (H) and stem diameter (D)) of eleven Aleppo pine seed sources planted in three sites using the restricted maximum likelihood method (REML). Survival was measured during the first spring, during and after the first summer, and during spring of the 3rd year. RGR-H and RGR-D were measured during first spring (1), first summer (2), second/third year (3), and overall (T, Total growth).

Traits	Components	1 st survival/ 1 st spring growth	2 nd survival/ 1 st summer growth	3 rd survival / 2 nd /3 rd year growth	4 th survival/ Total growth
Survival	Site (S)	97.7	98.1	98.4	99.1
	Seed source (S.S)	0.0	0.3	0.4	0.1
	S x S.S	2.3	1.7	1.3	0.8
RGR-D	Site (S)	94.4	99.8	91.9	92.1
	Seed source (S.S)	0.0	0.0	5.1	1.0
	S x S.S	5.6	0.2	3.0	6.9
RGR-H	Site (S)	99.8	96.9	84.6	92.4
	Seed source (S.S)	0.0	0.2	3.8	1.7
	S x S.S	0.2	2.9	11.6	5.9

Survival response

Seedling survival varied considerably among sites (Fig. 1). La Hunde seedlings had the highest final survival among sites (93%), with a mean survival time of 26.4 months over 28 and no significant differences in the Kaplan-Meier analysis. In the pairwise comparisons, however, the statistics differed significantly between the survival of 'Litoral levantino' (lowest survival at the end of this study, 85%) and other seed sources with final survival more than 93% (Fig. 1). Survival rate was maintained up to 94% throughout the first year (2010) at Granja d'Escarp site. However, survival decreased by the spring of 2012 (third year) to an average of 77%; the mean survival time in this site was 23.2 months out of 28 months. Survival among seed sources differed (p -value <0.01); 'Ibérico Aragonés' and 'H.S. El Serranillo' had lower survival (68%) than 'Litoral Levantino' and 'La Mancha' (84%) (Fig. 1). At Tramacastiel, survival decreased for all seed sources after the first winter of plantation (51%), and no additional mortality was observed during the first summer at this site. A further decrease till an average of 38% was recorded between October 2010 and May 2012. Differences among survival for seed sources were highly significant (p -value <0.001), with 'H.S. El Serranillo', 'Alcarria' and 'Levante interior' showing the highest survival after the first winter (about 60%) and 'Ibérico Aragonés' and 'Bética Septentrional/Sur' showing the lowest survival (about 35%) (Fig. 1). The mean survival time was 13.8 months out of 28 months.

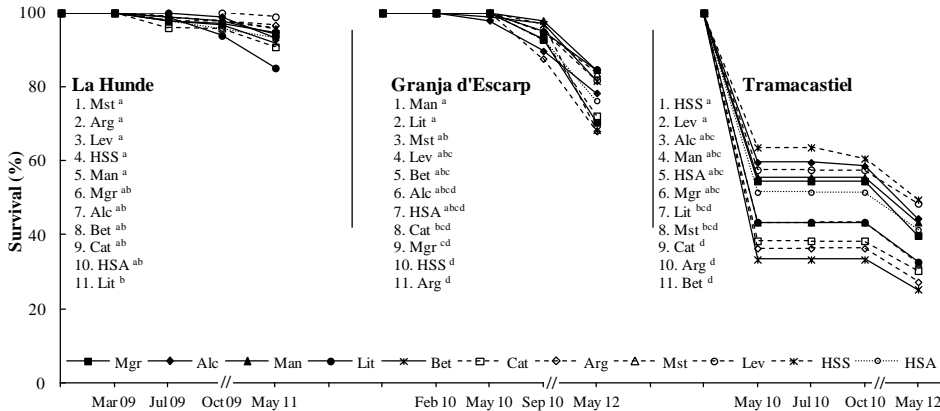


Fig. 1. Temporal survival of the eleven seed sources of Aleppo pine outplanted on the three trial sites. For each site, seed source abbreviations (see Table 1) followed by different letters indicate significant differences (p -value <0.05) in survival.

In general, ‘Levante Interior’, ‘La Mancha’ and ‘H.S. El Serranillo’, showed the best inter-site survival, whereas ‘Ibérico Aragonés’, ‘Bética Septentrional/Sur’ and ‘Cataluña’ showed the highest inter-site mortality. Significant correlations between climatic variables (Table 1) and survival were found in La Hundede and in Granja d’Escarp. In La Hundede, post-summer survival during the first year (2009) was positively correlated with altitude (p -value <0.01), negatively correlated with annual mean temperature (p -value <0.01) and negatively correlated with minimum daily temperature of the coolest month (p -value <0.01). Moreover, survival in May 2011 was negatively correlated with annual mean temperature (p -value <0.05). In Granja d’Escarp, latitude and survival in May 2012 were negatively correlated (p -value <0.05).

Growth response

Relative growth rates differed according to site and time (Fig. 2). During the first summer, RGR-H was greater on the coldest site,

Tramacastiel, than on La Hunde and Granja d'Escarp by at least $0.080 \text{ cm cm}^{-1} \text{ month}^{-1}$; for La Hunde and Granja d'Escarp, maximum RGR-H was reached during the first spring (RGR-H1: 0.038 and $0.020 \text{ cm cm}^{-1} \text{ month}^{-1}$ respectively), although, they were lower than those at Tramacastiel (RGR-H1: $0.057 \text{ cm cm}^{-1} \text{ month}^{-1}$). Consequently, RGR-HT at Tramacastiel was two to three times higher than La Hunde and Granja d'Escarp, respectively. RGR-D showed similar site and temporal patterns (Fig. 2). In the intra-site comparison of growth among seed sources (MANOVA), Wilks' Lambda statistic was significant ($p\text{-value} < 0.001$) for RGR-H and RGR-D in La Hunde and Granja d'Escarp, but not in Tramacastiel. ANOVA and Tukey tests showed that RGR-D differences were only due to the first spring rate (RGR-D1) at La Hunde and Granja d'Escarp and the RGR-DT (total) in Granja d'Escarp (Table 4, Fig. 2). Moreover, total growth rates (height and diameter) between 'Maestrazgo-Los Serranos' and 'Ibérico Aragonés' planted in Tramacastiel were significantly different. Thus, only height growth differed among seed sources. In La Hunde, the ranking was almost constant, with 'H.S. El Serranillo', 'H.S. Alaquàs' and 'Alcarria' showing higher performance (height and diameter growth) across time than 'Cataluña' and 'Ibérico Aragonés'. In Granja d'Escarp, temporal differences were less constant, although 'Bética septentrional/sur', 'La Mancha' and 'Maestrazgo-Los Serranos' showed better growth performance than 'Los Monegros Depresión del Ebro', 'Litoral Levantino', 'Cataluña' and 'Ibérico Aragonés'.

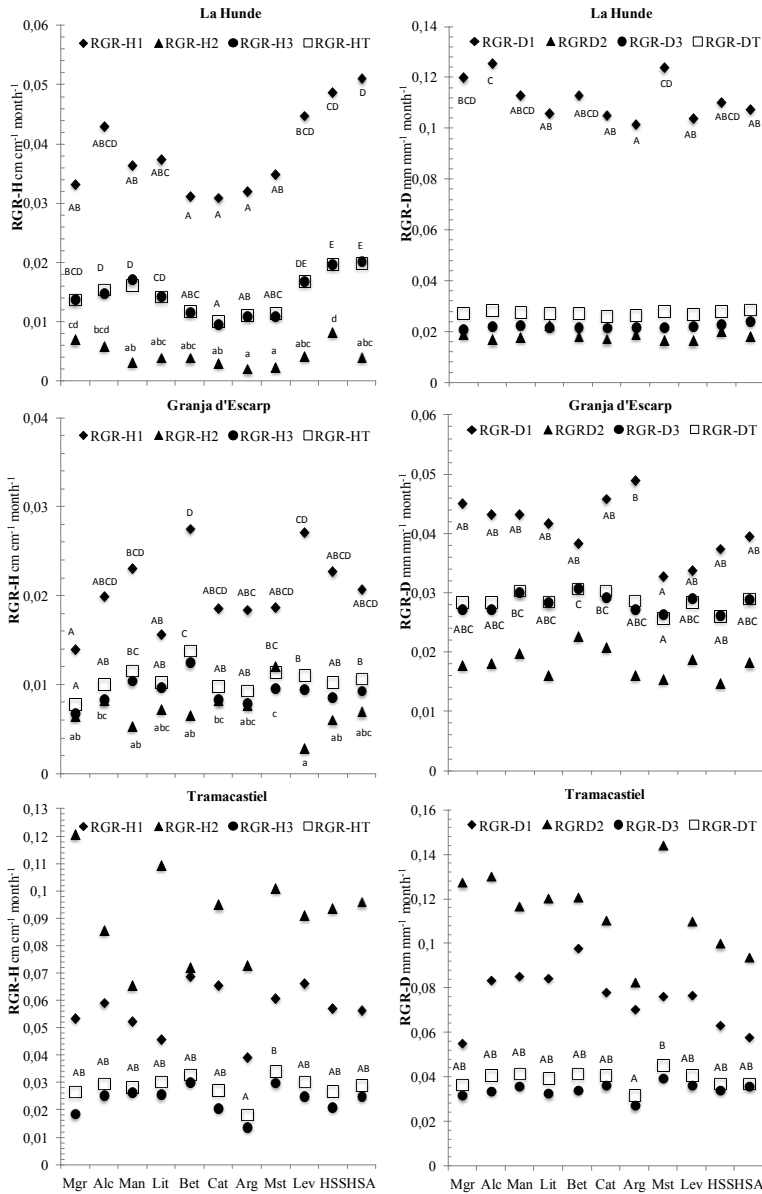


Fig. 2. Relative growth rates in height (RGR-H; $\text{cm cm}^{-1} \text{ month}^{-1}$) and diameter (RGR-D; $\text{mm mm}^{-1} \text{ month}^{-1}$) for the first spring (1), first summer (2), from first summer to 3rd May (3) and for the total study period (I) of eleven Aleppo pine seed sources out-planted on three contrasting sites. Within the same variable and site, different letters indicate significant differences at $p\text{-value} < 0.05$ (letters for RGR-H/D3 are not shown). Seed source abbreviations shown in Table 1.

Table 4. ANOVA summary (F-value, Mean Square Error (MSE), degrees of freedom of error (df) and significance: *p-value<0.05, ** p-value<0.01, *** p-value<0.001) of the relative growth rate (RGR) for height (H) or diameter (D) of eleven Aleppo pine seed sources planted in three sites. RGR-H and RGR-D were measured during first spring (1), first summer (2), second/third year (3), and overall (T, Total growth). ‡Homoscedasticity not achieved.

Site	Statistic	RGR-H				RGR-D			
		1 st spring	1 st summer	2 nd / 3 rd year	Total growth	1 st spring	1 st summer	2 nd / 3 rd year	Total growth
La Hunde	F-value	‡6.91***	‡5.59***	‡20.77***	‡23.78***	5.34***	0.78	1.27	1.45
	MSE(df)	6.2E-04(902)	5.2E-05(902)	5.1E-05(902)	4.0E-05(902)	1.2E-03(969)	3.1E-04(969)	4.4E-05(969)	3.7E-05(969)
Granja d'Escarp	F-value	‡4.83***	‡4.59***	5.23***	‡6.60***	2.22*	0.71	1.64	2.79**
	MSE(df)	2.9E-04(793)	9.8E-05(793)	3.3E-05(793)	2.6E-05(793)	8.6E-04(819)	6.3E-04(819)	1.0E-04(819)	7.0E-05(819)
Tramacastiel	F-value	0.48	0.95	2.08*	1.31	‡0.81	0.93	1.29	1.88
	MSE(df)	2.0E-03(180)	4.0E-03(180)	1.5E-04(180)	1.5E-04(180)	2.9E-03(181)	5.4E-03(181)	1.0E-04(181)	9.5E-05(181)

Significant correlations were found between climatic variables from Table 1 and growth on the three sites. In the core habitat, La Hunte, height growth during the first summer (RGR-H2) was significantly correlated with mean maximum daily temperature of the warmest month (p -value $<0.05^*$) whereas RGR-D2 showed opposite correlations to those found for post-summer survival in this site: altitude (-), annual mean temperature (+) and mean maximum daily temperature of the warmest month (+). In the marginal dry habitat of Granja d'Escarp, there were negative correlations between height growth and latitude (RGR-H1, p -value <0.05 ; RGR-H3 and RGR-HT, p -value <0.01) and height growth and longitude (RGR-H2, RGR-H3 and RGR-HT, p -value <0.05). In addition, diameter growth (RGR-D1, RGR-D3 and RGR-DT, p -value <0.05) was positively correlated with latitude transfer distance and negatively correlated with altitude and longitude transfer distance. In the marginal cold habitat of Tramacastiel, there was a negative correlation between height growth and latitude (RGR-H3, p -value <0.05) and between diameter growth and altitude (RGR-D2 and RGR-D3, p -value <0.05).

Physiological response

No significant differences were detected in the physiological variables among seed sources within each site (p -value >0.05). PSII maximal photochemical efficiency, estimated by the fluorescence parameter F_v/F_m , was unaffected by seed sources and ranged between 0.59 and 0.82; this ratio was more stable in Tramacastiel (0.79 to 0.82) than in La Hunte (0.59 to 0.70) and Granja d'Escarp (0.70 to 0.79). Regarding transpiration rates, water loss at date of measurement was higher in Tramacastiel ($3.39 \pm 0.3 \text{ mmol m}^{-2} \text{ s}^{-1}$) and La Hunte ($3.2 \pm 0.5 \text{ mmol m}^{-2} \text{ s}^{-1}$), while it was lower under the driest conditions of Granja d'Escarp ($1.09 \pm 0.2 \text{ mmol m}^{-2} \text{ s}^{-1}$). On average, PAR

was similar between Tramacastiel and La Hunde and lower in Granja d'Escarp. The net photosynthesis rate PN was similar among the three sites and ranged between 7.9 and 11.9 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

3.3.3. Multivariate screening of seed sources

Canonical discriminant analysis (CDA) was performed for each site on survival and growth traits (Fig. 3). In the three cases, the first canonical axis, Can1, had higher discriminant power (more than 50%) than the second one, Can2.

Seed sources outplanted at La Hunde were separated by Can1, accounting for 81.5% of the variability. RGR-H accounted for 84% of the variability whereas RGR-HT was responsible for 48% of the variability. A clear separation among seed sources can be observed according to this axis: 'Maestrazgo-Los Serranos', 'Cataluña', 'Bética septentrional/sur' and 'Ibérico Aragonés' are on the right side of the axis while 'H.S. El Serranillo' and 'H.S. Alaquàs', characterized by the highest growth rates, appeared on the opposite side. Separation on Can2 (10.3% of variability) was explained mostly by stem diameter variation (39.6%) from the whole variability ($r^2=0.87^{**}$), while RGR-H explained about 20% ($r^2=0.4^*$). 'Alcarria' and 'Maestrazgo-Los Serranos' manifested a high stem relative growth rates and appeared on the opposite side of 'Cataluña', and 'Ibérico Aragonés' seedlings.

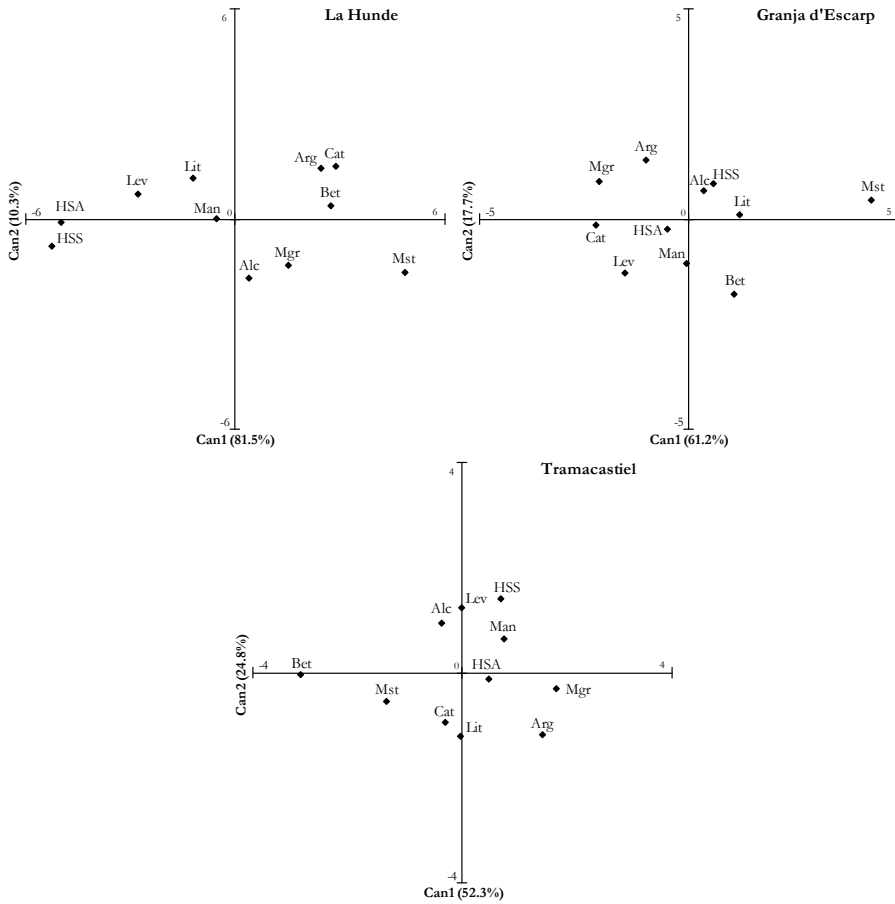


Fig. 3. The scatter-plot of the first and the second Canonical axes obtained by Canonical Discriminant Analysis on eleven Aleppo pine seed sources at the three trial sites in eastern Spain.

Can1 described around 61% of the whole variability of growth and survival observed in the dry and warm site of Granja d'Escarp. Around 46% of this variability was due to height growth (RGR-HT: 26% and RGR-H2: 19%), while 27% of the whole variability was due to survival variations among seed sources (survival time: 14.5% and rate: 13.5%). ‘Maestrazgo los Serranos’, ‘Litoral levantino’ and ‘Bética septentrional/sur’ manifested higher relative growth rates and appeared on the right of the axis, whereas

‘Cataluña’, ‘Los Monegros Depresión del Ebro’ and ‘Levante interior’, with lower performance, are on the left side. Can2 (17.7% of total variability) was described by total height relative growth rate (30%) and did not show a clear seed source separation.

At the cold Tramacastiel site, Can1 explained 52.3% of the total variability and was positively correlated to survival traits ($r^2=0.26$, 38% from 52.3% of variability) and negatively correlated to height ($r^2=-0.5^*$, 42% from 52.3% of variability) and stem relative growth rates ($r^2=-0.4^*$, 20% from 52.3% of variability). ‘Bética septentrional/sur’ seedlings had greater RGR-HT than ‘Los Monegros/Depression del Ebro’ and ‘Ibérico Aragonés’ seedlings. Can2 (24.8% of total variability) explained seedlings’ survival by 80% ($r^2=0.69^{**}$). ‘H.S. El Serranillo’ (high time and rate of survival) appeared close on the axis to ‘Levante interior’ and ‘La Mancha’ while ‘Litoral levantino’, ‘Cataluña’ and ‘Ibérico Aragonés’ seed sources characterized by low survival were opposite.

Dendrograms constructed using the Mahalanobis distances between pairs of seed sources revealed many similar associations (Fig. 4). Similar distances demonstrated by seed sources indicated similar performance of their seedlings in the site when measured variables showed significant differences. In the core habitat of La Hunde, seed sources are divided into two main groups. The first group was characterized by seed sources with higher height and moderate diameter growth (‘H.S El Serranillo’ and ‘H.S Alaquàs’) or by moderate height and higher diameter growth (‘Levante interior’, ‘Litoral levantino’ and ‘La Mancha’ subset). The second group was characterized by lower height growth and moderate diameter growth. ‘Alcarria’ and ‘Maestrazgo-Los Serranos’ emerged as single clusters in the second group, even though they had better survival and growth (high, moderate and low magnitudes are presented in Fig. 2).

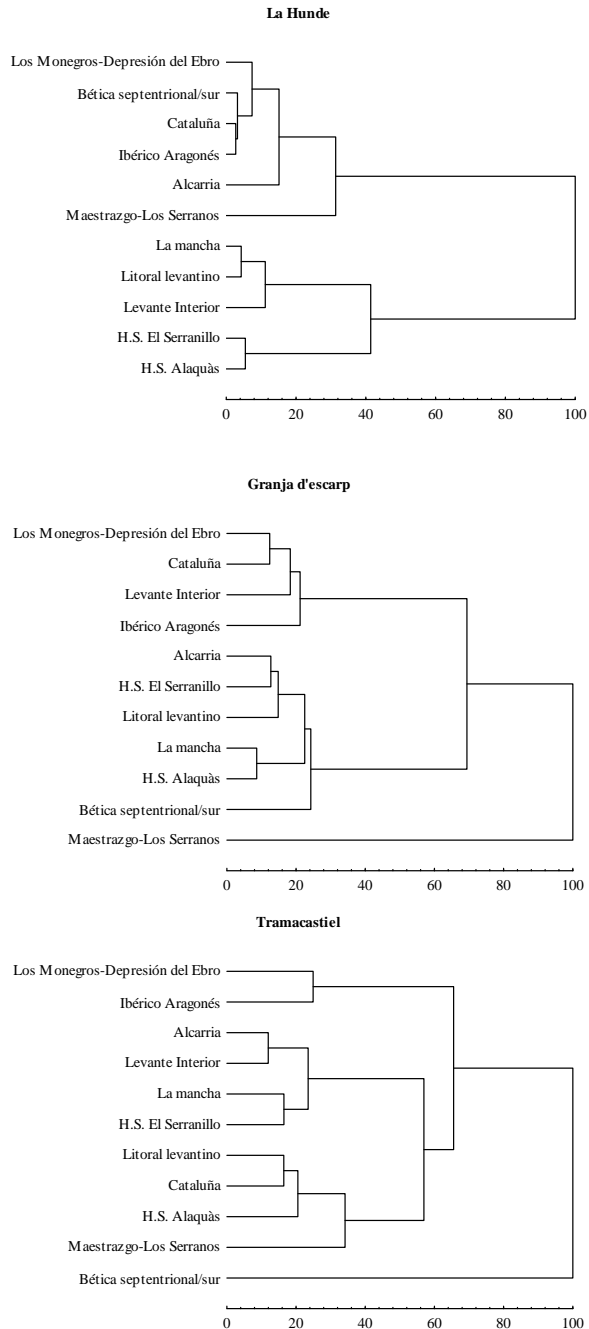


Fig. 4. Dendrograms based on Mahalanobis distances using clustering Ward's method among eleven Aleppo pine seed sources at the three trial sites in eastern Spain.

In Granja d'Escarp, 'Maestrazgo-Los Serranos' (good growth and survival scores) was separated from the other seed sources, leaving two groups. One group included seed sources with moderate to good performance ('Bética septentrional/sur', 'La Mancha' and 'Litoral levantino') and the other group included seed sources with bad to moderate performance ('Ibérico Aragonés', 'Los Monegros Depresión del Ebro' and 'Cataluña'). 'Levante interior', which had good survival and growth, appeared connected to the latter group, probably due to its low summer height growth.

In Tramacastiel four groups emerged. The first group comprised 'Ibérico Aragonés' and 'Los Monegros Depresión del Ebro' seed sources characterized by lower performance (survival and growth). The second set included 'Alcarria', 'Levante interior', 'La Mancha' and 'H.S El Serranillo', which manifested higher survival rates with good growth. The third set including 'Litoral levantino', 'Cataluña' and 'H.S Alaquàs' showed lower survival and growth than the previous group. The fourth cluster included the remaining seed source 'Bética septentrional/sur' which had the lowest survival rate in the site despite suitable growth.

3.4. Discussion

We analyzed the variability in growth and survival among eleven Aleppo pine seed sources and their phenotypic plasticity across three contrasting environmental sites in eastern Spain for subsequent assisted migration to expected climate change. Site was the most important factor affecting growth and survival reflecting that high phenotypic plasticity is widespread in this species. However, seed-source effects and the seed sources by site interaction explained low proportion of the variance, indicating a similar plastic response among seed sources. Phenotypic

plasticity allows species to live across a wider range of environments than those with stable phenotypes (van Kleunen and Fischer 2007; Aitken et al. 2008; Schiller and Atzmon 2009). Generally, tree species are considered to express moderate to high plasticity in their responses to environmental stress (Climent et al. 2008) and studies on some species suggest that plasticity can be highly trait dependent (Aitken et al. 2008; Benito-Garzón et al. 2011).

As expected, seedlings planted in its core habitat at La Hunde showed the highest final survival (93%) in contrast to seedlings grown at the two sites representing marginal habitats. Our results for Aleppo pine at La Hunde were similar to that previously reported for this area (del Campo et al. 2007a,b). Survival differences were minimal, although seed sources from colder and higher altitude provenances survived more (Tables 1 and 2). Growth difference among seed sources was significant at La Hunde, with trees from warmer provenances having faster growth rates than those from cooler provenances. Similar finding was found by Climent et al (2008). This trade-off between cold tolerance and growth potential has also been observed in different populations of other conifer species, such as *Pinus taeda* (Schmidting 1994) and *Pinus contorta* (Rehfeldt et al. 2002). *Pinus patula* populations differed significantly for seedling growth and exhibited a significant although weak altitudinal clinal pattern, with populations from lower altitudes having on average taller seedlings than populations from higher altitudes (Sáenz-Romero et al. 2011). Environmental stress response usually prompts plant cells to use energy that would otherwise be used for growth. So, surviving under stressful conditions usually results in a concomitant cost, that is, reduced growth (Aitken et al. 2008). Several descriptions of molecular or physiological processes show that a response to stress hinders growth and vice versa, both in model organisms and in plants (Mulet et al. 2006). In our case, provenances moved from slightly different

transfer distance metrics were the best performers. Longer growing seasons, as those observed in La Hunde in 2009 and 2011 (not shown), would have favoured seed sources from warmer provenances, although freezing events that likely occurred at this site could have decreased survival. In this sense, seed sources that were genetically improved (both seed orchards) or from this site ('Levante Interior') or from neighbouring regions with similar conditions ('La Mancha') could be the best choice for optimum reforestation results (early performance). Incidentally, these seed sources are grouped together according to their overall performance (Fig. 3,4). 'Litoral levantino' was also included in this group but survived poorly; thus, it is not recommended for this site.

The environmental conditions of the marginal dry site Granja d'Escarp led to a high differentiation among seed sources in survival and growth. In contrast to La Hunde, differences in survival among seed sources were related more closely to the ability of seedlings to cope with dry and warm conditions (Villar-Salvador et al. 2012). These responses could also be partially linked to latitude of seed sources (Table 1). Similarly, *Pinus sylvestris*, exhibits clinal trends associated with the latitude of seed origin (Persson 1994). Clinal variation in Aleppo pine in relation to latitude does occur (Agúndez et al. 1997; Climent et al. 2008). Growth rates were the lowest on this site. Our results show that growth performance of the seed sources were not opposite to that of survival (as occurred in La Hunde). In fact, correlations between performance and origin characteristics yielded the same result for either growth or survival: seed sources from lower latitudes performed best. This finding was expected as the distribution area of Aleppo pine in Spain presents a clinal increase of aridity towards the South, indicating that southern seed sources would perform better in drier and warmer sites (Ferrio and Voltas 2005; Aitken et al. 2008; Reich and Oleksyn

2008). In our data set, latitude and summer precipitation were significantly correlated (p -value <0.05 , not shown); moreover, in the specific harsh conditions of Granja d'Escarp (not representative from the larger Cataluña's provenance region, see tables 1 and 2) the home provenance survived and grew less than the other seed sources. Growth needs to be coupled to the functional traits allowing a plant to survive in water-restricted environments. As a consequence, seedlings may significantly reduce height and diameter growth as an adaptive strategy (Voltas et al. 2008). This is known to occur in other *Pinus* species, e.g. *P. sylvestris* and *P. taeda* (Cregg and Zhang 2001). Previous experiments involving the exposure of Aleppo pine to water stress resulted in decreased stem volume growth and aboveground biomass in association with decreased carbon assimilation rates (Villar-Salvador et al. 2012). Tognetti et al. (1997) reported similar results in Aleppo pine, with drought resistance (higher leaf conductance and transpiration rate) increasing from mesic to xeric sites of origin in water-stressed seedlings, but without geographical discrimination in well-watered seedlings. Less favourable growing conditions could enhance intraspecific differences between seed sources (Voltas et al. 2008). Populations are locally adapted when they have their highest relative fitness at their provenance sites and lower fitness in other parts of their range (Soto et al. 2010). Our results show that populations do not always present higher average fitness when grown under environmental conditions similar to those in their original provenance region. Multivariate grouping of seed sources at Granja d'Escarp is an example of how local provenances ('Cataluña', 'Ibérico Aragonés' and 'Monegros Depresión del Ebro') might be less suitable in environments expected to get drier and warmer due to climate change (Potter and Hargrove 2012; Williams and Dumroese 2013). Granja d'Escarp is characterized by lower precipitation (annual and summer) than the average

of its provenance region, a fact that could be the general case with climate change. Precipitation is the main factor influencing growth of pines species planted in semiarid Mediterranean conditions (Atzmon et al. 2004; Schiller and Atzmon 2009; Villar-Salvador et al. 2012).

In Tramacastiel, the high seedling mortality during the first winter, regardless of seed source, was important. This mortality was attributed to the minimal temperatures recorded immediately after outplanting in February (average: -3.2°C ; range -7.8 to 4.8°C) and March 2010 (average: 0.16°C ; range -7.2 to 4.1°C); such temperatures are known to affect establishment of Aleppo pine (Puértolas et al. 2005). Extreme winter temperatures cause frequent freeze-thaw events, which induce xylem embolism and frost drought (Mayr 2007). Our results indicate that a good choice of seed source can increase survival by 20%. Although no significant relationships were found between the environmental conditions of the seed source origin and survival on this site, ‘H.S. El Serranillo’, ‘Alcarria’ and ‘La Mancha’ yielded good survival and they are originally from continental areas with the lowest minimum temperatures during the coldest months. In fact, the ecological constraints of Tramacastiel (altitude, low winter temperature, continentality, etc) closely resemble those found for ‘H.S. El Serranillo’, ‘Alcarria’ and ‘La Mancha’ than those of the corresponding Aleppo pine region ‘Maestrazgo-Los Serranos’. In turn, growth differences among seed sources were minimal and correlations indicated reduced height growth (RGR-H3) with increasing origin latitude. A remarkable finding was the low mortality in the first summer coupled with very high RGR (H2 and D2), usually, mortality peaks and growth decreases for outplanted seedlings during the summer (del Campo et al. 2007a,b). Also, the sustained (long term) RGR-HT doubled and tripled that of La Hunde and Granja d’Escarpe, respectively. This is mainly attributed to the lack of summer drought and the subsequent

favourable growth conditions on this site thus pointing to its marked influence in the growth of the species (Matesanz and Valladares 2013). Ontogenetic variation might also contribute to the phenotypic variation of growth (Climent et al. 2008).

The previous knowledge (Agúndez et al. 1997; Atzmon et al. 2004; Climent et al. 2008; Voltas et al. 2008; Schiller and Atzmon 2009) on the geographic variation of *P. halepensis* has led to expect an ecotypic differentiation for survival and growth performance. Our results have shown an intraspecific variability in the expression of the measured traits: low survival differences along with high growth variation among seed sources were found in the core habitat against an opposite pattern in the marginal cold habitat. However, the harsh environmental conditions of the marginal dry habitat led to high differentiation for both survival and growth among seed sources. This is implying clearly the need to assist the migration of the species under predicted climate change conditions (Aitken et al. 2008; Williams and Dumroese 2013). Some traits might vary in their sensitivity to the environment being more plastic than others (Matesanz and Valladares 2013) as was the case in our experiment where not only phenotypic plasticity but also variation among seed sources was higher for the considered traits at each situation of core and marginal habitats. Ecological marginality can reduce ecosystem resilience under adverse climate conditions (Rehfeldt et al. 2002), although high phenotypic plasticity could be expected for species subjected to greater variation in precipitation and extreme temperatures (Sultan and Spencer 2002; van Kleunen and Fischer 2007).

