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Monitoring two REINFFORCE network arboreta Adaptation to climate change and abiotic factors

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Abstract:

Many forest ecosystems studies have correlated recent climate trends with changes in phenology, and indicated that climate warming appears to lengthen the growing season and increase tree growth rates. In addition, changes in climate variables may have direct influence on insect pests and pathogens affecting both their survival and development. Also, climate change is expected to increase the frequency of extreme weather events. Depending on the timing of spring warmth, early and accelerated leaf development has the potential to increase the frequency and magnitude of leaf damage from freezing events.

Forest ecosystems, store approximately 1200 gigatonnes of carbon. This is considerably more carbon than is present in the atmosphere (IPCC, 2007). Recently, the mutual interaction between forests and climate change has become one of the most important issues for research to address this concern, REINFFORCE program has established a network arboreta with the main objective of improving our general understanding of 35 species' capacity to cope with future climate changes by planting the same genetic material under 38 different climates from the south of Portugal to the south of Scotland (from latitude 37° to 58°).

In this regard, the aim of this study is to characterize in two REINFORCE arboreta (Lisbon and Sintra) the survival, growth, leaf phenology and tolerance to pests and cold, for 5 selected species (*Quercus robur* L., *Q. rubra* L., *Q. shumardii* Buckley., *Betula pendula* Roth. and *Acer pseudoplatanus* L.), relating their behavior with the origin of the propagating material. This, would allow to have information on the behavior of these species under the present climate conditions in the two studied arboreta, and if different provenances of the same species respond differently to the current conditions in the arboreta, or not. This type of study further provides information about the suitable species to be used in afforestation programs, where present study can be considered a preliminary work easing the way for further research regarding forests and afforestation.

Our study involved measurements of each of the following parameters: plant growth, morphological parameters, phenology and insects damage (which was carried out according to protocols defined under REINFFORCE project), and spring frost tolerance. This last parameter was estimated by measuring conductivity of leaf discs electrolyte leakage after applying controlled freezing treatment using a cryostat (Aralab, Lisbon, Portugal) for five different temperatures (−3, −5, −6.6, −9, and −10.5 C°).

In a comprehensive overview to the results, we have found an apparent relation between frost and the time of bud bursting and the sensitivity to insect respectively. *Betula pendula* was the earliest species to bud burst in both sites, being thus the highest exposed to spring frost. It was also the least species to be affected by insect damage which, in turn, indicates that the earlier

bud bursting for *B. pendula*, may have caused a noticeable gap between the bud bursting (where the leaves are the most sensible to insect attack) and between the development of the insects that attack the leaves. On the laboratory no significant difference were found among species on frost tolerance.

Resumo:

Os estudos em ecossistemas florestais têm correlacionado as recentes tendências climáticas com alterações fenológicas, indicando que o aquecimento climático parece aumentar o período da época de crescimento das árvores e as suas taxas de crescimento. , As alterações nas variáveis climáticas também podem ter influências diretas sobre as pragas de insetos e agentes patogénicos afetando tanto a sua sobrevivência como o seu desenvolvimento. Para além destes aspectos, com as alterações climáticas está previsto um aumento na frequência de acontecimentos meteorológicos extremos. Dependendo do "timing" das subidas de potencia o aumento da magnitude e frequência dos danos foliares feito por geadas.

Os ecossistemas florestais, armazenam aproximadamente 1200 giga toneladas de carbono. Isto é consideravelmente mais carbono do que o presente na atmosfera (IPCC, 2007). Recentemente, a interação mútua entre as alterações florestais e climáticas tornou-se num dos problemas prioritários da investigação neste sector, como resposta a esta preocupação, o projeto REINFFORCE estabeleceu uma rede de arboretos com o principal objetivo de melhorar o nosso conhecimento sobre a capacidade de adaptação de 35 espécies florestais às alterações climáticas, tendo para o efeito instalado o mesmo material genético em 38 diferentes climas desde Portugal até à Escócia (entre as Latitudes 37º até 58º).

Neste contexto, este estudo pretende caracterizar os dois arboretos REINFFORCE instalados em Lisboa e Sintra-, quanto à sobrevivência, crescimento, fenologia foliar e tolerância a pragas e às baixas temperaturas da Primavera, para as cinco espécies selecionadas (*Quercus robur* L., *Q. rubra* L., *Q. shumardii* Buckley., *Betula pendula* Roth. and *Acer pseudoplatanus* L.), relacionando o seu comportamento com a origem do material de propagação. Isto permite ter informação no comportamento destas espécies dentro das condições climáticas presentes nos dois arboretos estudados, e saber se as diferentes proveniências das mesmas espécies respondem de forma diferente às condições atuais dentro do arboreto, ou não. Este tipo de estudo disponibiliza informação relativamente ás espécies que mais se adequam aos programas de florestação, em que este estudo pode ser considerado um trabalho preliminar que facilitará a pesquisa nesta área.

O nosso estudo envolve medições dos seguintes parâmetros: crescimento da planta, , fenologia e dano feito por pragas (feitas de acordo com os protocolos definidos pelo projeto REINFFORCE), e ainda a tolerância a baixas temperatura na primavera. Este ultimo

parâmetro foi estimado medindo a condutividade electrica do exsudado de discos das foleares após terem sido submetidos num criostato (Aralab, Lisbon, Portugal) a baixas temperaturas (– 3, –5, –6.6, –9 e –10.5 C°).

Os resultados, demonstraram uma relação entre a resposta às baixas temperaturas e o início do abrolhamento dos gomos foliar e a sensibilidade aos insetos respetivamente. A *Betula pendula* foi a espécie que abrolhou mais cedo dois locais e por isso foi a mais exposta às baixas temperaturas da primavera. Também foi a espécie menos afetada por danos de insetos que, por sua vez, indica que o abrolhamento precoce da B. pendula, poderá criar um desfasamento temporal com o desenvolvimento dos insetos na fase em que a planta está mais suscetível a ataques destes. No laboratório não foram encontradas diferenças significativas entre as espécies relativamente à tolerância às baixas temperaturas da Primavera.

Introduction:

Climate change is now being accepted as one of the most important phenomenon affecting the future of the world's environment and natural systems and, in turn, human society.

Many scholars have published reports and presented significant evidence that climate changes over the past fifty years have affected several aspects of forest ecosystems. This included characteristics such as tree growth and dieback, invasive species problems, species distributions and migrations, seasonal patterns in ecosystem processes, demographics and extinctions (IPCC. 2007a)

In this respect, climate variation in Portugal has been similar to the variation occurred in the Mediterranean region. Future scenarios of climate change indicates a systematic 3 to 7 °C increase in average temperature and reduction in annual rain fall by 20 to 40 % . The combined outcome of droughts and high temperatures will cause lower carbon sequestration in some areas. This, in turn, will make species distribution to be mainly driven by the stress caused by the increase of arid and semi arid regions in the country (Santos *et al*. 2002)

Climate change programs like REINFFORCE created a physical and data infrastructure to study adaptation of forest trees to climate change in the European Atlantic temperate forests. Such program considered the Arboreta network as the means to measure the impact of climate change on species. Through this study, 35 species were planted in areas from south of Portugal to south of Scotland (from latitude 37° to 55° North). The main purpose behind planting the arboreta network has been to improve our understanding of the species capacity to cope with future climate by planting the same genetic material under 38 different climates (Orazio *et al* .2013, Orazio *et al*. 2009).

