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Comparisons between laboratory and field results of frost tolerance of pines from the southern USA and Mesoamerica planted as exotics

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An artificial freezing study was conducted with 14 pine species and varieties from Mexico and Central America, and the southern and western USA. The pines chosen represented major commercial plantation species in the Southern Hemisphere such as *Pinus caribaea* var. *hondurensis*, *P. taeda* (multiple sources), *P. patula* and *P. radiata*, as well as promising species such as *P. greggii*, *P. maximinoi* and *P. tecunumanii*. Seedlings were grown in environmentally controlled growth chambers in the North Carolina State University Phytotron, and conditions were designed to mimic actual climatic conditions at Curitiba, Brazil, and Sabie, South Africa, located at approximately 25° S latitude. Early autumn conditions were simulated using shortened photoperiods and lower temperatures to harden the trees before the actual freeze testing. There were two freeze experiments: one containing tropical and subtropical material using four temperature treatments (−3, −7, −10, and −14 °C), and one containing temperate and subtropical material using temperatures −7, −14, −21, and −28 °C. Needle segments were frozen, and damage assessed using the electrolyte leakage technique. Rankings of species, varieties and sources corresponded well with field results and expectations based on climate of the source origins. The rankings of pure species and varieties should be useful to predict frost tolerance of pine hybrids, and the methodology shows promise for future experiments to quantify cold tolerance and genetic variation among hybrid progeny.

Keywords: adaptability, cold hardiness, electrolyte leakage, frost tolerance, relative conductivity

Introduction

A number of tropical and subtropical pines in Mexico and Central America experience occasional subfreezing temperatures during winter nights in the mountains and high plateaus of Middle America. There is little reliable information on the duration and severity of these freeze events because weather stations are mainly distributed in agricultural areas and not on high mountains and remote locations (Sáenz-Romero et al. 2003). The ability of the tropical and subtropical pines to withstand subfreezing temperatures of limited duration in winter months is important in places such as northern Argentina, southern Brazil, northern Uruguay, and the highland areas of South Africa. Field trials in several of these regions have shown that subtropical species such as *Pinus maximinoi* and *Pinus tecunumanii* have great growth potential in exotic plantation forestry (Kietzka 1988, Hodge and Dvorak 1999, Dvorak et al. 2001, Gapare et al. 2001). However, the gains from improved growth need to be weighed against the risk of plantation failure because of an extreme freeze event, which occurs occasionally in these regions (see Picchi and Barrett 1967).

Our current knowledge about limits of cold tolerance of the Mesoamerican pines comes from observations made in the genetic field trials mentioned above, many of which have been established by Camcore (International Tree Conservation and Domestication, North Carolina State University) in tropical and temperate regions around the world. These observations suggest that some provenances of *P. tecunumanii* from the Chiapas highlands can withstand subfreezing temperatures better than populations from the highlands of Central America (Dvorak et al. 2000). However, it is unknown whether some populations of *P. maximinoi*, which grow in the same areas as *P. tecunumanii* in Central America and southern Mexico, also have the ability to withstand light frosts. *Pinus patula* var. *patula* and *P. greggii* var. *australis* appear to have about equal cold tolerance, but results vary somewhat depending on the planting site and the severity of freeze. Unfortunately, there are seldom any meteorological recording stations near to the genetic field trial sites to quantify the exact degree and duration of frost events. Better information would help foresters target pine species to the correct ecological niches for the development of plantations.

There is now an intensive effort to produce hybrid crosses within the Mexican pines, especially in the subsection *Oocarpae*, and between Mexican and southern USA pines in the subsection Australes, to promote better growth, wood quality and disease resistance of exotic plantation forests in the tropics and subtropics in frost-prone regions of the Southern Hemisphere (Camcore 2007, 2008). Knowledge about the frost tolerance limits of the pure species would also be very important in choosing the appropriate parental species for hybrid crosses and matching the hybrid progeny to the best climatic zone. Related to the question of where best to plant pure species (and hybrids) is the need to determine how quickly these species adapt to new temperature regimes in exotic environments. If the goal is to produce temperate × tropical pine hybrids with some degree of frost tolerance, does it make any difference if the temperate species pollen comes from selections in native environments or from genetic trials where the species is planted as exotic?