We measured different physiological parameters in the experimental process, but we were unable to observe significant differences among seed sources on outplanting sites. This is probably due to the fact that these measurements were performed in late spring, when environmental

conditions were favourable across all sites. The maximal photochemical efficiency of PSII (F_v/F_m), which can be used to detect the occurrence of photo-inhibitory damage in response to environmental stresses (Maxwell and Johnson 2000), ranged among trial sites from 0.70 to 0.82, slightly lower than the values obtained by Demming and Björkman (1987) for optimum growth conditions (from 0.78 to 0.85). Maki and Colombo (2001) and Percival (2004) noted that photosynthesis and, as a result, growth were not negatively affected when F_v/F_m values were more than 0.6, which suggest that our seedlings were not adversely affected at time of measuring.

Lack of physiological differences among seed sources has led us to use survival and growth as the main variables for multivariate analysis and screening. Survival is regarded as one of the key variables when analysing tree provenance trials because it indicates the adaptability of the provenance to the environment at the trial site. It should be noted that survival reflects only the conditions experienced during the first years of the trial and not necessarily the climatic extremes and conditions that may be experienced during the life-span of a tree (Ræbild et al. 2003). Regarding growth, tree height is considered an important variable in the evaluation of species and provenances (Bariteau 1992); height may be seen as a measure of the adaptability of trees to the environment, such that tall trees are better adapted to the site than short trees (Ræbild et al. 2003). This interpretation need not always be true, however, as examples exist where the tallest provenances are suddenly stressed and subsequently die-off (McDowell et al. 2008). Stem diameter is often used as a measure of the productivity of stands because it is correlated to radial growth and wood production (Ræbild et al. 2003). By using these variables we were able to determine which seed sources, from different provenances, may have the highest chance to succeed in a reforestation programme under the selection pressure of future

climate change throughout the assisted migration. Population structure is expected to determine species behaviour (Benito-Garzón et al. 2011), in this case, the use of plasticity and genetic differentiation among populations for adaptive transfers and assisted migration would be critical to improve efficiency in reforestation programs under conditions of global warming.

3.5. Conclusions

Our results indicate that ecological constraints present on our trial sites drove the overall response of seed sources, with those belonging to regions of similar ecological (climate) constraints performing better, independently of the proximity or vicinity between trial site and provenance regions. The use of local seed sources or qualified seeds seems more reliable on sites where conditions of core habitat remain and are present (i.e. local seed sources are best under local climate conditions). As climatic conditions (temperatures and precipitation) shift to more marginal habitats, however, seed sources from climatically similar regions could perform better than local sources, at least in the short term. In this sense, current plantations in areas already affected by global change (high and consistent deviation from historical climate records) could have better establishment.

The differences in survival and growth found in our study among some seed sources at each site during the experiment indicate a high phenotypic plasticity within this species and that these parameters were partly conditioned by the specific genetic status of each seed source. At the same time, some variables did not differ significantly among other seeds sources, indicating that the differences in genetic constitution were masked by the effect of site conditions. However, seed source and seed source by site interaction indicated a similar plastic response among seed sources.

The results suggest that it is difficult to predict the performance of Aleppo pine provenances under different natural sites from their known performance at a single location. However, this study only encompassed a very limited and specific set of likely future scenarios for reforestation with Aleppo pine, that coupled with the variation within this species and local adaptation understanding, provide evidence to screen a broader spectrum of conditions in order to provide specific recommendations and to significantly improve the ability of the selected trees to survive under unfavourable conditions. Even though the use of seedlings from groups of performing seed sources, we can conclude that ‘Levante interior’ and ‘La Mancha’ seedlings showed the best overall response and may be used for reforestation in contrasting environments similar to those tested here. Seed sources from southern provenances are more specifically suited for sites adversely affected by climate change. Also, the present study clearly emphasises the need for broad selection programs for Aleppo pine on the molecular level. The genomic approaches are becoming necessary to confirm our findings and to aid in selecting climatically appropriate genotypes.

Supplemental material

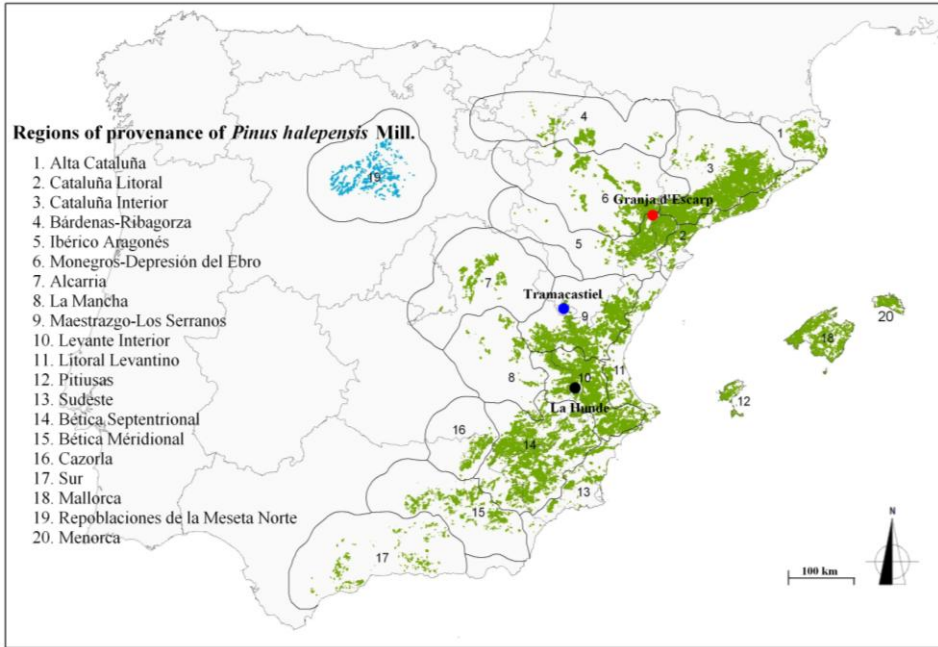


Fig. 1S. Regions of provenance of *Pinus halepensis* Mill. in Spain according to Alía et al. (2009). The three trial sites are indicated by bold colored points.

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Chapter two:

Addressing Genotype by Environment Interaction in *Pinus halepensis* towards Assisted Population Migration programs in response to climate change

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Abstract

Population assisted migration is becoming an important issue within the proactive adaptive silviculture oriented to global changes. However there is a considerable lack of information about genotype-environment interaction (GEI) and phenotypic plasticity in many non-commercial forest species, such as Aleppo pine, a key species in semiarid forest restoration programs. The objectives of this study were to (1) evaluate seed sources variation in their phenotypic plasticity and reaction norms in the broad context of GEI; and to (2) compare the efficiency of joint regression and Additive Main effect and Multiplication Interaction (AMMI) models in elucidating the seed sources adaptation pattern regarding height and diameter growth after out-planting in contrasting core and marginal habitats representing core-to-dry and cold-to-core drifts. Even though phenotypic plasticity was low, more plasticity was observed for height than for diameter along with differences in reaction norms for both traits. There were also significant environment, genotype and GEI effects. The AMMI model revealed higher capabilities than the joint regression analysis in determining seed sources adaptation across environments. Furthermore, specific adaptation to extreme environments was coupled with lower phenotypic plasticity. Besides, seed sources with higher plasticity performed better on the core conditions. 'Bética Septentrional' and 'La Mancha' seed sources suited more to the dry environment. However, 'Maestrazgo Los Serranos' seed source grew better under the cooler local conditions. 'Levante Interior' seed source was adapted to both drifts. These results make a significant contribution towards reforestation programs in the species, with practical implications for abiotic stress tolerance and assisted population migration in the context of climate change.

Keywords

Reforestation, Aleppo pine, seed sources, central and marginal habitats, relative growth rate, phenotypic plasticity, adaptation.

4.1. Introduction

Climate change is altering the environments in which all organisms develop with strong influence on the geographical distribution of plant species. Nevertheless its impact may vary significantly depending on the species' ability to adapt to the predicted climate conditions (de Luis et al. 2013). Plant species can adjust to these new conditions through phenotypic plasticity, adapt through natural selection or migrate to follow conditions to which they are adapted; these options are not mutually exclusive (Valladares 2008).

Aleppo pine (*Pinus halepensis* Mill.) is the most common pine species over the Mediterranean basin, where it plays an important role in forestry and in the conservation of the Mediterranean ecosystems. Its importance may also increase in the future due to the role of forest plantations for carbon sequestration in semiarid areas. In Spain, more than 85% of forest areas in the driest regions (south eastern) are dominated by this species (Agúndez et al. 1999). Similar relevance occurs in semiarid regions in Algeria, Morocco and Turkey (Pausas et al. 2004). Recent studies have shown the existence of geographically related trends in the intraspecific genetic differentiation of Aleppo pine, which indicates potentially large differences in abiotic stress tolerance among provenances (Agúndez et al. 1999; Voltas et al. 2008). This genetic diversity has to be considered in reforestation programs as establishing unsuitable seed sources may result in mal-adaptations, especially in cold and drought-prone sites (Zobel and Talbert 1984). However, the expected climatic change needs also to be considered, as reforestation guidelines focused on local seed sources may become irrelevant in the near future (Williams and Dumroese, 2013).

Population assisted migration could be needed in reforestation programs to select for the most tolerant genotypes and ecotypes to the

expected future abiotic stresses (Williams and Dumroese 2013; EFI 2009; FAO 2013). In this context, understanding the genotype by environment interaction is especially relevant for managing forest reproductive materials, as both its magnitude and its pattern have profound implications for breeding, screening, and seed deployment, particularly when using a broad range of genotypes and environments (Chambel et al. 2005). Either in locations (space) or time or both, the aim in breeding for wide adaptation is to obtain a genotype that performs well in nearly all environments. For specific adaptation, however, the intent is to achieve genotypes that perform well in a definite subset of environments within a target region (Baquedano et al. 2008).

Heterogeneous responses of species to climate variability across their range are directly connected to phenomena of local adaptation and phenotypic plasticity and are the basis of potential adaptability to future climate conditions (Valladares 2008). Climate change should in principle favour high levels of phenotypic plasticity (Rehfeldt et al. 2001). However, climate change involves simultaneous changes in two or more abiotic and biotic factors, which can be expected to impose restrictions on plastic responses to the environment (Valladares 2008). In a rapidly changing environment, narrowly adapted populations with low plasticity in important traits might undergo a high probability of extinction. However, little is known about the plasticity of many key plants, particularly of those of great longevity such as forest trees (Rehfeldt et al. 2001; Valladares et al. 2007).

The analysis of plasticity and adaptation, which also represents genotype reaction to environmental variation, has great potential for characterizing the relative performance of a genotype (families, hybrids, clones, etc.) under different environmental conditions (Viana and Cruz 2002). A common method to quantify populations' phenotypic plasticity

involves models of phenotype *versus* environment, where function parameters are taken as a measure of plasticity (Chambel et al. 2005). Norms of reaction are functions that relate phenotype and environment whose slope is phenotypic plasticity; they are of great interest as they deal with genotype by environment interaction in plant adaptation to dynamic environments, particularly in the context of global change and population assisted migration (Pigliucci 2005). In this sense, provenance trials are of fundamental importance when evaluating the phenotypic plasticity, the intraspecific variability in adaptive capacities and the patterns of genotype by environment interaction (GEI) across seed sources, thus guiding allocation of resources for reforestation improvement and conservation programs at a relatively low cost (Ræbild et al. 2003).

Only few studies treating genotype by environment interactions for *P. halepensis* have been recently reported through the analysis of plasticity in reproductive-vegetative relationships (Santos-del-Blanco et al. 2013) or in dendroclimatic response (de Luis et al. 2013). The present investigation is based on the understanding of the phenotypic plasticity of Aleppo pine vegetative traits under various growth conditions and the use of information about GEI structure, phenotypic plasticity and adaptation as a complementary selection strategy for identifying suitable seed sources for further introduction in specific areas in the context of assisted population migration toward predicted climate changes. The fundamental hypothesis of this work is that current core habitats for the species can shift towards more marginal habitats in which drier provenances could perform better than local seed sources (dry drift). Concomitantly, current marginal habitats for the species (high altitude belts) could be more suitable in the near future, as they became more central habitats (core drift). In this sense, it would be worth to know about the differences in provenance's response under probable low

temperatures and cold tolerance, as this is in fact the main risk in this species when provenances are moved away from their areas. Previous results on survival and growth have been presented in Taïbi et al. (2014).

Therefore, the main objectives of this study were (a) to identify the existence of ecotypic differentiation among *P. halepensis* seed sources under dry and core drifts, and (b) to determine whether different seed sources show divergence in their phenotypic plasticity for growth in response to abiotic stress. The expectation would be that seed sources will have some traits that are similarly plastic and some traits where the degree of plasticity varies among seed sources under the adverse conditions (van Tienderen 1997; Valladares et al. 2007).

To achieve these goals, we (1) evaluated seed sources variation in their phenotypic plasticity and the norm of reaction in the broad context of GEI and we (2) compared the efficiency of joint regression (Finlay and Wilkinson 1963) and AMMI (Additive Main effect and Multiplication Interaction model, Gauch 1992) in elucidating the pattern of the adaptation of eleven Aleppo pine seed sources regarding height and diameter growth under both drift situations; taking into account both the transplant shock (one year after planting) and the final establishment of seedlings at early growth stage (three years). This research could be valuable to address seed transfer zoning and to determine the suitable seed sources for reforestation programs and assisted population migration.

4.2. Material and methods

4.2.1. Plant material and trial sites

To achieve the aims of this study, eleven seed sources of *P. halepensis* covering most of the wide natural range of environments of this species in Spain were studied (table 1). The proposed assisted migration in this work analyses the drift from core habitat (La Hunde) to marginal dry habitat (Granja d'Escarp) and from marginal cold habitat (Tramacastiel) to core habitat (La Hunde) (table 1). The three study sites present similar soil characteristics (especially depth) and naturally regenerated Aleppo pine trees. General field methodology, survival rates and seed sources growth can be found in our previous work (Taïbi et al. 2014). Briefly, the control site of La Hunde (Ayora, Valencia) belongs to the central habitat of the species (Gandullo and Sanchez-Palomares 1994). In this area Aleppo pine forest is the most common vegetation type on the landscape. The Granja d'Escarp site (province of Lleida) represents the driest site and was chosen as a representative for the drift from current core to marginal dry habitats. This area has a dry continental Mediterranean climate, with cold winters and hot dry summers. The Tramacastiel site (province of Teruel) represents a current marginal cold habitat for the species that is becoming a core habitat (drift from marginal cold to core) and a more marginal habitat for other species such as *Pinus nigra* or *Juniperus thurifera*. The site is characterized by a continental mountain climate where frosts are common from November to April. Summer drought may be mitigated by altitude and storms.

Table 1. Trial sites and *Pinus halepensis* seed sources origin sites description (modified from Taïbi et al. 2014).

	ID	Coordinates	Altitude (m)	T _M (°C)	T _{MC} (°C)	T _{MW} (°C)	SP (mm)	AP (mm)
Seed sources								
H.S. Alaquàs ³	HSA	39°27'N-0°28'W	43	17.2	5	31.1	58	444
Litoral levantino ¹	Lit	39°45'N-0°18'W	200	16.5	4.8	30.3	64	599
Cataluña ^{2*}	Cat	41°46'N-2°46'E	333	13.9	1.3	29.3	128	714
Los Monegros- Depresión Ebro ¹	Mgr	41°53'N-0°52'W	401	14.2	0.9	32.3	79	399
Ibérico Aragonés ¹	Arg	40°47'N-0°04'W	555	14.0	1.5	30.2	91	497
H.S. El Serranillo ³	HSS	40°40'N-3°10'W	635	13.3	-0.4	33.0	46	380
Levante Interior ¹	Lev	39°05'N-1°12'W	673	14.4	2.6	30.5	67	524
Maestrazgo-Los Serranos ¹	Mst	39°47'N-1°11'W	763	13.6	1.4	30.0	93	546
Bética septentrional/sur ¹	Bet	37°55'N-2°55'W	789	14.5	1.9	31.9	55	410
La Mancha ¹	Man	39°55'N-2°14'W	856	13.1	-0.1	32.0	68	496
Alcarria ¹	Alc	40°24'N-2°24'W	869	12.4	-1.1	32.2	82	591
Trial sites								
La Hunde	HU	39°05'N-1°12'W	800	13.5	0.9	31	80	477
Granja d'Escarp	GE	41°23'N-0°20'E	310	14.2	1.0	31.3	47.7	353
Tramacastiel	TR	39°12'N-0°40'W	1150	11	-1.6	28.5	119	531

Note: T_M: annual mean temperature; T_{MC}: mean minimum daily temperature of the coolest month; T_{MW}: mean maximum daily temperature of the warmest month; SP: summer precipitation; AP: annual precipitation. Type of seed sources: 1, seed source (identified); 2, stand (selected); 3, seed orchard (qualified). *This is a composite seed source from Cataluña interior and Alta Cataluña seed sources.

On all sites the experimental layout was a randomized complete block design with nine blocks to alleviate site effects. Each seed source was represented by eleven trees per block and a total of ninety-nine trees per trial site. Planting was done in February 2009 in La Hundé, and in February 2010 in Granja d'Escarp and Tramacastiel. In each site, specific climate conditions during the study were characterized by a set of variables: Precipitation (P, mm); daily vapour pressure deficit (VPD, kPa); daily maximum, minimum and mean temperatures (T_{mx}, T_{mn}, T, °C) and monthly highest and lowest temperatures (TM_x, TM_n, °C). These variables were computed from records from the weather station network located in the vicinity of the sites. Further details on seed lots collecting, nursery growth, trial sites establishment and planting are described in Taïbi et al. (2014).

For all the seedlings, measurements of height and stem diameter at 1 cm above soil surface were assessed after the summer of the first year of outplanting (at the late growth period) in order to assess the post-planting shock stress effect, and in the spring of the third year of plantation to refer the final establishment. Growth response was expressed as the monthly relative growth rate in height (RGR-H) and diameter (RGR-D). Appending P or T to the RGR variable indicates the measurement period (P, post-planting and T, total). Analyses were conducted on RGR-H and RGR-D as growth variables using means of seed sources at the block level and by this way each seed source was represented by nine replicates for the considered growth variable in each environment.

4.2.2. Phenotypic plasticity and reaction norms

The proposed assisted migration was performed here on pair-sites; first to describe the drift from core to marginal dry habitat (La Hunte *vs.* Granja d'Escarp), then from marginal cold to core habitat (Tramacastiel *vs.* La Hunte). Due to this hypothesis (habitat drift), an analysis encompassing the three trial sites, even it was considered, is not shown in this paper.

The phenotypic plasticity of each growth trait (x : RGR-H and RGR-D) and seed source was estimated from the absolute difference (distance) in the values of x between two individuals of the same seed source grown in different environments using the relative distance plasticity index (RDPI) from Valladares et al. (2006). The RDPI is highly correlated with other indices of phenotypic plasticity commonly used in the literature (Valladares et al. 2006). A RDPI ranging from 0 (no plasticity) to 1 (maximal plasticity) can be obtained for each seed source as:

$$RDPI = \frac{\sum \frac{d_{ij \rightarrow i'j'}}{(x_{ij} + x_{i'j'})}}{n}, (1)$$

where n is the total number of distances. Each relative distance d was calculated as the absolute value of the trait distance between each two selected individuals i and i' of the same seed source belonging to two different environments j and j' , divided by the sum of their trait values. The overall standardized RDPI value, one per seed source and trait, is obtained by summing all relative distances obtained and dividing by the total number of distances.

Traits plasticity was also investigated by examining the reaction norms of seed sources under both drift situations. Reaction norms describe the set of phenotypes expressed by a single seed source across the different

environmental conditions (two in our case), and are representative of the amount of plasticity a genotype is able to express (Stearns 1992).

4.2.3. Genotype by environment interaction and adaptation

The existence of further ecotypic differentiation and specific adaptation for definite environments or drifts was also addressed. In this sense both the post-planting shock stress and the final establishment growth were considered and consequently, it resulted in four environments for each drift situation (2 sites x 2 assessments).

Usually, it is considered that GEI could be detected and quantified using two or more approaches together (Chahal and Gosal 2002) and thus, we used the commonly statistical methods applied to GEI in order to analyze adaptation patterns of the eleven Aleppo pine seed sources across the predicted drift situations: (1) analysis of variance, (2) concurrence or joint linear regression (Finlay and Wilkinson 1963), and (3) the additive main effects and multiplicative interaction (AMMI) model (Gauch 1992).

The percentage of variation explained by each one of the three factors (seed sources, environments and their interactions) was calculated with the variance components, assuming all the factors were random.

Analysis of variance

The *analysis of variance* (ANOVA) model is

$$y_{ij} = \mu + G_i + E_j + G_iE_j + \varepsilon_{ij}, \quad (2)$$

where y_{ij} is the mean of seed source i in environment j ; μ is the general mean; G_i is the mean of the i seed source; E_j is the mean of the j environment, G_iE_j is the seed source by environment interaction (GEI) component and ε_{ij} is the residual variation.

Joint regression analysis

The *joint regression analysis* is a simple linear regression that provides a conceptual model for genotype stability, being widely used in plant breeding. In this approach, the components of GEI are linearly related to environmental effects, which are represented by the average performance of all the seed sources tested for the trait under consideration (Finlay and Wilkinson 1963). The linear regression model is

$$y_{ij} = \mu + G_i + E_j + b_i E_j + \varepsilon_{ij}, \quad (3)$$

where y_{ij} is the mean of seed source i in environment j ; μ is the general mean; G_i is the mean of seed source i over all environments; E_j is the environmental index for environment j ($Y_{.j} - Y$); b_i is the slope of the regression specific for seed source i ; and ε_{ij} is the residual, which is assumed to be zero for the values averaged over replications. According to Finlay and Wilkinson (1963), the slope of regression was plotted against the mean RGR of the seed sources to determine the wide adaptability of each seed source. However, the interpretation has always to be associated with the seed source's RGR mean. When the regression coefficient of the seed source is 1.0 and the seed source has a RGR mean above the overall average, the seed source is well adapted to all the tested environments. If the mean RGR genotype is below the overall mean and the regression coefficient is 1.0, the seed source is poorly adapted to all environments. Regression coefficients above 1.0 indicate seed sources with a response above average to high performing environments. Regression coefficients below 1.0 provide a measure of below average responses to high performing environments. Because the regression is performed on the environmental mean, an ANOVA can be constructed according to Hardwick and Wood (1972) (for the joint regression analysis), where SS_{GEI} is partitioned into two

components related, respectively, to the heterogeneity of seed source regressions and the deviations from regressions (residual term).

Additive Main Effects and Multiplicative Interaction (AMMI)

The AMMI method uses the standard ANOVA procedure, separates the additive variance from the multiplicative variance (interaction), and then applies PCA to the interaction (residual) portion from the ANOVA analysis to extract a new set of coordinate axes which account more effectively for the interaction patterns following the model:

$$y_{ij} = \mu + G_i + E_j + \sum_{k=1}^n \lambda_k \alpha_{ik} \gamma_{jk} + \varepsilon_{ij}, \quad (4)$$

where y_{ij} is the observation on the i^{th} seed source in the j^{th} environment; μ is the overall mean; G_i is the i^{th} seed source effect; E_j is the j^{th} environmental main effect; n is the number of PCA axes considered in the model, λ_k is the eigen value of the n^{th} PCA axis and α_{ik} and γ_{jk} are scores for the i^{th} seed source and the j^{th} environment on the n^{th} PCA axis and ε_{ij} is the residual term which includes the experimental error (Gauch 1992). The SS_{GEI} was divided into n singular axes or main components of interaction (IPCA) that described the standard portion (effects of seed sources and environments) and the noise portion, which is the additional residue of unpredictable and not interpretable responses.

After selecting the AMMI model, the interpretation of wide or/and specific adaptation was accomplished by means of biplot representations (obtained by the combinations of the orthogonal axis of the IPCA's), which resulted in AMMI-2 biplot. This plots the interaction principal component axis (IPCA1) on abscissa and IPCA2 on ordinate for each seed source and site scores. The lower the IPCA value (absolute values), the lower its contribution to the GEI; therefore, the more stable the seed source. The ideal seed source is one with high performance and IPCA values close to

zero. An undesirable seed source has low stability associated with low performance (Gauch 1992). Seed source with an IPCA value close to zero suggests wide adaptation to the tested environments. A large seed source' IPCA reflects more specific adaptation to environments with IPCA score of the same size. The seed sources and environments with IPCA values of same sign show positive interaction suggesting specific adaptation of the seed sources in those environments. The reverse sign of IPCA value of seed sources and environments depicts negative interaction i.e. poor performance of seed sources in such environment (Chahal and Gosal 2002). Relationships of scaled IPCA scores of seed sources and environments and environmental variables at the collecting site of seed sources were investigated by simple correlation analysis.

Correlations between growth traits, the studied indices and the seed sources variables (geographic coordinates and climatic variables) were determined based on the coefficient of Pearson. Seed sources from seed orchards (Alaquàs and El Serranillo) were excluded in these analyses ($n = 9$) because they are grown from various sources. All statistical analyses were performed using the computing environment R (R Development Core Team, 2013).

4.3. Results

4.3.1. General performance

Mean RGR of both growth traits varied among seed sources in each paired-site combination (table 2). The highest RGR-H was manifested by both seed orchards and 'Lev' seed source in the dry drift and by 'HSA' and 'Lev' seed sources in the cold-to-core drift while the lowest values were expressed by 'Arg' and 'Mgr' seed sources either after the first year of out-planting or after the final establishment. For RGR-D, 'Alc', 'Bet' and 'Man'

seed sources performed higher values in the dry drift while ‘Bet’, ‘Mst’ and ‘Alc’ had the highest values in the cold-to-core drift. The lowest RGR-D values were manifested by ‘Arg’ and ‘HSS’ (table 2).

4.3.2. *Phenotypic plasticity*

Aleppo pine seed sources showed low values of the plasticity index, ranging from 0.07 to 0.35 for RGR-D and from 0.11 to 0.58 for RGR-H for both drifts. During the first year of out-planting, higher plasticity was observed for RGR-D (from 0.17 to 0.35) when compared to RGR-H (from 0.15 to 0.30) in the dry drift. On the contrary, plasticity was higher for RGR-H (from 0.30 to 0.50) than RGR-D (from 0.08 to 0.22) in the cold-to-core drift. High RGR-H plasticity was positively correlated with the origin mean temperature of the seed sources ($r=0.5$, $p\text{-value}<0.05$) in the dry drift and to summer precipitations ($r=0.5$, $p\text{-value}<0.05$) in the cold-to-core drift. In addition, it was correlated negatively with the daily mean maximum temperature of the warmest month ($r=-0.6$, $p\text{-value}<0.01$) of the seed sources origins in the core drift.

After the final establishment of the seedlings, ‘Bet’ seedlings manifested the highest values of RDPI while those of ‘Arg’ expressed the lowest ones (table 2). Besides, RGR-H plasticity was higher in both drift situations, noting that the higher RDPI values were recorded in the core drift. Both orchards manifested higher RDPI values for RGR-H in the dry drift; however they showed the lowest values of the index in the core drift for both growth traits (table 2).

Table 2. Mean performance, phenotypic plasticity and stability analyses of Aleppo pine seed sources as regard the two drift situations. RDPI indicates the relative distance plasticity index of Valladares et al. (2006). RDPI_p indicates the values of the index after the first year of the post-planting while RDPI_T indicates the final establishment.

Seed source	HU→GE (dry drift)				TR→HU (core drift)			
	Mean	bi	RDPI _p	RDPI _T	Mean	bi	RDPI _p	RDPI _T
Alc	0.0151	1.21 ¹⁰	0.21	0.26	0.0239	0.86	0.35	0.27
Arg	0.0119 ¹⁰	0.91	0.15 ¹¹	0.18	0.0173 ¹¹	0.82 ³	0.48 ³	0.20
Bet	0.0142	0.86 ³	0.20	0.16 ⁹	0.0240	1.11	0.55 ¹	0.58 ¹
Cat	0.0116 ¹¹	0.80 ¹	0.17 ⁹	0.12 ¹⁰	0.0231	1.31 ¹⁰	0.50	0.42
RGR-H HSA	0.0161 ²	1.13	0.19	0.37 ¹	0.0265 ¹	0.90	0.30 ¹¹	0.18 ¹¹
HSS	0.0169 ¹	1.32 ¹¹	0.22 ³	0.35 ²	0.0251	0.74 ¹	0.34 ¹⁰	0.18 ¹⁰
Lev	0.0157 ³	1.15 ⁹	0.15 ¹⁰	0.29	0.0264 ²	1.09	0.36	0.27
Lit	0.0132	0.88	0.30 ¹	0.23	0.0247	1.11 ⁹	0.40	0.32
Man	0.0143	0.85 ²	0.21	0.24	0.0211 ¹⁰	0.75 ²	0.34 ⁹	0.20
Mgr	0.0119 ⁹	0.90	0.23 ²	0.32 ³	0.0219 ⁹	0.97	0.35	0.26
Mst	0.0134	0.99	0.21	0.11 ¹¹	0.0257 ³	1.34 ¹¹	0.50 ²	0.46 ²
Alc	0.0371 ¹	1.14 ¹¹	0.25	0.10	0.0439 ³	1.09	0.17	0.17
Arg	0.0341	0.95	0.17 ¹¹	0.13 ²	0.0361 ¹¹	0.84 ³	0.21 ²	0.11 ¹¹
Bet	0.0355 ²	0.99	0.21 ¹⁰	0.10	0.0480 ¹	1.26 ¹¹	0.20	0.22 ¹
Cat	0.0342	0.91 ²	0.23	0.09 ¹⁰	0.0401	0.91	0.18	0.19 ²
RGR-D HSA	0.0345	0.96 ³	0.21 ⁹	0.10	0.0396 ⁹	0.84 ²	0.22 ¹	0.14
HSS	0.0335 ¹⁰	1.00	0.27	0.11	0.0381 ¹⁰	0.81 ¹	0.19	0.12 ¹⁰
Lev	0.0324 ¹¹	0.89 ¹	0.22	0.10	0.0407	0.94	0.08 ¹¹	0.19
Lit	0.0343	0.97	0.24	0.10	0.0424	1.01	0.13	0.17
Man	0.0355 ³	0.99	0.23	0.07 ¹¹	0.0416	0.99	0.13	0.15
Mgr	0.0354	1.11 ¹⁰	0.29 ²	0.14 ¹	0.0436	1.19 ¹⁰	0.10 ¹⁰	0.13
Mst	0.0338 ⁹	1.11 ⁹	0.35 ¹	0.11	0.0457 ²	1.12 ⁹	0.12	0.19

Mean: is the mean value for the two sites in each drift (including the two assessments within each trial).

* The exponent numbers above values indicate the seed source rank position in the considered scale of stability or of performance.

4.3.3. Norms of reaction

The graphic analysis of reaction norms revealed that the mean RGR's were not always parallel either during the first year of out-planting (Fig. 1) or after the final establishment (Fig. 2). During the first year, unlike the other seed sources, 'Bet', 'Mst', 'Man', 'Arg' and 'Cat' improved their RGR-H in the dry drift. Similar observation comes up for 'Alc', 'Man', 'Arg' and the seed orchards regarding RGR-D in the core drift (Fig. 1). At the end of monitoring, differences among seed sources appeared only under the dry drift: seedlings from 'Bet' improved their RGR-H while those from 'Cat' improved their RGR-D in contrast to the other seed sources (Fig. 2).