Many forest ecosystem studies have correlated recent climate trends with changes in phenology (the timing of seasonal activities of animals and plants) as well as with changes in

forest productivity (Rosenzweig *et al*. 2007). Such studies have indicated that climate warming appears to lengthen the growing season and increase tree growth rates in many boreal and temperate forests. However in Mediterranean region, the results of several studies have suggested that the warming has contributed to measurable reductions in productivity in some forests through interactions with drought, fire and biotic disturbance (Rosenzweig *et al*. 2007). But to the extent of our knowledge few studies addressed the effect of climate on the tree phenology in Mediterranean climates. In temperate regions, it was demonstrated that changes in plant phenology can affect ecological relationships, for example, by creating a mismatch between plant flowering time and the presence of insect pollinators (Post & Forschhammer, 2007, Rosenzweig *et al*. 2007).

In addition, changes in climate variables may have direct influence on insect pests and pathogens affecting both their survival and development. It also can have indirect influence brought by the interactions between host species and natural enemies (Santos *et al* .2002). Many herbivorous insect species can only develop on young plant material. For these species, the phenology of leaves growth or growing tips can be used to measure the shifts in the phenology of their larval development (Visser & Holleman, 2001). The physiology of insects and fungi is highly sensitive to temperature changes especially regarding its metabolic rate. Therefore, resource consumption tends to almost double with an increase in temperature of 10 °C (Gillooly *et al*. 2001, Clark & Fraser. 2004). There is further evidence that warmer temperature is generally better for insects development, in many species shortening its life cycle, even in climates that are already warm (Currano *et al*. 2008, Frazier *et al*. 2006). However, pestilence may tend to decrease in the warmer edges of contemporary distributions, as predicted by the model of climatic envelopes (Williams & Liebhold, 1995).Further, extreme hot temperature observed in some time periods may also cause important insect mortality (e.g. Santos *et al*. 2011).

Climate change indicators includes not only shifts in mean conditions but also changes in the frequency and timing of extreme weather events such as severe cold, spring frost and extended drought. Consequently, tree seedlings, as the potential future over story, are responding to the pressures of both mean, and extreme conditions as mean temperature and the occurrence of late spring frost affect the emergence, development, growth, and survival of plants (Fisichelli *et al*. 2014, Santos *et al*. 2002). Also, climate change is expected to increase the frequency of extreme weather events (Solomon *et al*. 2007, Marino *et al*. 2011). Specifically, and as a by-product of warmer temperatures, the occurrence of frost after leaf total flushing is projected to become more common phenomenon in some parts of the world (Meehl *et al*. 2000, Gu *et al*. 2008). This scenario has a number of important ecological implications. In particular, the newly developed leaves are sensitive to frost events as they lack the structural rigor necessary to prevent damage. Depending on the timing of spring

warmth, early and accelerated leaf development has the potential to increase the frequency and magnitude of leaf damage from freezing events (Norby *et al*. 2003, Inouye. 2008). This, in turn, lead to lasting effects that can include the loss of stored carbon and nutrients as well as reduced photosynthetic carbon gain (Gu *et al*. 2008, Martin *et al*. 2010).

Additional studies show that plant growth under elevated [CO2] level can lower the degree of freeze tolerance, making even the most freeze-tolerant species more vulnerable to potential frost damage at warmer freezing temperatures (Woldendorp *et al*. 2008).The most responsive species to these changes are likely to occur in the cool to cold climates at high latitudes and high altitudes where seasonal temperatures and the length of frost-free period are important determinants of the growing season (Chen *et al*. 1995). A freezing event will thus injure these freeze-tolerant plant species depending on the acclimation state of the plant which is also affected by elevated [CO2] levels (Loveys *et al*. 2006).

Even though it is quite important and informative to study all the 35 species in the two arboreta, it was not feasible during the time duration for our research, so we had to choose only 5 species. Against this background, the aim of this study is to characterize the survival, growth, physiological, pest attack and leaf phenology of 5 selected species (*Quercus robur* L.*, Q. rubra* L.*, Q. shumardii* Buckley.*, Betula pendula* Roth.*, Acer pseudoplatanus* L.). Each species is represented by several provenances, which is relevant to study the variability within the species, since adaptive traits are related to geographic origin. This study will allow to obtain information on the behavior of these species under the present climate conditions in the two studied arboreta, and if different provenances of the same species respond differently to the current conditions in the arboreta. This study can be considered a preliminary work which highlights the relevance of obtaining adequate information about the suitable species to be used in afforestation programs, easing the way for further research regarding forests and afforestation.

2. Materials and Methods:

1.   Field description:

The study took place in two arboreta; located in Lisbon and Sintra. Respectively each has different environmental and meteorological characteristics (table 1).The arboreta in Lisbon was planted in May 2012, and in Sintra It was planted in October 2012.For this study, 5 broadleaves species were chosen (*Q. robur, Q. rubra, Q. shumardii, B. pendula, A. pseudoplatanus*). *Q. robur* (Quro) is represented by four provenances, *B. pendula* (Bepe) and *A. pseudoplatanus* (Acps) are represented by three provenances each (but in Lisbon arboreta in only one provenance survived), *Q. rubra* (Quru) is represented by two provenances, and *Q. shumardii* (Qush) is represented by one provenance, relating the behavior to the origin of the propagating material(table 2).

Table 1 Characteristics of the study sites :

	Lisbon	Sintra
Altitude (m)	106	400
Climatic classification (Köppen classification)	Mediterenean climate Csa	Mediterenean climate Csb
Type of soil	leptosols	cambisols
pH	6.44	4.31
Mean T (°C) (60 years)	17.5	13.6
Mean T coldest month (°C) (60 years)	7.2	5.4
Mean Precipitation (mm) (60 years)	823	1019
Mean T (°C) (6 months)	14.85	13.94
Mean T coldest month (°C) (6 months)	10.39	10.40

Table 2 Distribution of the provenances for the studied species in the arboreta

Species	Provenances identification	Provenances codes	Latitude	Longitude	Altitude (m)
Q. rubra	France	Fest	47.797	5.10	400
	Litoral Vasco-Spain	Vana	43.308	-2.03	210
Q. shumardii	Texas-USA	Texa	31.168	-100.077	640
Acer pseudoplatanus	Spain /Vana/	Vana	42.734	-3.467	620
	Switzerland /Alps/	Alps	46.538	10.08	2250
	Wales-UK /Wale/	Wale	52.405	-4.03	15
Q. robur	France	Fran	46.211	2.20	380
	New forest, Hampsphire-UK	Unit	43.2	-2.43	140
	Navarro-Spain	Pago	50.857	-1.62	45
	Posavina-Croatia	Posa	44.473	16.46	1200
Betula pendula	France	Nord	48.39	5.98	470
	Kralova-Slovakia	Kral	48.352	17.32	240
	Wales-UK	Unit	52.405	-4.03	15

2. Measurements:

2.1 Survival:

Survival was evaluated previously in September 2013 (first summer after plantation in Sintra, and second summer after plantation in Lisbon), and during this study again in May 2014. Survival is expressed as the percentage of the living seedling for each provenance.

2.2 Growth:

Growth measurements were carried out previously on November 2013, included the height and the diameter for each seedlings. Height was measured (cm) for the tallest living plant branch/bud using extensive pole, with 1 mm precision. And diameter was measured at 2 cm from ground with a digital calliper, with 0.01 mm precision (mm), taking two crossed measurements.

2.3 Phenology:

Leaf buds were observed once a week from the last week of February 2014 until complete budburst. The phenological status of each seedling was evaluated weekly. For this assessment the best branch with highest numbers of buds in each direction (west, east, north and south) was chosen and tagged with the number of the seedling and the direction of the branch. Then, the buds in each of the tagged branches were observed every week to check the stage of development, following the phenology protocol defined under REINFFORCE project (Appendix 1). The collected data concerning phenology were organized in the form of the percentage of total bud bursting (stage 10) for each observation and provenance. All collected data were organized in excel tables.

2.4 Insect damage:

At the mid of April, when the leaves of all the seedlings have expanded, one field observation was done to asses insect damage. For that we applied the protocol of biotic damage defined under REINFFORCE project (Appendix 2). We documented the type of damage and the severity of it, as expressed by the proportion of attacked leaves per branch. Insects found on the foliage were collected for future identification.