There are several different methods to screen pine seedlings for frost tolerance in the laboratory (Burr et al. 1990). The electrolyte leakage (EL) method is one method that has been used successfully by a number of researchers to determine cold hardiness of seedlings (Burr et al. 1990, Yu et al. 2003, L′Hirondelle et al. 2006, Aldrete et al. 2008). It involves freezing needle tissue of seedlings at different levels of severity in the laboratory, followed by an assessment of injury using the amount of cellular electrolytes lost as a measure of membrane damage from the freezing treatment. Frozen tissue is thawed and put into a water solution, and subsequent electrolyte leakage is measured using electrical conductivity. The tissue is then killed completely with some kind of heat treatment, and electrical conductivity measured again. Relative conductivity of the damaged tissue is calculated as a ratio of the electrical conductivity of the damaged tissue relative to the maximum conductivity measured after the tissue is killed. A relative conductivity of 50% is often assumed to be a point where the plant cannot recover, and the temperature that produced that level of damage is called the $LT_{50.}$ The EL method seems to correlate well with field results. For example, Aldrete et al. (2008) used the EL method to determine that the maximum cold hardiness (LT_{50}) of *P. greggii* var. *greggii* and *P. greggii* var. *australis* was at temperatures −18°C and −12° C, respectively, which compares very well with our observations of local temperature extremes in Mexico where the two varieties naturally occur.

In this study, we use the EL method to quantify the frost tolerance of 14 pine species and varieties from Mexico, Central America, and the southern and western USA that are important in plantation forestry, and verify the EL laboratory findings with survival results from various genetic field trials in the subtropics. We also examine the cold hardiness of provenances of *P. taeda* sampled in native environments, and compare these to the cold hardiness of seed sources of the same species planted as exotics in different areas of the world. Finally, we discuss the ability of pine species to quickly adapt to exotic environments as a result of intercontinental germplasm transfer, which is an indication of how they also might adapt to future global climatic change.

Materials and methods

Species seed sources

The frost tolerance studies included 14 pines species and varieties from Mexico and Central America, and the southern and western USA (Table 1). The pines chosen represented major commercial plantation species in the Southern Hemisphere, such as *P. caribaea* var. *hondurensis*, *P. taeda*, *P. patula* and *P. radiata*, as well as promising species such as *P. greggii*, *P. maximinoi* and *P. tecunumanii.* These species were represented by different provenances from Camcore conservation collections in Middle America or California made in the last 20 years. The only exception was the *P. caribaea* var. *bahamensis* seed, which originally came from a collection made by the Oxford Forestry Institute. The number of provenances used to make a bulk species sample was dependent on seed availability and germination.

The *P. taeda* material came mostly from seed orchards of Camcore members with different genetic and climatic origin (Table 2). Alto Paraná (Argentina), Klabin and Inpacel (Brazil), and Sappi (South Africa) all provided seeds made up of a bulk of 20 different clones. Seeds were purchased from the Florida Department of Forestry′s seed orchard, which presumably represent the most tropical source of *P. taeda* in the USA. The North Carolina coastal plain source was provided by the North Carolina State University-Industry Tree Improvement Cooperative for *P. taeda,* and should be one of the most cold-hardy sources of the species. The *P. elliottii* material was a donation from the Cooperative Forest Genetics Research Program at the University of Florida, and was a bulk collection representing the entire range of the species.

For *P. radiata*, Camcore member CMPC Forestal Mininco also contributed a seedlot to represent Chilean commercial seeds, a mix made up of four separate seed sources collected between Concepción and Los Angeles, Chile.