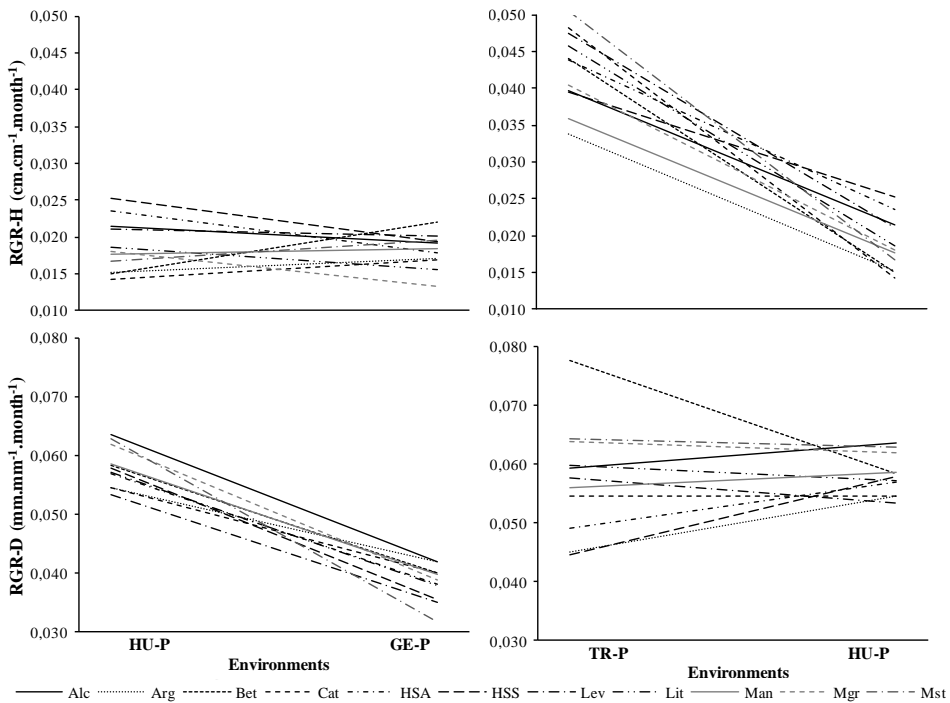


Fig. 1. Reaction norms for the the RGR-H and RGR-D of the tested Aleppo pine seed sources during the first outplanting year in three environments representing both drift situations. Values of the x-axis represent the mean value obtained for all seed sources at each site.

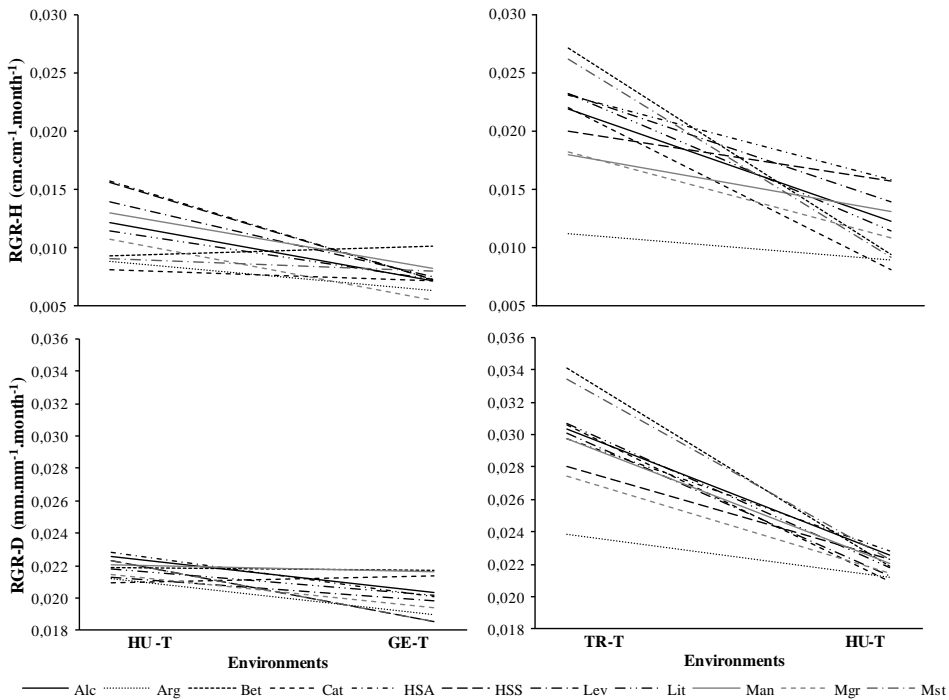


Fig. 2. Reaction norms for the RGR-H and RGR-D of the tested Aleppo pine seed sources outplanted three years in three environments representing both drift situations. Values of the x-axis represent the mean value obtained for all seed sources at each site.

4.3.4. Genotype by environment interaction (GEI)

Analysis of variance performed on both drifts showed that GEI effect was significant in RGR-H ($p\text{-value} < 0.05$) in the dry drift and for RGR-D in the cold-to-core drift. Most variation was attributed to the environment, which explained respectively 78% and 97% of the observed variability for RGR-H and RGR-D in the dry drift and about 90% for both traits in the core drift (tables 3 and 4), indicating that the tested environments were extremely diverse. The genotypic contribution was higher in the dry drift since it varied from 0.6% for RGR-D to 10.5% for RGR-H. However, it ranged between 3.7% and 4.5% for both traits in that order in the core drift. The interaction term (GEI) explained up to 11.6% of the total variance in

the dry drift (RGR-H) and up to 5.8% in the core drift (RGR-H). The regression term explained about 37% (in the dry drift) and 42% (in the core drift) for RGR-H along with 41% for RGR-D. The residual GEI was attributed to random deviations. The AMMI models have provided more detailed partitioning of the SS_{GEI} than linear regression since it accounted at least 94% with the two IPCA components (sum of IPCAs with significant differences, $p\text{-value} < 0.05$) while the regression model accounted no more than 44% of GEI terms, less than IPCA1 (50% vs. 44%) (tables 3 and 4).

Table 3. Analysis of variance, linear regression, and AMMI for Aleppo pine height of eleven provenances in the core-to-dry drift (Significance codes: 0 '***', 0.001 '**', 0.01 '*').

Sources	df	SS	MS	F value	p value	SS	MS	F value	p value
RGRH					RGRD				
ANOVA									
Gen	10	0.00117	0.00012	5.008	0.000 ***	0.00056	0.00006	0.740	0.687
Env	3	0.00868	0.00289	123.709	0.000 ***	0.09306	0.03102	410.776	0.000***
Gen:Env	30	0.00129	0.00004	1.840	0.005 **	0.00150	0.00005	0.664	0.913
Residuals	352	0.00823	0.00002			0.02658	0.00008		
Joint Regression									
Gen	10	0.00117	0.00012	7.468	0.000 ***	0.00056	0.00006	1.681	0.084
Env	3	0.00868	0.00289	184.485	0.000 ***	0.09306	0.03102	933.773	0.000 ***
Gen:Env	30	0.00129	0.00004	2.744	0.000 ***	0.00150	0.00005	1.509	0.046 *
Heterogeneity	10	0.00048	0.00005	3.039	0.001 **	0.00067	0.00007	2.008	0.031 *
Deviation	20	0.00081	0.00004	2.596	0.000 ***	0.00084	0.00004	1.259	0.204
Residuals	320	0.00502	0.00002			0.01063	0.00003		
AMMI									
Gen	10	0.00117	0.00012	7.468	0.000 ***	0.00056	0.00006	1.681	0.084
Env	3	0.00868	0.00289	28.807	0.000 ***	0.09306	0.03102	62.230	0.000 ***
Rep(Env)	32	0.00321	0.00010	6.404	0.000 ***	0.01595	0.00050	15.005	0.000 ***
Env:Gen	30	0.00129	0.00004	2.744	0.000 ***	0.00150	0.00005	1.509	0.046 *
IPCA1	12	0.00092	0.00008	4.89	0.000 ***	0.00089	0.00007	2.43	0.001 ***
IPCA2	10	0.00043	0.00004	2.74	0.002 **	0.00062	0.00006	1.86	0.049 *
Residuals	8	0.00004	0.00001	0.32	0.959	0.00002	0.00001	0.07	0.978
Residuals	320	0.00502	0.00002			0.01063	0.00003		

Table 4. Analysis of variance, linear regression, and AMMI for Aleppo pine height of eleven provenances in the cold-to-core drift (Significance codes: 0 '***', 0.001 '**', 0.01 '*').

Sources	df	SS	MS	F value	p value	SS	MS	F value	p value
RGRH					RGRD				
ANOVA									
Gen	10	0.00266	0.00027	1.867	0.048 *	0.00418	0.00042	3.321	0.000 ***
Env	3	0.05275	0.01758	123.679	0.000 ***	0.10405	0.03468	275.696	0.000 ***
Gen:Env	30	0.00341	0.00011	0.800	0.765	0.00571	0.00019	1.514	0.044 *
Residuals	352	0.05005	0.00014			0.04428	0.00013		
Joint Regression									
Gen	10	0.00266	0.00027	2.08	0.025 *	0.00418	0.00042	3.64	0.000 ***
Env	3	0.05275	0.01758	137.78	0.000 ***	0.10405	0.03468	302.32	0.000 ***
Gen:Env	30	0.00341	0.00011	0.89	0.633	0.00571	0.00019	1.66	0.018 *
Heterogeneity	10	0.00145	0.00014	1.13	0.335	0.00232	0.00023	2.02	0.030 *
Deviation	20	0.00197	0.00010	0.77	0.749	0.00339	0.00017	1.48	0.086
Residuals	320	0.04084	0.00013						
AMMI									
Gen	10	0.00266	0.00026	2.081	0.025 *	0.00418	0.00042	3.642	0.000 ***
Env	3	0.05275	0.01758	61.128	0.000 ***	0.10405	0.03468	146.605	0.000 ***
Rep(Env)	32	0.00921	0.00029	2.254	0.000 ***	0.00757	0.00024	2.062	0.000 ***
Env:Gen	30	0.00341	0.00011	0.892	0.633	0.00571	0.00019	1.660	0.019 *
IPCA1	12	0.00298	0.00025	1.95	0.028 *	0.00358	0.00029	2.60	0.002 **
IPCA2	10	0.00034	0.00003	0.27	0.987	0.00202	0.00020	1.76	0.046 *
Residuals	8	0.00009	0.00001	0.09	0.999	0.00012	0.00002	0.13	0.998
Residuals	320	0.04084	0.00013			0.03671	0.00012		

4.3.5. Adaptation analysis

The regression coefficients b_i calculated for each seed source showed the “response” or “sensitivity” to changing environments (table 2). Here, seed sources with higher RGR values, even for height or diameter, were associated to regression coefficients above the unit ($b_i > 1$). Seed sources with $b_i < 1$ (less responsive) in both growth variables were ‘Arg’, ‘Cat’ and ‘Lit’ in the dry drift along with ‘Arg’ and ‘Man’ in the cold to core drift. To determine adaptability, the slope of regression was plotted against the seed sources RGR’s mean (Fig. 3). ‘HSS’ seedlings in the dry drift, as well to ‘Mst’ seedlings in the cold to core drift, had higher RGR-H and higher slope than unity ($b_i > 1$), suggesting that these seed sources were well adapted to the good environments (HU). ‘Lit’ and ‘Man’ seed sources would be considered well adapted to the unfavourable environments respectively in the dry and core drifts as regards RGR-H. Concerning RGR-D, both orchards seem to be well adapted to the core habitat. Among seed sources near unity slope ($b_i = 1$) for RGR-H, ‘HSA’ and ‘Lev’ showed adaptability to most environments in the dry drift as was the case for ‘Lev’, ‘Lit’ and ‘Bet’ in the cold-to-core drift. By the same, for RGR-D, ‘Man’ and ‘Bet’ seed sources in the dry drift alike to ‘Mst’, ‘Alc’ and ‘Lit’ seedlings in the core drift were adapted to almost all the environments. On the contrary, ‘Cat’, ‘Mgr’ and ‘Arg’ showed $b_i < 1$ and low RGR-H’s in the dry drift alike to ‘Arg’ in the core drift, which means that they were poorly adapted to all the environments. Similarly, ‘Lev’ and ‘Arg’ seedlings were poorly adapted to all the environments respectively in the dry and the core drifts regarding RGR-D.

Seed sources' IPCA1 scores for RGR-H in the dry drift were correlated positively with the warmer temperature ($r=0.43$, $p\text{-value}<0.05$) and negatively with summer precipitation ($r=-0.43$, $p\text{-value}<0.05$) and altitude ($r=-0.4$, $p\text{-value}<0.05$). On the contrary, they were correlated positively with precipitation ($r=0.43$, $p\text{-value}<0.05$) and negatively with warmer temperature ($r=-0.48$, $p\text{-value}<0.05$) in the core drift. This indicated that these environmental variables induced height growth variation among the tested seed sources. As well, IPCA2 scores' were correlated positively with the longitudinal coordinates of seed sources' origins ($r=0.48$, $p\text{-value}<0.05$) in the dry drift, while in the core drift they were correlated negatively to longitude origins ($r=-0.63$, $p\text{-value}<0.05$) and positively with latitude ($r=0.67$, $p\text{-value}<0.05$) and altitude ($r=0.48$, $p\text{-value}<0.05$) origins, which indicates that the seed sources' separation in IPCA2 is related to their coordinates origins. As regards RGR-D, IPCA1 scores of seed sources were correlated positively with latitude ($r=0.3$, $p\text{-value}<0.05$) in the dry drift and altitude ($r=0.4$, $p\text{-value}<0.05$) in the core drift; while IPCA2 scores' were correlated positively with cooler temperature ($r=0.5$, $p\text{-value}<0.05$) and negatively with warmer temperature ($r=-0.4$, $p\text{-value}<0.05$) for both drifts.

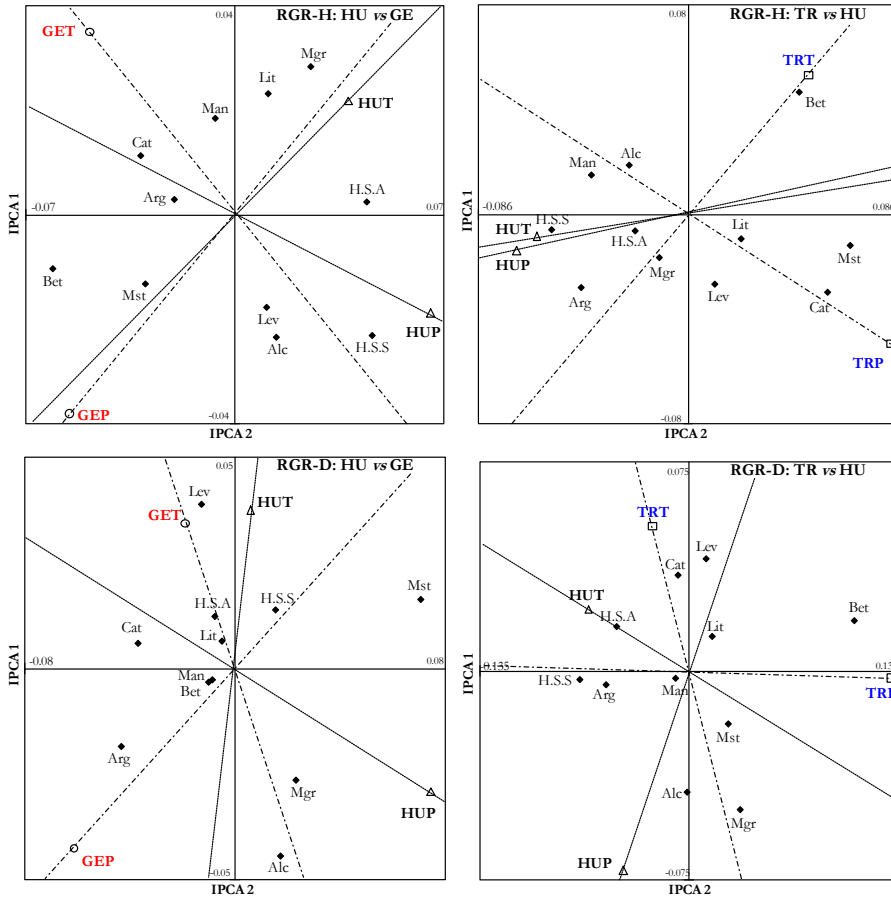


Fig. 4. AMMI2 biplot with the two first principal axes of interaction (IPCA1 and IPCA2) of RGR parameters of *Pinus halepensis* seed sources grown under different situations. The abbreviation indicates trial sites and seed sources as shown in Table 1.

Analysis of AMMI2 biplot generated on the basis of RGR-H indicated that dry and cold conditions had high contribution to the GEI as they were plotted on the opposite side of the core conditions (fig 4). A wide adaptation was observed for ‘Arg’ seedlings in the dry drift and for ‘Lit’, ‘Mgr’, ‘HSA’ and ‘Lev’ seed sources in the cold-to-core drift regarding their position closer to the zero with less contribution to the GEI. However, in the dry drift, ‘Bet’ and ‘Mst’ showed a large IPCA of the same sign to GE-P and

GE-T IPCAs' (drier conditions), similar to 'Cat' and 'Mst' in the colder site (TR-P). 'Man' is closer to GE-T IPCA', whereas HSS', 'Alc' and 'Lev' showed an opposite pattern, i.e. they were very sensitive to such conditions. Also, it is remarkable the positive relationship between 'Mgr' seed source and 'La Hunde' (HU-T), thus suggesting a specific adaptation to this core habitat. By the same, 'Bet' showed a specific adaptability to 'Tramacastiel' (TR-T) conditions. Seedlings orchards manifested positive interactions with 'La Hunde' conditions in both drift situations. As regards RGR-D, 'Man', 'Bet' and 'Lit' were closer to the zero in the dry drift much like 'Man' and 'Lit' in the core drift indicating their wide adaptation. 'Lev' and 'Cat' manifested a high contribution to the IPCA1 and explained a specific adaptability to TR-T. In addition, 'Lev' seedlings manifested good growth after the final establishment in the dry drift situation.

4.4. Discussion

The results presented in this work evidence a significant variation in growth performance among out-planted Aleppo pine seed sources depending on the environment, the genotype and their interaction (GEI). This finding reveals that matching genotypes and environments in assisted migration programs may require plasticity and adaptation analyses, as the relative phenotypic performance of genotypes may depend on the environment.

In general, the Aleppo pine seed sources tested in our study showed low phenotypic plasticity according to RDPI range established by Valladares et al. (2006); which is in accordance with previous results, ranging between 0.12 and 0.43 for physiological parameters of the same species under drought stress (Baquedano et al. 2008) or on other Mediterranean species such as *Quercus coccifera* (Balaguer et al. 2001), *Quercus robur* and *Fagus sylvatica*

(Valladares et al. 2000a, 2002). Balaguer et al. (2001), Gratani et al. (2003) and Valladares et al. (2002a and 2007) have suggested that the low phenotypic plasticity in Mediterranean forest trees constitutes an adaptation strategy to survive under the environmental stress frequent in the region.

The reaction norm was represented in an environment by phenotype plot, each line connecting the mean RGR value of a given seed source across environments (fig. 1 and 2). In our case, where only two environments were considered for each drift situation, the steepness of the slope indicated the level of plasticity for each seed source, thus showing genetic differences (Matesanz et al. 2010): differences were observed among the tested seed sources (separated lines with different slopes not always parallel) in both drifts during the first year of out-planting (fig. 1), with a significant environmental effect (the sloped lines indicated that the analysed growth traits were plastic) and significant interaction term (differently sloped lines indicated). Additionally, growth traits might differ in their sensitivity to the environment (Matesanz et al., 2010) as was the case in our experiment where not only reaction norms but also variation among seed sources were higher for RGR-H in the dry drift and for RGR-D in the cold-to-core drift, consistent with findings in different plant genera (Weiner et al., 2009 and references within). After the final establishment, difference among seed sources was manifested only under the dry drift where ‘Bet’ and ‘Man’ seed sources improved their RGR-H unlike the others (Fig. 2). This differential response reflects the particularity of their genetic response to environmental conditions’ shift between core and marginal dry habitats as was reported in similar studies of Balaguer et al. (2001) and Gratani et al. (2003). Variation in responses among seed sources was constrained in the marginal dry environment whereas differences were neatly expressed in the core site revealing the cryptic genetic variation of this species under drought (Santos-

del-Blanco et al. 2013). However, the opposite behaviour was observed in the cold-to-core drift where variation was more pronounced in the marginal cold habitat. Reaction norm diagrams are the most commonly used tool to visualize phenotypic plasticity; nevertheless this method measures only the amount of phenotypic plasticity and the existence of variations among genotypes, without measuring patterns (Valladares et al. 2007).

Either from the joint regression analysis or the combined ANOVA with AMMI, the environmental effect has shown an overwhelming influence on growth performance (tables 3 and 4). This means that the tested environments were extremely diverse and confirms previous findings on the environment as the major factor affecting Aleppo pine growth performance (del Campo et al. 2007). However, the significance of the GEI term justifies the need for investigating the nature of differential response of the seed sources to the experienced environments. The AMMI model identified two significant IPCA axes in our Aleppo pine drifts data and captured almost twice the SS_{GEI} captured by the joint regression analysis (94% vs. 44%). The statistical advantages of the AMMI model over the joint regression approach in dissecting GEI result is due to the removal of the additive parameter from the multiplicative component in the AMMI model, which overcomes one major statistical deficiency that is the dependency of the site and population indicators encountered in joint regression (Gauch 1992; Wu and Ying 2001). Consequently, AMMI model has been recommended to analyse complex GEI since it has been able to reveal the nature of interaction mainly if it captures more SS_{GEI} than joint regression (Nachit et al. 1992). Despite the above constraints, the joint regression analysis remains valuable for determining genotypic behaviour over environments since it demonstrates comparable results to the AMMI analysis and allows for the prediction of performance in untested environments (Barnes et al. 1984). It may also be

utilized to regress genotypes onto any other physically measurable feature of the environment (Matheson and Raymond 1986). Indeed it has been widely used in forestry experiments as in Clair and Einschmit (1986) for *P. abies* and in Dvorak and Ross (1994) for *P. tecunumanii* among others.

Plasticity and adaptation were different for RGR-H or RGR-D in many cases, as a consequence of contrasted tradeoffs between both growth rates while, interestingly, no significant effect of genotype on RGR-D variation was observed in the dry drift. Previous studies on some species suggested also that plasticity could be highly trait dependent (Namkoong and Kang 1990). Some traits might vary in their sensitivity to the environment being more plastic than others (van Tienderen 1997) as was the case in our experiment. The genotypic and the GEI effects were much higher for RGR-H than for RGR-D for both drifts. Besides, RGR-H plasticity was higher than RGR-D noting that the higher RDPI values were recorded in the core drift. In addition, the percentage of the explained variance of the genotypic and GEI effects was almost doubled in the dry drift compared to the core drift with reference to RGR-H. However, this relation was significantly inverted for RGR-D. Valladares et al. (2007) reported that limiting environmental conditions may induce differential plastic responses in tree species and influence features that may in turn affect plasticity for certain traits. Primary and secondary growth, represented here for height and diameter respectively, are dynamic processes resulting from a wide array of physiological processes and environmental conditions (Kozlowski and Pallardy 1997). In establishing seedlings, diameter growth is related to new root growth and is essential to ensure survival after transplanting. Root growth allows seedling to access soil water and nutrients and can be prioritized in the face of shoot growth as observed in seedlings of this species (Cuesta et al. 2010), a fact that might be enhanced under

limiting environmental conditions as both organs compete for the same resources. In our case, the results indicated lower variation among seed sources in diameter growth (coefficients of variation (CV) among seed sources at each site ranged between 2.8% and 9.4%) than in height growth (CV varying between 12.9% and 24.1%). This can be explained because the diameter of seedlings, being more sensitive to dry environmental conditions, is further fixed by the genetic control than height (Baquedano et al. 2008). Similar findings were reported by Harfouche (2000) on *P. pinaster* suggesting that the genotypic variation was more important in height than in diameter growth. It should be noted that height growth is usually considered as an important variable in the evaluation of species and provenances and it is a measure of the adaptability of trees to the environment, such that tall trees are better adapted to the site than short trees (Ræbild et al. 2003). Baquedano et al. (2008) suggested that selection by abiotic factors could drive genetic differentiation in the functional traits of populations occurring in contrasting environments.

The coefficient of regression (bi) was negatively correlated with the RGR's, i.e. the less plastic seed sources were those associated with relatively poor growth performance even for height or for diameter. Some seed sources appeared well adapted to favourable environments (RGR-H for 'HSS' and 'Mst') whereas other seed sources would be considered well adapted to the unfavourable environments (RGR-H for 'Lit' and 'Man' in the dry and core drift respectively). In addition, wide adaptation was also observed (RGR-H for 'Lev' in both drifts and RGR-D for 'Man' and 'Bet' in the dry drift and for 'Mst', 'Alc' and 'Lit' in the core drift). The differential responses of the tested Aleppo pine seed sources selected from a broad geographic area have reflected their differential adaptation strategies to natural environments of core and marginal habitats of this species which

reinforces the need of cautious selection of the seed source according to the target environments. This further reinforces the observation that productive seed sources were better adapted to productive sites, whereas less-productive seed sources performed better at poor sites (Wu and Ying 2001). Similar results have been also reported on *Picea abies* by Clair and Einschmit (1986).

According to AMMI analysis, the tested environments were more separated than the seed sources in the plot. In addition, marginal drier and cooler environments had higher contribution to the GEI in the opposite way in the biplot to the environments of the core habitat 'La Hunde' (fig. 4). This suggests that the significance of the tested drift from the core to the dry marginal habitat as well as from the cold-to-core habitat (Taïbi et al. 2014). Voltas et al. (2008) suggested that the less favourable growing conditions could enhance intraspecific differences between seed sources. Populations are locally adapted when they have their highest relative fitness at their provenance sites and lower fitness in other parts of their range. Our finding as regards to seed sources adaptation indicated that 'Bet' and 'Man' seedlings showed lower sensitivity to the post-planting stress shock and performed better later in the dry site 'Granja d'Escarp' indicating their specific adaptation to such conditions. In addition, these seed sources manifested the best RGR-H and RGR-D in the marginal dry habitat with good survival rates (Taïbi et al. 2014), although 'Man' grew less and 'Bet' died more in the marginal cold habitat (Taïbi et al. 2014). We should notice that 'Man' and 'Bet' seed sources manifested a wide adaptation regarding RGR-D in both drifts. This finding, in which seed sources from low latitudes performed better under dry conditions, was expected as the distribution area of Aleppo pine in Spain presents a clinal increase of aridity towards the South,

indicating that southern seed sources would perform better in drier and warmer sites (Taïbi et al. 2014 and references within).

An important finding is that Aleppo pine specific adaptation to adverse environmental conditions was coupled with low phenotypic plasticity responses either during the first year or after the final establishment. In the cited work, ‘Lev’ showed good overall performance in terms of survival and growth in the three sites, although it should be noted that its height growth was somewhat inhibited during the first year of out-plantation in ‘Granja d’Escarpe’ (Taïbi et al. 2014). This later seed source was characterised by medium plasticity within the range of RDPI observed values and manifested a wide adaption to all environments in both drifts according to the GEI analysis (fig. 4). High levels of phenotypic plasticity across a range of environments are usually conferred by the ability to plastically adjust the phenotype to each set of conditions and subsequently it is considered as a generalist adaptive strategy (Balaguer et al. 2001). However, the specific adaptation, which leads to ecotypic differentiation (Baquedano et al. 2008), involves a reduced phenotypic plasticity of populations limited to a restricted distribution range or to a particular habitat which exhibit narrow tolerance to environmental changes (van Tienderen 1997). Additionally, as it was expected, low plasticity in seed sources originating from adverse conditions (southern) was coupled with specific adaptation to cope with extreme abiotic stress (van Tienderen 1997; Valladares et al. 2007). Gratani et al. (2003) observed also in *Q. ilex* plants selected from different Italian provenances that seedlings from more xeric provenances had higher tolerances to drought. In addition, many authors have proposed that plant specialization in favourable environments is supposed to be linked to an increased plasticity, whereas the reverse can be expected for the opposite circumstances (Balaguer et al. 2001; Valladares et

al. 2000b, 2007). This finding supports the idea that specialization to adverse environments is linked to phenotypic stability and a conservative resource-use strategy, even when resources are temporarily abundant, in order to avoid the production of a plant too large or structures too expensive to be sustained once conditions deteriorate (Baquedano et al. 2008).

It should be stressed that early growth assessments for analysing ecotypic differentiation, phenotypic plasticity and seed sources adaptation' may not be reliable for assessing GEI at maturity, which implies the need to evaluate GEI at mature ages in forest trees (Chambel et al. 2005). In addition, deep studies at the molecular level on the relationship between adaptive traits and environments are required to delineate seed zones and achieve advanced understanding for more *P. halepensis* populations' reforestation and restoration in specific environments in the context of assisted population migration toward climate changes. However, the transfer of seed sources to the tested environments should be carried out with caution and carefully managed from the genetic conservation point of view.

4.5. Conclusion

Aleppo pine seed sources showed in general low phenotypic plasticity coupled with higher plastic response for RGR-H than for RGR-D. Nevertheless, plasticity was higher in the core drift in comparison to the dry drift. In addition, there were differences in seed sources reaction norms and their responses were not always parallel. However, the differential responses of the tested Aleppo pine seed sources from a broad geographic area reflect their differential adaptation strategies to core and marginal habitats represented here by dry and cold environments.

Significant variation existed for environment, seed sources and their interaction along with larger portion of the total sum of squares captured by environment and GEI, which reveals the influence of these factors in evaluating field performance of pine seed sources.

Either from the ANOVA, the joint regression analysis or the combined ANOVA with AMMI, the environmental effect has shown an overwhelming influence on growth performance. However, the significance of the GEI term justifies the need for investigating the nature of the seed sources growth differential responses to the assisted migration strategy of the species within its natural range of distribution with particular interest to core and marginal habitats specificity.

The AMMI model has been recommended to analyse complex GEI since it has been able to offer more information than joint regression bringing out the suitability of specific genotypes to specific conditions through the biplot. Furthermore, as it was expected, Aleppo pine specific adaptation to adverse marginal environmental conditions was coupled with low phenotypic plasticity responses either during the first year or after the final establishment of seedlings.

In this sense, 'Bet' and 'Man' seed sources showed specific adaptation to the dry environments. However, 'Mst' seedlings showed its local adaptation to the cooler conditions of 'Tramacastiel'. Nevertheless, seed sources with high phenotypic plasticity performed better in the core habitat. 'Lev' seed source manifested a wide adaption to all environments in both drifts situations.

These results were concomitants with those of field performance comparison among seed sources, which indicates the capabilities of our analysis as a complementary selection strategy to field performance screening for identifying the suitable Aleppo pine seed sources to the target

environment drifts. This study makes a contribution towards reforestation and breeding research programs in the species, with practical implications for abiotic stress tolerance and assisted population migration in the context of climate change.

4.6. References

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Chapter three:

On the Assisted Population Migration in *Pinus nigra ssp. salzmannii*: early plantation performance and Genotype by Environment Interaction of different seed sources in contrasting ecological sites

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Article submitted

Abstract

Forest ecosystems are likely to shift faster with climate change than their maximum natural rate at which they can migrate and establish. Population assisted migration constitutes an important issue within the proactive adaptive silviculture for climate change when carrying out reforestation programs or for conservation purposes. The objectives of this study were to (1) evaluate plantation performance and phenotypic plasticity of six seed sources of Salzman pine outplanted in three trial sites, testing a hypothetical northward migration and (2) compare the efficiency of joint regression and Additive Main effect and Multiplication Interaction model (AMMI) in elucidating the pattern of the adaptation of seed sources in each site. Seed sources have manifested low phenotypic plasticity, coupled with higher plastic response for height than for diameter, and many differences in survival and growth performance within and among sites. Seedlings outplanted in better site conditions did not differ in survival but in height growth; however, under climatic constraints, survival differed among seed sources and diameter growth was high. Among seed sources, the general best performers were 'H.S. El Serranillo' and 'Sistema Ibérico Meridional b'. The results support the hypothesis that northward migration of seed sources makes sense within limited transfer distances, and this was reinforced by the genotype by environment interaction (GEI) results. The AMMI models have provided details to analyse complex GEI since they have been able to reveal the nature of interaction through the biplot. The present study makes a contribution towards reforestation and breeding research programs in this species, with practical implications for abiotic stress tolerance, assisted population migration and adaptive silviculture for climate change.

