Intraspecific variability in cold tolerance in Pinus brutia *sampled from two contrasting provenance trials*

Akkın Semerci, Bora İmal & Carlos A. Gonzalez-Benecke

New Forests
International Journal on the Biology, Biotechnology, and Management of Afforestation and Reforestation

ISSN 0169-4286

New Forests
DOI 10.1007/s11056-020-09815-0

Springer

New Forests
https://doi.org/10.1007/s11056-020-09815-0

Intraspecific variability in cold tolerance in *Pinus brutia* sampled from two contrasting provenance trials

Akkın Semerci[1] · **Bora İmal**[2] · **Carlos A. Gonzalez-Benecke**[3]

Received: 30 April 2020 / Accepted: 6 October 2020

Abstract
Turkish red pine (*Pinus brutia* Ten.), is the most important tree species for afforestation in the Mediterranean basin due to its drought tolerance and fast growth rate. Cold damage to trees caused by harsh winter conditions is common on many sites in Turkey. Adaptation to climate change has been investigated primarily through the movement of species from warmer and drier climates, such as the Mediterranean *P. brutia*, to higher latitudes and cooler sites in central-north Turkey. In order to better guide species and provenances movement to new (and often harsh) environments for afforestation, the limits of tolerance to cold and drought should be better known. Thus, we designed an experiment to quantify the cold hardiness of nine *P. brutia* provenances originating from two different provenance trials in Turkey (Ankara, cold inner site; Antalya, warm Mediterranean site). Branches sampled at the end of January were exposed to cold temperatures between − 5 and − 40 °C. Visual damage observation, relative electric leakage and chlorophyll fluorometry (CF) screening methods were used to assess variation in cold hardiness among populations. Overall, *P. brutia* can tolerate winter temperatures up to − 16 °C. Even though there were significant differences on cold hardiness among populations, the operational application is limited due to the reduced magnitude of those differences. Measuring CF was the fastest and most easily replicated method to estimate cold hardiness and was as reliable as REL. We recommend that *P. brutia* should not be planted in cold areas where minimum annual temperatures are under − 16 °C. We also conclude that even though phenotypic plasticity exists for cold hardiness among the tested populations of *P. brutia*, the observed differences resulted from acclimation to the conditions of the provenance trial sites rather than from adaptation through natural selection.

Keywords Frost resistance · Turkish red pine · Electrolyte leakage · Chlorophyll fluorescence · Frost damage

✉ Akkın Semerci
akkin.semerci@gop.edu.tr

[1] Department of Forestry and Forest Products, Niksar Vocational School, Tokat Gaziosmanpaşa University, Tokat, Turkey

[2] Department of Forest Engineering, Faculty of Forestry, Çankırı Karatekin University, Çankırı, Turkey

[3] Department of Forest Engineering, Resources and Management, College of Forestry, Oregon State University, Corvallis, USA

Published online: 21 October 2020

Introduction

Climate projections for the Mediterranean basin suggest that the region will become warmer and drier with more frequent and extreme weather events (IPCC 2013, 2014). Historic data for Turkey indicated a marked temperature warming trend since the mid-1980s. The frequency of record minimum air temperature events observed in Turkey decreased from the 1950s to the present time, while the frequency of maximum air temperature events increased evidently in particular with the year of 2000 (Erlat and Türkeş 2015). However, regional climate projections suggests that the country will be warmer and in the period 2071–2100 precipitation will likely decrease along the Aegean and Mediterranean coasts and increase along the Black Sea coast of Turkey whereas Central Anatolia will show little or no change (Dalfes et al. 2007).

Temperate Mediterranean climates are characterized by mild, wet winters and pronounced dry summers. Regions at the limit of this climate type will typically experience cooler summer temperatures and a reduced summer drought. However, at higher latitude regions, these climates also tend to experience more rainfall and have colder minimum temperatures during wintertime. The milder summer temperatures and shorter drought, combined with colder winter conditions, provides both opportunities and challenges for landscape plant selection in these regions.

Even though drought is considered the main environmental constraint in Mediterranean ecosystems, frosts can be equally or more determinant in many areas. Cold hardiness of Mediterranean plant taxa has been a matter of concern (Boorse et al. 1998; Larcher 2000; Climent et al. 2009), as more drought tolerant species may be better adapted to future dry conditions but are thought to be more sensitive to frost events (Wieser et al. 2009). Climate change does not only mean an increase in air and water temperature, but also changes in rainfall and wind regimes; and increment in the likelihood and strength of extreme events such as droughts, storms and extreme temperatures. It is expected that global warming may shift species distribution to higher latitudes, even more than 250 km in some cases, where they will be more exposed to more extreme weather events (Park et al. 2016). Therefore, foresters need to obtain an accurate knowledge of economically important tree species capacity to cope with extreme weather events.

Adapting forest management activities to climate change may include changes in the composition and structure of forest stands, selection of adapted species and provenances or; if this is regarded as insufficient, assisted migration and, alternatively, substitution of native with non-native species (Bussotti et al. 2015).

Cold hardiness is an important trait reflecting adaptation to climate. It can be assessed by evaluating the freezing damage after natural frost events in field trials, but this method possesses limitations related to uncontrolled conditions and lack or repeatability, which in turn leads to low statistical power. A better solution is to subject samples to different freezing temperatures under controlled conditions and to evaluate the freezing damage in those samples (Burr et al. 1990).

Cold stress results in various observable or measurable symptoms of injury including death of whole plants, visible necrosis of specific tissues and organs; or less obvious cellular symptoms that can be detected by vital staining, osmotic responsiveness, chlorophyll fluorescence, or by measuring relative electrolyte leakage in the affected tissues. These measurements are often used to determine a minimum survival temperature, or to construct temperature response curves and interpolate the temperature resulting in 50% plant or tissue death, LT50 (Strimbeck et al. 2015). Visual observation (VO), relative

electrical conductivity (REL) and chlorophyll fluorometry (CF) are the most used methods for screening cold hardiness.

Turkish red pine (*Pinus brutia* Ten.) distributes naturally across a wide range of sites in the Eastern Mediterranean, ranging from 44° to 35° N, and from sea level to 1600 meters altitude. *P. brutia* forests are important for multi-purpose forestry and have a high economic and ecological value. *P. brutia* is the most important forest tree species in Turkey, covering approximately 5.6 million ha of natural forest areas (25% of the total forestland in the country) and yielding about 4.3 million m^3 $year^{-1}$ of wood production (31% of total wood production in the country). In 2015, 52.5 million seedlings of *P. brutia* were produced in Turkey (16% of the total forest tree seedlings production in the country) (OGM 2015). *P. brutia* forests have a high economic importance and represent the main source of wood and forest cover in some Mediterranean countries. The wood from this species is used for multiple purposes such as construction, carpentry, firewood and pulp and paper. In Turkey, Syria and Greece, *P. brutia* has additional economic importance due to the oleoresins that can be extracted and used in soap, nail polish and the pharmaceutical industry. Non-wood production of these pines is the honey produced in Greece and Turkey from the honeydew released by the sap-sucking insect *Marchalina hellenica* (Bacandritsos et al. 2004). *P. brutia* forests also hold a key role in providing important environmental services such as protection of soil and water resources, conservation of biological diversity, support to agricultural productivity, carbon sequestration, climate change mitigation and adaptation, and combating desertification.