Growing conditions and winter acclimation

The seeds were sown in environmentally controlled growth chambers in the North Carolina State University Phytotron. The seedlots were separated into two different experiments based on the climatic zones where they originated: a Temperate experiment containing temperate and subtropical sources, and a Tropical experiment containing the subtropical and tropical sources (Table 1). This was done partly for logistical reasons, in order to keep the needle harvesting and laboratory measurement work manageable, and partly for biological reasons, in that a different series of freezing temperatures were used for the Temperate and Tropical experiments. The seeds were sown in germination boxes filled with a commercial nursery medium (Metromix®) and covered with a dusting of vermiculite. The temperature during the germination phase was 28 °C. The temperate species, *P. taeda* and *P. elliottii*, were stratified before sowing by rinsing the seeds in cold water, draining the excess water, and placing the seeds in a plastic bag in the refrigerator for one month at 4 °C. Species in the temperate group were sown in March one month before the tropical species to account for expected differences in growth rate. There were a number of subtropical species/varieties common to both experiments: *P. patula* var. *patula*, *P. patula* var. *longipedunculata*, *P. greggii* var. *greggii*, *P. greggii* var. *australis*, *P. elliottii*, and *P. taeda* from Florida.

After germination, 60 germinants per species per experiment were planted into Ray Leach® supercell tubes (SC10) with 164 ml capacity filled with a mixture of sterilised composted pine bark, perlite and sand at a ratio of 3:1:1. The tubes were placed in trays with a capacity of 98 seedlings (7 × 14), and the 60 seedlings were arranged in three replications of 20 seedlings each in trays randomly positioned in the growth chamber. The goal was to raise seedlings that were approximately 25 cm shoot height at needle harvest time in mid-November. The seedlings were watered and fertilised weekly during the growth period using Hoagland′s solution and every two weeks with ammonium sulfate at a rate of 4.1 g m^{-2} to increase elemental nitrogen.

Table 1: Provenance and seed sources used in cold-hardiness tests of temperate/subtropical and subtropical/tropical pine species established in separate growth chambers. Common control species (*) were included in both experiments. Latitudes and elevations are mean values of ranges.

Species	State or region, country	Genetic composition	Latitude	Elevation (m)
Temperate/subtropical				
P. taeda	North Carolina, USA	North Carolina Coastal Plain	35°47′ N	121
*P. taeda**	Florida, USA	Clonal orchard	29°39′ N	46
P. taeda	Paraná, Brazil	Clonal orchard	24°08′ S	845
P. taeda	Santa Catarina, Brazil	Clonal orchard	27°30′ S	890
P. taeda	Missiones, Argentina	Clonal orchard	27°23′ S	54
P. taeda	Eastern South Africa	Clonal orchard	25°10′ S	1 200
*P. elliottii**	Florida, USA	Clonal orchards	31°15′ N	100
P. patula var. *patula*	Eastern Mexico	Five provenances	19°40′ N	2 348
P. patula var. *longipedunculata**	Southern Mexico	Two provenances	16°07′ N	2 472
P. radiata	California, USA	Three provenances	36°25′ N	128
P. radiata var. *binata*	Guadalupe Island, Mexico	One provenance	29°02′ N	1 200
P. radiata	Coastal Chile	Four seed sources	37°30′ S	160
P. greggii var. *greggii*	Northern Mexico	Five provenances	25°05′ N	2 412
P. greggii var. *australis*	Central Mexico	Six provenances	20°55′ N	1 667
Subtropical/tropical				
P. maximinoi	Central Guatemala	Five provenances	15°00′ N	1 686
P. pseudostrobus	Central Mexico	Eleven provenances	18°44′ N	2 100
P. tecunumanii	Central Guatemala (high elevation)	Three provenances	15°04′ N	1 893
P. tecunumanii	Chiapas, Mexico (high elevation)	Six provenances	16°28′ N	2 301
P. tecunumanii	South-eastern Honduras (low elevation)	Eight provenances	14°52′ N	971
P. oocarpa	North-western Mexico	Two provenances	26°50′ N	1 382
P. oocarpa	Central Honduras	Three provenances	14°01′ N	1 033
P. caribaea var. *hondurensis*	Northern Guatemala	Two provenances	15°57′ N	500
P. caribaea var. *bahamensis*	Bahama Islands	Nine provenances	26°01′ N	25