Keywords

Black pine, Salzman pine, genotype, survival, growth, phenotypic plasticity, adaptation, adaptive silviculture, reforestation.

5.1. Introduction

Climate change is one of the major challenges that may aggravate many threats to forest ecosystems and biodiversity in the next future since it is expected to drastically modify growing conditions for trees (Herrero et al. 2013). Greatest impacts are expected in the Mediterranean region, as transitional climatic zones between arid and humid regions of the world, where temperature is expected to increase between 2 and 5°C while precipitation is expected to decrease till 30% mainly in summer and autumn over the next century (Garrido et al. 2012). Nevertheless, climate change impacts on plant species may vary significantly depending on the species' ability to adapt to the predicted conditions (Sánchez-Salguero et al. 2013). Plant species respond to these new conditions through phenotypic plasticity, adapt through natural selection or migrate to follow conditions to which they are adapted giving that these options are not mutually exclusive (Willians and Dumroese 2013).

The rapidity of climate change, however, is expected to exceed natural migration rates and the expected northward migrations of plant populations could be subjected to the recurrence of extreme events and associated growth declines and mortality events (Linares and Tíscar 2011). Moreover, many tree species reach their southern distribution limit in the Mediterranean basin where drought constrains their ecosystem structure and function (Herrero et al. 2013). The theoretical limit between these species should quickly shift in elevation and in latitude from one vegetation stage to another with climate changes (Aitken et al. 2008). In forestry in particular, human-assisted population migration is proposed to counter long generation cycles and modest dispersal abilities of forest trees through selection for the most tolerant genotypes and ecotypes in reforestation programs to the expected future abiotic stresses (Willians and Dumroese 2013; Taïbi et al.

2014). However, this is in fact an important knowledge gap for the deployment of operational programs in adaptive silviculture (EFI 2009; FAO 2013).

Pine (*Pinus* spp.) has been extensively used for reforestation and land restoration in the Mediterranean regions and other parts of the world because it is stress tolerant, has pioneer features, and facilitates the long-term development of late-successional hardwoods (Pausas et al. 2004). Black pine (*Pinus nigra* Arnold) is one of the most important pines for mid to high elevation sites in southern Europe and especially the Mediterranean basin. The Salzmann pine (*Pinus nigra* ssp. *salzmannii*) is an endemic subspecies of black pine native to the Western Mediterranean basin and covers extensive areas of 350 000 ha in Spain (Rubio-Moraga et al. 2012). However, the species is expected to be severely affected by the impacts of global change (temperature increase, irregularity of precipitation, drought, fire...), so its regeneration, growth and mortality are seriously jeopardized in many ecological contexts (Banquet et al., 2013; Tiscar and Linares, 2011). Salzmann pine exhibits a high genetic diversity among populations most probably due to the geographic isolation and the fragmented (sub-) Mediterranean distribution of populations (Rubio-Moraga et al. 2012). Differences among provenances and populations, most likely, display differences in their phenotypic plasticity and adaptive capacity and at this point, provenance trials could reveal a diverse performance of provenances from various geographic origins (Varelides et al. 2001; Seho et al. 2010). This genetic diversity has to be considered in reforestation programs in a new context of changing climate, as guidelines focused on local seed sources may become irrelevant in the near future (Aitken et al. 2008 Breed et al. 2013; Williams and Dumroese 2013).

In this context, to what extent Salzman pine populations can be migrated so they are less sensitive to climate change and how they can be used in declining black pine plantation forests constitute serious forest management questions. At this point, besides populations survival and growth performance' assessment, understanding the phenotypic plasticity, adaptation and genotype by environment interaction are especially relevant for managing forest reproductive materials, as both their magnitude and their pattern have profound implications for breeding, screening, and seed deployment, particularly when using a broad range of genotypes and environments (Potter and Hargrove 2012). Even if the need to analyse Salzman pine for plantation in the Mediterranean region is seriously considered (Amodei et al. 2012), conservation issues have to be addressed in priority in order to develop effective reforestation policies and to cope with the new environmental cues from climate change (EFI 2009; United Nations et al. 2011; FAO 2013).

In our study, we hypothesized that Salzman pine seed sources from contrasted regions along its natural distribution in Spain, exhibiting local adaptations and ecotypic differentiation, will differ in their response (survival and growth) or adaptive capabilities when planted beyond their provenance region on contrasted trial sites reflecting different ecological constraints, considering a northward migration toward the environmental change. Six seed sources of Salzman pine (i.e. plant material from which forest reproductive material is derived) from three Spanish ecological regions (Alía et al. 2009) were tested in three contrasting trial planting sites in the distribution range of the species over three years. Considering the outlined intraspecific variability in performance and the potential differences in the response to climate, it is important to identify whether certain provenances of Salzman pine in Spain are better adapted to the current climate

conditions beyond their provenance regions. Such provenances might be also potential candidates for the substitution of indigenous conifer species suffering under climate change (Thiel et al. 2012). To achieve these goals, we (1) have evaluated inter and intra-site seed sources variation in their survival, growth and phenotypic plasticity and we (2) have compared the efficiency of joint regression (Finlay and Wilkinson 1963) and AMMI (Additive Main effect and Multiplication Interaction model, Gauch 1992) models in elucidating the pattern of the adaptation regarding height and diameter growth, taking into account both the transplant shock (one year after planting) and the final establishment of seedlings at early growth stage (three years). Selecting seed sources of higher performance (survival and growth) considering their phenotypic plasticity and adaptation through genotype by environment interaction studies was previously tested on *Pinus halepensis* (Taïbi et al. 2014) and this research approach could be valuable to address seedling adaptive transfer in ecological restoration and to determine the suitable seed sources for reforestation programs and assisted population migration.

5.2. Experimental methodology

5.2.1. Plant material

Six seed sources (basic materials) of *Pinus nigra* ssp. *salzmannii* covering most of the wide natural range of this species in Spain were selected. These seed sources correspond to five Spanish seed sources zones defined for the species (Alía et al. 2009) and one seed orchard (Table 1; Fig. 1). They can be grouped within three main provenance groups according to Alía et al. (2009): Mediterranean warm sub-humid (Alto Maestrazgo), cold continental (Sistema Ibérico Meridional, El Serranillo and Soria) and warm sub-dry (Béticas). Seeds were collected as open-pollinated bulk populations from

selected stands and grown as one-year-old container stock in a public nursery located in Centro Nacional de Mejora Genética Forestal de Alaquàs, Valencia (Spain) in 2009. Seedling lots were grown as one-year-old container stock under the standard conditions for the species (shade-house, 33 weeks, 200 cm³ container, sphagnum peat and a 15-4-7.5 slow release controlled fertilizer added at 7 g l⁻¹). During nursery culture, the different basic materials were located following a randomized block design with four replicates in order to avoid important differences in stock quality associated to microsite differences in the nursery benches.

Table 1. Site descriptions for the six seed sources of *Pinus nigra* ssp. *salzmannii* (from Alía et al. 2009). T_M : annual mean temperature; T_{MC} : mean minimum daily temperature of the coolest month; T_{MW} : mean maximum daily temperature of the warmest month; DM: drought months; SP: summer precipitation; AP: annual precipitation. The climatic variables shown for the trial sites are from both the historical value and the study period.

Seed source ^(class)	ID	Coordinates	Altitude (m)	T_M (°C)	DM	T_{MC} (°C)	T_{MW} (°C)	SP (mm)	AP (mm)
H.S. El Serranillo ¹	HSS	40°40'N-3°10'W	685	13.3	3.0	-0.4	33.0	65	504
Alto maestrazgo ²	MST	40°51'N-1°30'W	964	11.9	1.2	0.4	27.0	129	679
Soria ²	SOR	41°65'N-2°85'W	1094	9.7		-2.7	28.4	110	686
Sistema Ibérico Meridional b ² SMB		39°50'N-2°00'W	1100	10.7	1.8	-2.0	29.3	109	700
Sistema Ibérico Meridional a ² SMA		40°02'N-1°58'E	1215	10.8	1.8	-2.7	29.8	104	726
Cordilleras Béticas ²	BET	37°55'N-2°55'W	1350	11.8	2.7	-0.1	30,4	73	830
Trial sites									
Berga	BG	42°3'N 1°48'E	630	11.6		-2.1	28.8	239	750
2010;2011/12				10.3;12.1		-1.2;-1.1	28.6;28.0	322;358	944;793
Xert	XT	40°53'N 0°2'E	700	13.6		2.3	27.6	114	665
2010;2011/12				15.4;14.7		0.3;2.1	29.4;27.8	112;52	410;527
Tramacastiel	TR	39°12'N-0°40'W	1150	11		-1.6	28.5	119	531
2010;2011/12				10.3;11.9		-3.2;-1.8	30;29.5	162;94.1	586;371

Note: Class of seed source: 1, seed orchard (qualified; improved from Sistema Iberico Meridional); 2, stand (selected).

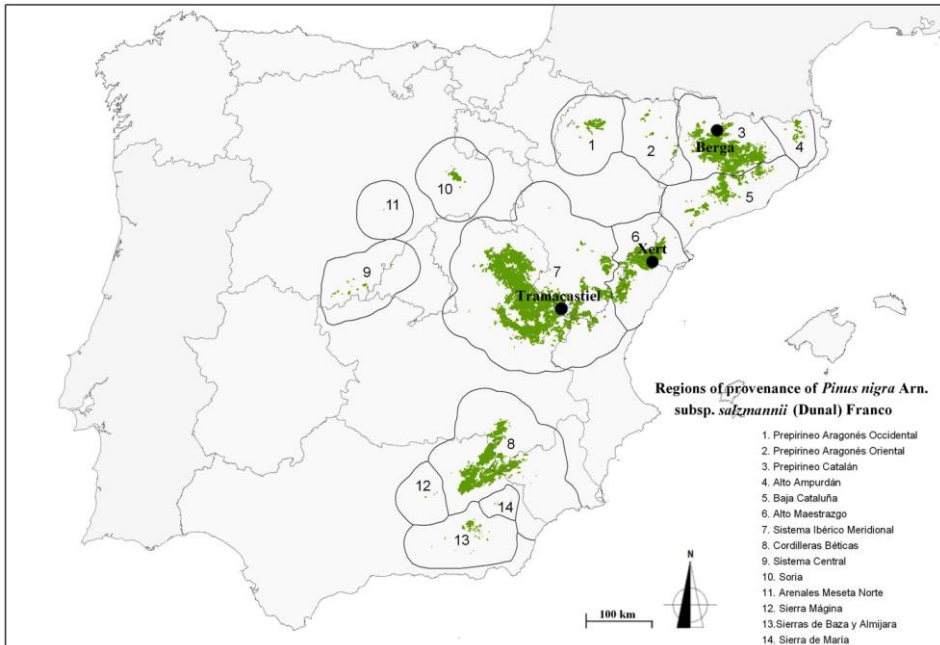


Fig. 1. Regions of provenance of *Pinus nigra* Arn. subsp. *salzmannii* (Dunal) Franco in Spain according to Alía et al. (2009). The three trial sites are indicated by bold colored points.

5.2.2. Trial sites selection and characterization

The study was performed on three different sites in Eastern Spain (Table 1). The objective in this selection was to test the seed sources in contrasting sites (good quality site, drought-prone site and high altitude site). The Berga site (Barcelona) is located in a valley between the Central Depression and the Catalan Pre-Pyrenees at an altitude of 670 m.a.s.l where it is protected from the north winds by the mountains. In this area, the soil type is mainly limestone while the climate is considered as a kind of transition between typical Mediterranean and sub-Mediterranean. The site is subjected to humid Mediterranean climate with continental tendency. Annual rainfall is comprised between 700 and 900 mm. The vegetation, grown on deep soils before the fire, was mainly composed of forest of *Pinus*

nigra ssp *salmanni* with the presence of *Q. humilis*, *Q. ilex*, *B. sempervirens*, *G. scorpius* and *R. ulmifolius*. Currently and after regeneration, the abundant species are *Q. humilis*, *Q. ilex* and *P. nigra*. The site belongs to the cool sub-humid provenances group (Alía et al. 2009) i.e., none of the seed sources tested belonged to this site, and can be considered a good quality site for this species (Gandullo and Sanchez-Palomares 1994).

The site Xert (Castellón, eastern Spain) is situated in one terraced hillside on a southeast facing slope at an altitude of 600 m.a.s.l. The site is characterized by marl-clay soil parent material. This area is the transition between two geologic periods; so the interface acts as a waterproof layer just below the limestone-dolomite dominating the higher parts. The climate is typically Mediterranean with hot summers and mild winters that favour vegetation of Mediterranean forest. The annual rainfall is around 600 mm. This hillside was occupied by *P. nigra*, *P. halepensis* and *Q. ilex*. After the fire of 2002, there was a great natural regeneration of *P. halepensis* isolated from specimens that survived the fire in sunny areas. The other natural vegetation is mainly composed of *R. officinalis*, *U. parviflorus* and *C. monspeliensis*. The site is located in the Mediterranean warm sub-humid group and hence, Alto Maestrazgo is the local seed source. However, the increasing frequency of summer droughts, together with its low altitude, is turning this site towards marginal for the species.

The Tramacastiel site is located in Monte UP 283, south of Teruel province (central-eastern Spain). The area belongs to the Jurassic and presents limestone-clay mother rocks with some sandstone presumably belonging to the surface as a result of quaternary glaciations and landslides. The soil is poor chalky-clay with little or low humus. The site is characterized by a continental mountain climate where frosts are common from November to April. Annual rainfall is around 531 mm and summer

drought is mitigated by altitude and storms. Before the 1994 fire, the area was occupied by a natural stand of *P. nigra* in the north and in the ravines and *P. pinaster* in the rest although, on many occasions, both species overlapped. Since the fire, little regeneration of both species has occurred except for some isolated survived specimens in sunny areas and in the vicinity of the ravines. The rest of the area is mainly composed of *R. officinalis*, *J. thurifera* and *J. oxycedrus* with presence of some other species as *G. scorpius*, *C. laurifolius*, *R. canina* and *T. vulgaris*. The site is located in the cold continental group (Alía et al. 2009) and hence several of the seed sources tested can be considered as suitable local provenances. Main ecological constraints of the site are severe frosts, precipitation irregularity and shallow soils.

5.2.3. Site preparation, planting works and experimental layout

The trials were established in the same year (February 2010) in the sites considered as suitable for forest field experimentation. Seedlings were out-planted using the techniques for operational reforestation programs. Before out-planting, sites were disked and each planting hole (0.5 x 0.5 x 0.4 meters) was opened with a backhoe excavator. On all sites the experimental layout was a randomized complete block design with three blocks. Each seed source was represented by thirty-three trees per block for a total of ninety-nine trees per experimental site (594 seedlings per site). Seedlings were out-planted in rows and spaced by 2.5 m apart.

5.2.4. Plantations monitoring and measurements

Survival and growth variables (expressed as the monthly relative growth rate in height, RGR-H, and stem diameter, RGR-D) were measured three times during the first year of plantation: after out-planting during the

spring growth, during summer drought period (July, after shoot elongation), and after summer (September-October, once the late growth period concluded). These measures were also done in the spring of the third year of plantation (May) for all sites. By this way, the first three years of growth and the first two summer periods were registered, which is considered enough to evaluate early plantation performance (Landis et al. 2010). Appending 1, 2, 3, or T to the RGR variables indicate the measurement period: 1 corresponds to growth from first to second assessment, 2 from second to third assessments, 3 from third to fourth assessments and T (total) from first to fourth assessments. RGR was preferred instead of absolute measures because there were slight differences among nursery stock at planting. Seedling height was measured to the nearest 0.5 cm from soil to the terminal bud and stem diameter at 1.0 cm above the soil surface. Survival was assessed by determining the status (alive, dead) of each individual.

5.2.5. Data analysis

Geographic and climatic variables with recognized influence on ecotypic variation of Mediterranean pines (Voltas et al. 2008) were selected (Table 1) to calculate the transfer distance from the trial site to the corresponding seed source native site (Aitken et al. 2008; Benito-Garzón et al. 2011). The Kaplan-Meier method (Kaplan and Meier 1958) was used to compare the survival functions of the different seed sources on a site basis. The statistics of LogRank, Breslow and Tarone-Ware were used to detect significant differences either among overall seed sources or for pair wise comparisons when significant differences appeared. Regarding growth, differences among seed sources were tested on the RGR-H and the RGR-D, determined every two consecutive measurements (Hoffmann and Poorter 2002), as well as the overall RGR value (last and first measurements). For

this purpose, a multivariate analysis of variance (MANOVA) using the Wilks' Lambda statistic was performed on all RGR variables (for height or for diameter) as dependent variables and block and seed sources as factors. Follow-up analyses (ANOVA) were performed to assess whether differences among groups on the population means for each dependent variable were significant. Post-hoc comparisons of means were made using Tukey's test. In all cases, data were examined to ensure normality and homogeneity of variances, indicating in the text when these assumptions are violated.

Analysis of *phenotypic plasticity, ecotypic differentiation and specific adaptation* for definite environments was addressed both on the post-planting shock stress of the first year (from plantation to the post-summer growth of the first year of out-planting) and the final establishment growth (after three years). This was done because post-planting response can be independent from that of the following years because it is affected by multiple factors such as stock quality, works execution, precipitation following plantation, etc. Consequently, it resulted in six environments (3 sites x 2 assessments) for the analysis. The *phenotypic plasticity* of each growth trait (x : RGR-H and RGR-D) and seed source was estimated from the absolute difference (distance) in the values of x between two individuals of the same seed source grown in different environments using the relative distance plasticity index (RDPI) from Valladares et al. (2006). A RPDI ranging from 0 (no plasticity) to 1 (maximal plasticity) can be obtained for each seed source as:

$$RDPI = \frac{\sum \frac{d_{ij \rightarrow i'j'}}{(x_{ij} + x_{i'j'})}}{n}, (1)$$

where n is the total number of distances. Each relative distance d was calculated as the absolute value of the trait distance between each two selected individuals i and i' of the same seed source belonging to different

environments j and j' , divided by the sum of their trait values. The overall standardized RDPI value, one per seed source and trait, is obtained by summing all relative distances and dividing by the total number of distances.

As regard *Genotype-Environment Interaction (GEI)*, the commonly statistical methods applied to GEI were performed to analyze adaptation patterns of the six black pine seed sources across the trial sites since it is recommended to use two or more approaches together (Chahal and Gosal 2002). Here, (1) analysis of variance, (2) concurrence or joint linear regression (Finlay and Wilkinson 1963), and (3) the additive main effects and multiplicative interaction (AMMI) model (Gauch 1992) were performed. The percentage of variation explained by each one of the three factors (seed sources, environments and their interactions) was calculated with the variance components, assuming all the factors were random.

The *analysis of variance* (ANOVA) model is

$$y_{ij} = \mu + G_i + E_j + G_iE_j + \varepsilon_{ij}, (2)$$

where y_{ij} is the mean of seed source i in environment j ; μ is the general mean; G_i is the mean of the i seed source; E_j is the mean of the j environment, G_iE_j is the seed source by environment interaction (GEI) component and ε_{ij} is the residual variation.

The *joint regression analysis* is a simple linear regression that provides a conceptual model for genotype adaptability, being widely used in plant breeding. In this approach, the components of GEI are linearly related to environmental effects, which are represented by the average performance of all the seed sources tested for the trait under consideration (Finlay and Wilkinson 1963). The linear regression model is

$$y_{ij} = \mu + G_i + E_j + b_iE_j + \varepsilon_{ij}, (3)$$

where y_{ij} is the mean of seed source i in environment j ; μ is the general mean; G_i is the mean of seed source i over all environments; E_j is the environmental index for environment j ($Y_{.j} - Y$); b_i is the slope of the regression specific for seed source i ; and ε_{ij} is the residual variation, which is assumed to be zero for the values averaged over replications. According to Finlay and Wilkinson (1963), the slope of regression was plotted against the mean RGR of the seed sources to determine the wide adaptability of each seed source. When the regression coefficient of the seed source is 1.0 and its RGR mean above the overall average, the seed source is well adapted to all the tested environments. If the mean RGR is below the overall mean and the regression coefficient is 1.0, the seed source is poorly adapted to all environments. Regression coefficients above 1.0 indicate seed sources with a response above average to high performing environments. Regression coefficients below 1.0 provide a measure of below average responses to high performing environments. Because the regression is performed on the environmental mean, an ANOVA can be constructed according to Hardwick and Wood (1972) (for the joint regression analysis), where SS_{GEI} is partitioned into two components related, respectively, to the heterogeneity of seed source regressions and the residual term.

The *additive main effects and multiplicative interaction (AMMI)* method uses the standard ANOVA procedure, separates the additive variance from the multiplicative variance (interaction), and then applies PCA to the interaction (residual) portion from the ANOVA analysis to extract a new set of coordinate axes which account more effectively for the interaction patterns following the model:

$$y_{ij} = \mu + G_i + E_j + \sum_{k=1}^n \lambda_k \alpha_{ik} \gamma_{jk} + \varepsilon_{ij}, \quad (4)$$

where y_{ij} is the observation on the i^{th} seed source in the j^{th} environment; μ is the overall mean; G_i is the i^{th} seed source effect; E_j is the j^{th} environmental main effect; n is the number of PCA axes considered in the model, λ_k is the eigen value of the n^{th} PCA axis and a_{ik} and γ_{jk} are scores for the i^{th} seed source and the j^{th} environment on the n^{th} PCA axis and ε_{ij} is the residual term which includes the experimental error (Gauch 1992). The $SS_{G \times E}$ was divided into n singular main components of interaction axes (IPCA) that described the standard portion (effects of seed sources and environments) and the noise portion, which is the additional residue of unpredictable and not interpretable responses. After selecting the AMMI model, the interpretation of wide or/and specific adaptation was accomplished by plotting IPCA1 on abscissa and IPCA2 on ordinate for each seed source and site scores. The lower the IPCA absolute values, the more stable the seed source. The ideal seed source is one with high performance and IPCA values close to zero suggesting wide adaptation to the tested environments. The undesirable one has low stability associated with low performance (Gauch 1992). A large seed source' IPCA reflects more specific adaptation to environments with IPCA score of the same size, the reverse sign of IPCA value depicts negative interaction i.e. poor performance of seed sources in such environment (Chahal and Gosal 2002).

Correlations between the measured (survival, growth traits, the studied indices) and the seed sources origin (geographic coordinates and climatic variables) variables were determined based on the coefficient of Pearson. The seed orchard 'H.S. El Serranillo' was excluded in these analyses because it is grown from various sources. All statistical analyses were performed using the computing environment R (R Development Core Team, 2013).

5.3. Results

Seedlings growth and survival were different among sites. The highest final survival rate and height growth were observed in Berga while the highest diameter growth was in Xert. Even seedlings grew well in Tramacastiel, this site was characterized by the highest mortality rates along the monitoring period.

5.3.1. Survival response

Seed sources survival varied significantly among sites (Fig. 2). The highest final survival rate among sites was observed in Berga (77%) with a mean survival time of 22.8 months over 28 in the Kaplan-Meier analysis. ‘Cordilleras Béticas’ seedlings had the lower survival (72.73%) while ‘Alto Maestrazgo’ seedlings had the higher survival (83.51%) without significant difference between seed sources (Fig. 2). In Xert, the survival rate was maintained up to 97% during the first year (2010) then decreased by the spring of 2012 (third year) to an average of 53%; the mean survival time in this site was 18.4 months out of 28 months. In this site, survival functions were not significantly different, although significant differences among seed sources were observed only for the third year (p -value <0.01): seedlings from ‘Sistema Ibérico Meridional a and b’ showed the highest survival (59.60% and 58.59% respectively) while those from ‘Cordilleras Béticas’ manifested the lowest survival (45%) (Fig. 2). In Tramacastiel, survival did not differ among seed sources; it showed a first decrease for all seed sources after the first summer of plantation (63%) and a further acute decrease between September 2010 and May 2012 (till an average of 33%). The mean survival time was about 13 months out of 28 months. In spite of not being significantly different, it is remarkable the changing patterns of the survival functions. ‘Cordilleras Béticas’ and ‘H.S. Serranillo’ marked the highest

survival (above 36%) whereas seedlings of ‘Sistema Ibérico Meridional a and b’ manifested the lowest survival (below 29%) (Fig. 2).

In general, ‘Alto Maestrazgo’ and ‘Sistema Ibérico Meridional b’ showed the best inter-site survival, whereas ‘Sistema Ibérico Meridional a’ showed the lowest inter-site survival.

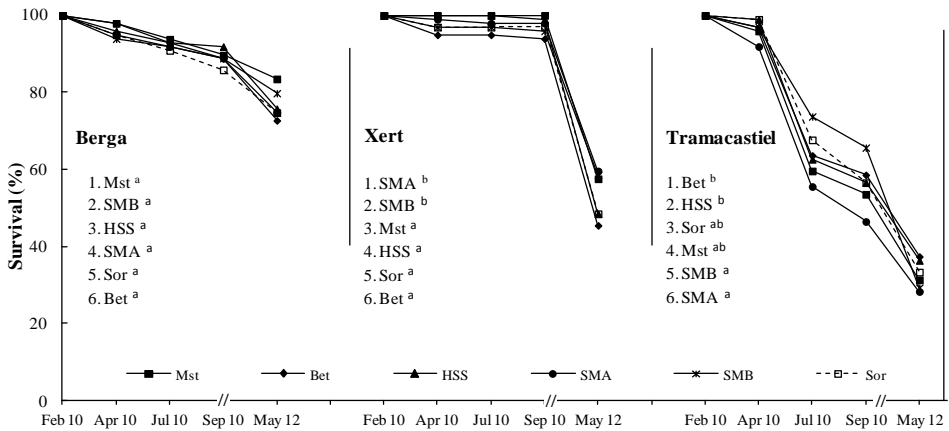


Fig. 2. Temporal survival of the six seed sources of Salzmann pine out-planted on the three trial sites. For each site, seed source abbreviations (see Table 1) followed by different letters indicate significant differences (p -value >0.05) in survival.

5.3.2. Growth response

Seedlings RGR differed according to site and time (Fig. 3). During the first spring of out-planting, RGR-H1 was greater on Tramacastiel than on Berga and Xert, whereas in the first summer the maximum growth was reached at Berga (RGR-H2: $0.095 \text{ cm cm}^{-1}\text{month}^{-1}$, more than twofold and eightfold comparing to Tramacastiel and Xert respectively). For the whole period, RGR-HT, was also higher in Berga ($0.046 \text{ cm cm}^{-1}\text{month}^{-1}$). Regarding the RGR-D, seedlings grown in Xert presented the highest values till the end of monitoring period (RGR-DT: $0.039 \text{ mm mm}^{-1}\text{month}^{-1}$) (Fig. 3).

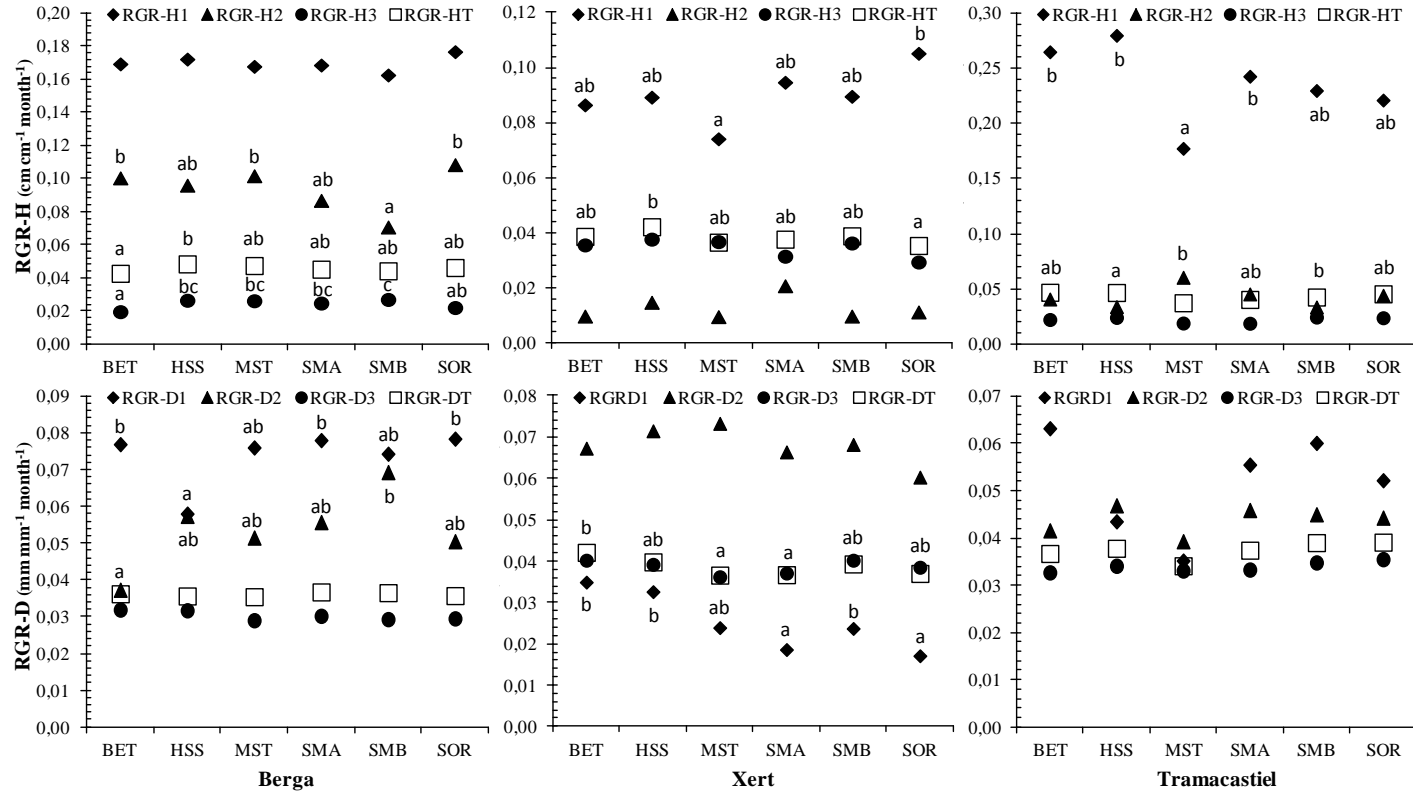


Fig. 3. Relative growth rates in height (RGR-H; $\text{cm cm}^{-1} \text{ month}^{-1}$) and diameter (RGR-D; $\text{mm mm}^{-1} \text{ month}^{-1}$) of *Pinus nigra* ssp. *salzmannii* seed sources out-planted on three contrasting sites. Within the same variable and site, different letters indicate significant differences at $p < 0.05$. Seed source abbreviation's shown in Table 1.

In the intra-site comparison of growth among seed sources (MANOVA), Wilks' Lambda statistic was significant (p -value <0.001) for RGR-H and RGR-D in Berga and Xert, but only for RGR-H in Tramacastiel. ANOVA and Tukey tests showed that RGR-D differences were only due to the rate of the first spring (RGR-D1, poor performance of 'H.S. El Serranillo') and the first summer (RGR-D2, 'Sistema Ibérico Meridional b' performed better than 'Cordilleras Béticas') at Berga and to the RGR-D1 and the RGR-DT in Xert ('Cordilleras Béticas' showed superior rate; Table 2, Fig. 3). Height growth, differences among seed sources appeared after the first summer in Berga, whereas in Xert and in Tramacastiel there were differences in the first spring. In these two sites, 'Alto Maestrazgo' seedlings marked the lowest growth rates in the early establishment stage (RGR-H1), although it improved later on. In Xert, temporal differences were less marked, with 'H.S. Serranillo' showing better height growth (total) than 'Soria'. On the contrary, 'H.S. Serranillo' showed lower summer growth (RGR-H2) than 'Alto Maestrazgo' and 'Sistema Ibérico Meridional b' in Tramacastiel. In Berga, 'H.S. El Serranillo' was the first of the ranking for RGR-HT (and RGR-H3) on the opposite of 'Cordilleras Béticas' with no significant differences among the other seed sources.