The capability of *P. brutia* to grow on a wide range of soils and elevations, as well as its high productivity potential, make it one of the most promising pine species for plantations in Mediterranean basin. Furthermore, its ability to withstand aridity and continentalism, and regeneration post wildfires, makes it an exceptional forest species within the fragile Mediterranean ecosystems. *P. brutia* has been identified as a target species for intensive forestry and tree breeding programs in Turkey (Koski and Antola 1993). Provenance trials were carried out by the Turkish Forest Research Institute, with a set of 26 trial sites planted between 1988 and 1989 throughout Turkey. Cold damage events have been reported in the study site established in the cooler Central Anatolia region, unfortunately intensity and quantity of cold damage in these provenance trials were not evaluated (Cengiz et al. 1999). Additionally, cold browning and mortality has been observed after harsh winters in some small-scale plantations in the same area. Therefore, better understanding intra-specific variation in the tolerance to low temperatures is essential for seed transfer between geographic regions.

Historical records and modeling of future temperature and precipitation of the Central Anatolia region in Turkey, show that there is a consistent trend of increasing minimum and maximum temperatures (Kızılelma et al. 2015). Additionally, species distribution models indicate that climatically suitable areas for *P. brutia* trees are expected to shift in a near future to higher altitudes and toward the north and northeastern regions of Turkey (Yalçın 2012). Therefore, future climate may provide an opportunity to introduce *P. brutia* to these regions.

When introducing a species to cooler sites, the limits of cold tolerance should be well understood. Artificial freezing tests are a good tool to simulate different levels of cold severity, which can then be used to identify the onset temperatures that start to cause damage (Lu et al. 2003). With this information it is possible to compare the threshold temperatures of different genotypes (species and provenances) with climatic conditions of a particular region and predict the potential risks associated with seed movement. The objectives of this study were to: (1) quantify and rank cold hardiness, measured as LT50, for 9

P. brutia provenances growing in two contrasting provenance trial sites in Turkey, and (2) to compare three screening methods (VO, REL and CF) commonly used to assess cold hardiness.

Materials and methods

Provenances and sample collection

This study was based on two *P. brutia* provenance trials that were established in 1988 in Turkey. The two selected study sites (Antalya, ANT, warmer southern coast; Ankara, ANK, colder north inland) are part of a total of 26 testing sites covering the range of distribution of the species in Turkey (Fig. 1). Each provenance test included 50 provenances from entire geographic range of the species. At each site, a randomized complete block design with three replications (blocks) was applied. Each provenance was represented by 16 trees in each block. At year 5, across all provenances, the ANT site had a mean survival of 100% while the ANK site had a survival of 76% (Cengiz et al. 1999).

We studied 9 provenances that survived 5 years at both the ANK and ANT provenance trial sites (Table 1): Anamur (ANA), Bafra (BAF), Bayramiç (BAY), Bucak (BUC), Gölhisar (GOL), Gülnar (GUL), Kaş (KAS) and Tarsus (TAR), which performed with high survival rate (85–96%), and a Northern Cyprus provenance (CYP) which performed with low survival rate (19%). Air temperature data from provenances and study sites were obtained from the Turkish Meteorology Service using data from the nearest weather station available. Data spanned between 1960 (ANA and TAR) and 1985 (ANK) to 1992 (BAY) and 2015 (ANA, BAF, CYP, KAS, ANK and ANT).

The whole-plant freeze testing method (Burr et al. 2001) was used to estimate frost damage. Distal parts of the branches of approximately 20 cm length were frozen at different

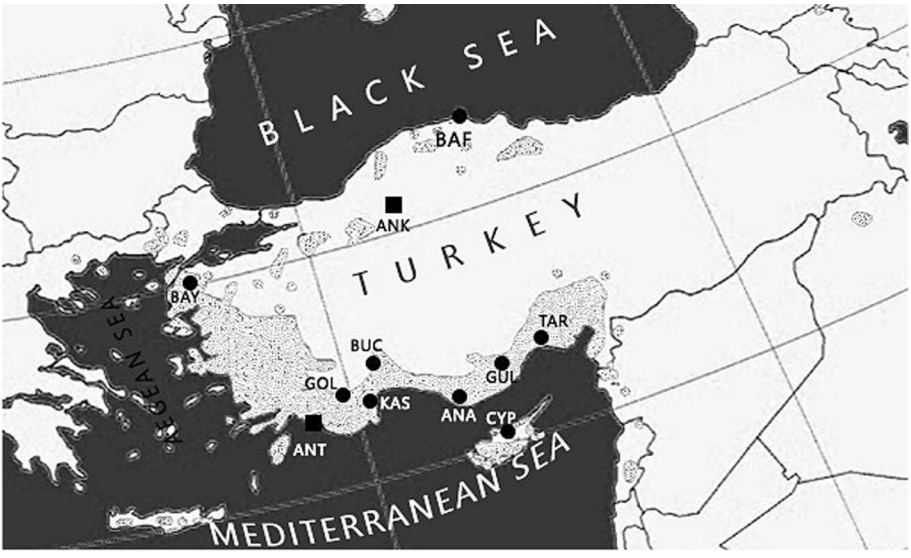

Fig. 1 Provenance trials (black square) and provenances (black circle) used in this study. Grey areas indicate natural distribution areas of *P. brutia*

Table 1 Site characteristics of *P. brutia* provenance origin and study sites and survival rates at year 5 the Ankara site

Label	Location	Enterprise-district	Lat.	Lon.	Elevation (m)	Survival (%)
Provenances						
ANA	Mersin	Anamur–Anamur	36° 05′	32° 41′	650	85
BAF	Samsun	Bafra-Yakakent	41° 39′	35° 27′	100	87
BAY	Çanakkale	Bayramiç-Karaköy	39° 50′	25° 55′	400	95
BUC	Burdur	Bucak–Bucak	37° 30′	30° 41′	800	96
CYP	Lefkoşa	Cyprus-Güzelyurt	35° 18′	33° 03′	200	19
GOL	Burdur	Gölhisar-Gölhisar	37° 04′	30° 32′	1100	88
GUL	Mersin	Gülnar-Pembecik	36° 14′	33° 15′	650	96
KAS	Antalya	Kaş-Lengüme	36° 24′	29° 30′	720	96
TAR	Mersin	Tarsus-Cehennemdere	37° 07′	34° 31′	800	89
Provenance trial sites						
ANK	ANKARA	İlyakut	40° 03′	32° 28′	980	76
ANT	ANTALYA	Finike-Yazır	36° 30′	30° 07′	950	100

temperatures in a programmable freezer. We collected 1-year old branchlets (~ 20 cm length) from the southern middle part of the crown (fully exposed to sun) from 10 trees per provenance. Sample collection was carried out using a ladder and a telescopic tree pruner between the 12th and 13th of January in 2016 at both the ANT and ANK sites. Dominant trees distributed over all three blocks were chosen. Immediately after branchlets excision, samples were placed in plastic bags and sprayed with distilled water while kept in the dark at + 4 °C and transported to the laboratory.

Cold hardiness tests and experimental design

Two days after harvesting the twigs, which were always kept moist at low temperature, were inserted in a low-temperature freezer equipped with a programmable controller (Vötsch Industrietechnik, Model: VT3 4034). The freezing rate was kept to 5 °C per hour (Burr et al. 2001) until each target temperature (− 5, − 10, − 12.5, − 15, − 17.5, − 20, − 22.5, − 25, − 30 and − 40 °C) was reached. Each target temperature was achieved independently to keep each treatment group at the target temperature for 5 h. After that, thawing was done by increasing temperature by 5 °C per hour until they reached room temperature.