Table 2: Details on the geographic origin of the *P. taeda* provenances included in the cold-hardiness study

Company or institution	State or province, country	Origin of material
NC Tree Improvement	North Carolina, USA	Coastal Plain of North Carolina
Florida Department of Forestry	Florida, USA	Central Florida
Inpacel (now Arauco Florestas)	Paraná, Brazil	Local selections in Paraná and São Paulo states of unknown origin
Klabin	Santa Catarina, Brazil	Local selections originally from South Carolina coastal plain and South Africa
Alto Paraná	Misiones, Argentina	Local selections originally from Marion County, Florida
Sappi	KwaZulu-Natal, South Africa	Local selections originally from Zimbabwe and South Africa of unknown origin

Fertilisation and watering regimes were tapered off and terminated during the last six weeks prior to needle harvest to encourage hardening off.

The environmental conditions for raising the seedlings in the growth chambers were designed to mimic actual climatic conditions at Curitiba, Brazil, and Sabie, South Africa, located at approximately 25° S latitude where subfreezing temperatures are occasionally experienced each year. This included six months of growing conditions that represented normal spring, summer and early autumn in the subtropics followed by a six-week hardening-off period that reflected late autumn before a winter freeze. The initial settings for the first six months were day- and night-time temperatures of 24 °C and 15 °C, respectively, with a photoperiod of 13.5 h. The hardening-off period was for six weeks divided into two three-week segments. For the first three weeks, day- and night-time temperatures were 18 °C and 10 °C, respectively, with a photoperiod of 11.5 h. For the second three-week period, the seedlings from the two experiments were then moved into two separate and smaller growth chambers that could accommodate lower night-time temperatures. Day- and night-time temperatures of 10 °C and 4 °C, respectively, were used with a 10.5 h photoperiod.

Freezing treatments and electrolyte leakage measurement

For each freezing experiment, there were four test temperatures: for the Tropical experiment the temperatures were −3, −7, −10, and −14 °C; for the Temperate experiment the temperatures were −7, −14, −21, and −28 °C. For each experiment, there were two separate runs for each temperature, and the four temperatures were randomised for each run. Foliage samples were taken by harvesting one needle from each of the 20 trees per species per replication, cutting the needles to a uniform length of 4 mm, and placing them in a 16 ml sample tube. For each temperature run, there was a treatment sample that was subjected to the target freezing temperature, and an unfrozen control sample that was maintained in the cold room at 4 °C during the freezing treatment.

The freezing treatments all followed the same time schedule regardless of target temperature (*T*) (e.g. see Figure 1). The samples were removed from the cold room where they were maintained at 4 °C, and placed into a programmable Scientemp Corporation model 34-09A freezer at 0 °C. Chilling then began at a rate of cooling not faster than 0.25*T* per 15 min. After 1 h, the freezer was at the target temperature *T* where it was then maintained for 3 h. The temperature was then raised, reversing the cooling process used earlier. After 1 h of controlled warming the freezer was back at 0 °C, and the samples were removed and placed back in the cold room at 4 °C. After 3 h, 14 ml of distilled deionised water was added to the sample tubes for both the freezing treatment and the companion control tube. The tubes were then placed onto a gyratory shaker set at 100 revolutions min^{-1} for 16 h. Electrical conductivity (EC) was measured with an Oakton CON6/TDS6 handheld conductivity meter. Following EC measurements, the tubes were tightly capped and placed in a laboratory oven at 85 °C for 2 h to completely kill the tissue. Following this treatment, EC was again measured.

For each species–replicate–temperature–run combination, relative conductivity (RC_t) of the freezing treatment for a given temperature *t* was calculated as:

$$RC_t = (EC_{\text{treatment}} / EC_{\text{dead}}) \times 100$$

where $EC_{\text{treatment}}$ = the electrical conductivity of the sample after the freezing or control treatment, and EC_{dead} = the electrical conductivity of the sample after the heat treatment to kill all of the tissue.