2.5 Spring frost tolerance:

Frost tolerance was evaluated in two species *Q. robur* and *B. pendula* by the end of May. Each species was represented by three provenances as showed in table 2. Seven seedlings from each provenance were sampled. Five fully expanded leaves from each seedling were collected, and a composite sample with one leaf disc per each seedling and per provenance was prepared in a vial tube. Three tubes per provenance and per species were placed in each freezing bath. Frost treatment was induced in a cryostat (Aralab, Lisbon, Portugal) with three baths containing an aqueous ethylene glycol solution. A controlled freezing program followed a constant cooling and thawing rate of 4 C°/ h and 2 h exposure to five different target freezing temperatures (–3, –5, –6.6, –9, –10.5 C°). When the temperature of the bath was at –2 C°, about 0.5 g of finely crushed ice (from deionized water) was added to each tube to make contact with the leaf discs avoiding super cooling. The temperatures in the different baths were controlled by using thermocouples sensors connected to a data logger (DL2, Delta-T). After the freezing treatment, 15 ml of deionized water was added to each tube, the tubes were then kept for 24 hours at water bath at 25 C°. After this period electrolyte conductivity was measured in each tube (T1)

with a K220 conductivity meter (Consort, Turnhout, Belgium). The samples were then boiled in an autoclave at 120 C° for 10 min and held, afterwards at 25 C° for 4 h before measurement of maximum electrolyte conductivity (T2). Relative injury (RI) was expressed as a ratio of electrolyte conductivity measured after freezing treatment relative to maximum electrolyte conductivity, RI = (T1/T2) *100 (Rocha et al .2013, Costa e Silva et al. 2008). We used RI in order to calculate TL50 for each provenance (which is an assessment of the temperature that causes death to 50 % of plants).

2.6. Statistical analysis:

For each variable we calculated the average, and standard error. Two-way ANOVA were used to compare height, diameter, phenology stage and frost damage to assess the effects of species and provenances within species. To test differences in survival rate between the two sites, on the proportion of attacked leaves (insect damage), the differences of bud burst percentage, and the differences of frost damage percentage we used generalized liner model, with Binomial distribution, considering the factors species and provenances, nested within species. Same model was used to test the difference in survival rates considering the species and provenances within species as factors. We also used Mann-Whitney test to compare phenology stage considering the site factor. To compare the phenology stage for all the species we used Kruskal-Wallis test, followed by Mann-Whitney test to compare the differentness between each pair of species. All

analysis were done using statistical software (SPSS).

3. Results :

3.1. Survival:

In Lisbon, *Q. robur* Pago had the highest survival rate in 2013 (the second summer after plantation) 50% . From 2013 to 2014 mortality was low increasing slightly to 52.5%. Thus most of the plants died in the first summer. A similar pattern was also observed for the other species and provenances, with mortality above 50% in the first summer and decreasing thereafter. Mortality was extreme for *A. pseudoplatanus* Vana since all plants had died during the first summer. Also for *A. pseudoplatanus* Alps the survival rate dropped from 33.3 % in 2013 to 0 % in 2014.

In contrast in Sintra, survival rates were high on the first observation (soon after planting) and decreased steeply in 2014 (after the first summer in the field). We had 100 % survival rate for *A. pseudoplatanus* Alps and *A. pseudoplatanus* Van in 2013 and dropped to 66.6 and 83.3 % in 2014. *Q. rubra* Fest had the smallest rate in both years (50 and 25 %).

When applying generalized linear with binary logistic distribution, to test the difference in survival rates from 2013 to 2014 between species in Lisbon, we found no significant difference (Z= 1.63, d.f.=4, P=0.651). We also found that provenance was not a significant factor for *B. pendula* (Z= 0.237, d.f.=2, P=0.626), *Q. robur* (Z= 3.85, d.f.=3, P=0.277), and for *Q. rubra* (Z= 0, d.f.=1, P=1).

In Sintra, we also did not find significant difference between species (Z= 4.23, d.f.=4, P=0.375), and no significant difference between provenances for *A. pseudoplatanus* (Z= 4.29, d.f.=2, P=0.117), *B. pendula* (Z= 5.695, d.f.=2, P=0.058), *Q. robur* (Z= 2.877, d.f.=3, P=0.411), and for *Q. rubra* (Z= 3.601, d.f.=1, P=0.058).

Table 3 Survival rate per provenance for five species: *Acer pseudoplatanus* (Acsp), *Betula pendula* (Bepe), *Quercus robur* (Quro), *Quercus rubra* (Quru), and *Quercus shumardii* (Qush). Observed in Lisbon and Sintra (November 2013 and May 2014)

Species and provenances	Lisbon		Sintra	
	September 2013	May 2014	September 2013	May 2014
Acer Wale	41.6	33.3	83.3	75
Acer Alps	33.3	0	100	66.66
Acer Van	0	0	100	83.3
Bepe Kral	32.2	27.7	91.6	55.5
Bepe Nord	23.07	19.4	88.8	83.3
Bepe Unit	34.14	33.33	91.6	66.6
Quro Fran	46.15	36.11	72.2	55.5
Quro Pago	52.5	50	69.4	27.7
Quro Posa	50	36.11	63.8	44.4
Quro Unit	50	44.44	66.6	41.6
Quru Fest	33.3	33.33	50	25
Quru Vana	25	25	83.3	16.6
Qush Texa	33.3	25	83.3	50

3.2. Growth:

In Lisbon, the provenance *B. pendula* Unit was the highest (17.9 cm), while *Q. rubra* Vana and *Q. rubra* Fest were the smallest 6 , 6.8 cm respectively. On the other hand *Q. robur* Unit showed the biggest diameter (4.3 cm) while *Q. rubra* Fest showed the smallest one (0.7 cm) (Fig. 1 and 2).