Relative electrolyte leakage

Membrane injury was determined by measuring the electrical conductivity of the incubation solution where the ions leaked from the cells after artificial freezing. After thawing the twigs at each freezing treatment, sections of approximately 1 cm in length were taken from the middle section of 20 needles and added to vials containing 15 ml of deionized water. Subsequently, the vials were shaken in an orbital shaker (WiseShake, Model: SHO-2D) at 100 rpm speed at room temperature for 20 h. Afterwards, the initial electrical conductivity reading (C1, μS cm^{-1}) was measured with an electrical conductivity meter (WTW inoLab pH/Cond, Model: Level 1). Finally, the samples were autoclaved (Nüve Laboratory

Equipment Model: OT 90) at 121 °C for 1 h, held at 25 °C for 4 h where the final electrical conductivity was measured (C2, μS cm^{-1}). The relative cell damage at each target freezing temperature, or relative electrolyte leakage (REL), was computed as REL = (C1/C2) × 100.

Chlorophyll fluorometry

Chlorophyll fluorescence (CF) measurements provide information about the overall photosynthetic potential of the plant and its responses to stress or disturbances (Mohammed et al. 1995). The ratio of variable chlorophyll fluorescence to maximum chlorophyll fluorescence (Fv/Fm) is linearly correlated with the quantum yield of net photosynthesis. The parameter Fv/Fm was used for CF as it has been found to be highly correlated with low temperature tolerance (Rose and Haase 2002; Strand and Öquist 2006; Corcuera et al. 2011). High values of Fv/Fm reveal undamaged tissue, while low values are indicative of freezing damage. Measurements of Fv/Fm were performed using a Handheld chlorophyll fluorometer (Opti-Sciences, Model: OS30p). After thawing the twigs at each freezing treatment, needles were acclimated to dark for at least 30 min before they were measured.

Visual observation

Freeze-injured tissue typically develops a brown or yellowish color from the oxidation of polyphenols (Lindén, 2002). In this procedure, the tissue is allowed to develop symptoms of damage for several days (commonly 1–2 weeks) after freezing before scoring the damages into discrete classes (Burr et al. 2001). After thawing, the remaining twigs of each freezing treatment were left for 14 days at greenhouse temperature (15–20 °C) to allow visible signs of freezing damage to develop. To improve visibility of symptoms, water was sprayed on samples every 3 days. Cold injury was assessed visually by recording the percentage of discolored needles (Burr et al. 1990; Prada et al. 2014) at two times: 7 and 14 days after freezing tests. Due to poor correlations of observations performed after 7 days, only the data collected after 14 days was used for further evaluations. Visual scoring in needles was done by using a scale of 0 to 5 depending on the percentage of the foliar area damaged (0 = 0%, no damage; 1 = 1–20%; 2 = 21–40%; 3 = 41–60%; 4 = 61–80%; 5 = 81–100% of foliar area damaged). For statistical analyses, the median value was used from each category (0, 10, 30, 50, 70 and 90%, respectively).

Statistical analysis

A non-linear model reflecting the relationship between temperature (expressed as positive values) and tissue damage (assessed using either REL, CF or VO) was fitted for each sample. After testing several sigmoidal curves, the model proposed by Kreyling et al. (2012) was selected:

$$Y_T = Y_{min} + \frac{Y_{max} + Y_{min}}{1 + e^{k \cdot (T_m - T)}} \quad (1)$$

where Y_T is the tissue damage (either, REL, CF or VO) at temperature T, Y_{min} is the lower horizontal asymptotic value of the response variable, Y_{max} is the upper horizontal asymptotic value of the response variable, k represents the steepness of the response curve, and

T_m is the inflection point of the curve, which corresponds to LT50 (Pinheiro and Bates, 2000).

A non-linear model fitting was used to estimate LT50 and analysis of variance was used to test the effects of provenances and sites on LT50 (PROC NLIN and PRCO GLM; SAS Institute Inc., Cary, NC, USA).

Results

Long-term minimum temperature (percentile 5 of minimum monthly temperatures for 27–55 years of observations) of provenance and study sites is displayed in Fig. 2. KAS provenance has the highest temperatures with no frosts during 51 years of temperature records. The ANA and CYP (provenance), and ANT (study) sites showed slightly lower temperatures during winter, but only reached mild frosts. During winter, provenance sites BAF and TAR reached minimum temperature above − 10 °C, while provenance sites BAY, BUC and GUL showed minimum temperature ranging between − 15 and − 10 °C. The ANK (study) and GOL (provenance) sites had the lowest temperatures, reaching below − 15 °C during January and February.

The ANK site was much colder than the ANT site during branch sampling, corroborating the historical meteorological data for these sites (Fig. 2). During winter until branch sampling date (January 12–13 of 2016), there were 38 frost days and the minimum temperature reached − 10.8 °C at the ANK site. However, at the ANT site, there were no frost days and the minimum temperature reached 0.9 °C (Fig. 3).

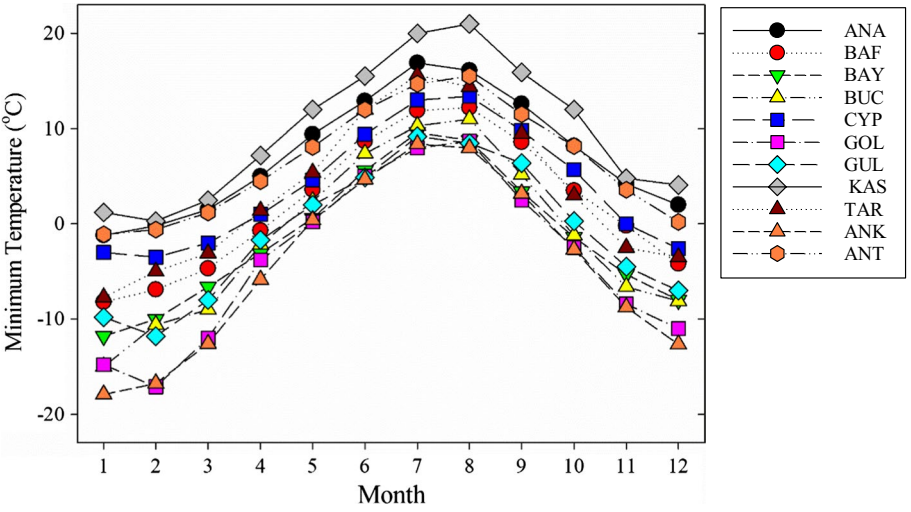

Fig. 2 Minimum temperature for provenance origin and study (Ankara, ANK, orange triangle; Antalya, ANT, orange hexagon) sites. Each symbol represents the 5th percentile of minimum monthly temperatures of 27–55 years of observations. Description of provenances is provided in Table 1

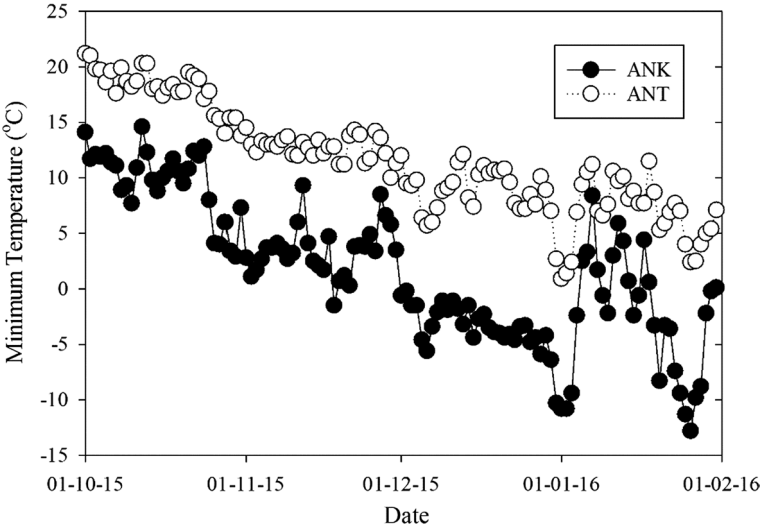

Fig. 3 Daily minimum temperature for provenance study sites (Ankara, ANK, filled circle figures; Antalya, ANT, open circle) during winter when sampling was carried out

Cold hardiness variations among provenances

Overall, the three methods used to assess frost damage on *P. brutia* showed similar results, indicating that lethal damage in *P. brutia* needles started below −16 °C (Fig. 4). Frost hardiness of *P. brutia*, expressed as LT50 values, ranged from −21.4 (with REL) to −16.1 °C (with VO) (Table 2). There was a sharp increase in damage expressed as relative electrolyte leakage (REL), chlorophyll fluorescence (CF) and visual observation of percent damage (VO) between −15 and −20 °C (Fig. 4).