The RC_t values were used to calculate an injury index for a given temperature *t* (Flint et al. 1967):

$$\text{Injury}_t = [(RC_t - RC_0) / (1 - RC_0/100)] \times 100$$

where RC_0 = the relative conductivity of the control treatment. The injury index is intended to correct for inherent differences among species or replications for the amount of electrolyte leakage that takes place in the control (unfrozen) sample.

Comparison of artificial freeze results with field study results

Results from the artificial freezing study were compared to four Camcore trials that included some of the same species and provenances as the laboratory trial. These field tests were established in South Africa and Uruguay and experienced subfreezing temperatures shortly after their establishment (Table 3).

Results

Mean values for relative conductivity and injury

Relative conductivity values and injury indices were lower than expected in both experiments. In the tropical experiment, the highest damage observed was for *P. caribaea* var. *hondurensis* at temperature −14 °C (RC_{14} = 60.0%, injury_{14} = 57.3%). However, all other species–temperature combinations had a mean injury less than 50.0%, and the mean injury_{14} across all species was 37.5%. Results were similar in the temperate experiment: the highest damage observed was for *P. elliottii* at temperature −21 °C (RC_{21} = 36.1%, Injury_{21} = 31.7%), substantially lower than 50% for both variables. For *P. elliottii*, very similar damage values were observed for temperatures −14 °C and −28 °C (injury_{14} = 30.6% and injury_{21} = 30.4%). In general, injury indices were typically just a few percentage points lower than the corresponding RC values. Since injury index values were less than 50.0%, it was not possible to calculate reliable LT_{50} values. The data were then analysed treating each temperature as a specific freezing treatment.

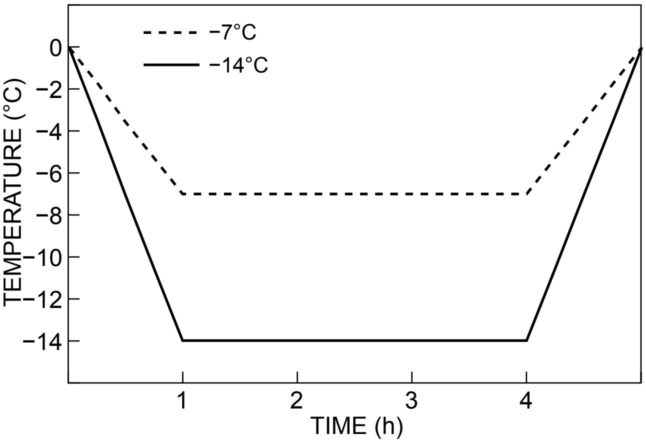

Figure 1: Diagram of freezing temperature regime for the −7 °C and −14 °C treatments. Other temperature treatments (−3, −10, −21 and −28 °C) followed a similar pattern

ANOVA by temperature

An analysis of variance was conducted for each temperature using the following model:

$$\text{Injury}_{ijk} = \mu + \text{species}_i + \text{rep}_j + \text{run}_k + \text{species*rep}_{ij} + \text{species*run}_{ik} + \text{rep*run}_{jk} + e_{ijk}$$

with species treated as a fixed effect, and all other effects random, using SAS Proc GLM (SAS Institute 2000–2004).

There were significant differences in RC among the unfrozen control samples for species and run, reflecting differences in electrolyte leakage. This confirms the need to use injury index to correct for baseline differences in electrolyte leakage. In analyses for injury index, the rep*species and run*species interaction terms were uniformly non-significant. Thus, these terms were dropped, and each temperature was analysed with a reduced model:

$$\text{Injury}_{ijk} = \mu + \text{species}_i + \text{rep}_j + \text{run}_k + \text{rep*run}_{jk} + e_{ijk}$$

with species treated as a fixed effect, and all other effects random. Effects were considered statistically significant at $p < 0.05$.