Nevertheless when applying two-way ANOVA to compare difference in height between species, we found significant difference between species (F=8.99, d.f.=4,89, P<0.001), we had no significant difference for *B. pendula* provenances (F=0.66, d.f.=2,28, P=0.52), *Q. robur* provenances (F=1.36, d.f.=3,43, P=0.26), and for *Q. rubra* provenances (F=0.25, d.f.=1,4, P= 0.64).We also found no significant difference in diameter between species (F=01.93, d.f.=4,114, P= 0.11), and we had no significant difference for *B. pendula* provenances (F=0.98, d.f.=2,30, P=0.38), *Q. rubra* provenances (F=0.21, d.f.=1,3, P= 0.67), but we found significant difference between provenances within species in *Q. robur* (F=4.74, d.f.=3,68, P= 0.005)
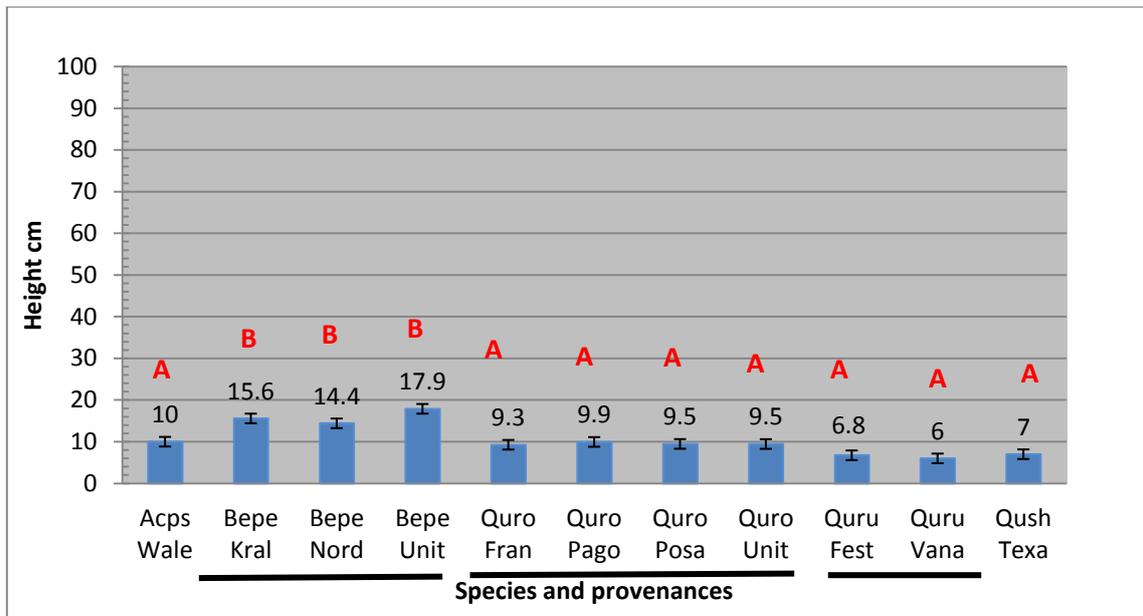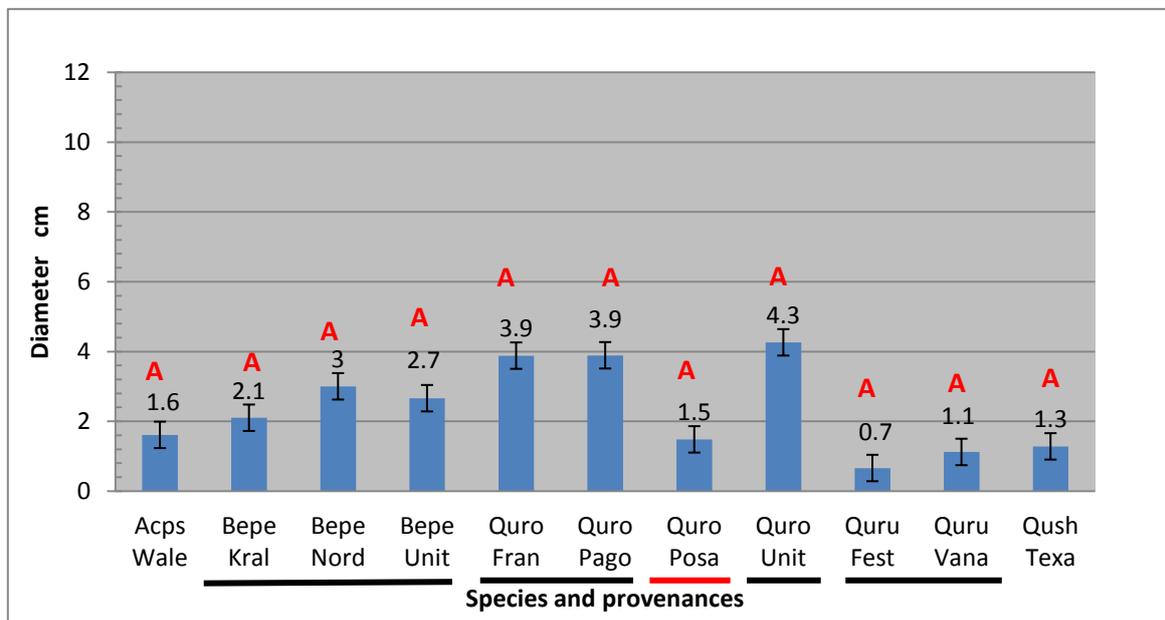
Figure 1 Mean height and standard error per provenance for five species: *Acer pseudoplatanus (*Acsp), *Betula pendula* (Bepe), *Quercus robur* (Quro), *Quercus rubra* (Quru), and *Quercus shumardii* (Qush). Observed in Lisbon (November, 2013). The same letter means no significant difference between species, different letter means significant differences between species (α= 0,05). The same color line, within species, correspond no significant difference in Provenances within species (α= 0,05).

Figure 2 Mean diameter and standard error per provenance for five species: *Acer pseudoplatanus* (Acsp), *Betula pendula* (Bepe), *Quercus robur* (Quro), *Quercus rubra* (Quru), and *Quercus shumardii* (Qush). Observed in Lisbon (November, 2013). The same letter means no significant difference between species. Different color line, within species, correspond to significant difference in Provenances within species (α= 0,05). The same color line, within species, correspond to no significant difference in Provenances within species (α= 0,05).

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In Sintra, the provenance *B. pendula* Nord was the heighest (37.9cm), while *Q. rubra* Fest was the smallest (8.6 cm). On the other hand *B. pendula* Kral showed the biggest diameter (8.6 cm) while *Q. rubra* Vana showed the smallest one (2.1 cm). (Fig. 3 and 4)

After applying one way ANOVA test we found significant difference for height between species (F=21.63, d.f.=4,181, P<0.001), we had no significant difference for *B. pendula* provenances (F=0.99, d.f.=2,72, P=0.37), *Q. robur* provenances (F=1.42, d.f.=3,59, P=0.24), or *Q. rubra* provenances (F=1.22, d.f.=1,15, P= 0.28) and *A. pseudoplatanus* provenances (F=0.17, d.f.=2,20, P= 0.83). We found significant difference for diameter between species (F=6.15, d.f.=4,213, P<0.001), we had no significant difference for *B. pendula* provenances (F=0.34, d.f.=2,81, P=0.70), *Q. robur* provenances (F=1.34, d.f.=3,75, P=0.268), and *A. pseudoplatanus* provenances (F=0.87, d.f.=2,27, P= 0.43), but we found significant differences for *Q. rubra* provenances (F=4.99, d.f.=1,15, P= 0.04)
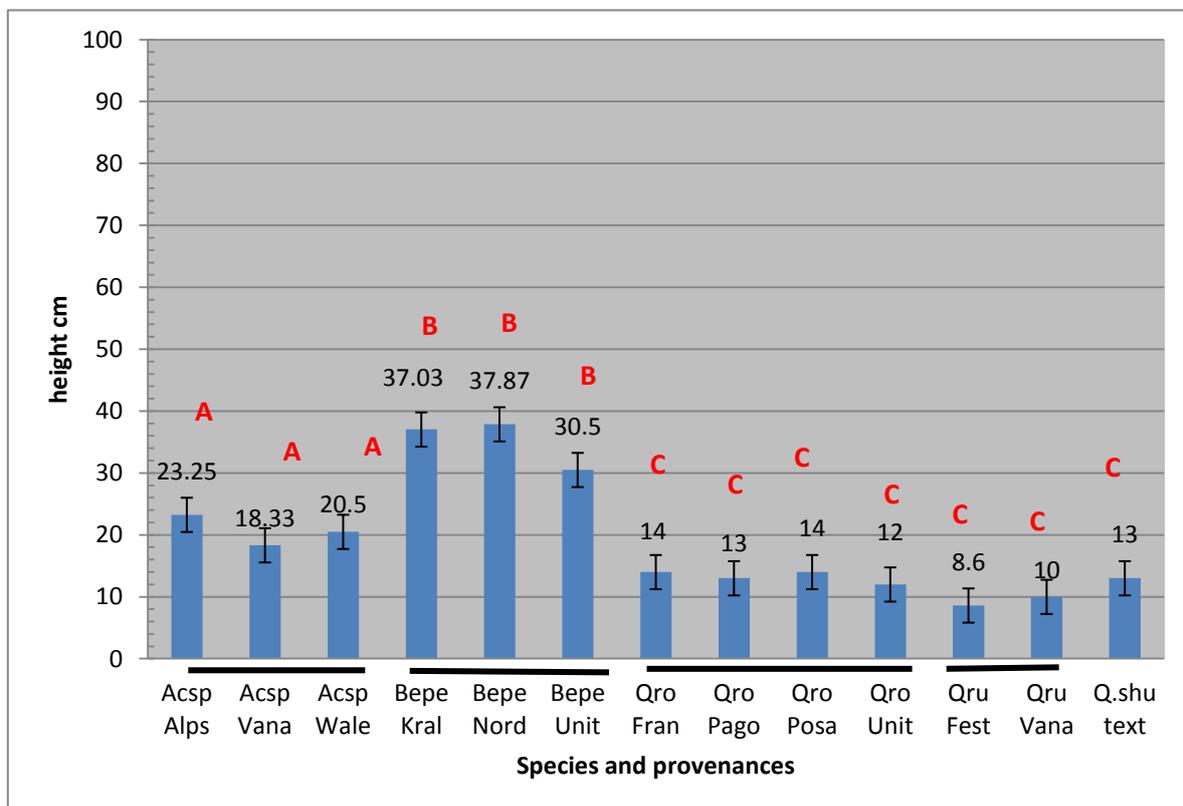