Table 2. ANOVA summary (F-value, Mean Square Error (MSE), degrees of freedom of error (df) and significance: *p-value<0.05, ** p-value<0.01, *** p-value<0.001) of the relative growth rate (RGR) for height (H) or diameter (D) of six *Pinus nigra* ssp. *salzmannii* seed sources planted in three sites. RGR-H and RGR-D were measured during first spring (1), first summer (2), second/third year (3), and overall (T, Total growth). †Homoscedasticity not achieved.

		RGR-H				RGR-D			
Site	Statistic	1 st spring	1 st summer	2 nd &3 rd year	Total growth	1 st spring	1 st summer	2 nd &3 rd year	Total growth
Berga	F-value	1.09	2.93*	5.97***	2.49*	2.68*	2.1	1.15	0.22
	MSE (df)	6E-03 (431)	4E-03 (431)	0.00 (431)	0.00 (431)	2E-03 (431)	3E-03 (431)	9.29E-05 (431)	8.16E-05 (431)
Xert	F-value	2.80*	0.74	2.76*	2.33*	3.35**	0.77	1.14	2.74*
	MSE (df)	3E-03 (293)	1E-03 (293)	0.00 (293)	0.00 (293)	1E-03 (293)	3E-03 (293)	0.00 (293)	8.36E-05 (293)
Trama-castiel	F-value	2.88*	1.19*	1.09	2.23	1.53	0.58	0.29	0.61
	MSE (df)	1.50E-02 (173)	2E-03 (173)	0.00 (173)	0.00 (173)	3E-03 (173)	1E-03 (173)	0.00 (173)	0.00 (173)

Significant correlations were found between geographic and climatic variables of the seed sources origin (Table 1) and growth on the three sites. In Berga, height growth (RGR-H3 and RGR-HT) showed significant negative correlations with altitude (p-value<0.01) and the mean maximum daily temperature of the warmest month (p-value<0.01). In Xert, there were negative correlations of height growth with latitude (RGR-H3 and RGR-HT, p-value<0.01) and longitude (RGR-H2, p-value<0.05). However, there were positive correlations with mean minimum daily temperature of the coldest month (RGR-H3, p-value<0.05). In addition, diameter growth (RGR-D1 and RGR-DT, p-value<0.05) was negatively correlated with latitude and

summer precipitation and positively correlated with altitude (RGR-DT, p -value <0.05) and mean maximum daily temperature of the warmest month (RGR-D1, RGR-D3 and RGR-DT, p -value <0.05). In Tramacastiel, significant correlations were established only during the first spring growth; growth was correlated positively with altitude and mean maximum daily temperature of the warmest month and negatively with summer precipitation (RGR-D1 and RGR-H1, p -value <0.01).

5.3.3. Phenotypic plasticity

The tested seed sources showed low values of the plasticity index, ranging from 0.07 to 0.31 for RGR-D and from 0.09 to 0.33 for RGR-H. At the end of the first year of out-planting, higher plasticity was observed for RGR-H (from 0.26 to 0.33) when compared to RGR-D (from 0.18 to 0.31). Plasticity was negatively correlated with the daily mean minimum temperature of the coldest month (p -value <0.01) for RGR-H and positively with latitude (p -value <0.05) for RGR-D seed sources origin. After the final establishment of the seedlings, for RGR-D, ‘H.S. El Serranillo’ and ‘Soria’ seed sources manifested the highest values of RDPI while those of ‘Alto Maestrazgo’ expressed the lowest ones. As regard RGR-H, ‘Soria’ seedlings manifested the highest plasticity value on the contrary to ‘H.S. El Serranillo’ and ‘Sistema Ibérico Meridional b’ (Table 3). The higher plasticity values estimated for RGR-D were correlated negatively with the daily mean maximum temperature of the warmest month (p -value <0.05) and positively with summer precipitation (p -value <0.05) of the seed sources origin. Regarding RGR-H, the higher plasticity values were positively correlated with cooler temperature (annual mean temperature, p -value <0.05 ; mean maximum daily temperature of the warmest month, p -value <0.05).

Table 3. Mean performance, phenotypic plasticity and stability analyses of *Pinus nigra* ssp. *salzmannii* seed sources. RDPI indicates the relative distance plasticity index of Valladares et al. (2006). RDPI_p indicates the values of the index after the first year of the post-planting while RDPI_T indicates the final establishment.

Seed sources	RGR-D				RGR-H			
	Mean	<i>bi</i>	RDPI _p	RDPI _T	Mean	<i>bi</i>	RDPI _p	RDPI _T
Bet	0.0371	0.80	0.22 ¹	0.09	0.0609	1.09	0.33 ¹	0.12
HSS	0.0376	0.54	0.18 ⁶	0.12 ¹	0.0632 ¹	1.03	0.29	0.09 ⁵
Mst	0.0350 ⁶	1.02	0.21	0.07 ⁶	0.0550 ⁶	0.98	0.33 ²	0.12
SIMa	0.0377	1.12	0.21	0.09	0.0595	1.01	0.27	0.09 ⁶
SIMb	0.0384 ¹	1.30	0.21	0.11	0.0557	0.84	0.27	0.11
Sor	0.0359	1.31	0.31 ¹	0.11	0.0609	1.05	0.26 ⁶	0.15 ¹

* The exponent numbers above values indicate the seed source rank position in the considered scale of stability or of performance.

5.3.4. Genotype by environment interaction (GEI)

Analysis of variance showed that GEI effect was significant only for RGR-H. Most variation was attributed to the environment, which explained respectively 79% and 95% of the observed variability for RGR-D and RGR-H (Table 4), indicating the heterogeneity of the tested environments. The genotypic contribution was about 1.5% for RGR-H to 4.5% for RGR-D. The interaction term (GEI) explained up to 3% of the total variance for RGR-H and up to 17% for RGR-D. The regression term explained about 30% for RGR-H and 54% for RGR-D while the residual GEI was attributed to random deviations. The AMMI models have provided more detailed partitioning of the SS_{GEI} than linear regression since it accounted at least 95% for RGR-H and 85% for RGR-D with the two IPCA components (sum of IPCAs with significant differences, p -value<0.05) while the regression model accounted no more than 54% of GEI terms (Table 4).

Table 4. Analysis of variance, linear regression, and AMMI for growth (height and diameter) of six *Pinus nigra* ssp. *salzmannii* seed sources (Significance codes: 0 ‘***’, 0.001 ‘**’, 0.01 ‘*’).

Sources		df	SS	MS	F value	p value	SS	MS	F value	p value	
		RGR-H					RGR-D				
ANOVA	Gen	5	0.00278	0.00056	3.631	0.003 **	0.00045	0.00009	1.171	0.323	
	Env	5	0.19080	0.03816	24.88	0.000 ***	0.00838	0.00168	22.02	0.000 ***	
	Gen:Env	25	0.00530	0.00021	1.383	0.010*	0.00180	0.00007	0.945	0.542	
	Residuals	288	0.04416	0.00015			0.02191	0.00008			
Joint Regression	Gen	5	0.00278	0.00056	4.803	0.000 ***	0.00045	0.00009	1.222	0.299	
	Env	5	0.19080	0.03816	32.91	0.000 ***	0.00838	0.00168	22.97	0.000 ***	
	Gen:Env	25	0.00530	0.00021	1.829	0.010 **	0.00180	0.00007	0.986	0.486	
	Heterogeneity	5	0.00152	0.00030	2.620	0.049 *	0.00098	0.00020	2.673	0.046 *	
	Deviation	20	0.00378	0.00019	1.631	0.047 *	0.00082	0.00004	0.564	0.934	
	Residuals	35	0.02783	0.00012			0.01751	0.00007			
AMMI	Gen	5	0.00278	0.00056	4.802	0.000 ***	0.00045	0.00009	1.222	0.300	
	Env	5	0.19080	0.03816	11.21	0.000 ***	0.00838	0.00168	18.25	0.000 ***	
	Rep(Env)	48	0.01633	0.00034	2.935	0.000 ***	0.00441	0.00009	1.258	0.136	
	Env:Gen	25	0.00530	0.00021	1.828	0.011 *	0.00180	0.00007	0.986	0.487	
	IPCA1	9	0.00283	0.00031	2.711	0.005 **	0.00120	0.00013	1.821	0.065	
	IPCA2	7	0.00220	0.00031	2.712	0.010 **	0.00033	0.00005	0.651	0.713	
	Residuals	9	0.00027	0.00003	0.259	0.985	0.00027	0.00003	0.411	0.928	
	Residuals	240	0.02783	0.00012			0.01751	0.00007			

5.3.5. Adaptation analysis

The less responsive or/and sensitive seed sources to the changing environments (with regression coefficients $b_i < 1$) were 'Alto Maestrazgo' regarding RGR-H and 'Cordilleras Béticas' along with 'H.S. El Serranillo' regarding RGR-D (Table 4, Fig 4). To determine adaptability, the slope of regression was plotted against the seed sources RGR's mean (Fig. 4). 'H.S. El Serranillo' seedlings had higher RGR-H and a slope near the unity ($b_i = 1$), suggesting that this seed source was well adapted to all the tested environments. 'Soria', 'Cordilleras Béticas' and 'Sistema Ibérico Meridional a' seed sources would be considered well adapted to the most of environments. On the contrary, 'Alto Maestrazgo' and 'Sistema Ibérico Meridional b' seed sources were poorly adapted to all the environments as regards RGR-H. Concerning RGR-D, both seed sources from the Sistema Ibérico Meridional seem to be well adapted to the good environments. However, 'H.S. El Serranillo' and 'Cordilleras Béticas' seed sources showed $b_i < 1$ and high RGR-D's which means that they were well adapted to the unfavourable environments. 'Alto Maestrazgo' and 'Soria' seed sources were poorly adapted to all the environments.

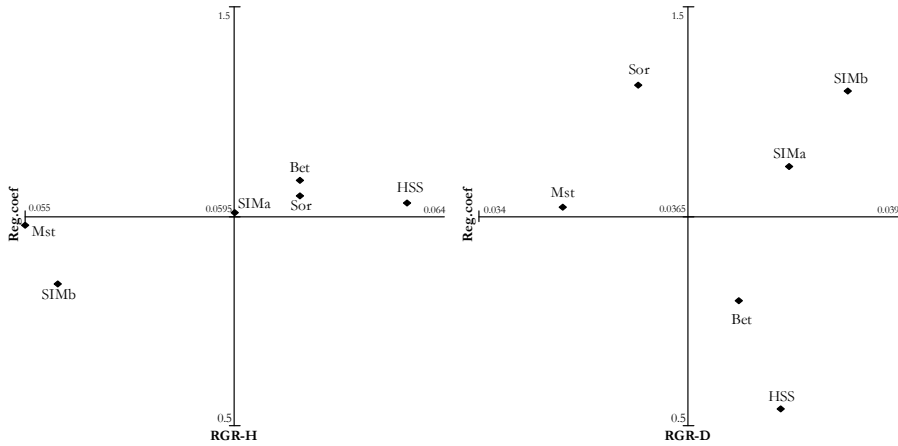


Fig. 4. Seed sources growth's adaptation to the experienced environments as plotted by Finlay and Wilkinson model as mean growth to regression coefficient. The abbreviation indicates seed sources as shown in table 1. The vertical line is the general mean growth of the experiment and the horizontal line is $b_i = 1$.

The AMMI results were graphically interpreted by plotting the IPCA1 scores against the IPCA2 scores to explore also wide and/or specific adaptation (Fig. 4). The tested environments were more separated than the seed sources in the plot indicating the higher influence of the environmental conditions on seed sources growth variation among the tested environments. Correlations between seed sources' IPCAs scores and the environmental variables of their habitats origin were significant only for IPCA1; for RGR-H, seed sources' IPCA1 scores were correlated positively with summer precipitation (p -value <0.01) and latitude (p -value <0.05) and negatively with the daily mean maximum temperature of the warmest month (p -value <0.05). This indicated that height growth variation among the tested seed sources was induced mainly by these environmental variables. Regarding RGR-D, seed sources' IPCA1 scores were correlated positively with summer precipitation (p -value <0.05) and negatively with the annual mean temperature (p -value <0.01). Analysis of AMMI2 biplot generated on

the basis of RGR indicated that the sites had high contribution to the GEI as they were plotted on the opposite side of each other (Fig. 5).

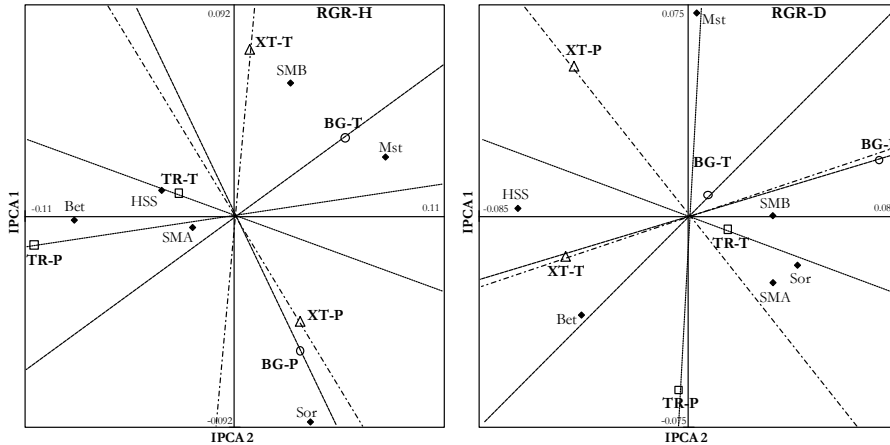


Fig. 5. AMMI2 biplot with the two first principal components of interaction (IPCA1 and IPCA2) of RGR-H and RGR-D of *Pinus nigra* ssp. *salzmannii* seed sources grown under different situations (after one year: P, and three years: T). The abbreviation indicates trial sites and seed sources as shown in Table 1.

A wide adaptation was observed for ‘Sistema Ibérico Meridional a’ and ‘H.S. El Serranillo’ seed sources concerning RGR-H regarding their position closer to the zero with less contribution to the GEI. ‘Cordilleras Béticas’ seedlings showed a large IPCA of the same sign to TR-P and TR-T IPCAs’ (higher altitude), similar to ‘Soria’ seed sources manifesting a large IPCA of the same sign to the first out-planting year conditions in Berga and Xert (BG-P and XT-P). Also, it is remarkable the positive relationship between ‘Sistema Ibérico Meridional b’ and ‘Alto Maestrazgo’ seed sources and XT-T and BG-T sites conditions suggesting a specific adaptation. As regards RGR-D, ‘H.S. El Serranillo’ and ‘Cordilleras Béticas’ seed sources showed a specific adaptability to Xert conditions (XT-P, XT-T). ‘Sistema Ibérico Meridional b’ seed source was closer to the zero indicating its wide

adaptation while ‘Sistema Ibérico Meridional a’ and ‘Soria’ manifested a specific adaptability to TR-T.

5.4. Discussion

We analyzed among six Salzman pine seed sources the variability in growth and survival along with their phenotypic plasticity and adaptation across three environments in eastern Spain for subsequent assisted migration to expected climate change. During the study period, the sites showed contrasting environmental conditions as hypothesized in this work, with Berga acting as a core habitat, while Xert and Tramacastiel showed constraints related either to summer droughts or frost occurrence (Table 1). Accordingly, the results evidenced a significant variation among seed sources, revealing that matching genotypes, environments and their interactions in assisted migration programs may require plasticity and adaptation analyses.

Seedlings out-planted in Berga showed the highest final survival (77%), without significant differences between seed sources. Similar results were reported by Perez et al. (2005) in the sub-humid north-western Spain, with survival rates above 85% and no differences among 22 provenances. In contrast, seedlings grown at Xert and Tramacastiel showed high mortality (53% and 33%, respectively) and differences among seed sources’ performance (in the former site). Summer drought is a key factor affecting Salzman pine seedlings survival (Tiscar-Oliver 2007; Tiscar and Linares 2011), especially in the drier regions of Spain, and this can explain particularly the important decrease of survival in Xert after 2010 (with less than 50% of the expected summer rain, see table 1) as well as in the second and third years in Tramacastiel. Summer precipitations below 52 mm fall out of the ecological range of this parameter for this species in Spain (Gandullo

and Sanchez-Palomares 1994). However, there was also an important survival decrease in the spring of 2010 in Tramacastiel, which was attributed to the minimal temperatures recorded after outplanting in February (average: -3.2°C ; range -7.8 to 4.8°C) and March 2010 (average: 0.16°C ; range -7.2 to 4.1°C). Kreyling et al. (2012) suggested that low temperatures may pose a serious threat which naturally determines the poleward range limits of black pine since that extreme winter temperatures cause frequent freeze-thaw events, which induce xylem embolism and frost drought (Mayr 2007). This dual stress (drought and frost) made that survival patterns among seed sources were not consistent (eg. ‘Sistema Ibérico Meridional a, b’ and ‘Cordilleras Béticas’).

Relative growth rates also differed among seed sources according to site and time, and the ranking varied among the sites, as reflected in the strong interaction component in the analysis (Table 4). Among sites, seedlings from ‘H.S. El Serranillo’ (which is improved from ‘Sistema Iberico Meridional’) and ‘Sistema Ibérico Meridional b’ showed higher growth and good survival. Differences in early growth performance among *Pinus nigra* provenances of different geographic origins have been almost always observed in trials with different results depending on the sites (Varelides et al. 2001 and references within). It is to be noted that height growth differed more among seed sources than diameter growth within each site. This can be explained by the fact that seedlings diameter, being frequently more sensitive to change in environmental conditions, is further fixed than height in establishing seedlings (Baquedano et al. 2008). However, there were specific differences among sites, with height growth being higher in Berga while in Xert diameter growth was the highest and height growth rates were the lowest. Higher diameter growth, which is related to root growth (Mexal and Landis 1990), can be considered as a response to water stress on this

site, as this is essential to ensure survival after transplanting (Grossnickle 2005). Several studies have shown that black pine growth is sensitive to water deficit during spring and summer and to high summer temperatures (Martin-Benito et al. 2010; Linares and Tiscar 2010). Seedlings may significantly reduce height and diameter growth as an adaptive strategy (Voltas et al. 2008). This is known to occur also in other pine species, e.g. *P. sylvestris* and *P. taeda* (Cregg and Zhang 2001) and *P. halepensis* (Taïbi et al. 2014).

It can be drawn from the survival and growth response that under good site conditions (Berga in our case), seed sources differences were mainly related to the height growth, and the improved seedlings from the seed orchard ‘H.S. El Serranillo’ showed the highest growth rate, whereas those seedlings associated to large transfer distance from their site origin to the trial (‘Cordilleras Béticas’) performed worse. This trade-off between tolerance of provenances from low and high latitudes and growth performance has been also reported in other conifer species such as *Pinus taeda* (Schmidting 1994) and *Pinus contorta* (Rehfeldt et al. 2002). On the opposite, when environmental constraints operate, survival (and growth) differences came up, and the hypothesis of northward migration became supported: seed sources from the warm sub-dry group (‘Cordilleras Béticas’) survived more in the upper-north continental site (especially after the drought of years 2011-2012) than local provenances (Sistema Ibérico Meridional), and the seed sources from this region (Tramacastiel belongs to Sistema Ibérico Meridional) survived more in the upper-north warm sub-humid site than local provenances (Alto Maestrazgo). Again in this case, long transfer distances and frost tolerance appear as interacting factors affecting survival of the southernmost provenance. However, it is noticeable that the diameter growth of ‘Cordilleras Béticas’ was the highest in Xert and

that some correlations obtained here (temperatures and summer precipitation of seed sources origins), were also indicative of an acceptable response of this seed source in this site. As stated before, water stress leads to more root growth (diameter growth) to ensure survival after transplanting. The Tramacastiel site acted as a marginal site where a compromise between frost and drought tolerance has determined establishment success. Therefore, referring to the early pine performance, seed sources not belonging to the target site could be selected for facing current climate irregularities in different environments (Taïbi et al. 2014). Similar clinal trends were exhibited by *P. sylvestris* (Persson 1994) and *P. halepensis* (Agúndez et al. 1997; Climent et al. 2008; Taïbi et al. 2014).

Either from the joint regression analysis or the combined ANOVA with AMMI, the environmental effect has shown a great influence on seedlings performance variation (Table 4). This means that the tested environments were extremely diverse and confirms findings that environment is the major factor affecting Salzman pine growth performance (Amodei et al. 2012). However, the significance of the GEI term for RGR-H justifies the need for investigating the nature of differential response of the seed sources to the experienced environments. Significant genotype by environment interactions (GEI) have been frequently reported in *Pinus nigra* provenance trials and were attributed to the broad natural distribution of the species in a wide range of environments leading to distinct races (Varelides et al. 2001 and references within), although other studies did not report significant interactions (Perez et al. 2005). The removal of the additive parameter from the multiplicative component in the AMMI model overcomes one major statistical deficiency that is the dependency of the site and population indicators encountered in joint regression (Gauch 1992; Wu and Ying 2001) and thus the AMMI models have provided more

detailed partitioning of the SS_{GEI} than linear regression since they accounted 95% for RGR-H and 85% for RGR-D with the two significant IPCA axes while the joint regression model accounted no more than 54% of GEI terms. Therefore, the AMMI models have been recommended to analyse complex GEI since it have been able to reveal the nature of interaction mainly if it captures more SS_{GEI} than joint regression (Nachit et al. 1992). Even with, the joint regression analysis remains valuable for determining genotypic behaviour over environments since it demonstrates comparable results to the AMMI analysis and allows for the prediction of performance in untested environments (Barnes et al. 1984).

Regarding phenotypic plasticity, and according to RDPI range established by Valladares et al. (2006), the tested Salzmänn pine seed sources showed low phenotypic plasticity for both growth traits which concurs with previous results of pine species such as *Pinus halepensis* (Baquedano et al. 2008) or on other Mediterranean species such as *Quercus coccifera* (Balaguer et al. 2001), *Quercus robur* and *Fagus sylvatica* (Valladares et al. 2000a, 2002). Some traits might vary in their sensitivity to the environment being more plastic than others (Aitken et al. 2008; Benito-Garzón et al. 2011; Matesanz and Valladares 2013) as was the case in our experiment where height growth plasticity was higher than of that diameter among sites. The facts that higher plasticity values were correlated positively with cooler temperatures and summer precipitation and negatively with warmer temperatures (Table 3) corroborates the theory that limited phenotypic plasticity is beneficial in stressful environments (Chambel et al. 2005). The seed orchard 'H.S. El Serranillo' showed less height growth plasticity than the other seed sources after the final establishment since it performed higher in Berga and Xert while no significant difference among seed sources was noted in Tramacastiel (Table 2). Balaguer et al. (2001) and Valladares et al. (2002 and

2007) have suggested also that the low phenotypic plasticity in Mediterranean forest trees constitutes an adaptation strategy to survive under the environmental stress frequent in the region.

The differential responses of the tested Salzman pine seed sources selected from a broad geographic area have reflected their differential adaptation strategies to natural environments of this species which reinforces the need of cautious selection of the seed source according to the target environments (Wu and Ying 2001). Regarding height growth, the seed orchard ‘H.S. El Serranillo’ was well adapted to all the tested environments and this observation was confirmed previously by their good growth and survival.

According to AMMI results, our finding as regards to RGR-H seed sources adaptation indicated a wide adaptation for ‘Sistema Ibérico Meridional a’ and ‘H.S. El Serranillo’ to all the environments. However, ‘Cordilleras Béticas’ seedlings from the lower latitude in the South of Spain, where drought is more frequent, were specifically adapted to the poor conditions encountered in Tramacastiel of shallow soil and adverse climate by performing high growth and survival in the site. Similar results were found for *Pinus halepensis*, where Southern provenances performed better in marginal habitats (Taïbi et al. 2014). Besides, ‘Sistema Ibérico Meridional b’ seedlings were specifically adapted to Berga and Xert sites. This was explained also by their higher growth and survival on both sites. Furthermore, ‘Alto Maestrazgo’ seedlings were specifically adapted to Berga site, thus supporting the northward migration hypothesis. To reduce maladaptation and, ultimately, avoid extirpation from the landscape, plant populations must be able to adapt by a combination of phenotypic plasticity (i.e., acclimation), evolutionary adaptation (i.e., natural selection), and migration at rates adequate to keep pace with geographic shifts in climatic

habitat (Aitken et al. 2008). It should be noted that high levels of phenotypic plasticity across a range of environments are usually conferred by the ability to plastically adjust the phenotype to each set of conditions and subsequently it is considered as a generalist adaptive strategy (Balaguer et al. 2001). However, the specific adaptation, which leads to ecotypic differentiation (Baquedano et al. 2008), involves a reduced phenotypic plasticity of populations limited to a restricted distribution range or to a particular habitat, which exhibit narrow tolerance to environmental changes (van Tienderen 1997). Many authors have proposed that plant specialization in favourable environments is supposed to be linked to an increased plasticity, whereas the reverse can be expected for the opposite circumstances (Balaguer et al. 2001; Valladares et al. 2000b, 2007).

It should be stressed that early growth assessments for analysing ecotypic differentiation, phenotypic plasticity and seed sources adaptation' may not be reliable for assessing GEI at maturity, which implies the need to evaluate GEI at mature ages in forest trees (Chambel et al. 2005). As well, studies at the molecular level on the relationship between adaptive traits and environments are required to define seed zones and achieve advanced understanding for more Salzmänn pine populations' reforestation and restoration in specific environments in the context of assisted population migration toward climate changes. However, the transfer of seed sources to the tested environments should be carried out with caution and carefully managed from the genetic conservation point of view.

5.5. Conclusions

Six Salzmann pine seed sources outplanted in three environments in eastern Spain were analysed regarding their variability in survival and growth along with phenotypic plasticity and adaptation for subsequent assisted migration toward climate change.

Seedlings out-planted in better site conditions did not differ in survival performance but in height growth; however, under climate constraints, survival differed among seed sources and diameter growth was higher (Xert). Among seed sources, the general best performers were 'H.S. El Serranillo' and 'Sistema Ibérico Meridional b' with higher growth and good survival. The differences in survival and early growth performance among the Salzmann pine seed sources support the hypothesis that northward migration of seed sources makes sense within limited transfer distances. This hypothesis has been reinforced by the genotype x environment interaction survey carried out in this work. Seed sources have manifested low phenotypic plasticity coupled with higher plastic response for height than for diameter. Either from the joint regression analysis or the combined ANOVA with AMMI, the environmental effect has shown a great influence on seedlings performance variation. However, the differential responses of the tested Salzmann pine seed sources from a broad geographic area reflect their differential adaptation strategies to the experienced environments and thus, the justification to investigate the nature of these differential responses to the assisted migration strategy. The AMMI models have provided more detailed partitioning of the SS_{GEI} than linear regression, therefore, they have been recommended to analyse complex GEI since they have been able to reveal the nature of interaction through the biplot.

The present study makes a contribution towards reforestation and breeding research programs in the species, with practical implications for

abiotic stress tolerance, assisted population migration and adaptive silviculture for climate change. Furthermore, the genomic approaches are becoming necessary to confirm our findings in order to select for more resistant genotypes at the molecular level.

5.6. References

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Chapter four:

Physiological, biochemical and proteomic response of Aleppo pine seed sources to water and cold stress as a contribution for Assisted Population Migration in the species

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Article in préparation

Abstract

The response of different populations to water and temperature resources is of prime importance in their poleward range shifts towards climate change which imply the need to consider these parameters in assisted migration programs. The aim of this study was to compare the intraspecific variability among nine Aleppo pine in response to drought and cold stress through the analysis of several physiological and biochemical parameters such as water potential (Ψ_w), transpiration (E), stomatal conductance (gs), photosynthesis (A_{net}), soluble sugars and proteins patterns. Difference among seed sources was observed for photosynthetic pigments and soluble sugars concentrations under both stresses. Drought tolerance was related to the decrease in needle glucose and fructose and the increase of sucrose concentration however, the cold tolerance was associated to the decrease of glucose and the increase of sucrose and fructose concentrations. In addition, differential response among drought stressed seedlings was observed at the proteomic level and most of the identified proteins were related to the transcriptional machinery and sugar metabolism. The presence of enzyme related to the sulphur amino-acids metabolism “5-methyl-tetrahydropteroyl-triglutamate-homocysteine methyl-transferase” could be the limiting factor for drought stress in Aleppo pine. The results of this study agree with our previous results regarding survival and growth performance on these seed sources tested under real field conditions simulating drought and cold stresses. In this sense, ‘Bet’ and ‘Man’ seed sources showed specific adaptation to drought whereas ‘Lev’ and ‘Mst’ seedlings were more suited to cold stress.

Keywords

Pinus halepensis, drought, low temperature, water potential, transpiration, photosynthesis, soluble sugars, proteins, 1D PAGE.

6.1. Introduction

The fate of many forest ecosystems will depend on the ratio of the rate of the climate changes to the rate of their adaptation to such changes. It is widely predicted that plant species will respond to climate change by poleward range shifts (Parmesan and Yohe 2003). Since the speed of climate change is expected to exceed natural migration rates of many plant species (Thomas et al. 2004), human-assisted migration is proposed to counter long generation cycles and modest dispersal abilities of forest trees (Williams and Dumroese 2013).

The response of different populations to basic resources such as water and temperature is of prime importance in assisted migration programs (Aitken et al. 2008). Phenotypic plasticity and the adaptive potential of forest trees are determined by their high genetic diversity, allowing forest trees to develop local adaptations to environmental stressors (Kreyling et al. 2012). Interspecific variation in key functional traits along environmental gradients can explain adaptive patterns related to tolerance to limiting resources (Sánchez-Gómez et al. 2010). This is also expected to occur at an intraspecific level which would explain adaptive variation patterns among different populations along the geographic range of species. Hence, characterization of that variation within forest tree species is important to understand the interaction and significance of evolutionary forces and to carry out appropriate and genetically sound breeding programs (White et al. 2007) and seedling adaptive transfer in ecological restoration, conservation of genetic resources and assisted migration (Williams and Dumroese 2013).

Response to abiotic stress environments can induce a wide number of responses in plants ranging from readjustment of metabolic and biochemical processes leading to physiological, morphological and finally phenological and/or developmental adaptations (Debnath et al. 2011). Different

responses in morphology and development can be reliably addressed in provenance trials as shown in this species (Taïbi et al. 2014). However, responses involving changes in short time spells need to be studied under controlled experiments.

Aleppo pine (*Pinus halepensis* Mil.) is an important forest tree in the Mediterranean region (Quezel and Médail 2003). In light of predictions of climate change in the Mediterranean region, some concern has raised about the ability of *P. halepensis* to persevere in large afforestations in the future (Maestre and Cortina 2004). Natural populations experience several simultaneous environmental stresses that have interactive effects. Yet, the importance of winter and summer conditions on forest trees is often overlooked, especially in the ecology of temperate regions (Kreyling et al. 2012). Summer drought and winter minimum temperature have strong implications for forest tree species distributions by often determining their southern (Taïbi et al. 2014) and northern (Kreyling et al. 2012) boundaries in their poleward shifts and range limits. Besides, cold and drought stress remain a serious threat to Aleppo pine survival and growth particularly in its earliest developmental stages for establishment which imply the need to address serious investigations on both stresses among the intraspecific variability of the species for assisted migration implementation (Taïbi et al. 2014).