Species effects were significant at all temperatures except −3 °C in the Tropical experiment, and at all temperatures in the Temperate experiment (Table 4). Mean injury was very low at temperatures −3 °C and −7 °C in the Tropical experiment, and also at −7 °C in the Temperate experiment (the

Table 3: Location of Camcore field trials where subfreezing temperatures caused various levels of mortality in species/provenances during winter. Freeze information is estimated. See text for more details

Test Code	Company	Location	Country	Latitude	Elevation (m)	Freeze information
20-18-02E	Mondi	Commonage	South Africa	31°02′ S	1 480	Several nights of −5 to −8°C
33-18-02C1	Mondi	Rush Valley	South Africa	30°51′ S	1 782	Annual winter freezes of −5 to −10°C; snow
33-18-02C2	Mondi	Bendoran	South Africa	31°27′ S	1 500	Annual winter freezes of −5 to −10°C; snow
99-11-01A	Weyerhaeuser	Tacuarembó	Uruguay	31°44′ S	140	−7°C reached for five consecutive nights

Table 4: Summary statistics of ANOVA by temperature

Temp. (°C)	Mean injury		R^2	Species differences	
	All species	Common species[1]		*F*-test	*p*-value
Tropical experiment					
−3	0.4	0.4	0.27	0.90	0.5623
−7	13.0	12.9	0.40	3.05	0.0010
−10	27.9	28.0	0.54	4.39	0.0001
−14	37.5	28.6	0.64	8.16	0.0001
Temperate experiment					
−7	6.5	5.4	0.53	4.18	0.0001
−14	23.4	25.3	0.49	2.12	0.0246
−21	23.8	25.5	0.52	4.43	0.0001
−28	25.3	27.2	0.77	2.21	0.0458

[1] Species/varieties common to both experiments were *P. patula* var. *patula*, *P. patula* var. *longipedunculata*, *P. greggii* var. *greggii*, *P. greggii* var. *australis*, *P. elliottii*, and *P. taeda* from Florida

average for all species was 0.4, 13.0 and 6.5%, respectively). For all other temperature treatments in both experiments, average injury was substantially higher, ranging from 25.3% to 37.5% (Table 4).

Comparison of mean injury across the six common species represented in both the Temperate and Tropical experiments showed very similar levels of injury for all temperatures from −14 °C to −28 °C, with an average injury of 26.9%, ranging only from 25.3 to 28.6% (Table 4). The data suggest that the temperature treatments −3 and −7 °C are measuring a very low level of injury, and that all other treatments are measuring similar levels of freeze damage. Least square means for injury were calculated for each species by temperature using SAS Proc Mixed (SAS Institute 2000–2004), and the probability of differences among all possible pairs was also calculated. For the Temperate experiment the correlations among species LS means for −14, −21, and −28 °C ranged from $R = 0.55$ to 0.89, and all were statistically significant. Similarly, in the tropical experiment, the correlation between species LS means for −10 and −14 °C was $R = 0.58$, which was statistically significant.

Combined ANOVA across temperatures

The next phase of analysis was a combined ANOVA across temperatures −10 and −14 °C in the Tropical experiment and across −14, −21 and −28 °C in the Temperate experiment. The following model was used for analysis:

$$\text{Injury}_{ijkl} = \mu + \text{species}_i + \text{temp}_l + \text{species*temp}_{il} + \text{rep}_j + \text{run}_k + \text{rep*run}_{jk} + e_{ijk}$$

with species, temp, and species*temp treated as fixed effects, and all other effects random. In the Temperate experiment, species differences were highly significant, whereas temperature and the species*temperature interaction were not significant (Table 5). In the Tropical experiment, temperature effects were highly significant (Table 5), because there was substantially higher injury at −14 °C than for −10 °C ($\text{injury}_{14} = 37.5\%$, $\text{injury}_{10} = 27.9\%$; Table 4). Species differences were also highly significant. Species*temperature interaction was statistically significant, but with a much lower *F*-statistic than for species (Table 5), which suggested that the species effect was predominant.