Figure 3 Average heights and standard errors per provenance for five species: *Acer pseudoplatanus* (Acsp), *Betula pendula* (Bepe), *Quercus robur* (Quro), *Quercus rubra* (Quru), and *Quercus shumardii* (Qush). Observed in Sintra (November, 2013).

The same letter means no significant difference between species, different letter means significant differences between species (Prob α= 0,05). The same color line, within species, correspond to no significant difference in Provenances within species (Prob α= 0,05).
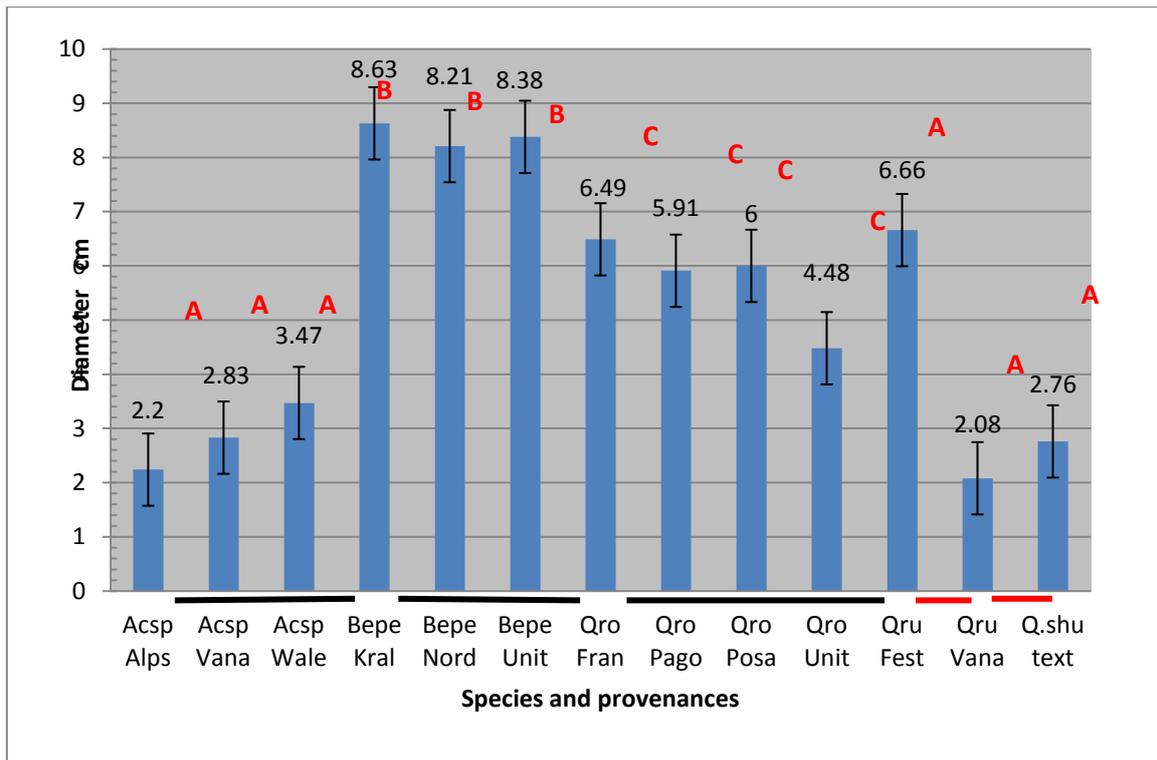
Figure 4 Average diameter and standard errors per provenance for five species: *Acer pseudoplatanus* (Acsp), *Betula pendula* (Bepe), *Quercus robur* (Quro), *Quercus rubra* (Quru), and *Quercus shumardii* (Qush). Observed in Sintra (November, 2013). The same letter means no significant difference between species, different letter means significant differences between species (Prob α= 0,95). Different color line, within species, correspond to significant difference in Provenances within species (Prob α= 0,05). Same color line, within species, correspond to no significant difference in Provenances within species (Prob α= 0,05).

3.3. Phenology:

All the provenance of *B. pendula* have an early bursting comparing with the other species, while the two provenances of *Q. rubra* showed the latest bursting (Fig 5).

In this regard, the two way ANOVA, showed significant difference among the four provenances of *Q. robur* (F= 3.56, d.f.=3,35, P=0.024), while the provenances of the other species showed no significance difference *Q. rubra* (F= 0.042, d.f.=1,17, P=0.839), *B. pendula* (F= 1.81, d.f.=2,26, P=0.183). On the other hand there is significant difference between the 5 studied species (F= 18.22, d.f.=4,104, P <0.001). We also used Kruskal-Wallis test to compare the phenology stage between species and found also significant differences (Z= 72.64, d.f.= 4, P<0.001).

The study also compared the percentage of bud bursting for each of the provenance between the two locations of the study (Lisbon and Sintra) at (3 and 4 March). Six provenances, *A. pseudoplatanus* Wales, *B. pendula* Nord, *B. pendula* Unit, *Q. robur* Pago, *Q. robur* Posa, *Q. robur* Unit and *Q. rubra* Vanna, were earlier to burst in Sintra in comparison to Lisbon. Three provenances *Q. robur* Fran, *Q. rubra* Fest, and *Q. shumardii* showed similar bud burst timing in both locations. Only *B. pendula* Kral was earlier to sprout in Lisbon than Sintra (Fig. 6).

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Nevertheless, when applying Mann-Whiteny test to compare the phenology stage in both sites at (3 and 4 March, 2014) no significant difference was found (Z= -1.044, d.f.= 1, P= 0.296).
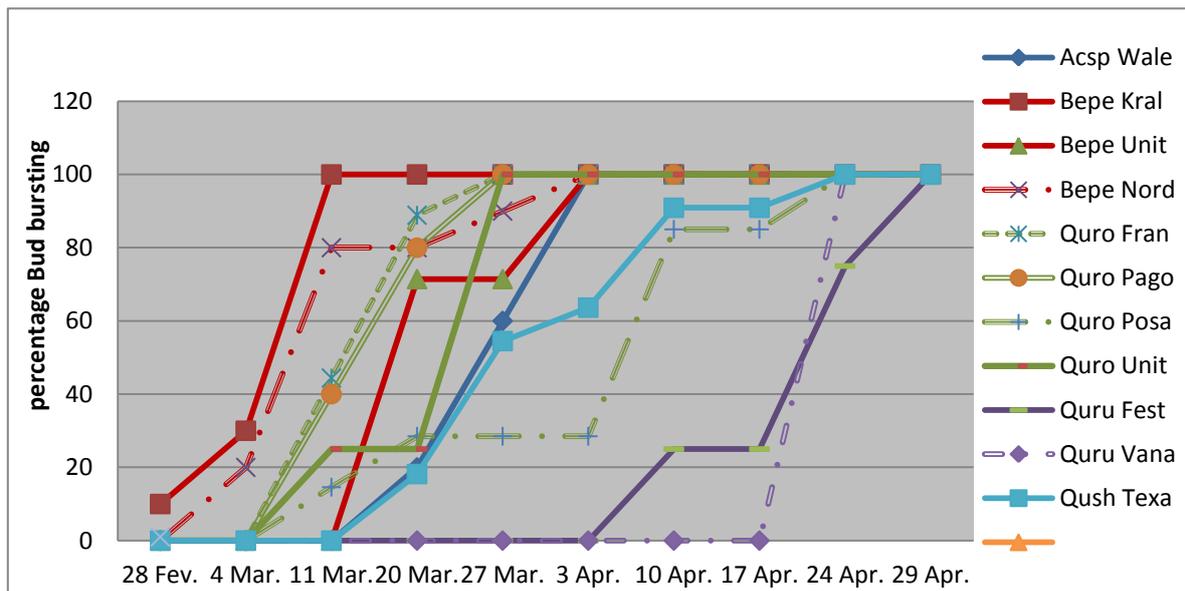