Plants can further cope with different environmental stressors by similar responses at the cellular and molecular level when these stressors trigger similar signal transduction pathways (Corcuera et al. 2011). Drought and cold stress, for instance, lead to similar physiological responses in a coniferous forest tree aiming to prevent its cellular dehydration (Blodner et al. 2005). Even though the genetic controls of the protective processes in conifers are complex and not yet enough understood (Holliday et al. 2008),

data has been summarized on the increased concentrations of chemical components involved in preventing intra-cellular damages as the unsaturated lipids (Bakht et al. 2006), soluble carbohydrates, hydrophilic polypeptides, antioxidants and chaperones in the membranes (Bigras et al. 2001).

Aleppo pine prevents water stress damage by stomatal closure before strong changes occur in leaf water potential (Tognetti et al. 1997; Baquedano and Castillo 2006). As a consequence of stomatal closure, carbon assimilation can be completely inhibited (Martinez-Ferri et al. 2000), thereby increasing the risk of oxidative stress. The impairment of photosynthetic carbon assimilation by stomatal closure affects the metabolic balance in plants (Pinheiro et al. 2011). Soluble sugars are among the most drought-responsive metabolites increasing due to starch hydrolysis or impairment of starch production (Rodriguez-Calcerrada et al. 2011). In fact, soluble sugars correlate negatively to photosynthesis (Franck et al. 2006). Specific responses involve also nitrogen (N)-containing compounds, e.g., amino compounds and proteins (Hu et al. 2012).

In fact, the seedling stage is a critical part of the tree's life cycle because seedlings have great susceptibility to resource limitations that affect survival, establishment and growth (Leck et al. 2008). In this work, the intraspecific variability of *Pinus halepensis* through nine seed sources from southern Spain, previously tested for survival and growth performance under field conditions over three years for assisted migration purpose in response to climate change (Taïbi et al. 2014), was tested for its drought and cold hardiness under controlled conditions.

Only few studies have been addressed on Aleppo pine response to drought (Baquedano et al. 2008) and cold (Fernández et al. 2002) stress at the morphological and physiological levels on seedlings stage but without encompassing the wide range of the variability of the species.

We hypothesized that (1) drought and cold hardiness differ between seed sources, with expected seed sources' local adaptation, and that (2) intraspecific differences in selected functional traits (water potential, transpiration, photosynthesis, gas exchange, water use efficiency and soluble sugars) exist in the studied species at the seedling stage. In addition, (3) regional variation correlates to intraspecific variation in functional traits linked to stress tolerance. We further expected that (4) differences in drought and cold hardiness between provenances are physiologically related to the concentration of soluble carbohydrates of the needles.

Our objectives were (1) to assess the intraspecific variability in water relations and photosynthesis in response to drought and cold stress and (2) to determine whether the stress tolerance was related to the original climate of the populations. Photosynthetic gas exchange traits (net photosynthesis, A_{net} ; stomatal conductance, g_s ; transpiration, E) were compared with the concentration of soluble sugars to examine the physiological performance of the stressed seed-lings. Water-use efficiency (WUE) was examined to determine its response to stress. A further determination of potential proteins that could be useful as markers of resistance or/and sensitivity was also discussed

6.2. Materials and methods

6.2.1. Plant material

Seeds from nine *Pinus halepensis* seed sources (basic materials) covering most of the climatic and ecologic regions of the natural range of this species in Spain with a wide spectre of molecular and phenotypic variation (Climent et al. 2008; Voltas et al. 2008) were selected and germinated in the laboratory. These seed sources corresponding to eight Spanish provenances defined for the species (Alía et al. 2009) and one seed orchard (Table 1),

have been tested previously in field provenance trials over three years (1+3 years) for survival and growth performance (Taïbi et al. 2014).

Table 1. Site descriptions for the nine seed sources of *Pinus halepensis* Mill. (from Alía et al. 2009). T_M : annual mean temperature; T_{MC} : mean minimum daily temperature of the coolest month; T_{MW} : mean maximum daily temperature of the warmest month; DM: drought months; SP: summer precipitation; AP: annual precipitation.

Seed source ^(class)	ID	Coordinates	Altitude (m)	T_M (°C)	T_{MC} (°C)	T_{MW} (°C)	SP (mm)	AP (mm)
H.S. Alaquàs*	HSA	39°27'N-0°28'W	43	17.2	5	31.1	58	444
Litoral levantino ¹	Lit	39°45'N-0°18'W	200	16.5	4.8	30.3	64	599
Los Monegros- Depresión Ebro ¹	Mgr	41°53'N-0°52'W	401	14.2	0.9	32.3	79	399
Ibérico Aragonés ¹	Arg	40°47'N-0°04'W	555	14.0	1.5	30.2	91	497
Levante Interior ¹	Lev	39°05'N-1°12'W	673	14.4	2.6	30.5	67	524
Maestrazgo-Los Serranos ¹	Mst	39°47'N-1°11'W	763	13.6	1.4	30.0	93	546
Bética septentrional/sur ¹	Bet	37°55'N-2°55'W	789	14.5	1.9	31.9	55	410
La Mancha ¹	Man	39°55'N-2°14'W	856	13.1	-0.1	32.0	68	496
Alcarria ¹	Alc	40°24'N-2°24'W	869	12.4	-1.1	32.2	82	591

Note: Class of seed source: 1, seed source (identified); *, seed orchard (qualified).

6.2.2. Experimental conditions and treatments

Seedlings were cultivated for 25 weeks in a Sphagnum peat vermiculite-pine bark mix (3:1:1 v/v), in 200 cm³ cell tray (Forest Pot® 200 trays). Each 16-cm-deep plastic tray consists of 54 cells providing a density of 360 seedlings m⁻². After germination, seedlings were obtained accounting for 5 replicates per seed source per tray were arranged in a complete random block design, with nine blocks. Each block contained one tray i.e. one replicate for each seed source. Seedlings of different seed sources were randomized within the block.

Seedlings were watered to full capacity three times a week to avoid water stress once by complete Hoagland's nutrient solution containing all essential macro and micro-nutrients to avoid nutrient deficiency. Seedlings were grown during six months in a phytotron under controlled conditions set at a 24°C/16°C day/night temperatures, relative humidity from 70 per cent and a photoperiod of 16 hours (200 μmol m⁻² s⁻¹).

Trays were grouped randomly and arranged into three treatments. Healthy plants of similar size for each seed source were randomly assigned to each of the three treatments; (C) control treatment well watered conducted under the same previous cited growth conditions, (D) drought stress treatment applied by stopping irrigation, (T) low temperature treatment conducted into a growth chamber of constant temperature set at 6°C and similar growing conditions to those of the other treatments. Seedlings were left for three weeks.

6.2.3. Measurements

6.2.3.1. Water potential

Seedling water potential (Ψ), an indicator of plant water status at the moment of measurement, was measured with a Scholander-type pressure bomb on five seedlings per treatment (model PMS- 1000, PMS Instruments, Corvallis, OR, USA).

6.2.3.2. Photosynthetic gas exchange

In each seedling, measurements were conducted on instantaneous determinations of net CO₂ assimilation (A_{net} , $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), stomatal conductance (g_s , $\text{mmol H}_2\text{O cm}^{-2} \text{ s}^{-1}$), transpiration E ($\text{mmol}^{-1} \text{ H}_2\text{O cm}^{-2} \text{ s}^{-1}$), instantaneous water-use efficiency (WUE; $\mu\text{mol CO}_2 \text{ mmol}^{-1} \text{ H}_2\text{O}$) calculated as assimilation divided by transpiration A/E , and intrinsic water-use efficiency (iWUE; $\mu\text{mol CO}_2 \text{ mol}^{-1} \text{ H}_2\text{O}$) calculated as assimilation divided by stomatal conductance A/g_s , were estimated under conditions of saturating light ($1500 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$), 25 °C for the dry treatment and 6°C for the cold treatment, and ambient CO₂ ($380 \mu\text{mol mol}^{-1} \text{ CO}_2$). In addition, air and needle temperatures were conducted at mid-morning using a portable photosynthesis open-system (Model LI-6400, LI-COR Biosciences Inc., Lincoln, NE, USA), maintaining the relative humidity in the chamber at $55 \pm 5\%$. Air temperature and humidity measurements were performed to ensure that the conditions inside the chamber were similar. The area of the needles in the chamber was the projected needle area and the whole chamber area was completely covered with needles without gaps in between. All gas-exchange measurements were adjusted for needle surface area to scale gas exchange measurements per unit of total photosynthetic area.

6.2.3.3. Chlorophyll fluorescence

The photochemical parameters, Φ_{PSII} and F_v/F_m , provide valuable information about the electron transport system and the conversion and dissipation of the excess excitation energy into heat and can be used as indicators of plant stress (Maxwell and Johnson 2000). Φ_{PSII} and F_v/F_m reflect the actual and maximum potential efficiency of the excitation energy captured by open PSII centers, respectively. Φ_{PSII} indicates the proportion of absorbed energy that is used in photochemistry. The maximum quantum efficiency of PSII photochemistry (F_v/F_m) was determined on the needles during the morning, using an open gas exchange system (LI-6400; LI-COR, Inc., Lincoln, NE, USA) with an integrated fluorescence chamber (LI-6400-40 leaf chamber fluorometer; LI-COR).

Following dark adaptation for 2 h, the minimum fluorescence (F_0) was determined by a measuring light of about $0.5 \mu\text{mol photon m}^{-2} \text{s}^{-1}$, and the maximum fluorescence (F_m) was determined by a 0.8-s saturating flash of about $10,000 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ in the dark-adapted needles. F_v/F_m was calculated as $(F_m - F_0)/F_m$ (Maxwell and Johnson 2000). Five replicates per seed source and treatment were randomly obtained from different individuals after three weeks of treatments.

6.2.3.4. Photosynthetic pigments

Total chlorophylls (Chl_{a+b}), chlorophyll a (Chl_a), chlorophyll b (Chl_b), and carotenoids (Car) were determined spectrophotometrically using 80% acetone as a solvent (Lichtenthaler, 1987). Five replicates per seed source and treatment were obtained from the needles of different individuals during midday.

6.2.3.5. Soluble sugars

Soluble sugars were determined by grinding 0.2 g of needles fresh weight in liquid nitrogen with a mortar and pestle, and then the homogenized powder was re-suspended in 1 ml water. The samples were incubated at 95°C for 10 min, cooled on ice and centrifuged at 4°C for 5 min to remove debris, and the supernatants were filtered through Sep-Pak Plus C-18 solid phase cartridges (Waters). The soluble sugar fraction (mono and oligosaccharides) was separated by chromatography in an HPLC anion exchange column (Hamilton RCX-10, 250mm long, 4.1 mm i.d.), coupled to a pulsed electrochemical detector (Waters 464). Elution was carried out in an isocratic flux of 100 mM NaOH, and quantification was done by peak integration and comparison with sugar standards (1 mM aqueous solutions of glucose, sucrose or fructose) (Vicente et al. 2004).

6.2.3.6. Extraction, separation and determination of proteins

Changes of proteins patterns were also tested under induced drought and cold stress in the aim to determine which proteins could have a potential use as biomarkers for stress tolerance or sensitivity facilitating further future screening of seed sources under similar environmental conditions. Unfortunately, the study of proteins patterns under cold stress is not yet completely achieved and thus only those related to drought stress will be presented. For this purpose, proteins were extracted from roots and needles, from the control and drought treatments, from the most vs. the less responsive seed sources, e.g. those showing opposite response to drought. One gram of plant material powder with the addition of 0.1 g of polyvinylpyrrolidone (PVPP) was extracted with 10 mL of extraction buffer (5% sucrose, 4% SDS and 5% 2-ME) for 10 min at room temperature with gentle stirring, followed by centrifugation at 10,000 g. The clear

supernatant was heated at 100°C for 3 min and then cooled at room temperature.

Proteins were precipitated by 8 volumes of cold acetone. After at least 1 h at -20°C, the mixture was centrifuged at 10,000 g. The pellet was re-suspended in 5 mL of extraction buffer and centrifuged at 10,000 g. After washed once or twice with 80% cold acetone, the pellet was precipitated by adding 4 volumes of cold acetone, and then was lyophilized and stored at -0°C (He et al. 2007). The concentrations of proteins extracted were determined using the Bradford method. Proteins coprecipitating were excised from a 5-20% gradient gel. Gels were visualized by staining with Coomassie Brilliant Blue with some modifications (Loewith et al. 2002). Proteins bands which were present in only one treatment were analyzed using MALDI-TOF mass spectrometry. 1D PAGE was used to separate proteins as this technique allowed to compare different seed sources from different treatments in the same gel.

6.2.4. Statistical analysis

All data were subjected to two-way analysis of variance (ANOVA) to determine differences among seed sources and stress treatments in comparison with the control for each variable then one-way analysis of variance was performed within each treatment. The significant differences between means were determined using Tukey's test at $P < 0.05$ level. In all cases, data were examined for normality and homogeneity of variances and identified for any violations of assumptions. Correlations between the measured and the seed sources origin (geographic coordinates and climatic variables) variables were determined based on the coefficient of Pearson. The seed orchard 'H.S. Alaquàs' was excluded in these analyses because it is grown from various sources.

Multivariate screening of seed sources was carried out to make additional comprehensive assessments of the overall response. Canonical discriminant analysis was used to summarize the within-treatment among seed sources variability. The multivariate generalized distances of Mahalanobis were computed later on the same variables used for the multivariate analysis and were used to cluster seed sources into groups with similar performance. The agglomerative hierarchical clustering procedure based on Ward's method was used. All statistical analyses were performed using the computing environment R (R Development Core Team, 2013).

6.3. Results

Analysis of variance indicated that both stresses, in comparison with the control treatment for all the seed sources, induced significant variations in Ψ_w , A_{net} , WUE, iWUE, fructose and carotenoid concentrations (Tables 2,3). In addition, drought stress induced significant difference in Fv/Fm and sucrose concentration however, cold stress changes the levels of g_s , E , photosynthetic pigments and glucose concentrations (Tables 2, 3). Furthermore, photosynthetic pigments and soluble sugars concentrations variation' among the seed sources was significant for both stresses. Variation among seed sources under control conditions was significant only for chlorophyll pigments and sucrose concentrations (p -value<0.05). Besides, the effect of drought stress induced significant differences among seed sources for Fv/Fm, photosynthetic pigments, glucose and fructose concentrations (p -value<0.05). Unlike under drought stress, Fv/Fm and chlorophyll concentrations were not affected by the cold stress (Tables 2,3).

Table 2. ANOVA summary performed on each stress in comparison with the control (F: F-value; P: *p-value<0.05, ** p-value<0.01, *** p-value<0.001) of the measured traits of nine Aleppo pine seed sources subjected to three treatments (control, drought and cold). Stress: variation induced by the stress factor, SS: variation induced by seed sources variability, S*SS: variation induced by the interaction.

Variation	Drought stress						Cold stress					
	Stress		SS		S*SS		Stress		SS		S*SS	
	F	P	F	P	F	P	F	P	F	P	F	P
Ψ_w	666.3	0.00***	0.45	0.885	1.19	0.331	11.47	0.002**	1.34	0.255	0.83	0.583
Fv/Fm	8.71	0.006**	1.48	0.199	3.50	0.005**	3.77	0.060	1.46	0.207	1.70	0.133
A_{net}	4.18	0.049*	0.50	0.844	0.28	0.970	4.13	0.050*	0.17	0.993	0.81	0.597
g_s	1.34	0.255	0.33	0.947	0.65	0.732	43.89	0.00***	0.54	0.816	1.34	0.257
Φ_{PSII}	1.75	0.194	0.62	0.755	1.82	0.106	0.11	0.738	1.48	0.199	0.88	0.544
E	1.54	0.223	0.32	0.952	0.63	0.747	50.21	0.00***	0.55	0.811	1.34	0.258
WUE	5.65	0.023*	1.65	0.146	0.75	0.650	41.51	0.00***	0.95	0.492	0.59	0.776
iWUE	6.73	0.014*	1.56	0.172	0.71	0.682	33.29	0.00***	0.96	0.480	0.59	0.783
Glucose	0.24	0.630	3.88	0.002**	5.25	0.00***	110.10	0.00***	3.80	0.003**	2.16	0.056
Fructose	35.91	0.00***	2.57	0.025*	2.74	0.019*	6.99	0.012*	3.49	0.005**	1.27	0.291
Sucrose	15.86	0.00***	1.26	0.296	2.08	0.064	0.44	0.510	1.72	0.128	2.11	0.061*
Chl a	0.08	0.781	9.21	0.00***	4.39	0.001**	44.65	0.00***	2.28	0.045*	1.74	0.124
Chl b	0.49	0.487	5.96	0.00***	5.01	0.00***	24.78	0.00***	2.28	0.044*	3.87	0.002**
Chl t	0.00	0.946	8.57	0.00***	4.82	0.00***	41.26	0.00***	2.34	0.040*	2.38	0.037
Car	6.04	0.019*	6.69	0.00***	2.25	0.047*	8.81	0.005**	2.61	0.024*	2.45	0.032

Table 3. ANOVA summary performed for the provenance factor on each of the three treatments (F: F-value; P: *p-value<0.05, ** p-value<0.01, *** p-value<0.001) of the measured traits of nine Aleppo pine seed sources.

	Control		Drought		Cold	
	F	P	F	P	F	P
Ψ_w	0.88	0.554	0.83	0.585	1.20	0.350
Fv/Fm	2.08	0.097	2.70	0.038*	1.50	0.224
A_{net}	0.27	0.967	0.74	0.656	1.16	0.374
g^s	0.44	0.881	0.60	0.766	1.21	0.348
Φ_{PSII}	1.12	0.399	1.31	0.301	1.23	0.335
E	0.42	0.895	0.61	0.755	1.21	0.347
WUE	0.73	0.661	1.71	0.163	0.69	0.696
iWUE	0.74	0.656	1.63	0.186	0.63	0.746
Glucose	1.25	0.328	9.03	0.000***	4.78	0.003**
Fructose	1.32	0.298	4.48	0.004**	3.89	0.008**
Sucrose	2.84	0.033*	1.73	0.159	1.97	0.111
Chl a	2.59	0.047*	12.35	0.000***	1.32	0.295
Chl b	7.00	0.000***	5.05	0.002**	0.84	0.580
Chl t	3.86	0.009**	9.05	0.000***	1.08	0.418
Car	2.61	0.046*	6.50	0.000***	2.46	0.054*

6.3.1. Water potential

Taking all seed sources together, mean Ψ_w exhibited significant reductions after seedlings treatment by low temperatures comparing to the control treatment (-1.56 ± 0.09 compared with -1.34 ± 0.04 MPa, respectively), moreover, decrease was by threefold under drought stress treatment (-3.34 ± 0.16 MPa). Seed sources from lower altitudes exhibited lower water potential under cold stress (p-value<0.05) (Table 4).

6.3.2. Photosynthetic gas exchange and water use efficiency

On the other hand, values of E and g_s were more than twice as large for cold-stressed seedlings (0.863 ± 0.08 and 0.038 ± 0.01 $\text{mmol}^{-1} \text{H}_2\text{O cm}^{-2} \text{s}^{-1}$, respectively) as for control seedlings (0.376 ± 0.10 and 0.017 ± 0.01 $\text{mmol}^{-1} \text{H}_2\text{O cm}^{-2} \text{s}^{-1}$, respectively) (p -value < 0.01). As well, there were significant differences in E and g_s between control seedlings and drought-stressed seedlings (0.289 ± 0.05 and 0.013 ± 0.0 $\text{mmol}^{-1} \text{H}_2\text{O cm}^{-2} \text{s}^{-1}$, respectively).

A_{net} under low temperature treatment was significantly greater by 15% than in control (1.025 ± 0.12 compared with 0.893 ± 0.27 $\mu\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1}$, respectively) while it was significantly lower by 40% under drought treatment (0.541 ± 0.13 $\mu\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1}$) (p -value < 0.05).

WUE as well as $i\text{WUE}$ were significantly different among treatments, with greatest values in the control lot (2.35 ± 0.33 $\mu\text{mol CO}_2 \text{mmol}^{-1} \text{H}_2\text{O}$ and 51.48 ± 7.55 $\mu\text{mol CO}_2 \text{mol}^{-1} \text{H}_2\text{O}$ respectively) compared with the drought (1.87 ± 0.53 $\mu\text{mol CO}_2 \text{mmol}^{-1} \text{H}_2\text{O}$ and 40.50 ± 10.9 $\mu\text{mol CO}_2 \text{mol}^{-1} \text{H}_2\text{O}$ respectively) and cold (1.23 ± 0.14 $\mu\text{mol CO}_2 \text{mmol}^{-1} \text{H}_2\text{O}$ and 28.59 ± 3.39 $\mu\text{mol CO}_2 \text{mol}^{-1} \text{H}_2\text{O}$ respectively) treatments (p -value < 0.01). This trend was accompanied by increases in mesophyll CO_2 concentrations in cold (329.86 ± 5.3 $\mu\text{mol mol}^{-1}$) and drought (312.29 ± 16.8 $\mu\text{mol mol}^{-1}$) treatments comparing to the control seedlings (294.86 ± 12.4 $\mu\text{mol mol}^{-1}$) (p -value < 0.01).

6.3.3. Chlorophyll fluorescence

Maximal efficiency of PSII (F_v/F_m) was similar (~ 0.76) in both control and cold treatments (p -value > 0.05), but it decreased significantly under drought stress (p -value < 0.01). Quantum yield of non-cyclic electron transport (Φ_{PSII}) difference was not significant among treatments (p -

value >0.05). Significant difference among seed sources in the establishment of F_v/F_m was observed under drought conditions. Higher F_v/F_m values were correlated positively with altitude (p-value <0.05) and negatively with mean annual temperature (p-value <0.01) and minimum daily temperature of the coolest month of seed sources' origin (p-value <0.01). Highest values were recorded by 'Alc' and 'Mgr' while the lowest value was of 'Lit' (Table 4).

Table 4. Summary of the variation within and among treatments for water potential (Ψ_w), transpiration (E), net photosynthesis (A_{net}), stomatal conductance (gs), water (WUE) and internal water (iWUE) use efficiency, maximum quantum efficiency of PSII photochemistry (F_v/F_m) and quantum yield of non-cyclic electron transport (Φ_{PSII}).

	Control	Drought	Cold	Control	Drought	Cold
Ψ_w (MPa)				E ($\text{mmol}^{-1} \text{H}_2\text{O cm}^{-2} \text{s}^{-1}$)		
Alc	-1.02 ± 0.15	-3.23 ± 0.55	-1.52 ± 0.68	0.34 ± 0.10	0.38 ± 0.22	0.85 ± 0.16
Arg	-1.17 ± 0.17	-3.25 ± 0.24	-1.77 ± 0.63	0.27 ± 0.19	0.28 ± 0.09	0.69 ± 0.65
Bet	-0.93 ± 0.06	-3.56 ± 0.12	-1.30 ± 0.17	0.47 ± 0.29	0.28 ± 0.05	0.88 ± 0.18
Hsa	-1.25 ± 0.24	-3.20 ± 0.42	-1.75 ± 0.24	0.31 ± 0.10	0.25 ± 0.12	0.84 ± 0.24
Lev	-1.17 ± 0.29	-3.52 ± 0.32	-1.42 ± 0.46	0.43 ± 0.23	0.23 ± 0.04	0.86 ± 0.52
Lit	-1.07 ± 0.22	-3.45 ± 0.53	-1.85 ± 0.17	0.23 ± 0.07	0.35 ± 0.17	0.98 ± 0.44
Man	-1.30 ± 0.17	-3.30 ± 0.39	-1.53 ± 0.32	0.55 ± 0.37	0.28 ± 0.12	0.91 ± 0.31
Mgr	-1.12 ± 0.22	-3.43 ± 0.06	-1.80 ± 0.71	0.39 ± 0.21	0.30 ± 0.09	0.92 ± 0.63
Mst	-1.15 ± 0.31	-3.10 ± 0.10	-1.15 ± 0.13	0.39 ± 0.22	0.26 ± 0.15	0.83 ± 0.41
A_{net} ($\mu\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1}$)				gs ($\text{mmol}^{-1} \text{H}_2\text{O cm}^{-2} \text{s}^{-1}$)		
Alc	0.64 ± 0.25	0.26 ± 0.15	1.08 ± 0.29	0.016 ± 0.01	0.016 ± 0.01	0.037 ± 0.01
Arg	0.68 ± 0.66	0.71 ± 0.42	0.99 ± 0.82	0.012 ± 0.01	0.013 ± 0.00	0.030 ± 0.03
Bet	1.07 ± 0.90	0.67 ± 0.19	1.19 ± 0.30	0.022 ± 0.01	0.013 ± 0.00	0.037 ± 0.01
Hsa	0.70 ± 0.21	0.50 ± 0.14	1.00 ± 0.50	0.014 ± 0.01	0.012 ± 0.01	0.037 ± 0.01
Lev	1.36 ± 0.89	0.55 ± 0.37	0.79 ± 0.31	0.019 ± 0.01	0.011 ± 0.00	0.039 ± 0.03
Lit	0.52 ± 0.23	0.64 ± 0.57	1.07 ± 0.77	0.010 ± 0.00	0.016 ± 0.01	0.043 ± 0.02
Man	0.98 ± 0.71	0.52 ± 0.45	1.00 ± 0.34	0.026 ± 0.02	0.013 ± 0.01	0.039 ± 0.01
Mgr	1.12 ± 0.97	0.50 ± 0.20	1.18 ± 0.66	0.018 ± 0.01	0.014 ± 0.00	0.041 ± 0.03
Mst	0.97 ± 0.53	0.53 ± 0.48	0.92 ± 0.41	0.018 ± 0.01	0.012 ± 0.01	0.036 ± 0.02

WUE ($\mu\text{mol CO}_2 \text{ mmol}^{-1} \text{ H}_2\text{O}$)				iWUE ($\mu\text{mol CO}_2 \text{ mol}^{-1} \text{ H}_2\text{O}$)		
Alc	1.98±0.71	0.69±0.23	1.27±0.24	42.89±16.65	16.81±5.80	29.46±5.18
Arg	2.41±1.25	2.43±1.19	1.46±0.71	53.84±29.35	53.33±25.93	34.03±16.32
Bet	2.09±0.49	2.42±0.32	1.36±0.30	45.63±10.29	51.02±6.62	31.84±6.66
Hsa	2.32±0.36	2.12±0.55	1.14±0.39	50.55±08.24	45.20±9.88	25.92±9.36
Lev	3.04±0.37	2.22±1.27	1.26±0.73	67.09±08.87	47.31±26.73	28.92±17.35
Lit	2.35±0.97	1.71±0.83	1.00±0.56	51.10±21.05	37.45±15.97	23.02±13.18
Man	1.99±1.10	1.69±0.96	1.13±0.29	42.97±24.43	35.83±19.80	26.21±7.08
Mgr	2.43±1.20	1.84±1.10	1.33±0.32	52.98±26.12	40.05±23.07	30.84±7.85
Mst	2.57±0.87	1.75±0.82	1.15±0.38	56.25±21.90	37.50±17.61	27.10±9.11
Fv/Fm				Φ PSII		
Alc	0.745±0.01	0.766±0.01 ^b	0.748±0.02	0.12±0.05	0.22±0.03	0.14±0.05
Arg	0.760±0.01	0.755±0.01 ^{ab}	0.758±0.03	0.13±0.03	0.19±0.07	0.18±0.08
Bet	0.743±0.03	0.756±0.01 ^{ab}	0.752±0.02	0.11±0.05	0.17±0.12	0.07±0.01
Hsa	0.763±0.01	0.739±0.04 ^{ab}	0.761±0.02	0.18±0.03	0.17±0.05	0.22±0.13
Lev	0.766±0.01	0.751±0.01 ^{ab}	0.721±0.05	0.18±0.11	0.18±0.06	0.13±0.07
Lit	0.775±0.00	0.723±0.04 ^a	0.731±0.04	0.15±0.02	0.13±0.08	0.19±0.09
Man	0.773±0.01	0.754±0.01 ^{ab}	0.733±0.04	0.14±0.03	0.17±0.04	0.16±0.04
Mgr	0.769±0.01	0.761±0.01 ^b	0.771±0.01	0.18±0.06	0.15±0.05	0.20±0.14
Mst	0.766±0.01	0.751±0.01 ^{ab}	0.767±0.02	0.14±0.02	0.17±0.03	0.14±0.05

6.3.4. Photosynthetic pigments

Cold stress induced a significant reduction for all photosynthetic pigments (p -value <0.01) while drought stress induced only significant decrease of carotenoids concentration (p -value <0.05) (Table 5). Chl a concentration decrease was significant only under cold stress (8.32 ± 1.03 mg g^{-1} FM against 11.30 ± 1.6 mg g^{-1} FM in the control). Chl b concentration decreased under cold stress (3.18 ± 0.44 mg g^{-1} FM in comparison to control 4.19 ± 0.97 mg g^{-1} FM). The decrease in the carotenoid concentration was significant under both stresses (p -value <0.01). The decrease of chlorophyll pigments under the cold treatment was correlated negatively with the maximum daily temperature of the warmest month of seed sources' origin (p -value <0.05).

Table 5. Summary of the variation within and between treatments for the concentration of photosynthetic pigments; chlorophyll a (Chl a), chlorophyll b (Chl b), total chlorophyll (Chl t) and carotenoid (Car).

	Control	Drought	Cold	Control	Drought	Cold
Chl a (mg g ⁻¹ FM)				Chl b (mg g ⁻¹ FM)		
Alc	8.36±2.16	4.82±0.74 ^a	8.42±0.37	2.99±1.44 ^{ab}	2.02±0.96 ^a	3.41±0.73
Arg	10.17±0.32	9.82±0.04 ^b	8.36±1.69	2.75±0.38 ^a	3.90±0.37 ^{abc}	3.48±1.54
Bet	11.85±0.08	10.22±0.31 ^b	8.14±0.10	5.10±0.07 ^{cd}	3.52±0.39 ^b	3.14±0.02
Hsa	13.18±2.33	12.98±2.03 ^{bc}	8.15±1.20	5.36±0.44 ^d	4.82±1.77 ^{abc}	3.08±0.64
Lev	12.16±0.38	11.28±1.07 ^b	9.93±1.88	4.43±0.02 ^{abc}	4.23±0.48 ^{abc}	3.28±0.50
Lit	12.53±1.74	10.54±0.87 ^b	9.00±1.07	5.07±0.70 ^{cd}	3.59±0.49 ^{ab}	3.19±0.36
Man	9.61±1.11	15.65±3.31 ^c	7.33±1.45	3.40±0.16 ^{abc}	6.75±2.08 ^c	2.75±0.45
Mgr	12.11±2.51	11.82±1.36 ^{bc}	6.42±1.00	4.79±0.02 ^{bcd}	5.22±0.55 ^{bc}	2.35±0.49
Mst	11.72±2.54	13.44±0.60 ^{bc}	9.15±3.04	3.87±1.02 ^{abc}	5.19±0.42 ^{bc}	3.89±1.43
Chl a+b (mg g ⁻¹ FM)				Car (mg g ⁻¹ FM)		
Alc	11.35±3.59 ^a	6.83±1.70 ^a	11.83±1.11	1.45±0.30 ^a	1.22±0.32 ^a	1.84±0.27
Arg	12.93±0.07 ^{ab}	13.72±0.36 ^{ab}	11.84±3.23	2.09±0.41 ^{ab}	1.98±0.47 ^{abcd}	2.20±0.39
Bet	16.94±0.15 ^{ab}	13.74±0.10 ^{ab}	11.28±0.12	2.50±0.10 ^{ab}	1.38±0.11 ^{ab}	1.71±0.48
Hsa	18.53±2.77 ^b	17.80±3.80 ^{bc}	11.23±1.84	2.74±0.83 ^b	2.66±0.56 ^d	2.06±0.48
Lev	16.58±0.38 ^{ab}	15.51±1.54 ^{bc}	13.21±2.38	2.29±0.21 ^{ab}	1.41±0.14 ^{abc}	2.32±0.68
Lit	17.60±2.45 ^{ab}	14.14±1.36 ^b	12.19±1.43	2.36±0.30 ^{ab}	2.59±0.56 ^d	2.17±0.01
Man	13.01±0.95 ^{ab}	22.40±5.39 ^c	10.08±1.90	2.10±0.44 ^{ab}	2.49±0.26 ^{cd}	1.26±0.43
Mgr	16.90±2.52 ^{ab}	17.04±1.90 ^{bc}	8.77±1.49	2.54±0.25 ^{ab}	2.33±0.07 ^{bcd}	1.52±0.23
Mst	15.59±3.56 ^{ab}	18.64±1.02 ^{bc}	13.04±4.47	2.05±0.12 ^{ab}	1.67±0.54 ^{abcd}	2.13±0.02

6.3.5. Soluble sugars

Differential accumulation pattern of glucose in needles was observed among Aleppo pine seed sources subjected to drought stress (Table 6); 'Lit', 'Mst', 'HSA' and 'Mgr' seedlings manifested a significant increase of glucose concentrations in comparison to the control treatment and subsequently marked in that order the highest values while this concentration decreased in the other seed sources (p -value <0.05). However, significant increase was noted regarding needles fructose concentrations for all the tested seed sources with respect the control (p -value <0.01). In addition, almost of seed sources showed significant increase of sucrose concentrations under drought except 'Alc' and 'Man' (p -value <0.05).