Species LS means and ranking of common-control species

Overall species LS means were calculated for the combined temperature treatments in the Temperate and Tropical experiment, and a comparison of the LS means for the six common species/varieties in the two experiments showed a very strong relationship ($R = 0.95$, Figure 2). Both experiments gave the same ranking for the six common species/varieties, and these rankings corresponded well to the known frost tolerance from field trials. For example, *P. greggii* var. *australis* had higher injury than *P. greggii* var. *greggii* in both experiments in this study (Figure 2). This reflects the fact that *P. greggii* var. *australis* is native to lower latitudes and lower elevations (21° N and 1 200 to 2 000 m elevation) than *P. greggii* var. *greggii* (around 25° N and 2 000 to 2 500 m). Artificial freeze testing experiments have also demonstrated significantly better frost tolerance for the northern source, *P. greggii* var. *greggii* (Aldrete et al. 2008), and results from Camcore progeny tests confirm this in the field. Similarly, *P. patula* var. *longipedunculata* had higher injury than *P. patula* var. *patula* in both experiments in this study (Figure 2). This corresponds to expectations from the native distribution (*P. patula* var. *longipedunculata* is found at latitudes 16–18° S in the Sierra Madre del Sur mountain range, whereas *P. patula* var. *patula* is found at latitudes 17–22° N in the Sierra Madre Oriental mountain range. Lastly, the *P. elliottii* collection used in this study represents the entire range of the species, covering latitudes 29–32° N, whereas the Florida source *P. taeda* originates from the most southern part of that species range, primarily Marion, Levy and Dixie counties, between latitudes 29–30° N. Thus, one might expect the Florida source *P. taeda* to be more frost susceptible, and this agrees with the higher injury observed for Florida *P. taeda* relative to *P. elliottii* in both experiments in this study.

Species rankings: Tropical experiment

Figure 3 shows the least square means for the Tropical experiment. For species with multiple varieties or multiple

Table 5: Summary statistics of ANOVA combined across temperature for the Tropical species experiment (temperatures −10 and −14 °C) and the Temperate species experiment (temperatures −14, −21 and −28 °C)

Effect	Tropical experiment		Temperate experiment	
	F-test	*p*-value	*F*-test	*p*-value
Temperature	24.90	0.0001	0.87	0.4233
Species	9.03	0.0001	6.88	0.0001
Species × temperature	2.35	0.0062	0.91	0.6001

sources, the rankings generally are in line with expectations derived from the native range/collection sites or results from field studies.

The highest injury observed in this study (injury = 48.1%) was for *P. caribaea* var. *hondurensis*, which is found at the lowest latitudes and lowest elevations (0–700 m) of any species in the study. This variety rarely experiences any frost in its native range. The closely related *P. caribaea* var. *bahamensis* is also found in tropical climates, but in artificial freeze studies, Duncan et al. (1996) observed that var. *bahamensis* has more frost tolerance than var. *hondurensis*. In the same study, Duncan et al. (1996) also found both varieties of *P. caribaea* to be more frost susceptible than *P. elliottii*, which agrees with the current results (for var. *hondurensis* vs *P. elliottii*, injury$_{10–14}$ = 48.1% vs 34.9%, difference significant at $p = 0.0003$; for var. *bahamensis* vs *P. elliottii*, injury$_{10–14}$ = 43.4% vs 34.9%, significant at $p = 0.0175$).

Different sources of *P. tecunumanii* and *P. oocarpa* showed ranks consistent with their geographic origin: *P. oocarpa* from Honduras had more injury than *P. oocarpa* from central Mexico (injury$_{10–14}$ = 42.6% vs 28.2%, $p = 0.0001$). Low-elevation *P. tecunumanii* from Honduras had more injury than high-elevation material from *Guatemala* (39.3% vs 35.0%, $p = 0.2284$), whereas high-elevation *P. tecunumanii* from Mexico had significantly less frost injury (Injury$_{10–14}$ = 27.2% vs 39.3%, $p = 0.0008$ and 27.2% vs 35.0%, $p = 0.0275$).

The related species *P. maximinoi* and *P. pseudostrobus* had LS means for injury of 32.3% and 21.4%, respectively, different at $p < 0.0001$. This is consistent with their native range. The *P. maximinoi* source in this study is from central Guatemala, around latitude 15° N, and elevations of 800 to 1 500 m. In comparison, *P. pseudostrobus* is found at latitudes 17–21° N and elevations of 1 600 to 3 200 m, and frost events are common (Perry 1991).