Figure 5 Total bud burst percentage per provenance for each observation in Lisbon.
BK= *B. pendula* Kral, BN=*B. pendula* Nord, BU= *B. pendula*.Unit, QUF= *Q. robur* Fran, QOP= *Q. robur* Pago, QOPO= *Q. robur* Posa, QOU= *Q. robur* Unit, A= A. psuedoplatanus Wale, QS= *Q. shumardii* Texa, QUF= *Q. rubra* Fest, QUV= *Q. rubra* Vana. The same letter means no significant difference between species, different letter means significant differences between species (Prob α= 0,05).
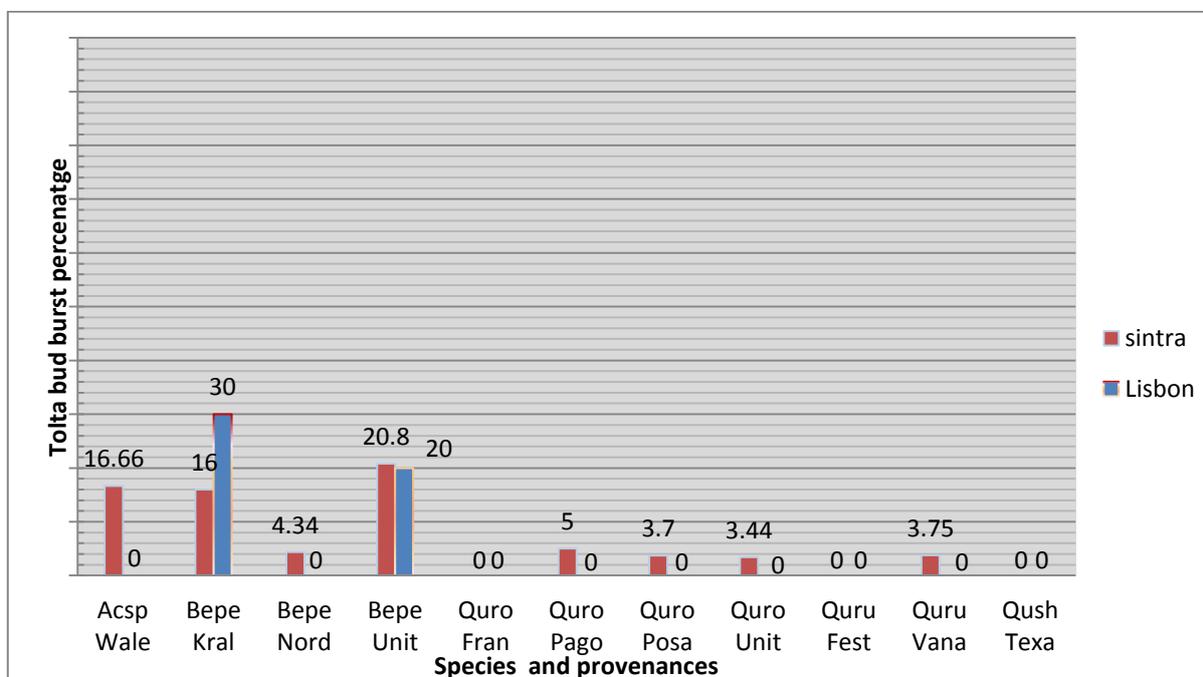

Figure 6 Percentage of total budburst for several provenances of five species: Acer pseudoplatanus (Acsp), Betula pendula (Bepe), Quercus robur (Quro), and Quercus rubra (Quru). Observed in Lisbon and Sintra(3 and 4 March respectively, 2014)

3.4. Insect damage:

After calculating the percentage damage for each of the species and analyzing the mean value of the probability of suffering leaf damage, using a logistic regression, we found that *Q. robur* (which is the only native species in the study area) has the highest percentage of damaged leaves. The proportion of leaves with damage observed on *Q. robur* in Sintra was significantly higher than all other species (Table 4 and 5). In Lisbon, *Q. robur* also presented high values of damage (40%) but which were similar to *Q. rubra* (Tables 4 and 5). *B. pendula* on the other hand, (native species in Portugal but exotic in the study areas) showed the lowest damage percentage (9-18%)(Table 4).

All species differ significantly with each other in Lisbon except between *A. pseudoplatanus* and *Q. shumardii* (P =0.523)(Table5). In Lisbon, we observed that *B. pendula* has significantly lower damage than all the other species (Table 4 and 5), and *Q. shumardii* was significantly different than the congeners *Q. robur* and *Q.rubra* (Table5). The damage type consisted mainly on chewed leaves, which had the highest relative percentage for all the species. Leaf miners, sap feeders, galls and skeletizers showed a lower percentage (Table 6).

The comparison between the average native species with the average sum of the exotic species also revealed a significant difference for Lisbon (Z= 130.56 , d.f. = 1, P<0.001), and for Sintra (Z = 304.41, d.f. = 1, P< 0.001).

After fitting a generalized linear model (with provenance effect nested within species effect), provenances of *B. pendula* in Lisbon did not differ on the insect damage (Z = 2.25 , d.f. = 1, P= 0.13), nor did the provenances of *Q. robur* (Z = 3.61 , d.f. = 1, P= 0.057), or *Q. rubra* (Z = 0 , d.f. = 1, P= 1.0). Yet, for Sintra, provenances of *Q. robur* differed significantly (Z = 4.32 , d.f. = 1, P= 0.037). Still, there were no significant differences observed among the provenances of *B. pendula* and *Q. rubra* (Z = 2.58 , d.f. = 1, P= 0.1) (Z = 3, d.f. = 1, P= 0.83).

Most of damage leaves in all species are due to leaf chewing insects (Table 6).**Table 4** Percentage of damage (average ± standard error) observed in Lisbon and Sintra for each tree species. Acps= Acer pseudoplatanus, Bepe= Betula pendula, Quro= Quercus robur, Quru= Quercus rubra, Qush= Quercus shumardii. Within each column values followed by the same letter do not differ significantly.

Species	Lisbon	Sintra
Acps	0.31 ± 0.055 [bc]	0.44 ± 0.021 [c]
Bepe	0.09 ± 0.012 [a]	0.18 ± 0.007 [a]
Quro	0.40 ± 0.013 [c]	0.49 ± 0.014 [d]
Quru	0.40 ± 0.048 [c]	0.31 ± 0.027 [b]
Qush	0.26 ± 0.031 [b]	0.41 ± 0.038 [c]

Table 5 Statistical comparison between the species in Sintra and Lisbon
Acps= *Acer pseudoplatanus*, Bepe= *Betula pendula*, Quro= *Quercus robur*, Quru= *Quercus rubra*, Qush= *Quercus shumardii*.