The model selected (Eq. 1) for LT50 determinations proved to be a good fit. All parameter estimates from model fitting were significant at $P < 0.05$ (data not shown). Using the REL method R^2 ranged between 0.918 and 0.966, and CV ranged between 0.126 and 0.212. Using the CF method R^2 and CV ranged between 0.897 and 0.989, and 0.02 and 0.249, respectively. With the VO method R^2 ranged between 0.856 and 0.994, and CV ranged between 0.062 and 0.312 (Table 2).

Using the REL method LT50 ranged between −19.3 and −21.4 °C at the ANK site, and between −16.8 and −20.6 °C at the ANT site (Table 3). Using the CF method LT50 ranged between −18.3 and −20.7 °C at the ANK site, and between −16.8 and −20.5 °C at the ANT site. However, using the VO method, LT50 ranged between −18.3 and −20.1 °C at the ANK site, and between −16.1 and −19.6 °C at the ANT site (Table 3).

At the ANK site, independent of the methods employed, the three provenances more resistant to cold (more negative LT50) were the same: CYP, GOL and KAS. When using the REL method the cold resistance ranking was led by CYP, followed by GOL and KAS (average LT50: −21.4, −20.7 and −20.3 °C, respectively). Using the CF method the most resistant provenance was KAS, followed by GOL and CYP (average LT50: −21.2, −20.7 and −20.3 °C, respectively). Using the VO method, the most resistant provenance was GOL, followed by CYP and KAS (average LT50: −20.1, −19.9 and −19.6 °C, respectively). However, the provenance least resistant to cold (less negative LT50) was BAF.

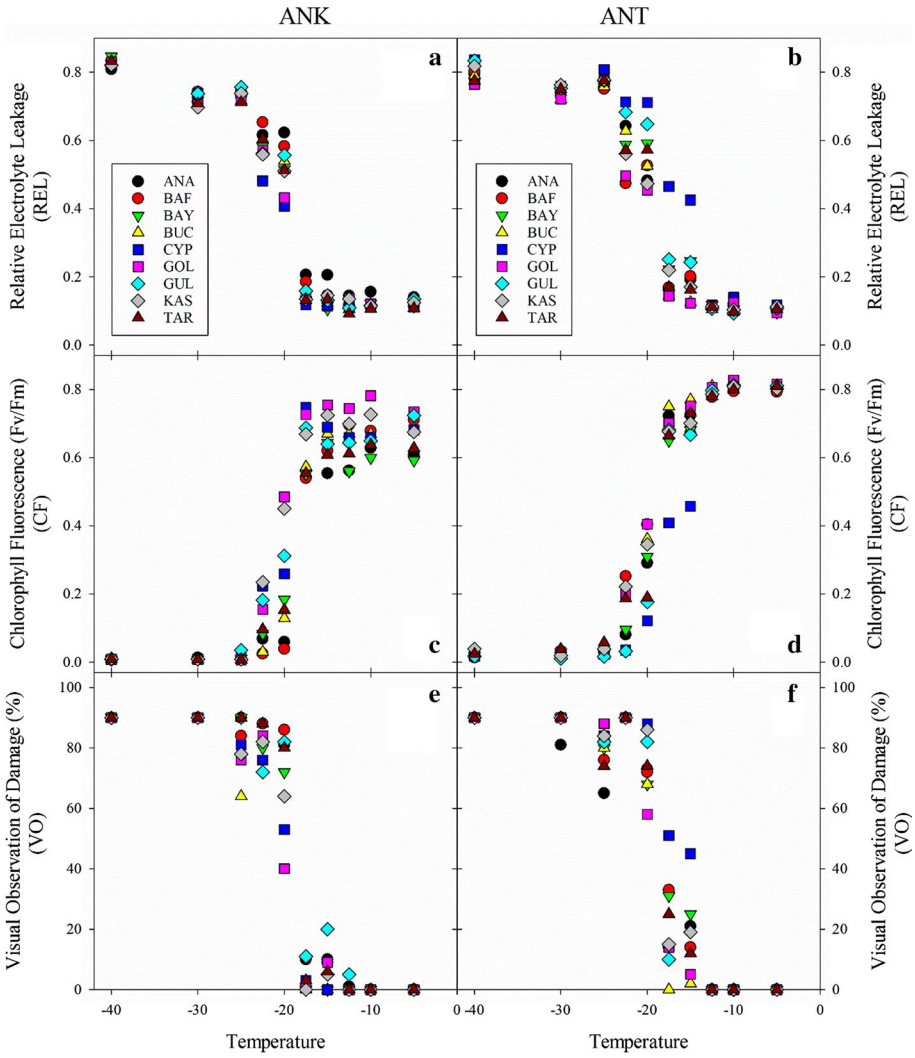

Fig. 4 Relationships between temperature and relative electrolyte leakage (REL) (**a**, **b**), chlorophyll fluorescence (CF) (**c**, **d**) and visual observation of percent damage after 2 weeks (VO) (**e**, **f**) of *Pinus brutia* provenances experiments on sites located in Ankara (ANK, left) and Antalya (ANT, right) regions of Turkey. Each symbol represents the mean value of 10 observations. Error bars were not included to facilitate visual comparisons. Description of provenances is provided in Table 1

That ranking was held by the REL (− 19.3 °C) and CF (− 18.3 °C) methods. Using the VO method, the provenance with larger LT50 was GUL (− 18.3 °C) (Table 3).