On the other hand, the glucose concentration significantly decreased under cold stress (p -value <0.05). 'Lev' seedlings had manifested the highest glucose concentration among seed sources whilst 'Bet' seedlings were characterized by the lowest concentration (Tables 6). Regarding fructose concentration, 'Arg', 'Lev' and 'Lit' showed significant increases whereas the other seed sources manifested significant decreases of its concentrations. Furthermore, almost of seed sources had manifested a significant decrease of sucrose concentration under the cold stress except for 'Bet', 'Mst' and 'Lit' which they increased their needles concentration of sucrose and marked respectively the highest values (p -value <0.05).

Table 6. Summary of the variation within and between treatments for the concentration of soluble sugar concentrations (glucose, fructose and sucrose).

Seed source	Glucose mg ml ⁻¹			Fructose mg ml ⁻¹			Sucrose (µg ml ⁻¹)		
	Control	Drought	Cold	Control	Drought	Cold	Control	Drought	Cold
Alc	2.55±0.34	2.01±0.22 ^{ab}	1.07±0.06 ^{abc}	3.06±0.51	4.51±0.56 ^c	2.39±0.30 ^{ab}	0.26±0.09	0.19±0.17	0.09±0.11
Arg	2.67±0.30	1.89±0.34 ^a	1.32±0.10 ^{abc}	2.94±0.39	3.11±0.37 ^{ab}	3.27±0.52 ^b	0.09±0.03	0.77±0.53	0.02±0.02
Bet	2.51±0.25	1.77±0.28 ^a	0.53±0.41 ^a	2.57±0.41	3.45±0.40 ^{abc}	1.96±0.27 ^a	0.17±0.05	1.06±1.03	0.49±0.48
Hsa	2.77±0.43	3.00±0.32 ^{bcd}	1.19±0.24 ^{abc}	3.42±0.17	3.89±0.23 ^{abc}	2.55±0.48 ^{ab}	0.21±0.09	0.25±0.04	0.02±0.03
Lev	2.65±0.37	2.23±0.13 ^{abc}	2.16±0.71 ^c	2.79±0.31	3.85±0.55 ^{abc}	2.97±0.25 ^{ab}	0.15±0.11	1.01±0.73	0.04±0.04
Lit	2.52±0.55	3.48±0.53 ^d	1.92±0.36 ^{bc}	2.85±0.75	4.37±0.38 ^{bc}	2.96±0.27 ^{ab}	0.11±0.02	0.73±0.52	0.11±0.09
Man	2.70±0.30	2.08±0.57 ^{ab}	1.58±0.70 ^{abc}	3.43±0.59	2.93±0.48 ^a	2.78±0.87 ^{ab}	0.27±0.05	0.04±0.01	0.08±0.09
Mgr	2.01±0.58	2.25±0.07 ^{abc}	1.03±0.13 ^{ab}	2.47±0.82	3.29±0.36 ^{abc}	1.80±0.31 ^a	0.17±0.01	0.28±0.01	0.05±0.00
Mst	2.23±0.50	3.23±0.47 ^{cd}	1.12±0.07 ^{abc}	2.68±0.40	3.88±0.56 ^{abc}	2.16±0.32 ^{ab}	0.13±0.04	0.90±0.45	0.38±0.38

6.3.6. Multivariate screening of seed sources

In order to select the distinct groups of seed sources according to their performance in each treatment, the canonical discriminant analysis complemented by cluster analysis were performed on the measured traits (Fig. 1, 2). Regarding the three treatments, the first canonical axis (Can1) had higher discriminant power (more than 60%) than the second one (Can2). Control seed sources were separated by Can1, accounting for 74% of the variability. A clear separation among seed sources can be observed according to this axis relative to their internal concentrations of photosynthetic pigments mainly Chl b: 'Bética septentrional/sur', 'H.S. Alaquàs', 'Los Monegros Depression del Ebro', 'Levante Interior', and 'Litoral levantino' are on the right side of the axis while 'Maestrazgo-Los Serranos' and 'Ibérico Aragonés', characterized by the lowest concentrations, appeared on the opposite side. Separation on Can2 (12% of variability) was explained mostly by sucrose and Chl a concentrations variation. 'Alcarria' and 'La Mancha' manifested higher concentrations of these molecules and appeared on the opposite side of 'Ibérico Aragonés', 'Levante Interior' and 'Litoral Levantino' seedlings.

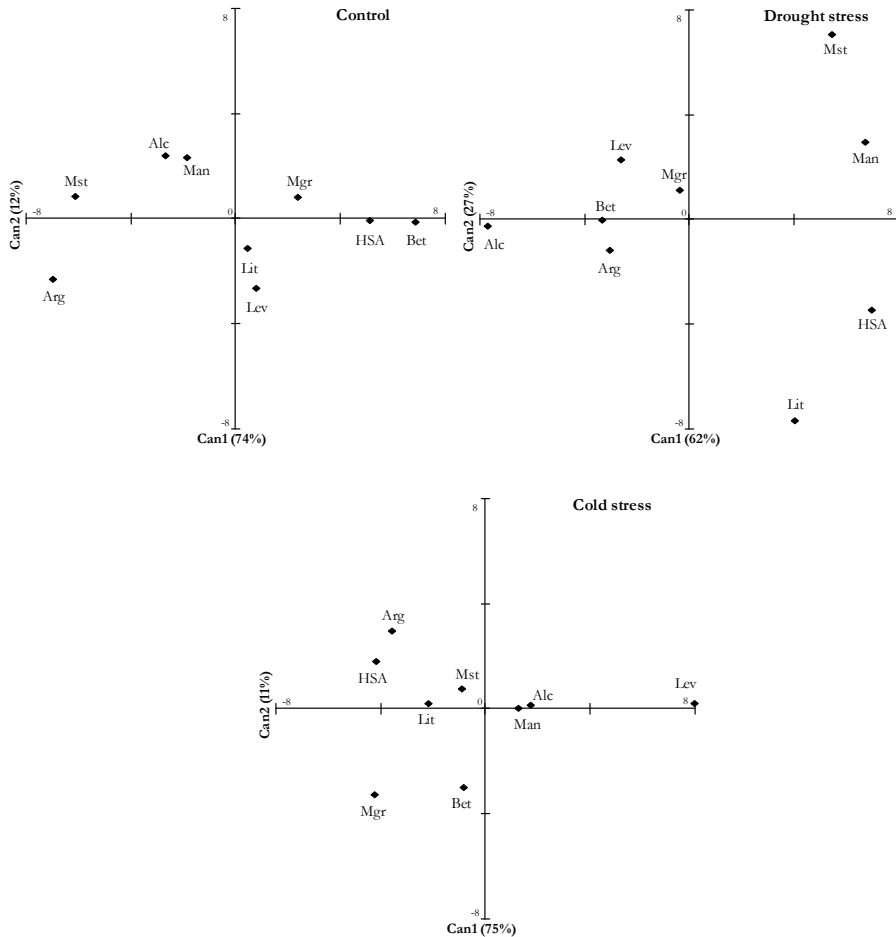


Fig. 1. The scatter-plot of the first and the second Canonical axes obtained by Canonical Discriminant Analysis on nine Aleppo pine seed sources.

Can1 described around 62% of the whole variability observed among seed sources subjected to drought stress. The main variability was due to the concentrations of glucose and Chl a variation in the needles among seed sources. ‘H.S. El Alaquàs’, ‘La Mancha’, ‘Maestrazgo los Serranos’ and ‘Litoral levantino’ manifested higher concentrations and appeared on the right of the axis, whereas ‘Alcarria’, ‘Bética Septentrional/Sur’ and ‘Ibérico Aragonés’, with lower concentrations, are on the left side. Can2 (27% of

total variability) separated seed source according to Fv/Fm; ‘Litoral levantino’ and ‘H.S. El Alaquàs’ seed sources with low values appeared on the opposite side to ‘Maestrazgo los Serranos’, ‘La Mancha’ and ‘Levante Interior’.

Under the cold stress, Can1 explained 75% of the total variability and was positively correlated to glucose needles concentrations ($r^2=0.14$, p -value <0.05) and negatively correlated to E and g_s ($r^2=-0.1^*$, p -value <0.05). ‘Levante Interior’, characterized by the lowest Fv/Fm along with the highest concentrations of Chl a, Car and glucose, appeared faraway on the axis to ‘Alcarria’ and ‘La Mancha’ and on the opposite side to the other seed sources. Can2 (11% of total variability) explained seedlings’ survival by 80% ($r^2=0.69^{**}$). ‘Los Monegros/Depression del Ebro’ and ‘Bética septentrional/sur’ seedlings had lower concentrations of glucose and fructose appeared on the opposite side to the other seed sources.

Dendrograms constructed using the Mahalanobis distances between pairs of seed sources revealed many similar associations (Fig. 2). Similar distances demonstrated by seed sources indicated similar performance of their seedlings under similar conditions. Under control conditions, the first group was characterized by seed sources with high sucrose and low Chl a and Car concentrations (‘Alcarria’ and ‘La Mancha’) on the opposite of the subset which included ‘Bética septentrional/sur’ and ‘H.S. Alaquàs’ with low chlorophyll pigments concentration and the subset of ‘Ibérico Aragonés’ and ‘Maestrazgo-Los Serranos’ with low sucrose concentration. The subset included ‘Levante Interior’ and ‘Litoral levantino’ was characterized by high concentration of Chl a.

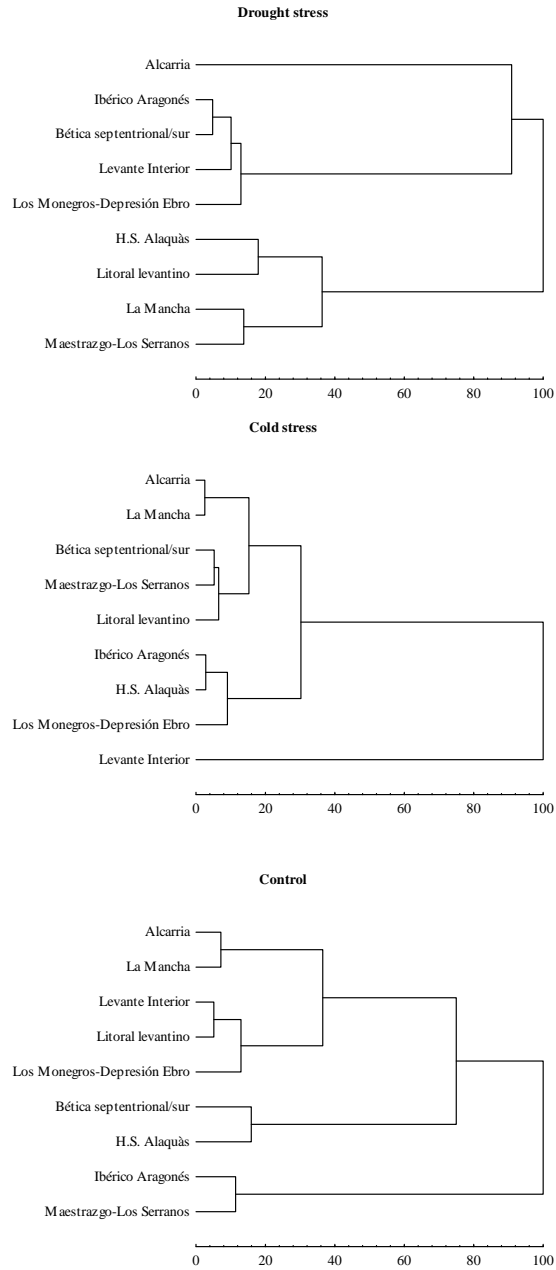


Fig. 2. Dendrograms based on Mahalanobis distances using clustering Ward's method among nine Aleppo pine seed sources tested under different conditions of drought and low temperature.

Under drought stress, 'H.S. El Alaquàs' and 'Litoral levantino' seed sources characterized by low F_v/F_m values were grouped with 'La Mancha' and 'Maestrazgo los Serranos' of high F_v/F_m ratios manifesting together high needles concentrations of soluble sugars and photosynthetic pigments along with low transpiration rates. 'Litoral levantino' seedlings manifested high transpiration and low concentrations of soluble sugars and photosynthetic pigments among seed sources of this group. The second group included 'Ibérico Aragonés', 'Bética septentrional/sur', 'Levante Interior' and 'Los Monegros Depresión del Ebro' seed sources, even with lowest water potential values, characterized by low concentrations of soluble sugars and photosynthetic pigments. 'Levante Interior' seedlings distinguished from this group by their lowest transpiration rates and high concentrations of soluble sugars and photosynthetic pigments. 'Alcarria' seedlings manifested the lowest photosynthetic rates and the highest transpiration rates along with low levels of soluble sugars and photosynthetic pigments.

Under the cold stress, the first set included 'Alcarria' and 'La Mancha' which manifested higher survival rates with good growth. The second set regroups 'Bética septentrional/sur', 'Maestrazgo-Los Serranos' and 'Litoral levantino' seedlings characterized by high concentrations of sucrose and total chlorophyll. The third group comprised 'Ibérico Aragonés', 'H.S. Alaquàs' and 'Los Monegros Depresión del Ebro' seed sources characterized by low water potential and high. 'Levante interior' seed source which had the lowest photosynthetic rates along with high levels of glucose, fructose, Car and Chl a appeared in a separate cluster.

6.3.7. Proteins

Changes of proteins patterns were also tested under induced by drought and cold stress in the aim to determine which proteins could have a potential use as biomarkers for stress tolerance or sensitivity facilitating further future screening of seed sources under similar environmental conditions. Unfortunately, the study of proteins patterns under cold stress is not yet completely achieved and thus only those related to drought stress are summarized in the Table 7.

Table 7. Proteins of differential expression identified in response to drought (according to our previous results of seed sources' adaptation).

Seed source	m/z	Score	NCBI/TrEmbl	Protein family
Needles				
	694,4	170	<i>Arabidopsis thaliana</i> Q9SJU4	Probable fructose-bisphosphate aldolase 1, chloroplastic
	706,4	86	<i>Lupinus luteus</i> P50345	60S acidic ribosomal protein P0
	694,4	356	<i>Picea sitchensis</i> ABK24240	Unknown(~plastidic aldolase)
	694,4	531	<i>Picea sitchensis</i> ACN40657	Unknown(~fructose-bisphosphate aldolase)
Adapted under drought	890,5	413	<i>Coelogyne sp.</i> Tran T-37 AGL73005	ATP synthase beta subunit, partial (chloroplast)
	472,8	483	<i>Picea sitchensis</i> ADE76480	Unknown (~Histone H2A-like protein)
	881,4	134	<i>Pinus pinaster</i> CAC83359	Histone H2B
	511,8	249	<i>Picea sitchensis</i> ABK23173	Unknown (~40S ribosomal protein S13)
	480,3	247	<i>Picea sitchensis</i> ABK22887	Unknown
Roots				
Adapted under control	609,9	102	<i>Oryza sativa</i> Japonica Group BAA02152	eukaryotic initiation factor 4A
	513,3	99	<i>Juniperus ashei</i> AAK85129	elongation factor
	940,2	86	<i>Picea sitchensis</i> ABR16781	Unknown (~3-ketoacyl-CoA thiolase 2, peroxisomal-like)
	622,9	53	<i>Oryza sativa</i> Japonica Group AAN60990	Putative Nonclathrin coat protein gamma - like protein
	445,8		<i>Solanum lycopersicum</i> AAG31638	Elongation factor 2

Mal-adapted under control	577,9	79	<i>Arabidopsis thaliana</i> NP_564878	60S ribosomal protein L10-3
	626,4	56	<i>Picea sitchensis</i> ABR17995	Unknown
	712,4	123	<i>Picea sitchensis</i> ABK24378	Unknown (~ATP synthase subunit beta)
	896,1	116	<i>Picea sitchensis</i> ABK24331	Unknown (~malate dehydrogenase)
	983,5	85	<i>Oryza sativa</i> Japonica Group AAN60990	malate dehydrogenase
Adapted under drought	600,3	252	<i>Plectranthus scutellarioides</i> Q42662	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase
	636,8	127	<i>Oryza sativa</i> subsp. indica A2YWQ1	Heat shock protein 82
	527,3	95	<i>Arabidopsis thaliana</i> P51818	Heat shock protein 81-3
	630,3	166	<i>Citrus sinensis</i> XP_006488105	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase, chloroplastic-like isoform X1
	782,4	55	<i>Pinus radiata</i> AEW09360	hypothetical protein UMN_6182_01, partial
	950,2	98	<i>Picea sitchensis</i> ACN40706	Unknown (~amidase 1-like)
	804,4	93	<i>Zea mays</i> ACG43057	succinate dehydrogenase flavoprotein subunit, mitochondrial
	589,3	80	<i>Pinus banksiana</i> AFU73844	(-)-beta-pinene synthase
	837,4	102	<i>Picea sitchensis</i> ABK23116	Unknown (~chloroplast photosystem I subunit)
	472,8	83	<i>Picea sitchensis</i> ABK27024	Unknown (~histone H2A)

It is to be noted that the qualitative difference under the control treatment was observed only between roots samples, 10 bands were identified without potential function. Under drought stress, 10 bands in the roots and 9 bands in the needles were identified in the samples belonging from seed sources adapted to this constraint ('Man' and 'Bet'). Several proteins overrepresented in the drought adapted seed sources were determined. Most of them were of unknown function or related to the transcriptional machinery and sugar metabolism. Interestingly, enzyme related to sulphur amino-acids metabolism was detected.

6.4. Discussion

Aleppo pine (*Pinus halepensis* Mill.), as a pioneer tree species in the Mediterranean region, has to deal with fluctuations in temperature and water soil availability during his life cycle (Baquedano et al. 2008). This study documents variation in physiological traits related to transpiration, photosynthesis, gas exchanges and soluble sugars accumulation among Aleppo pine seed sources.

Water potential exhibited significant reductions in Aleppo pine seedlings under drought and low temperature stresses. The Ψ_w values ranging from -3.1 to -3.6 MPa on drought stressed seedlings are relatively high value in comparison with other arid coniferous tree species such as *Callitris rheomboidea* (-4.3 MPa, Brodribb and Cochard 2009), and *Juniperus scopulorum* (-5.5 MPa, McDowell et al. 2008). High Ψ_w may be related to high sensitivity of the xylem to embolism or to a wide safety margin taken by the plant, since cavitation occurs when Ψ declines below a threshold value (Klein et al. 2011). Tognetti et al. (1997) and Baquedano and Castillo (2006) reported previously that this water-saving strategy of this species prevents damage by stomatal closure before strong changes occur in needle water potential. Similarly to drought stress, the decrease in water potential may result from the movement of water out of the cells or an accumulation of solutes (Fernández et al. 2002). In addition, after exposure to low temperature, low water potentials have been recorded in several conifer species including *Pseudotsuga menziesii* (Mirb.) Franco (Ritchie and Shula 1984), *Abies amabilis* Douglas ex Forbes (Teskey et al. 1984), and *Picea mariana* (Mill.) (Colombo 1986). Less favourable growing conditions could enhance intraspecific differences between seed sources (Voltas et al. 2008). Seed sources from lower altitudes exhibited lower water potential under drought. Similar clinal trends associated with the latitude of seed origin have

been exhibited previously for this species (Agúndez et al. 1997; Climent et al. 2008) or for other pines e.g. *Pinus sylvestris* (Persson 1994). The capacity to adjust water potential is usually related to the resistance to drought. Through this mechanism, plants are able to maintain turgor potential, the capacity for growth and photosynthesis, and the ability to tolerate more negative water potential and lower water availability (Villar-Salvador et al. 1997). This demonstrated clearly the selective role of climate variables in assessing adaptive variation to climate (Voltas et al. 2008) and determining populations' fitness in this species (Climent et al. 2008).

Due to the severe stomatal control under drought, there were significant decrease in E and g_s . However, Klein et al. (2011) reported that stomata closed in Aleppo pine seedlings at a leaf water potential (Ψ_w) of -2.8 MPa suggesting an isohydric stomatal regulation. This could explain clearly the fact the E and g_s were more than twice as large for cold-stressed seedlings as for control since cold stressed seedlings Ψ_w varied between -1.15 and -1.85 MPa. This fact could explain the observed increase of A_{net} of cold stress seedlings. However, as a consequence of stomatal closure under drought, carbon assimilation through photosynthesis A_{net} was significantly reduced. Similar observation were reported by Epron et al. (1992), Schwanz et al. (1996) and Martinez-Ferri et al. (2000) suggesting that A_{net} can be reduced or inhibited completely and thereby the risk of oxidative stress increases.

The cellular changes induced by either drought or cold stresses include responses leading to the excess accumulation of toxic compounds especially reactive oxygen species (ROS) and thus inducing to oxidative stress (Suzuki and Mittler 2006). Under drought and especially heat conditions, the reaction catalyzed by ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) can lead to the production of H_2O_2 as a consequence of

increases in its oxygenase reactions (Kim and Portis 2004). On the other hand, cold conditions can create an imbalance between light absorption and light use by inhibiting the activity of the Calvin-Benson cycle; enhanced photosynthetic electron flux to O₂ and over-reduction of the respiratory electron transport chain can also result in ROS accumulation which causes oxidative stress (Hu et al. 2008). Plants have evolved a variety of responses to extreme temperatures those minimize damages and ensure the maintenance of cellular homeostasis (Kotak et al. 2007). Alonso et al. (2001) reported that Aleppo pine minimizes this kind of oxidative stress by decreasing the level of needle pigments; a process that was previously seen as damage. This report is in agreement with our results manifesting the decrease in Chl a, Chl b and Car in stressed seedlings needles. Reduction of pigments concentration, as a typical symptom of oxidative stress, could be the result of either slow synthesis or fast breakdown (Liu et al. 2011). The decreases of pigments concentration did not cause any decrease of F_v/F_m , which indicated that pigments breakdown was not accompanied by the decreasing of the maximum photochemical efficiency. Other authors explained this phenomenon as a photo-protection mechanism through reducing light absorbance by decreasing pigments concentration (Galmés et al. 2007; Elsheery and Cao 2008). Higher concentrations of Chl a against Chl b was also considered as a decreased emphasis on light collection in relation to the rates of PSII photochemistry (Demmig-Adams and Adams 1996). Drought stress imposed an increase of Chl a against Chl b which could be explained as a decrease of peripheral light-harvesting complexes. Since Car played an important role in photo-protection (Demmig-Adams and Adams 1996; Adams et al. 1999; Munné Bosch and Peñuelas 2003), their low decrease indicated a higher need to photo-protection phenomenon (Baquedano and Castillo 2006; Elsheery and Cao 2008).

Chlorophyll fluorescence, expressed as the Fv/Fm ratio and Φ_{PSII} , represents a good indicator of photosystem II efficiency and it has been used to estimate long term responses to cold and drought stress assays (Bolh ar-Nordenkampf and  quist 1993). Maximal efficiency of PSII (Fv/Fm) didn't change under cold stress but it decreased significantly under drought. Similar results were obtained on this species subjected to drought (Navarro et al. 2004) and on other species e.g. *Malus domestica* where Fv/Fm was affected only by water stress (Major et al. 1994). However, quantum yield of non-cyclic electron transport (Φ_{PSII}) difference wasn't significant.

The difference among drought stressed seed sources is consistent with the results of Colom et al. (2003) who observed small but significant differences between populations in Fv/Fm and effective PSII quantum yield (Φ_{PSII}) at increasing light intensities. Aranda et al. (2005) also found differences among populations of *Quercus suber* during periods of low winter temperatures, when greater reductions in Fv/Fm caused the highest population variance. The low variation in the photochemical parameters attributed to the population effects found in this experiment is in agreement with Lopez et al. (2009) for *Pinus canariensis* and Baquedano et al. (2008) for *Pinus halepensis*. Water use efficiency (WUE) has been reported to be positively correlated to the harsh conditions of the native habitat (Anderson et al. 1996). However, the reverse pattern has also been found (Nguyen-Queyrens et al. 1998). This might be reflecting constraints and trade-offs that arise when multiple interacting factors co-occur. Here, WUE as iWUE decreased under both stresses without significant correlation with native conditions of seed sources.

The impairment of photosynthetic carbon assimilation by stomatal closure under drought conditions (Reddy et al. 2004) affects the metabolic balance in plants (Pinheiro et al. 2011). Soluble sugars are among the most

drought-responsive metabolites increasing due to starch hydrolysis or impairment of starch production (Correia et al. 2006; Rodriguez-Calcerrada et al. 2011). In fact, soluble sugars correlate negatively to photosynthesis (Franck et al. 2006).

Differential accumulation pattern of soluble sugars among seedlings under drought; 'Lit', 'Mst', 'Hsa' and 'Mgr' seedlings manifested significant increase of glucose concentrations on the opposite to the others while fructose has been increased for all seed sources. In drought-stressed plants, sugars have a flexible role and multiple functions as energy substrate, cellular osmolytes and signal molecules (Rodriguez-Calcerrada et al. 2011). Thus, the effect of drought on sugars cannot be readily described and depends on the species and the degree of stress experienced by the plant. The increase in proportion of soluble carbohydrates reflected an adaptation that maintains intracellular osmotic concentration. Cold hardiness in forest trees is also positively related to soluble carbohydrate concentration. Total soluble carbohydrate concentration represents the pool of carbon that can be used either to enhance cold hardiness via hydrolysis to soluble carbohydrates, or to support metabolic processes (Thomas et al. 2004). From the other side, glucose and fructose concentration decreased under cold stress. Furthermore, in conifer foliage, increase of sucrose concentration appears to happen primarily by conversion of starch to sugar, mainly sucrose, but with smaller concentrations of other sugars, including the oligosaccharides raffinose and stachyose, which often show the greatest proportional increase during hardening (Hinesley et al. 1992). In this study, almost all seedlings showed significant decrease of sucrose concentrations except 'Alc' and 'Man' under drought and 'Bet', 'Mst' and 'Lit' under cold stress. The decreases in sucrose may be due to respiratory depletion, as observed in *Pinus* and *Picea* seedlings (Ögren 1997), or phloem export.

Specific responses to drought also involve nitrogen (N)-containing compounds, e.g., amino compounds and proteins (Hu et al. 2012). The existence of qualitative difference among seed sources at the proteomic level under drought stress explains their differential response. This difference could be related to the site of origin and the identified proteins could be used as specific molecular markers for the considered seed source under drought. Our results are in agreement with previous researches reported on other forest species (He et al. 2007; Valero et al. 2011). The presence of unknown fragments could be due to the lack of complete proteomic database regarding forest species or to the denaturation of Rubisco under drought constraint (Polmion et al. 2006). Most of the identified proteins are related to the transcriptional machinery and sugar metabolism indicating that the ability to maintain cellular homeostasis and carbohydrate metabolism under stress conditions may be a determinant factor under stress conditions. The presence of enzyme related to the sulphur amino-acids metabolism is determinant for drought stress tolerance (Freeman et al. 2004; Mulet et al. 2004) although the enzyme which becomes the limiting factor could change in different species (Mulet et al. 2004). Our data indicates that *5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase* enzyme could be the limiting factor for drought stress in Aleppo pine. The differential response among seed sources at this molecular level could be related to their phenotypic plasticity (Alía et al. 2003).

The intraspecific variability among Aleppo pine seedlings tested under drought and cold stress was in agreement with our previous results of survival and growth on the same seed sources tested under real field conditions simulating drought and cold stresses (Taïbi et al. 2014). In this sense, ‘Bet’ and ‘Man’ seed sources showed specific adaptation to drought whereas ‘Lev’ and ‘Mst’ seedlings were more suited to cold stress.

Population variation was displayed exclusively under stress. Moreover, the fact that only variables related to photosynthesis pigments and soluble sugars varied among seed sources indicates the high plasticity of the species in the expression of these traits under various conditions. This might conform well to an adaptive mechanism involved in maximizing plant performance under stress. This finding is in line with a previous study (Corcera et al. 2011) and suggests that population differentiation (Warren et al. 2005) and population selection (Baquedano et al. 2008) take place under adverse conditions.

6.5. Conclusions

Aleppo pine seed sources showed many differences in water potential, photosynthesis, fructose and carotenoid concentrations under induced drought and cold stress. With respect to control conditions, drought stress induced significant difference in F_v/F_m and sucrose concentration however, cold stress changes the levels of g_s , E , photosynthetic pigments and glucose concentrations. Furthermore, difference among seed sources was observed for photosynthetic pigments and soluble sugars concentrations under both stresses. Differences among seed sources subjected to drought were significant for F_v/F_m , photosynthetic pigments, glucose and fructose concentrations. However, F_v/F_m and chlorophyll concentrations were not affected under cold stress. E , g_s and A_{net} decrease under drought stress was due to stomatal closure unlike to cold stress. The observed decrease in photosynthetic pigments concentration under both stresses occurred as a photo-protection mechanism to reduce oxidative stress damages and it did not affect F_v/F_m . Maximal efficiency of PSII (F_v/F_m) did not change under cold stress but it decreased significantly under drought.

Soluble sugars accumulation showed different patterns among seedlings under both stresses; drought tolerance was related to the decrease of glucose and fructose concentrations and the increase of sucrose concentration in needles however, the cold tolerance was associated to the decrease of glucose concentration and the increase of sucrose and fructose concentrations. In addition, differential response among drought stressed seedlings was observed at the proteomic level. Most of the identified proteins were related to the transcriptional machinery and sugar metabolism indicating that the ability to maintain cellular homeostasis and carbohydrate metabolism under stress conditions may be a determinant factor under stress conditions. The presence of enzyme related to the sulphur amino-acids metabolism “*5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase*” could be the limiting factor for drought stress in Aleppo pine. The results of this study complete our previous results regarding survival and growth performance on these seed sources tested under real field conditions simulating drought and cold stresses. In this sense, ‘Bet’ and ‘Man’ seed sources showed specific adaptation to drought whereas ‘Lev’ and ‘Mst’ seedlings were more suited to cold stress.

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General discussion

7. General discussion

Forest ecosystems response to increased temperature or changes in precipitation regimes strongly depends on the geographic or bioclimatic region (IPCC 2012). The negative impacts on forest productivity are expected to be severe in more continental or Mediterranean forest ecosystems where growth is already water limited (Lindner et al. 2010). In these dry regions, high temperatures and drought conditions will most likely reduce forest productivity and facilitate tree mortality (Allen et al. 2010).

Native conifer species will suffer under increased temperatures along with reduced precipitation in summer, and might be replaced by more competitive tree species, especially in Western and Central Europe (Kölling 2009). In contrast, warmer winter temperatures have been found to reduce the frost hardening of trees, especially in the continental regions of Europe with harsh frost events during the cold months (Lindner et al. 2010). Moreover, trees are expected to become more vulnerable to late frost events in spring as warmer summer temperatures (Kramer et al. 2000). However, climate change impacts on forest ecosystems are strongly species dependent according to the dominant key tree species and can even differ in severity within the distribution range of one single species as site conditions, phenotypes, and regional climatic changes may differ (Lindner et al. 2010).