Species		Lisbon		Sintra	
		P-Value	Chi- square	P-Value	Chi- square
Acps	Bepe	0,000	16,358	0,000	138,919
	Quro	0,153	2,044	0,031	3,910
	Quru	0,242	1,370	0,000	12,383
	Qush	0,414	0,668	0,523	0,407
Bepe	Acps	0,000	16,358	0,000	138,919
	Quro	0,000	287,858	0,000	400,164
	Quru	0,000	39,364	0,000	22,129
	Qush	0,000	29,102	0,000	36,023
Quro	Acps	0,153	2,044	0,031	3,910
	Bepe	0,000	287,858	0,000	400,164
	Quru	0,934	0,007	0,000	35,354
	Qush	0,000	14,868	0,041	3,948
Quru	Acps	0,242	1,370	0,000	12,383
	Bepe	0,000	39,364	0,000	22,129
	Quro	0,934	0,007	0,000	35,354
	Qush	0,016	6,042	0,036	4,662
Qush	Acps	0,414	0,668	0,523	0,407
	Bepe	0,000	29,102	0,000	36,023
	Quro	0,000	14.868	0,041	3,948
	Quru	0.016	6.042	0,036	4,662

Table 6 The percentage of each damage type in Lisbon and Sintra.
CH= Chewed, SF= Sap-feeders , NEC= Necroses, Gall=Gall , LF= leaf roller, SK= Skeletizers
Lis= Lisbon, Sin= Sintra, Acps= Acer pseudoplatanus, Bepe= Betula pendula, Quro= Quercus robur, Quru= Quercus rubra, Qush= Quercus shumardii.

Damage type %		CH		SF		NEC		GALL		LF		SK	
Site		Lis	Sin	Lis	Sin	Lis	Sin	Lis	Sin	Lis	Sin	Lis	Sin
Species	Acps	100	84.8	0	14.4	0	0.8	0	0	0	0	0	0
	Bepe	98.7	97.1	1.3	2.8	0	0.2	0	0	0	0	0	0
	Quro	85.5	96.1	10.4	3.6	0.7	0.1	0.9	0.1	0.5	0	0.2	0
	Quru	99.2	98.2	0	1.8	0.8	0	0	0	0	0	0	0
	Qush	93.4	92.9	4.6	7.1	2	0	0	0	0	0	0	0

3.5. Spring frost tolerance:

Both species showed similar RI when subjected to negative temperatures ranging from -3 to -10.5 (Fig. 7). Nevertheless, frost damage was somewhat higher for *B. pendula* provenances which, in turn, indicates that this species is more sensitive to frost than *Q. robur*. (41.39+- 1.73 *vs*. 39.05+-1.87). Accordingly, the study calculated the TL50 for each of the provenances, which are ranked as follow*:*

Q. robur Pago ,Q. robur Posa, Q. robur Unit, B. pendula Nord, B. pendula Kral and *B. pendula Unit*, -7.12, -7.15,-7.26, -7.18, -7.15 and -6.82 C°.

We did not find statistical effect of provenance on frost damage, within each species, *Q. robur* (F= 0.241, d.f.= 2,30, P= 0.787), *B. pendula* (F=1.19, d.f.= 2,30, P= 0.317). Moreover, after fitting a generalized linear model (with provenance effect nested within species effect), using a fixed temperature -6.6 (which is the closest to the TL50), we also found no significant effect for the provenances within species on frost damage (Z = 0.42 , d.f. = 1, P= 0.51).
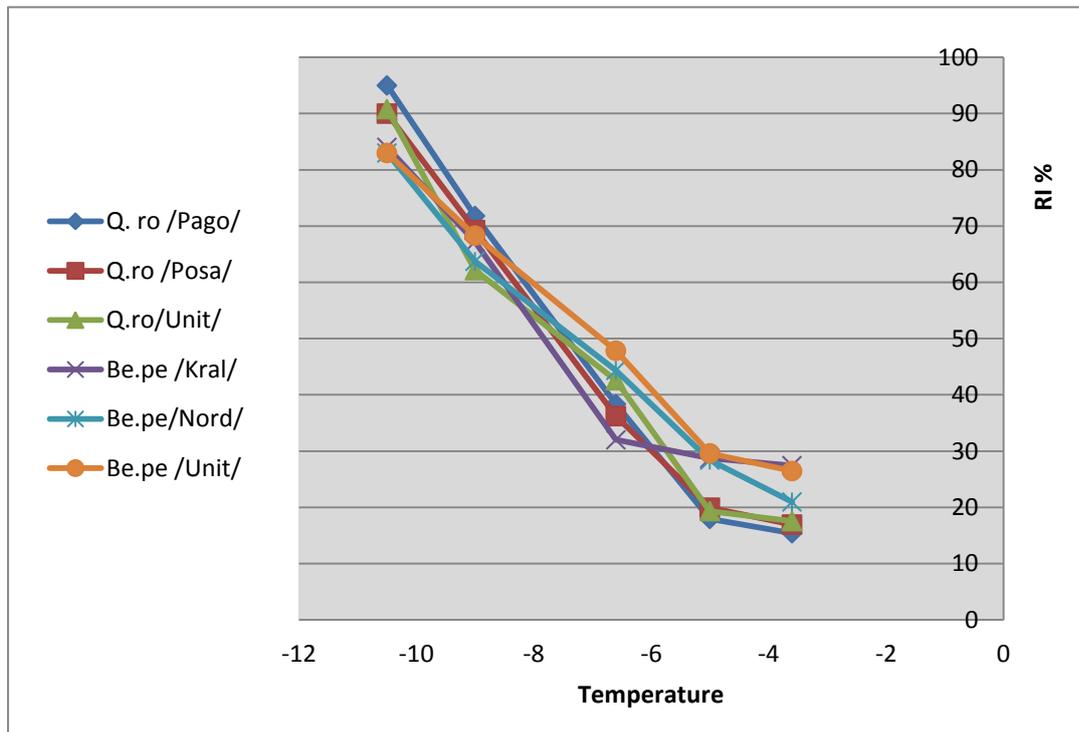

Figure 7 Response of each provenance to the different freezing temperatures that. RI is the relative electrolyte conductivity RI= (T1/T2)*100, that we calculated by dividing the first electrolyte conductivity after the freezing test T1 to the final electrolyte conductivity after using the autoclave T2.

4. Discussion:

4.1. Survival:

The results showed no significance difference between provenances for all the studied species, and in both sites. On the other hand, survival rates were high between both evaluations (September 2013 and May 2014) in Lisbon, while in Sintra we noticed a drop in the survival rates between the two observations. Since Sintra arboreta was planted in the Autum of 2012 the effect of transplant shock was observed during the first summer after plantation, the seedlings were still fragile and more sensitive to drought. The effect of drought on vegetation under warmer conditions can be severe, as highlighted by recent regional-scale woody plant die-off across the southern western United States (Breshears *et al*. 2005 and Mueller *et al*. 2005).

4.2. Growth:

Betula pendula is considered to be a typical pioneer tree species with fast early growth with a sympodial height growth pattern (Heräjärvi. 2001). This species is adapted to continental temperate climatic conditions of relatively hot summers and cold winters (Forestry Compendium by CABI). Our results are constant with these previous studies, as *B. pendula* also has the maximum height among the studied species 1 year after plantation. *Quercus robur,* on the other hand, has the biggest diameters. We may consider that this might be due to the fact that the species is the most adapted species to the local climate conditions since it is native species in the studied area.