At the ANT site, the three provenances that showed more resistance to cold were GOL, BAF and KAS (average LT50: − 20.6, − 20.5 and − 20.3 °C, respectively). That ranking was held by the REL and CF methods. Using the VO method, the three provenances more resistant to cold were BUC, GOL and ANA (average LT50: − 19.6, − 19.1 and − 18.5 °C, respectively). However, the provenance least resistant to cold was CYP. That ranking was

Table 2 Average fit statistics for LT50 determined with relative electrolyte leakage (REL), fluorescence (CF) and visual damage observation (VO) methods for *Pinus brutia* provenances experiments on sites located in Ankara (ANK) and Antalya (ANT) regions of Turkey

Site	Provenance	REL		CF		VO	
		R^2	CV	R^2	CV	R^2	CV
ANK	ANA	0.918	0.169	0.927	0.249	0.992	0.082
	BAF	0.966	0.131	0.982	0.114	0.976	0.128
	BAY	0.958	0.154	0.958	0.158	0.994	0.042
	BUC	0.933	0.180	0.986	0.002	0.931	0.233
	CYP	0.950	0.170	0.897	0.213	0.988	0.078
	GOL	0.964	0.140	0.989	0.079	0.969	0.177
	GUL	0.923	0.178	0.949	0.170	0.856	0.312
	KAS	0.949	0.164	0.969	0.130	0.965	0.167
	TAR	0.947	0.170	0.955	0.164	0.990	0.062
ANT	ANA	0.966	0.128	0.987	0.094	0.899	0.250
	BAF	0.899	0.212	0.975	0.120	0.945	0.169
	BAY	0.933	0.170	0.979	0.109	0.918	0.196
	BUC	0.953	0.155	0.980	0.088	0.985	0.096
	CYP	0.952	0.126	0.978	0.127	0.965	0.079
	GUL	0.930	0.169	0.979	0.117	0.932	0.165
	KAS	0.961	0.139	0.941	0.154	0.933	0.148
	TAR	0.944	0.167	0.974	0.113	0.951	0.178

Description of provenances is provided in Table 1. All *P* values were < 0.05

R^2: coefficient of determination; CV: coefficient of variation (RMSE/mean)

held by the three methods (average LT50: −16.7, −16.8 and −16.1 °C, for REL, CF and VO methods, respectively) (Table 3).

Even though the material collected from the ANK site (colder north inland) generally showed lower levels of damage from artificial freezing than the needles collected at the ANT site (warmer southern coast) (Table 3), there was a strong interaction between provenance and site effects (G×E Interaction) on LT50 ($P < 0.0001$; Table 4). The interaction was driven by changes in ranking across sites by BAF ($P = 0.056$), CYP ($P < 0.0001$) and GUL ($P = 0.036$) provenances (*P* values for differences in LT50 using REL). That effect was observed independent if REL or CF methodologies were employed, which indicates the response of the provenances to the controlled freezing test was also dependent on site condition (when using CF, the *P* values for the differences across sites for BAF, CYP and GUL were 0.044, < 0.0001 and 0.024, respectively). Interestingly, when using VO the P-values for the differences across sites for GUL was non-significant ($P = 0.949$), but yes for KAS ($P = 0.014$).

Overall, there was no correlation between LT50 and altitude or latitude of provenances origin (Fig. 5). Nevertheless, at the ANT site, according to REL screening method results, provenances from continental inner and colder Black sea region (GOL and BAF) withstand lower levels of cold (−20.6 and −20.5 °C, respectively) than those from coastal Mediterranean populations (CYP and GUL) with LT50 averaging −16.8 and −18.5 °C, respectively (Table 3). Similarly, at the ANT site, CF and VO results indicated that CYP provenance is less tolerant to frost than the other populations tested. According to REL screening method

Table 3 Average LT50 (°C), measured with relative electrolyte leakage (REL), chlorophyll fluorescence (CF) and visual observation of percent damage (n = 10) after 2 weeks (VO) for *Pinus brutia* provenances experiments on sites located in Ankara (ANK) and Antalya (ANT) regions of Turkey

Site	Provenance	REL	CF	VO
ANK	ANA	− 19.4 (0.41)b	− 18.6 (0.46)dc	− 18.8 (0.34)ab
	BAF	− 19.3 (0.35)b	− 18.3 (0.46)d	− 19.2 (0.23)ab
	BAY	− 20.1 (0.53)ab	− 19.2 (0.37)bdc	− 19.6 (0.42)ab
	BUC	− 19.9 (0.35)ab	− 18.9 (0.42)dc	− 18.6 (0.24)ab
	CYP	− 21.4 (0.55)a	− 20.3 (0.34)abc	− 19.9 (0.33)a
	GOL	− 20.7 (0.46)ab	− 20.7 (0.45)ab	− 20.1 (0.43)a
	GUL	− 19.9 (0.55)ab	− 20.2 (0.71)abc	− 18.3 (0.73)b
	KAS	− 20.3 (0.45)ab	− 21.2 (0.52)a	− 19.7 (0.32)ab
	TAR	− 19.9 (0.26)ab	− 19.2 (0.26)bcd	− 19.0 (0.29)ab
ANT	ANA	− 19.9 (0.39)ab	− 19.6 (0.38)ab	− 18.5 (0.30)ab
	BAF	− 20.5 (0.54)ab	− 20.5 (0.50)a	− 17.8 (0.49)abc
	BAY	− 19.1 (0.57)ab	− 19.4 (0.52)a	− 17.5 (0.67)bc
	BUC	− 19.7 (0.28)ab	− 20.1 (0.44)a	− 19.6 (0.18)a
	CYP	− 16.8 (0.88)c	− 16.8 (0.89)b	− 16.1 (0.72)c
	GOL	− 20.6 (0.35)a	− 20.3 (0.35)a	− 19.1 (0.32)ab
	GUL	− 18.5 (0.29)bc	− 18.9 (0.33)ab	− 18.3 (0.54)ab
	KAS	− 20.3 (0.50)ab	− 20.0 (0.54)a	− 18.1 (0.46)ab
	TAR	− 19.7 (0.45)ab	− 19.3 (0.65)a	− 18.1 (0.54)abc

Description of provenances is provided in Table 1

Values in parenthesis indicate standard error. Letters indicate significant differences within each site at $\alpha = 0.05$

Table 4 Results of analysis of variance (ANOVA) testing the effects of provenance, site and their interaction on LT50, measured with relative electrolyte leakage (REL), chlorophyll fluorescence (CF) and visual observation of percent damage after 2 weeks (VO), of *Pinus brutia* provenances experiments on sites located in Ankara and Antalya regions of Turkey

Method	Provenance	Site	Provenance × site
REL	0.0180	0.0032	< 0.0001
CF	0.0003	0.4188	< 0.0001
VO	0.0170	< 0.0001	< 0.0001

results, *P. brutia* trees from CYP showed 27% increased cold hardiness when growing at the ANK (cold) site (LT50 = − 16.8 °C) when compared with the ANT (warmer) site (LT50 = − 21.4 °C) (Table 3). Other provenances, except for KAS, showed small changes in cold hardiness (between 1 and 8%) when growing on ANK trial site.

Comparison of screening methods

Even though there was a strong correlation between the methods used to determine frost tolerance across provenances, that relationship was different across sites and testing

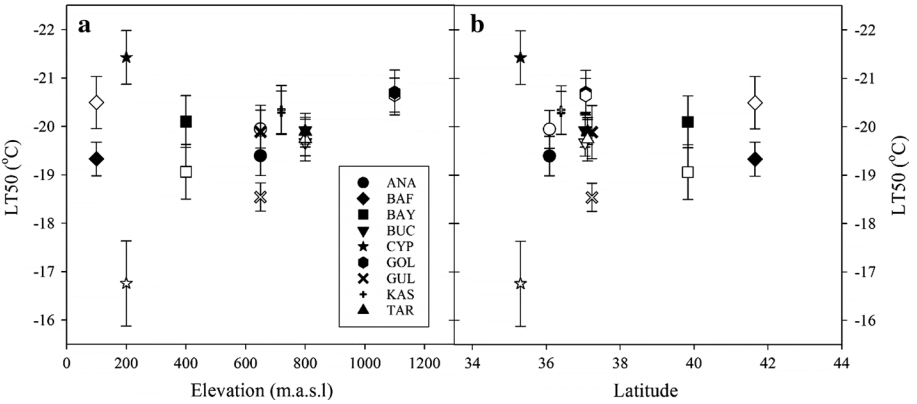

Fig. 5 Relationship between LT50 (determined using relative electrolyte leakage) and **a** elevation and **b** latitude of *Pinus brutia* provenances experiments on sites located in Ankara (ANK, filled symbols) and Antalya (ANT, open symbols) regions of Turkey. Each symbol represents the mean value of 10 observations. Error bars represent standard error

methods ($P < 0.0001$; Fig. 6). Overall, across provenances, the slope and R^2 of the relationship between LT50 determined with REL and CF were 1.074 and 0.49 at the ANK site, and 0.887 and 0.92 at the ANT site, respectively. The mean difference in LT50 estimated with both methods were −0.48 and −0.03 °C, at both the ANK and ANT sites (corresponding 2.4 and 0.2% underestimations). Larger discrepancies were observed when comparing LT50 determined with REL and VO. The slope and R^2 of those relationships were 0.659 and 0.49 at the ANK site, and 0.595 and 0.49, at the ANT site, respectively. The mean difference in LT50 estimated with both methods was −0.87 and −1.34 °C, at the ANK and ANT sites (corresponding 4.3 and 6.9% underestimations), respectively.