Assisted population migration has gained more interest during last decade (Williams and Dumroese 2013). It is understood in this thesis in a broad manner, either as a way of improving forest plantation success (selecting non-native seed sources more resistant to abiotic stress) or for conservation purpose as a means of avoiding predicted species extinction as

a result of various forms of disturbance including climate change. However, both the species chosen and the context of the work fit better in the former meaning. The later approach is proposed as a conservation strategy for long life cycle species with poor dispersal abilities in highly modified landscapes subject to the effects of climate change (Shirey and Lamberti 2010).

In the current study, assisted populations migration is discussed as a proactive silviculture management approach to mitigate the impacts of future climate change on two abundant pine tree species in the south east of Spain, Aleppo pine (*Pinus halepensis* Mill.) and Black pine (*Pinus nigra* ssp. *salzmannii*). This thesis introduces an interdisciplinary approach to developing a sustainable proactive management strategy and discusses the need for understanding and developing integrated information and management options with the use of the intraspecific variability from different geographical regions among the range of distribution of each species. Observations were made on field outplanted seed sources and under controlled phytotron conditions to encompass all the possible levels of differential response.

1. Survival

Survival is regarded as one of the key variables when analysing tree provenance trials because it indicates the adaptability of the provenance to the environment at the trial site. It should be noted that early survival assessment reflects only the conditions experienced during the first years of the trial and not necessarily the climatic extremes and conditions that may be experienced during the life-span of a tree (Ræbild et al. 2003). Survival is of crucial importance for seedlings establishment success in their native or the new target environments throughout assisted migration strategy.

Obviously, survival is at its maximum for seedlings grown under most non-limiting conditions. Aleppo pine seedlings out-planted in La Hude showed the highest final survival (93%). Similar results were reported previously by del Campo et al. (2007a,b). Survival differences were minimal even if seed sources from colder and higher altitude provenances survived more; freezing events that likely occurred at this site could have decreased survival of seed sources from warmer provenances. Furthermore, black pine seedlings out-planted in Berga showed the highest final survival (77%), without significant differences between seed sources. Similar results were reported for this species by Perez et al. (2005) in the sub-humid north-western Spain, with survival rates above 85% and no differences among 22 provenances.

However, pine seedlings grown under environmental conditions characterized by severe spring and summer drought suffer significantly high mortality (Martínez-Vilalta and Piñol 2002). The dry environmental conditions of the site Granja d'Escarp led to a high differentiation among Aleppo pine seed sources survival which was related to the seedlings ability to cope with dry and warm conditions (Villar-Salvador et al. 2012). This differential response could be partially linked to latitude of seed sources origins as reported by Agúndez et al. (1997) and Climent et al. (2008) or as reported by Persson (1994) for *Pinus sylvestris*. Similarly, black pine seedlings grown at Xert showed high mortality (53%) and significant differences among them. Summer drought has been reported as a key factor affecting Salzman pine seedlings survival especially in the dryer regions of Spain (Tiscar-Oliver 2007; Tiscar and Linares 2011). In this site, long transfer distances and frost tolerance appear as interacting factors affecting survival of the southernmost provenance.

Moving southern seed sources up to northern areas (or from low to high altitudes), may pose winter survival a serious threat to pine trees establishment as cold extremes, which naturally determine the poleward range limits of forest trees (Thiel et al. 2012). There was an important survival decrease during the first winter (2010) for Aleppo pine and during the first spring (2010) for black pine in Tramacastiel, which was attributed to the minimal temperatures recorded after outplanting in February (average: -3.2°C; range -7.8 to 4.8°C) and March 2010 (average: 0.16°C; range -7.2 to 4.1°C). This fact, together with harsh site conditions and precipitation irregularity, affects seedling establishment, although summer mortality, which usually peaks for outplanted seedlings (del Campo et al. 2007a,b), was remarkably low in Aleppo pine. Under these conditions, a proper choice of seed source can increase Aleppo pine survival by 20% as observed in our results. Puértolas et al. (2005) suggested that low temperatures are known to affect Aleppo pine establishment alike to Kreyling et al. (2012) study on black pine suggesting that low temperatures may pose a serious threat which naturally determines its poleward range limits. Extreme winter temperatures cause frequent freeze-thaw events, which induce xylem embolism and frost drought (Mayr 2007).

From our overall results, it seems that under good site condition or core habitats, survival differences among different seed sources are not expected to differ substantially. However, when climate conditions are extremer and far from those typically found on core habitats, seed sources from regions where those conditions are met more frequently performed better than local genotypes.

2. Growth

Tree height is considered usually as an important variable in the evaluation of species and provenances (Bariteau 1992); height may be seen as a measure of the adaptability of trees to the environment, such that tall trees are better adapted to the site than short trees (Ræbild et al. 2003). This interpretation need not always be true, however, as examples exist where the tallest provenances are suddenly stressed and subsequently die-off (McDowell et al. 2008).

In addition, stem diameter is often used as a measure of the productivity of stands because it is correlated to radial growth and wood production (Ræbild et al. 2003). By using these variables (height and diameter) we will be able to determine which seed sources, from different provenances, may have the highest chance to succeed in a reforestation programme under the selection pressure of future climate change throughout the assisted migration.

Growth response differed significantly among seed sources under favourable sites conditions (La Hunte for Aleppo pine and Berga for Black pine). Aleppo pine seedlings from warmer provenances had faster growth rates than those from cooler provenances. Similar finding was reported by Climent et al (2008). Growth differences among Black pine seed sources were mainly related to the height growth. In our case, provenances from both species moved from slightly different transfer distance metrics were the best performers whereas those associated to large transfer distance from their site origin to the trial performed worse.

Ecological marginality can reduce ecosystem resilience under adverse climate conditions (Rehfeldt et al. 2002), although high phenotypic plasticity could be expected for species subjected to greater variation in precipitation and extreme temperatures (van Kleunen and Fischer 2007). Precipitation is

the main factor influencing growth of pines species planted in semiarid Mediterranean conditions (Atzmon et al. 2004; Schiller and Atzmon 2009; Villar-Salvador et al. 2012). Aleppo pine growth rates were the lowest on the dry site Granja d'Escarp. The home provenance grew less than the other seed sources; moreover seed sources from lower latitudes performed best. This finding was expected as the distribution area of Aleppo pine in Spain presents a clinal increase of aridity towards the South, indicating that southern seed sources would perform better in drier and warmer sites (Ferrio and Voltas 2005; Aitken et al. 2008; Reich and Oleksyn 2008). Voltas et al. (2008) reported that seedlings may significantly reduce height and diameter growth as an adaptive strategy to survive in water-restricted environments. This is known to occur in other *Pinus* species, e.g. *P. sylvestris* and *P. taeda* (Cregg and Zhang 2001). Previous experiments involving the exposure of Aleppo pine to water stress resulted in decreased stem volume growth and aboveground biomass in association with decreased carbon assimilation rates (Villar-Salvador et al. 2012). Tognetti et al. (1997) reported similar results in Aleppo pine, with drought resistance increasing from mesic to xeric sites of origin in water-stressed seedlings, but without geographical discrimination in well-watered seedlings.

Several studies have shown that black pine growth is sensitive to water deficit during spring and summer and to high summer temperatures (Martin-Benito et al. 2010; Linares and Tiscar 2010). Black pine diameter growth was the highest in Xert while height growth rates were the lowest. Higher diameter growth could be related to root growth (Mexal and Landis 1990) and can be considered as a response to water stress on this site as this is essential to ensure survival after transplanting (Grossnickle 2005). Voltas et al. (2008) reported that less favourable growing conditions could enhance intraspecific differences between seed sources. Populations are locally

adapted when they have their highest relative fitness at their provenance sites and lower fitness in other parts of their range (Soto et al. 2010).

In the cold site Tramacastiel, growth differences among seed sources were minimal for both species. The site acted as a marginal site where a compromise between frost and drought tolerance has determined establishment success. Reduced Aleppo pine height growth was correlated with increasing origin latitude. Also, the sustained (long term) height growth doubled and tripled than that of La Hunde and Granja d'Escarp, respectively, which is mainly attributed to the lack of summer drought (Matesanz and Valladares 2013). Black pine growth was correlated positively with altitude and negatively with summer precipitation during the first spring. Therefore, referring to the early pine performance, seed sources not belonging to the target site could be selected for facing current climate irregularities in different environments (Taïbi et al. 2014). Similar clinal trends were exhibited by *P. sylvestris* (Persson 1994) and *P. halepensis* (Agúndez et al. 1997; Climent et al. 2008; Taïbi et al. 2014).

Assisted migration is a sensitive strategy because it might disrupt conservation objectives and paradigms and raises scientific, policy, and ethical questions (McLachlan et al. 2007). The intentional movement of species in response to climate change does not come without ethical, economical, legal, political, and ecological issues (Schwartz et al. 2012). Assisted range expansion refers to the movement of a species outside its current natural distribution which may pose greater risk of ecological disturbance because of the unknown interactions complexity of the introduced species with the native species (Leech et al. 2011).

However, assisted migration in the form of assisted population expansion, as the case of this study, represents the movement of seed within a species current range to encourage the use of seed sources of native

species from climates anticipated to exist at the target environment in the future. Depending on the magnitude of the migration distance and the size of the current seed zone, this form of assisted migration may involve little movement of seed beyond its current seed transfer limit (Leech et al. 2011; Williams and Dumroese 2013). Assisted migration refers here to reforesting sites using seed sources of the same species preadapted to future climates to ensure that plantations remain healthy and productive in future climates. Natural selection has provided forest trees populations with a suite of adaptive traits best suited to its climate (e.g., cold hardiness, drought resistance) and local pests and disease thus, identifying and maintaining these natural adaptations through assisted populations' migration to areas predicted to occupy their ancestral climates may offer an effective and practical alternative (Vitt et al. 2010).

Preliminary research on most commercial forest tree species demonstrates that target migration distances would be short, occurring within current ranges of those species (O'Neill et al. 2008; Gray et al. 2011). Moving the species or population before the recipient site is suitable and inappropriate matching of the seed source with the outplanting site in a projected area could increase establishment failure (Vitt et al. 2010). By moving trees north out of their local habitat, we would generally expect an increasing risk of frost damage in autumn due to delayed growth cessation (Gray et al. 2011). Further, this compromises seedlings survival and the timing of the onset of dormancy and frost hardiness suggesting the potential risks involved with the northward transfers of planting material.

In operational forestry, the emphasis is usually on maintaining adaptation of plantations. Consequently, required migration distances are shorter, potentially less risky and, for the most part, supported through extensive provenance testing. Migrations being considered within forest

management involve movement between similar biogeoclimatic units and are usually within species' existing ranges. Climatic migration distances under discussion in forest operations translate to geographic distances of approximately 200-400 m up in elevation or up to 300 km northward (Ukrainetz et al. 2011).

3. Phenotypic plasticity

Phenotypic plasticity is a way to cope with environmental heterogeneity that is particularly adequate for sessile organisms (Canale and Henry 2010). It allows species to live across a wider range of environments than those with stable phenotypes (van Kleunen and Fischer 2007; Aitken et al. 2008; Schiller and Atzmon 2009). Generally, tree species are considered to express moderate to high plasticity in their responses to environmental stress (Climent et al. 2008). The importance of phenotypic plasticity as a buffer against extinction has not been widely appreciated. It might need to be considered for assisted population migration programs to manage forest ecosystems. Plastic responses of plants to contrasting environments have been frequently reported as adaptive (Valladares and Pearcy 1998), but this is not always the case (van Kleunen and Fischer 2005). Differences among species and populations in their plasticity may reflect differential selective pressures on plasticity, differential limitations acting upon the maximization of plasticity, or a combination of both (Valladares et al. 2007).

Seed sources from both tested species showed low phenotypic plasticity for both growth traits with respect to RDPI range established by Valladares et al. (2006). Comparable results were obtained on Aleppo pine by Baquedano et al. (2008) and on other Mediterranean species such as *Quercus coccifera* (Balaguer et al. 2001), *Quercus robur* and *Fagus sylvatica* (Valladares et al. 2000a, 2002). It should be noted that, even the values of

the considered index of plasticity ranged among 0.1 and 0.5, this plasticity shouldn't be considered as low since it concerns a complex trait such as growth. In addition, height growth plasticity was higher than that of diameter among sites for both species. This fact was reported also indicating that some traits might vary in their sensitivity to the environment being more plastic than others (Aitken et al. 2008; Benito-Garzón et al. 2011; Matesanz and Valladares 2013). Various pieces of evidence suggest that global change should in principle favor high levels of phenotypic plasticity in plants (Parmesan 2006). However, global change often involves simultaneous changes in two or more abiotic and biotic factors, which may impose restrictions on plastic responses to the environment. Balaguer et al. (2001), Gratani et al. (2003) and Valladares et al. (2002a and 2007) have suggested that phenotypic plasticity in Mediterranean forest trees constitutes an adaptation strategy to survive under the environmental stress frequent in the region.

4. Genotype by environment interactions

The genotype by environment interactions (GEI) can lead to the choice of seed sources for planting at a particular site. As a result, interest in studies of genotype by environment interaction has increased in forest tree improvement programs (White et al. 2007). This kind of studies could offer valuable information for managing assisted population migration to the target environments.

Through our results, the environmental effect has shown a great influence on seedlings performance variation of both species. This means that the tested environments were extremely diverse and confirms findings that environment is the major factor affecting Aleppo pine (del Campo et al. 2007) and Salzmänn pine (Amodei et al. 2012) growth performance in the

Mediterranean region. Significant genotype by environment interactions have been frequently reported in *Pinus halepensis* (Santos-del-Blanco et al. 2013; de Luis et al. 2013) and *Pinus nigra* (Varelides et al. 2001 and references within) provenance trials and were attributed to the broad natural distribution of the species in a wide range of environments. However, the significance of the GEI term justifies the need for investigating the nature of seed sources response to the experienced environments. Approaches currently used to gain an understanding of the causes of GE interactions can be categorized into two strategies;

The first involves the use of factorial regression models based on two-way GE tables with concomitant variables which could either be environmental factors, genotypic traits, or combinations thereof (Baril et al. 1995). According to the joint regression analysis of Finlay and Wilkinson (1963), the differential responses of the tested seed sources from both species that were selected from a broad geographic area have reflected their differential adaptation strategies to natural environments of core and marginal habitats of these species which reinforce the need of cautious selection of the seed source according to the target environments. This further reinforces the observation that productive seed sources were better adapted to productive sites, whereas less-productive seed sources performed better at poor sites as reported by Clair and Einschmit (1986) and Wu and Ying (2001).

The second strategy involves the correlation of genotypic or environmental scores derived from additive main effects and multiplicative interaction (AMMI) analysis to genotypic or environmental covariates (Van Eeuwijk et al. 1995). In our study, the removal of the additive parameter from the multiplicative component in the AMMI model overcomes one major statistical deficiency that is the dependency of the site and population

indicators encountered in joint regression (Gauch 1992; Wu and Ying 2001) and thus the AMMI models have provided more detailed partitioning of the sum of squares of the interaction among the tested seed sources of both species than linear regression and has been recommended to analyse complex GEI. Even though, the joint regression analysis remains valuable for determining genotypic behaviour over environments since it demonstrates comparable results to the AMMI analysis and allows for the prediction of performance in untested environments (Barnes et al. 1984). It may also be utilized to regress genotypes onto any other physically measurable feature of the environment (Dvorak and Ross 1994).

Both strategies, although different in approaches, have been shown to produce similar results in our study as reported by Vargas et al. (1999). Many studies have utilized the second strategy due to the production of principal components analysis (PCA)-based biplots, which allow for easy visualization of responses and relationships. In addition, biplots produced from AMMI analysis can be enriched with covariates to allow for easier interpretation, provided there are strong correlations between AMMI IPCA scores and the covariates.

Voltas et al. (2008) suggested that the less favourable growing conditions could enhance intraspecific differences between seed sources. Populations are locally adapted when they have their highest relative fitness at their provenance sites and lower fitness in other parts of their range. Our finding as regards to Aleppo pine seed sources adaptation indicated that ‘Bet’ and ‘Man’ seedlings showed specific adaptation to the dry site. However, ‘Mst’ seedlings showed its local adaptation to the cooler conditions of ‘Tramacastiel’. Nevertheless, seed sources with high phenotypic plasticity performed better in the core habitat. ‘Lev’ seed source manifested a wide adaption to all environments in both drifts situations. An

important finding is that Aleppo pine specific adaptation to adverse environmental conditions was coupled with low phenotypic plasticity responses either during the first year or after the final establishment.

For Black pine seed sources adaptation, ‘Sistema Ibérico Meridional a’ and ‘H.S. El Serranillo’ indicated wide adaptation for to all the tested environments. However, ‘Cordilleras Béticas’ seedlings from the lower latitude in the south of Spain, where drought is more frequent, were specifically adapted to the poor conditions encountered in Tramacastiel. Besides, ‘Sistema Ibérico Meridional b’ seedlings were specifically adapted to Berga and Xert sites. Furthermore, ‘Alto Maestrazgo’ seedlings were specifically adapted to Berga site, thus supporting the northward migration hypothesis.

It should be noted that high levels of phenotypic plasticity across a range of environments are usually conferred by the ability to plastically adjust the phenotype to each set of conditions and subsequently it is considered as a generalist adaptive strategy (Balaguer et al. 2001). However, the specific adaptation, which leads to ecotypic differentiation (Baquedano et al. 2008), involves a reduced phenotypic plasticity of populations limited to a restricted distribution range or to a particular habitat, which exhibit narrow tolerance to environmental changes (van Tienderen 1997).

5. Physiological response

The study of physiological variables could explain well the response patterns of the field variability observed within both species. Evaluation of forest trees either in field or under controlled growth conditions might need from the physiological analyses including water relations, photosynthesis, chlorophyll fluorescence, nutrients analysis, pigments, etc. (Vilagrosa et al. 2007).

However, the measure of these variables conducted during the experimental process on both species was unable to observe significant differences among seed sources on outplanting sites (unpublished data). This was probably due to the fact that these measurements were performed in late spring, when environmental conditions were favourable across all sites. The maximal photochemical efficiency of PSII (F_v/F_m), which can be used to detect the occurrence of photo-inhibitory damage in response to environmental stresses (Maxwell and Johnson 2000), ranged among trial sites from 0.70 to 0.82, slightly lower than the values obtained by Demming and Björkman (1987) for optimum growth conditions (from 0.78 to 0.85). Maki and Colombo (2001) and Percival (2004) noted that photosynthesis and, as a result, growth were not negatively affected when F_v/F_m values were more than 0.6, which suggest that our seedlings were not adversely affected at time of measuring.

Thus, in order to address the physiological level of plant response, a complementary study was conducted under controlled phytotron conditions in the aim to analyse variation in physiological traits related to transpiration, photosynthesis, gas exchanges and soluble sugars accumulation among Aleppo pine seed sources subjected to drought or cold stress.

In this case, a clear pattern was observed offering more details and information of use for seed sources selection. Water potential decreased as

reported by Tognetti et al. (1997) and Baquedano and Castillo (2006) under drought and by Fernández et al. (2002) under low temperature. Unlike cold stress, the severe stomatal control under drought leads to E , g_s and A_{net} decrease (Martinez-Ferri et al. 2000). The cellular changes induced by either drought or cold stresses include responses leading to oxidative stress (Suzuki and Mittler 2006). Alonso et al. (2001) reported that Aleppo pine minimizes this kind of oxidative stress by decreasing the level of needle pigments. The decrease of pigments content is a photo-protection mechanism through reducing light absorbance by decreasing pigments content (Elsheery and Cao 2008). Chlorophyll fluorescence, expressed as the F_v/F_m ratio and Φ_{PSII} , represents a good indicator of photosystem II efficiency and it has been used to estimate long term responses to cold and drought stress assays (Bolh ar-Nordenkampf and  quist 1993). Maximal efficiency of PSII (F_v/F_m) did not change under cold stress but it decreased significantly under drought as reported by Navarro et al. (2004) on drought stressed seedlings. The low variation in the photochemical parameters attributed to the population effects found in this experiment is in agreement with Lopez et al. (2009) for *Pinus canariensis* and Baquedano et al. (2008) for *Pinus halepensis*. Aranda et al. (2005) found also differences among populations of *Quercus suber* during periods of low winter temperatures, when greater reductions in F_v/F_m caused the highest population variance. The intraspecific variability among Aleppo pine seedlings tested under drought and cold stress was in agreement with our previous results of survival and growth on the same seed sources tested under real field conditions simulating drought and cold stresses (Ta ibi et al. 2014). In this sense, ‘Bet’ and ‘Man’ seed sources showed specific adaptation to drought whereas ‘Lev’ and ‘Mst’ seedlings were more suited to cold stress.

6. Molecular response

The physiological analyses generate much information but at the same time many ambiguities and questions which might need for more explanations. Here, molecular studies as proteomic, transcriptomic and metabolomic researches demonstrate high efficiency in the characterization of the physiological status of forest trees mainly those subjected to stress (Abril et al. 2011).

The *metabolomic approach* was addressed in this study on drought and cold stressed Aleppo pine seedlings comparing with control conditions to analyse the variation patterns of photosynthetic pigments and soluble sugars. Photosynthetic pigments were indicators of photosynthesis activity and oxidative stress damage of stressed seedlings (Alonso et al. 2001). Soluble sugars are frequently used to assess variation in carbon balances among conifer populations, although their variation may only be partially attributable to the balance of carbon source: sink (Rodríguez-Calcerrada et al. 2011). Soluble sugars are among the most drought-responsive molecules increasing due to starch hydrolysis or impairment of starch production (Rodríguez-Calcerrada et al. 2011). In fact, soluble sugars correlate negatively to photosynthesis (Franck et al. 2006). In drought-stressed plants, sugars have a flexible role and multiple functions as energy substrate, cellular osmolytes and signal molecules (Rodríguez-Calcerrada et al. 2011). Thus, the effect of drought on sugars cannot be readily described and depends on the species and the degree of stress experienced by the plant. Numerous studies showed an association between sugar accumulation or concentration, especially of oligosaccharides, and low temperature acclimation or tolerance in conifers (Ögren 1997). The increase in proportion of soluble carbohydrates reflected an adaptation that maintains intracellular osmotic concentration. Cold hardiness in forest trees is positively related to soluble

carbohydrate concentration (Thomas et al. 2004). Total carbohydrate concentration represents the pool of carbon that can be used either to enhance cold hardiness via hydrolysis to soluble carbohydrates, or to support metabolic processes. From the other side, the glucose content significantly decreased under cold stress. The raffinose contributes in maintaining membrane integrity during freezing and thawing. Some of the fluctuations in sugar concentration corresponded to fluctuations in environmental temperature, as reported by others (Hinesley et al. 1992). The decreases in sucrose may be due to respiratory depletion, as observed in *Pinus* and *Picea* seedlings (Ögren 1997), or phloem export.

The *proteomic approach* was addressed through the analysis of proteins patterns changes under induced drought and cold stress in the aim to determine which proteins could have a potential use as biomarkers for stress tolerance or sensitivity facilitating further future screening of seed sources under similar environmental conditions. Specific responses to drought also involve nitrogen (N)-containing compounds, e.g., amino compounds and proteins (Hu et al. 2012). Proteins extracted from roots and needles, from the control and drought treatments, from the most vs. the less responsive seed sources, e.g. those showing opposite response to drought. Qualitative difference of proteins bands among seed sources under the same treatment were analyzed using MALDI-TOF mass spectrometry. 1D PAGE was used to separate proteins as this technique allowed to compare different seed sources from different treatments in the same gel. The existence of qualitative difference among seed sources at the proteomic level under drought stress explained their differential response as reported in previous research on other forest species (Jorge et al. 2006; He et al. 2007; Valero et al. 2011). The presence of unknown fragments could be due to the lack of complete proteomic database regarding forest species or to the denaturation

of Rubisco under drought constraint (Polmion et al. 2006). Most of the identified proteins are related to the transcriptional machinery and sugar metabolism indicating that the ability to maintain cellular homeostasis and carbohydrate metabolism under stress conditions may be a determinant factor under stress conditions. This result could explain the pattern of variation in soluble sugars accumulation among seed sources under different treatments. The presence of enzyme related to the sulphur amino-acids metabolism is determinant for drought stress tolerance (Freeman et al. 2004; Mulet et al. 2004) although the enzyme which becomes the limiting factor could change in different species (Mulet et al. 2004). Alía et al. (2003) reported that the differential response among seed sources at this level could be related to their phenotypic plasticity.

It is important to understand forests dynamics from a multidisciplinary perspective. The integration of the previous cited methodologies in a multidisciplinary approach is essential as an effective framework for forest-based climate change mitigation and adaptation to facilitate the creation of a guideline model that simultaneously preserves natural forests, guarantees environmental services as well as secures sustainable forest resources.

The content of this thesis provides the tools and methods for understanding the multidisciplinary approach of sustainably managed forest ecosystems towards climate changes through assisted population migration. The present study makes a contribution towards reforestation and breeding research programs in the species, with practical implications for abiotic stress tolerance, assisted population migration and adaptive silviculture towards future climate change.

The integrated approach addressed in this study illustrates clearly the need to consider all the possible analysed aspects through the monitoring of

seedlings performance under real conditions of field, investigation on phenotypic plasticity and adaptation via genotype by environment interactions studies, physiological and molecular evaluation at very specific levels.

The efficiency of the proposed measures appears in their consistency, complementarity and ability to explain better the differential response observed within the conducted intraspecific variability of the tested species for sustainable forest management.

Main findings and conclusion

8. Main findings and conclusion

In this study, assisted populations migration was introduced as a proactive silviculture management approach to mitigate the impacts of future climate change on two recurrent pine species in the south east of Spain; Aleppo pine (*Pinus halepensis* Mill.) and Black pine (*Pinus nigra* ssp. *salzmannii*). The thesis analyses an interdisciplinary approach and discusses the need for understanding and developing information and management options with the use of the intraspecific variability from different geographical regions among the range of distribution of the species.

In general, the findings of this study demonstrate clearly that the selective use of within-species variability has a potential contribution to alleviate adverse climate change impacts on forest ecosystems. For both species, differences in the response or sensitivity of different seed sources were found to be very specific towards the northwards shifts in response to climate change. This implies that certain seed sources are able to cope better with specific climate perturbations than others.

Referring to the early pine performance (survival and growth) under real field conditions, seed sources not belonging to the target site could be selected for facing current climate irregularities in different environments. For both species, provenances moved from slightly different transfer distance metrics were the best performers whereas those associated to large transfer distance from their site origin to the trial site performed worse.

The main problem facing the northwards pine migration is the high expected seedlings mortality due to freezing events and drought stress mainly for seedlings belonging to warmer provenances. Growth response

differed significantly among seed sources under favourable conditions; seedlings from warmer provenances had faster growth rates than those from cooler ones. Pine seedlings growth rates were the lowest on the dry site and seedlings from lower latitudes performed best. However, under cold conditions, differences among seed sources were minimal.

For both species, seed sources showed low to moderate phenotypic plasticity for both growth traits (height and diameter) across the trial sites. Besides, height growth plasticity was higher than that of diameter. The environmental effect has shown a great influence on seedlings performance variation of both species. In addition, significant genotype by environment interactions (GEI) has been reported. The significance of the GEI term justifies the need for investigating the nature of seed sources response to the experienced environments.

According to the joint regression analysis of Finlay and Wilkinson (1963), the differential responses of the tested seed sources that were selected from a broad geographic area have reflected their differential adaptation strategies to natural environments. This finding reinforces the need of cautious selection of the seed source according to the target environments.

The removal of the additive parameter from the multiplicative component in the additive main effects and multiplicative interaction (AMMI) model overcomes one major statistical deficiency that is the dependency of the site and population indicators encountered in joint regression and thus the AMMI models have provided more detailed partitioning of the sum of squares of the interaction among the tested seed sources than linear regression and have been recommended to analyse complex GEI.

An important finding is that specific adaptation to adverse environmental conditions was coupled with low phenotypic plasticity responses. However, many specific adaptations among provenances to particular environments conditions appeared which could lead to ecotypic differentiation.

In order to address the physiological and the molecular levels of seed sources response, a complementary study was conducted under controlled conditions on seedlings subjected to induced drought or cold stress. A clear pattern was observed offering more details and information of use for seed sources selection. Aleppo pine seed sources showed many fluctuations in water potential, photosynthesis, fructose and carotenoid contents under drought and cold stress. With respect to control conditions, drought stress induced significant difference in chlorophyll fluorescence and sucrose content however, cold stress changed the levels of transpiration, stomatal conductance, photosynthetic pigments and glucose contents. Furthermore, difference among seed sources was observed for photosynthetic pigments and soluble sugars contents under both stresses. Differences among seed sources subjected to drought were significant for chlorophyll fluorescence, photosynthetic pigments, glucose and fructose contents. However, chlorophyll fluorescence and pigments were not affected under cold stress. The decrease in transpiration, stomatal conductance and photosynthesis under drought stress was due to stomatal closure unlike to cold stress. The decrease in photosynthetic pigments contents under both stresses occurred as a photo-protection mechanism to reduce oxidative stress damages and it did not affect chlorophyll fluorescence. Soluble sugars accumulation showed different patterns among seedlings under both stresses; drought tolerance was related to the decrease of glucose and fructose and the increase of

sucrose contents in needles. However, the cold tolerance was associated to the decrease of glucose and the increase of sucrose and fructose contents.

In addition, differential response among drought stressed seedlings was observed at the proteomic level. Most of the identified proteins were related to the transcriptional machinery and sugar metabolism indicating that the ability to maintain cellular homeostasis and carbohydrate metabolism under stress conditions may be a determinant factor under stress conditions. The presence of enzyme related to the sulphur amino-acids metabolism “*5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase*” could be the limiting factor for drought stress in Aleppo pine. The results of this study explain our previous results regarding survival and growth performance under real field conditions simulating drought and cold stresses.

The present study makes a contribution towards reforestation and breeding research programs in pine species, with practical implications for abiotic stress tolerance, assisted population migration and adaptive silviculture towards future climate change. The efficiency of the proposed integrated approach addressed in this study appears in the consistency, complementarity and ability to explain better the differential response observed within the conducted intraspecific variability of the tested species for sustainable forest management.

Limitations and challenges

9. Limitations and challenges

Throughout this study, increase and diversification of trial sites might be needed to encompass the wide natural variability of environments. Besides, the use of more variation either among or within-species is of crucial importance for best understanding of the biodiversity dynamic towards the predicted shifts.

The timing and interaction of several climate factors in a changing climate, especially on the background of warmer average conditions and longer growing seasons, proved to be of importance, as they can magnify or reduce the impact of each single factor, and need more attention in future climate change experiments.

Likewise non-climatic factors, such as soil properties or biotic factors, such as herbivory or inter-specific competition interact with a changing climatic environment. Especially the integration of biotic antagonists and other disturbances will be one of the major challenges of future climate change experiments.

In addition, it should be stressed that early growth assessments for analysing ecotypic differentiation, phenotypic plasticity and seed sources adaptation' may not be reliable for assessing genotype by environment interaction at maturity, which implies the need to evaluate genotype by environment interaction at mature ages in forest trees (Chambel et al. 2005). However, the transfer of seed sources to the tested environments should be carried out with caution and carefully managed from the genetic conservation point of view.

Nonetheless, the maintenance and support of intra-specific diversity is important in the face of changing environmental conditions, and should consequently be included in future climate change and adaptation research, as the adaptation capability of species might else be misinterpreted.

It could be demonstrated that some of the effects of climate extremes on the performance of plant species can be substantially delayed. Ecological inertia must be considered. Responses can become evident one year after the actual extreme event took place, as the events may alter e.g. soil processes that can have delayed and long-term impacts on ecosystems, what underlines the importance of the establishment of long-term experiments and observations.

Bioclimatic models coupled with species genetic information in a geographic information system (GIS) can be of great importance to identify current and projected distributions (e.g., Rehfeldt and Jaquish 2010; McLane and Aitken 2012; Notaro et al. 2012).

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11. References

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