4.3. Phenology:

The strong relationship that has been found between winter temperature and dormancy release implies that even small changes in winter temperature can have large impacts on the timing of bud burst. As a reaction-to global warming, the chilling requirement might not be fully met, and thus bud burst could then be delayed. Alternatively in an environment where the chilling requirement is presently far exceeded, bud burst could occur earlier than at present (Heidi .1993). Our results regarding *Betula pendula* are further consistent with these expectancies. Since its three provenances have the earliest bud burst compared to the other species and as mentioned in the previous paragraph, *B. pendula* is known to be among fast growing species and also was well adapted to the climatic conditions of the studied site as evaluated by survival and growth. *Quercus rubra* is considered to be a late species for bud sprout and flowering time in its native range (Vieitez *et al* 2012), our study confirms that the species keep this late bud burst pattern in the introduced regions of Sintra and Lisbon.

When comparing the results of our observations on the 3 and 4 of March 2014, in Lisbon and Sintra there were no significant differences between the provenances in the two studied sites. This means that the combination of photoperiod and temperature required for bud bursting were similar in both sites. Unfortunately we could not compare the percentage of the total bud burst between the two sites, due to the lack of data regarding Sintra, and that is why we can't know the effect of the site on bud burst.

4.4 Insect Damage:

Insect damage was relatively low for all species, yet it tended to be higher for the native species *Q. robur,* In fact this species has overall significantly higher values of leaf damage than the exotic ones. This is consistent with the theory of enemy free hypothesis (Keane and Crawley, 2002) which says that outside its native range plants will be benefit of a release from their native natural enemies. In agreement, it would be expected that exotic plants in the areas of study to behave according to the theory of free enemy and thus suffer less damage than native plants. The phylogenetic isolation mechanism is necessary in order to occur the escape from old enemies by the plants (Keane and Crawley, 2002). Nevertheless, *Q. rubra* and *Q. shumardii* also had higher levels of damage by

herbivores even though they are exotic species in the area. This result is justified due to the fact that these two oak species share close phylogenetic relationship with *Q. robur*, and thus are expected to have similar physical and chemical characteristics. Thus it is expected that the herbivores which feed on *Q. robur*, may also consume other oaks and expand its host range to feed on exotic oak species when in contact with them. Most of the specialist invertebrates attack not just one individual species but families or genus of plants Pylogenetically close plant species share similar defense mechanisms and therefore are vulnerable to attacks by the same specialist herbivores (Ali and Agrawal, 2012).

B. pendula damage has been significantly lower than the other species in the study. We think this is mainly because *B. pendula* unlike *Quercus* sp, does not have any other native plant species phylogenetically close in the areas of study. This justifies the fact that it has been less susceptible to the local specialist herbivore species and therefore only vulnerable to local generalist species that represent a minor impact in the studied sites.

Moreover, our findings indicate that *B. pendula* has also low resistance compared with, *A. pseudoplatanus*. This species suffered higher damage than *B. pendula*. A native species *A. monspeliensis* may share the same herbivores with *A. pseudoplatanus*. The difference between the two species may also be explained by the fact that the method of damage assessment, proportion of damage leaves, may not be ideal to compare damage between plants with large leaf anatomical differences. In effect, since *A. pseudoplatanus* has large leaves, the number of leaves per sampling unit is lower. On the contrary, *B. pendula*, has extremely small and numerous leaves. On the other hand, the larger leaves increase the probability of each leaf having any king of minimal damage than the small leaves of the *B. pendula*. If we had assessed the area of damage in the leaves compared with the total leaf area, the results would probably have been different, with *A. pseudoplatanus* showing less damage.

As a result, we propose that future observations of herbivores damage should also use methods based on the ratio of leaf area damaged by herbivores and the total leaf area weighted by the number of leaves used for each specimen tree, so that anatomical differences of plants would not compromise the results and would make comparisons possible between different plants.

Phenology may also play a role on insect damage. From our results, *B. pendula* was the first species to budburst. This may have occurred in a period when the activity of chewing insects was still low. If the leaves of *B. pendula* were already matured when the feeding activity insects of insects was higher, then the leaf toughness and the amount of defensive compounds of these mature leaves could make them less edible for the insects (van Asch & Visser, 2007). In fact early budburst might be a way of plants to escape herbivores (Bertheau *et al*. 2009).

4.5.Spring frost tolerance:

A number of relevant cold studies showed that *B. pendula* and *Q. robur* have high cold tolerance during winter, the minimum lowest temperature can reach for *Q. robur* as low as -40, and -35 for *B. pendula (*Maliouchenko *et al*, 2007 and Olalde *et al*, 2002 *)*. However, the purpose behind doing this test at the end of May was to simulate the occurrence of late spring frost. In order to test a hypothesis arguing that the seedling which we used for the test will be very sensitive to frost taking into consideration that the leaves were still young, thin and not harden yet.

The performed test showed that both species were highly tolerant to cold, the results have been similar for both species, and the chosen provenances are not a significant factor in these results. Yet, *B. pendula* was a little more sensitive to frost than *Q. robur* which also confirm other relevant studies concerning the species tolerance to frost during the coldest month of the year *(*Maliouchenko *et al*, 2007 and Olalde *et al*, 2002 *)*. Nevertheless *B. pendula* (from UK /Unit/) was the most sensitive to frost comparing with the other two provenances, also this provenance was the latest sprouting between provenances of *B. pendula.* Thus, we can assume that provenances originate from Atlantic climate might be more prone to cold damage than the other provenances from continental regions. Aldrete *et al* (2008) has argued in his study that the seedlings coming from the North and colder regions can harden further more than the seedling coming from relatively less cold areas, which also can explain the results of this study.

In a more comprehensive overview of the results we have so far, an apparent relation can be found between frost and the time of bud bursting and the sensitivity to insect. By tracking the behavior of *Betula pendula* through the different tests we undertook, we observed that it was the earliest species to bud burst in both sites. It was also the species with lower insect damage which, in turn, indicates that the change in phenology for *B. pendula* (which is expressed by the early bud bursting) may have caused a noticeable gap between the bud bursting (where the leaves are the most sensible to insect attack) and between the development of the insects that attack the leaves. This concept has been also supported by several previous studies such as (Gillooly *et al*. 2001; Clark and Fraser 2004).

Additional cold hardiness is considered an important factor related to the physiological condition of the seedlings (Langvall. 2011). Cold hardiness and timing of bud set and bud break are important processes that provide protection of seedlings against low temperatures. In this respect, there is evidence that cold hardiness can be related to the timing of bud burst (Aldrete *et al*. 2008). This explains why *B. pendula* which comes from UK /Unit/, was the latest provenance to bud burst. When doing the freezing test, it was the most sensitive to frost comparing to the other two provenances of *B. pendula* which sprouted earlier.

5. Conclusion

In our study, we found that provenances within the species was not a significant factor for most of the traits analyzed. However the number of individuals assessed as well as the number of provenances was quite low to allow us to generalize results. Indeed the REINFFORCE arboreta value lies in its global information considering the 38 sites and 35 species, therefore it is not realistic to conduct final conclusion on a few provenances and species. Nevertheless, our results suggest some relevant information.

Phenology varied among species and among provenances for *B. pendula*. Also species tend to keep same pattern of phenology observed in its native range as observed for *Q. rubra*. Further, phenology may play a relevant role on insect damage and frost damage. Altogether, these results point out the relevance for studies on the phenology of trees regarding climate change and biotic and abiotic risk factors.

From our results, *B. pendula* was the first species to budburst. This may have occurred in a period when the activity of chewing insects was still low. If the leaves of *B. pendula* were already matured when the feeding activity insects of insects was higher, then the leaf toughness and the amount of defensive compounds of these mature leaves could make them less edible for the insects.

Since species differ significantly on leaf area, we further propose that future observations of herbivores damage should also use methods based on the ratio of leaf area damaged by herbivores, so that anatomical differences of plants would not compromise the results and would make comparisons possible between different plants.

In general it is still also too early to conduct final results considering that the arboreta network needs a long period of time to provide conclusive results. However further studies can continue this research depending on our results and conclusion to study much deeper this subject by studying more species of the arboreta.

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