In general, LT50 determined with REL was more stable across provenances, and VO was less precise than REL and CF methods. The coefficient of variation of the LT50 estimates determined with the three methods for each provenance at each site is shown in Fig. 7.

Discussion

Cold hardiness variations among provenances

Tree species have different levels of resistance to low temperatures. This characteristic is related to how the species are distributed (George et al. 1974; Sakai and Larcher 1987). Based on that, a classification of the climate based on minimum temperatures has been used to categorize the frost resistance of each species. These climate classifications are known as plant hardiness zones. The U.S. Department of Agriculture (USDA) refined the method and adopted 11 hardiness zones, where *P. brutia* is classified in Zone 7, having a minimum temperature of −17.7 °C as its zonal limit (Bannister and Neuner 2001). Similarly, Atalay et al. (1998) reported that −17.8 °C is the lowest mean air temperature of the coldest month for the area of natural distribution of *P. brutia* in Turkey. In our experiments, frost hardiness (e.g. LT50) of *P. brutia* ranged between −21.4 (using REL method, in CYP provenance) to −16.1 °C (using VO, in CYP provenance). Hence, our determinations of

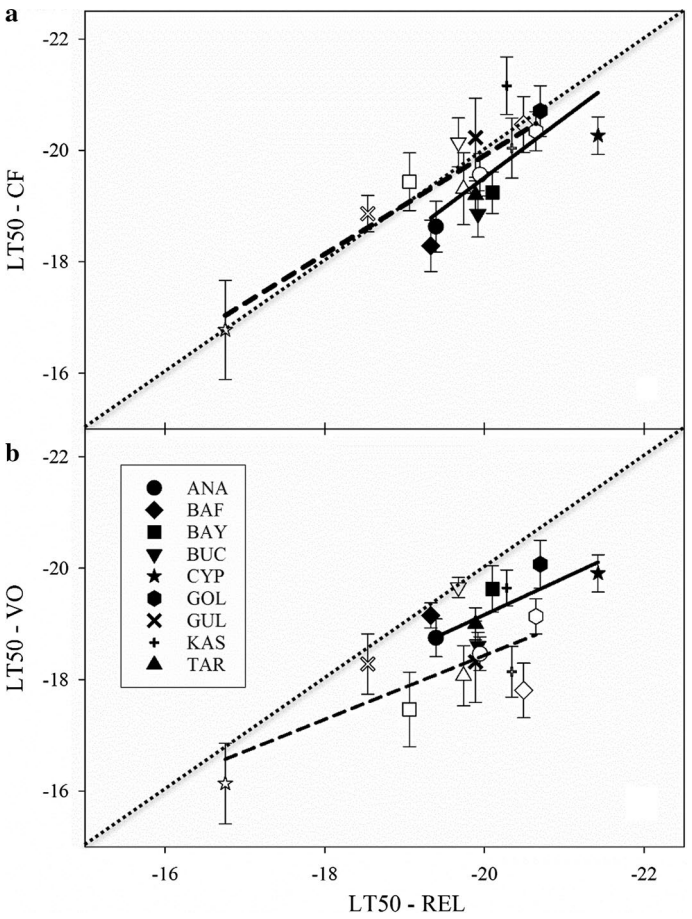

Fig. 6 Relationship between LT50 determined using relative electrolyte leakage (REL) and **a** chlorophyll fluorescence (CF), and **b** visual observation of percent damage after 2 weeks (VO) of *Pinus brutia* provenances experiments on sites located in Ankara (ANK, filled symbol) and Antalya (ANT, open symbol) regions of Turkey. Each symbol represents the mean value of 10 observations. Error bars represent standard error. Dotted line represents 1:1 relationship

frost resistances of *P. brutia* are consistent with the climatic zones made by the USDA. Kandemir et al. (2008) also reported that visible cold damage *in P. brutia* started at − 15.2 °C in a common garden experiment established in Central Anatolia of Turkey. Our finding is consistent with Climent et al. (2009) and Yildiz et al. (2014) which found the threshold in artificial freezing of 32-week-old secondary needles in *P. brutia* to be approximately − 17 and − 15 °C. According to our results and previous work on cold hardiness of *P. brutia*, we can advise practitioners that this species should not be planted in areas where the coldest annual temperature is lower than − 16 °C. It has been reported that resistance to cold temperatures can be influenced by the genetic origin or provenance (Sakai and Weiser 1973; Glerum 1985; Bigras et al. 2001; Malmqvist et al. 2018). These findings are consistent with our result which indicated significant variation among provenances in LT50. Other previous studies in Turkey also reported that *P. brutia* shown variation in frost resistance across populations (Kandemir et al. 2008; Yildiz et al. 2014).

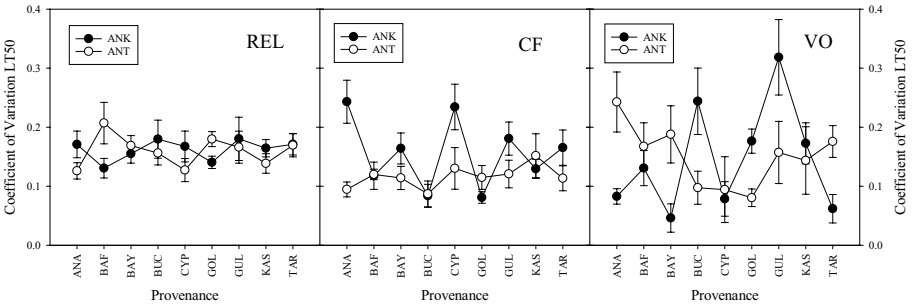

Fig. 7 Coefficient of variation for LT50 was determined using relative electrolyte leakage (REL; left panel), chlorophyll fluorescence (CF; center panel) and visual observation of damage after 2 weeks (VO; right panel) of *Pinus brutia* provenances experiments on sites located in Ankara (ANK, filled circle) and Antalya (ANT, open circle) regions of Turkey. Each symbol represents the mean value of 10 observations. Error bars represent standard error. Description of provenances is provided in Table 1

The significant interaction between provenance and site (G×E interaction) indicate that the differences in cold tolerance were related to both, site and genetic structure of the populations. The observed interaction was driven by changes in cold tolerance across sites by the BAF, CYP and GUL provenances (Tables 3, 4). That may be the result of the manifestation of phenotypic plasticity in those provenances growing under different soil and climate conditions between the trial sites. A similar observation was also reported for Norway spruce (*Picea abies*) by Gömöry et al. (2010), which found that site and provenance had a significant effect on cold hardiness. The authors indicated that provenances sampled from colder experimental sites were more cold tolerant than samples taken from a warmer experimental site.

There was a trend towards GOL provenance having high level of frost resistance across sites. Similarly, Yildiz et al. (2014) also reported that GOL provenance was the most frost resistant of the provenances in their study. GOL provenance comes from higher altitude (1100 m.a.s.l.). In a study addressing genetic differentiation in *P. brutia* populations in Turkey, Kurt et al. (2012) found that a population (Hacibekar) was highly differentiated from all others. That population is located at high altitude (1000 m.a.s.l) at about 40 km distance from GOL provenance origin site. Our findings are also consistent with Kandemir et al. (2008) who reported that Gölhisar and Çameli populations, located more inland and at higher elevations (800–1100 m.a.s.l), presented higher levels of frost tolerance. GOL support the general idea that higher frost resistance can be generally found in populations from higher altitudes (Bannister and Neuner 2001). Kurt et al. (2012) indicated that genetic differentiation among altitudinal groups was higher than among transects and local adaptation to environmental gradients related to altitude in *P. brutia*. Nevertheless, in our study we did not observe any clear altitudinal trend in cold hardiness when comparing the various provenances. This result consistent previous study of Yildiz et al. (2014) that which also did not observe any clear altitudinal trend when comparing the various provenances in *P. brutia.*

According to our results, at the ANT site the CYP provenance was less tolerant to frost than all others. Evidence of that was the evaluation of growth and survival performed at the same study sites by Cengiz et al. (1999) at age 5 years. The authors reported that survival of the CYP provenance at the ANK site was 19%. The CYP provenance sampled from the colder ANK increased cold hardiness by 27% when compared to samples taken at the warmer Mediterranean ANT site (Tables 3, 4). This result indicates that the CYP

provenance has a higher degree of phenotypic plasticity than the other provenances used in this study. Eliades et al. (2018) evaluated genetic and morphoanatomical diversity of *P. brutia* populations from Cyprus by using isoenzyme analysis and measuring morphoanatomical traits of needless and cones. The authors concluded that *P. brutia* growing in Cyprus is a peripheral population with high genetic and morphoanatomical diversity, despite its small geographical distribution within an island. They also concluded that the high phenotypic plasticity is important for adapting to changing environmental conditions, allowing the species to occupy a large geographic range in the island.

Comparison of screening methods

The three methods compared in this study (REL, CF and VO) were well correlated and VO gave comparable results (Fig. 6). This finding is consistent with Burr et al. (2001), who reported that electrolyte leakage and visible injury of needles of boreal conifers exposed to −18 °C had a strong correlation ($R^2 = 0.95$). The same authors also showed a strong correlation ($R^2 = 0.97$) between visual observation and chlorophyll fluorometry for Douglas fir and *Pinus contorta*. Peguero-Pina et al. (2008) compared chlorophyll fluorescence, electrolyte leakage, visual scoring, and the normalized difference vegetation index in cold hardiness study in *Pinus sylvestris*; which indicated that chlorophyll fluorescence and electrolyte leakage gave similar results, in contrast to the overestimation of visual scoring.

In our study, REL showed more stable results with lower variability, especially when compared with VO (Fig. 7). Even though results can be obtained in 2 days, the REL method is more labor intensive than the other two methods compared in this study.

The CF method showed more accurate and precise results than the VO method on the other hand similar to REL. Even though CF measure functioning of photosystem II and REL methods determine damage at tissue membrane, both methods yielded similar results. The major advantages of chlorophyll fluorescence over electrolyte leakage are the shorter time to completion and the larger numbers of samples that can be handled at each time (Burr et al. 2001). In our study, chlorophyll fluorescence was a useful screening toll because cold stress can be detected prior to visible signs of deterioration in a less laborious and less time-consuming way.

Conclusions

Even though we found significant differences in frost tolerance among the tested provenances of *P. brutia*, the significant Provenance × Site interaction indicates that some provenances showed different cold resistance when growing at different sites. The result of the significant interaction in our study possibly are the result of the manifestation of phenotypic plasticity where the CYP provenance was the most cold sensitive provenance but had higher plasticity than other provenances used in this study. According to our results, the most sensitive population can withstand up to −16.8 °C (±0.9 °C) and the most tolerant can handle a temperature of −20.6 °C (±0.4 °C). Therefore, from a practical standpoint, we recommend that *P. brutia* should not be planted in areas where the minimum annual temperature is below −16 °C. According to our results, CF is the most recommendable method for assessing cold hardiness, since it was the fastest among the three tested methods and it was more objective than the visual observation and as reliable as the REL method.

Acknowledgements We thank the General Directorate of Forestry and all the individuals linked to the establishment and maintenance of the provenance trials in Turkey. We are grateful to Prof Dr. Gülen Özalp and Prof Dr. Doğanay Tolunay from Istanbul University-Cerrahpaşa Faculty of Forestry who provide valuable suggestions and laboratory facilities.

References

Atalay İ, Sezer İ, Çukur H (1998) Kızılçam (*Pinus brutia* Ten.) ormanlarının ekolojik özellikleri ve tohum nakli açısından bölgelere ayrılması. The ecologic properties of red pine forests and their regioning in terms of seed transfer. Orman Bakanlığı Orman Ağaçları ve Tohumları Islah Araştırma Müdürlüğü Yayın No: 6, Ege Üniversitesi Basım Evi, 108 s, İzmir (in Turkish with English summary)

Bacandritsos N, Saitanis C, Papanastasiou I (2004) Morphology and life cycle of *Marchalina hellenica* (Gennadius) (Hemiptera: Margarodidae) on pine (Parnis Mt.) and fir (Helmos Mt.) forests of Greece. Annals de la Société Entomologique de France 40:169–176

Bannister P, Neuner G (2001) Frost resistance and the distribution of conifers. New For 30:167–184

Bigras FJ, Ryyppö A, Lindstom A, Stattin E (2001) Cold acclimation and deacclimation of shoots and roots of conifer seedlings. In: Bigras FJ, Colombo SJ (eds) Conifer cold hardiness. Kluwer Academic Publishers, Dordrecht, pp 57–88

Boorse GC, Ewers FW, Davis SD (1998) Response of chaparral shrubs to below-freezing temperatures: acclimation, ecotypes, seedlings vs. adults. Am J Bot 85:1224–1230. https://doi.org/10.2307/2446631

Burr KE, Tinus RW, Wallner SJ, King RM (1990) Comparison of three cold hardiness tests for conifer seedlings. Tree Physiol 6:351–369. https://doi.org/10.1093/treephys/6.4.351

Burr KE, Hawkings CDB, L'Hirondelle SJ, Binder WD, George MF, Repo T (2001) Methods for measuring cold hardiness of conifers. In: Bigras FJ, Colombo SJ (eds) Conifer cold hardiness. Kluwer Academic, Dordrecht, pp 369–401

Bussotti F, Pollastrini M, Holland V, Brüggemann W (2015) Functional traits and adaptive capacity of European forests to climate change. Environ Exp Bot 111:91–113. https://doi.org/10.1016/j.envexpbot.2014.11.006

Cengiz Y, Işık F, Keskin S, Genç A, Tosun S, Aksoy C, Doğan B, Özpay Z, Örtel E, Gürgen D, Dağdaş S, Uğurlu S (1999) Kızılçam (*Pinus brutia*) orijin denemeleri: beş yıllık sonuçlar. *Pinus brutia* provenance trails in Turkey: fifth year results. Technical bulletin no: 11, Southwest Anatolia Forest Research Institute, Antalya, Turkey (in Turkish with English summary)

Climent J, Costa e Silva F, Chambel MR, Pardos M, Almeida MH (2009) Freezing injury in primary and secondary needles of Mediterranean pine species of contrasting ecological niches. Ann For Sci 66:407. https://doi.org/10.1051/forest/2009016

Corcuera L, Gil-Pelegrin E, Notivol E (2011) Intraspecific variation in *Pinus pinaster* PSII photochemical efficiency in response to winter stress and freezing temperatures. PLoS ONE 6:e28772. https://doi.org/10.1371/journal.pone.0028772

Dalfes HN, Karaca M, Şen ÖL (2007) Climate change scenarios for Turkey. In: Güven Ç (ed) Climate change and Turkey: impacts, sectoral analyses, socio economic dimensions. United Nations Development Program, Ankara, pp 13–19

Eliades N-GH, Aravanopoulos FA, Christou AK (2018) Mediterranean islands hosting marginal and peripheral forest tree populations: the case of *Pinus brutia* Ten. in Cyprus. Forests 9:514. https://doi.org/10.3390/f9090514

Erlat E, Türkeş M (2015) Observed changes in the frequencies of record maximum and record minimum air temperatures in Turkey during the period 1950–2014 and their connections with atmospheric conditions. Aegean Geogr J 24:29–55 **(in Turkish with English abstract)**

George MF, Burke MJ, Pellett HM, Johnson AG (1974) Low temperature exotherms and woody plant distributions. HortScience 9:519–522

Glerum C (1985) Frost hardiness of coniferous seedlings: principles and applications. In: Duryea ML (ed) Evaluating seedling quality: principles, procedures, and predictive abilities of major test. Forest Research Laboratory, Oregon State University, Corvallis, pp 107–123

Gömöry G, Foffová E, Kmet J, Longauer R, Romšáková I (2010) Norway spruce (*Picea abies* [L.] Karst.) provenance variation in autumn cold hardiness: adaptation or acclimation? Acta Biologica Cracoviensia Series Botanica 52:42–49. https://doi.org/10.2478/V10182-010-0022-8

IPCC (2013) Climate change 2013: the physical science basis. In: Stocker TF, Qin D, Plattner G-K, Tignor M, Allen SK, Boschung J, Nauels A, Xia Y, Bex V, Midgley PM (eds) Contribution of working group I to the fifth assessment report of the intergovernmental panel on climate change. Cambridge University Press, Cambridge

IPCC (2014) Climate change 2014 synthesis report contribution of working groups I, II and III to the fifth assessment report of the intergovernmental panel on climate change. In: Pachauri RK, Meyer LA (eds) Core writing team. IPCC, Geneva

Kandemir GE, Kaya Z, Temel F, Önde S (2008) Genetic variation in cold hardiness and phenology between and within Red pine (*Pinus brutia* Ten.) populations: implications for seed transfer. Silvae Genet 59:2–3

Kızılelma Y, Çelik MA, Karabulut M (2015) Trend analyses of temperature and precipitations in Central Anatolia. Turk Geogr Rev 64:1–10 **(e-ISSN 1308-9773 (in Turkish with English abstract))**

Koski V, Antola J (1993) Turkish national tree breeding and seed production program for Turkey (1994–2003). Prepared with cooperation ENSO Forest Development Inc. and Forest Tree Seeds and Tree Breeding Research Institute, Ankara, Turkey

Kreyling J, Wiesenberg GLB, Thiel D, Wohlfart C, Huber G, Walter J, Jentsch A, Konnert M, Beierkuhnlein C (2012) Cold hardiness of *Pinus nigra* Arnold as influenced by geographic origin, warming, and extreme summer drought. Environ Exp Bot 7378:99–108. https://doi.org/10.1016/j.envexpbot.2011.12.026

Kurt Y, González-Martínez SC, Alía R, Isik K (2012) Genetic differentiation in *Pinus brutia* Ten. using molecular markers and quantitative traits: the role of altitude. Ann For Sci 69:345–351. https://doi.org/10.1007/s13595-011-0169-9

Larcher W (2000) Temperature stress and survival ability of Mediterranean sclerophyllous plants. Plant Biosyst 134:279–295. https://doi.org/10.1080/11263500012331350455

Lindén L (2002) Measuring cold hardiness in woody plants. Dissertation, University of Helsinki

Lu P, Joyce DG, Sinclair RW (2003) Geographic variation in cold hardiness among eastern white pine (*Pinus strobus* L.) provenances in Ontario. For Ecol Manag 178:329–340. https://doi.org/10.1016/S0378-1127(02)00481-4

Malmqvist C, Wallertz K, Johansson U (2018) Survival, early growth and impact of damage by late-spring frost and winter desiccation on Douglas-fir seedlings in southern Sweden. New For 49:723–736. https://doi.org/10.1007/s11056-018-9635-7

Mohammed GH, Binder WD, Gillies SL (1995) Chlorophyll fluorescence: a review of its practical forestry applications and instrumentation. Scand J For Res 10:383–410

OGM (2015) Orman Varlığımız. Orman Genel Müdürlüğü Yayınları, Ankara **(in Turkish)**

Park SJ, Kim DW, Kim JH, Chung JH, Lee JS (2016) Future disaster scenario using big data: a case study of extreme cold wave. Int J Des Nat Ecodyn 11:362–369

Peguero-Pina JJ, Morales F, Gil-Pelegrin E (2008) Frost damage in *Pinus sylvestris* L. stems assessed by chlorophyll fluorescence in cortical bark chlorenchyma. Ann For Sci 65:813. https://doi.org/10.1051/forest:2008068

Pinheiro JC, Bates DM (2000) Mixed-effects models in S and S-plus. Springer, New York

Prada E, Alía R, Climent J, Raquel D (2014) Seasonal cold hardiness in maritime pine assessed by different methods. Tree Genet Genomes 10:689–701. https://doi.org/10.1007/s11295-014-0714-4

Rose R, Haase D (2002) Chlorophyll fluorescence and variations in tissue cold hardiness in response to freezing stress in Douglas-fir seedlings. New For 23:81–96

Sakai A, Larcher W (1987) Frost survival of plants. Springer, Berlin

Sakai A, Weiser CJ (1973) Freezing resistance of trees in North America with reference to three regions. Ecology 54:118–126

Strand M, Öquist G (2006) Inhibition of photosynthesis by freezing temperatures and high light levels in cold-acclimated seedlings of Scots pine (*Pinus sylvestris*). I. effects on the light-limited and light-saturated rates of CO assimilation. Physiol Plant 64:425–430

Strimbeck GR, Schaberg PG, Fossdal CG, Schröder WP, Kjellsen TD (2015) Extreme low temperature tolerance in woody plants. Front Plant Sci 6:884. https://doi.org/10.3389/fpls.2015.00884

Wieser G, Matyssek R, Luzian R, Zwerger P, Pindur P, Oberhuber W, Gruber A (2009) Effects of atmospheric and climate change at the timberline of the Central European Alps. Ann For Sci 66:402. https://doi.org/10.1051/forest/2009023

Yalçın S (2012) Modeling the current and future ranges of Turkish pine (*Pinus brutia*) and oriental beech (*Fagus orientalis*) in Turkey in the face of climate change. Dissertation, Middle East Technical University

Yildiz D, Nzokou P, Deligoz A, Koc I, Genc M (2014) Chemical and physiological responses of four Turkish red pine (*Pinus brutia* Ten.) provenances to cold temperature treatments. Eur J For Res 133:809–818. https://doi.org/10.1007/s10342-014-0798-2