Species rankings: Temperate experiment

The rankings for the species in the temperate experiment (Figure 3) also agreed with expectations. The highest injury was observed for *P. elliottii* from Florida (injury$_{14–21–28}$ = 30.6%), followed closely by *P. patula* var. *lonigpedunculata* (28.5%) and *P. taeda* from Florida (27.8%). The Florida source *P. taeda* had significantly higher injury than the other five *P. taeda* sources ($p < 0.003$ in all cases). For the five non-Florida sources of *P. taeda*, there were no significant differences among the LS means for injury; however, the rankings did seem to correspond to the climate of the source (see latitude and elevation in Table 1). For example, the lowest injury observed in this experiment was the *P. taeda* from North Carolina (injury$_{14–21–28}$ = 18.2%).

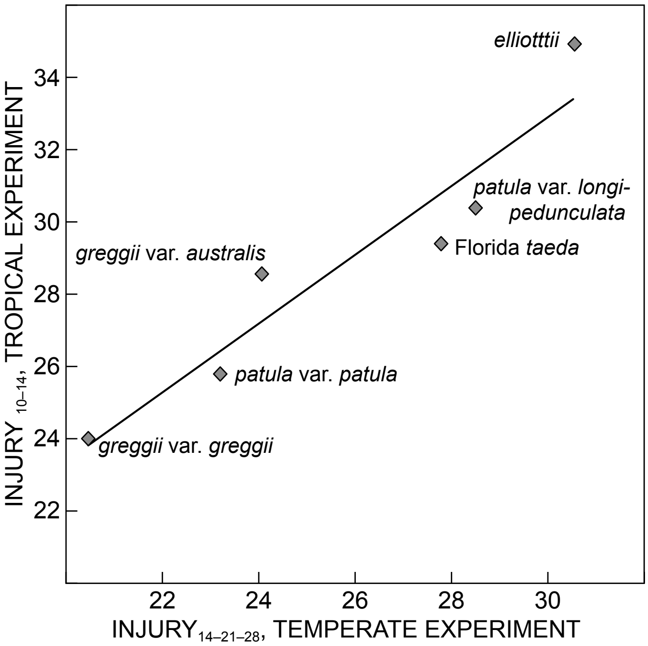

Figure 2: Relationship between Injury$_{14–21–28}$ from the Temperate experiment and Injury$_{10–14}$ from the Tropical experiment for six species/varieties common to both experiments

As a group, the non-Florida *P. taeda* sources were more frost tolerant than the three *P. radiata* sources. There was some indication that *P. radiata* var. *binata* and *P. radiata* from Chile might be slightly more frost tolerant than the *P. radiata* from California, but these differences were not statistically significant ($p = 0.26$ and 0.16, respectively).

Correspondence with field results

Survival data from three genetic trials in South Africa and one in Uruguay are presented in Table 6. There was fairly good agreement between the injury index and the field survival on these sites; for example, at the Commonage site in South Africa, *P. patula* var. *patula* had 92% survival compared to 40% survival for *P. patula* var. *longipedunculata.* This study detected clear and significant differences in injury that agree with field survival. At the Rush Valley and Bendoran sites in South Africa, *P. greggii* var. *greggii* had better survival than *P. greggii* var. *australis* and *P. patula* var. *patula*, as predicted by the injury index in this study (Figures 2 and 3). Finally, at the Tacuarembó site in Uruguay, which experienced a severe freeze event lasting several days, the survival of the high-elevation *P. tecunumanii* from Mexico (34%) was better than high-elevation and low-elevation *P. tecunumanii* and *P. maximinoi* from Central America, in general agreement with the injury index measured in this study. In addition, *P. caribaea* var. *hondurensis,* with the highest injury index in this study, had the lowest survival at this site.

Discussion

In many artificial freezing studies, electrolyte leakage is measured using electrical conductivity. Electrolyte leakage

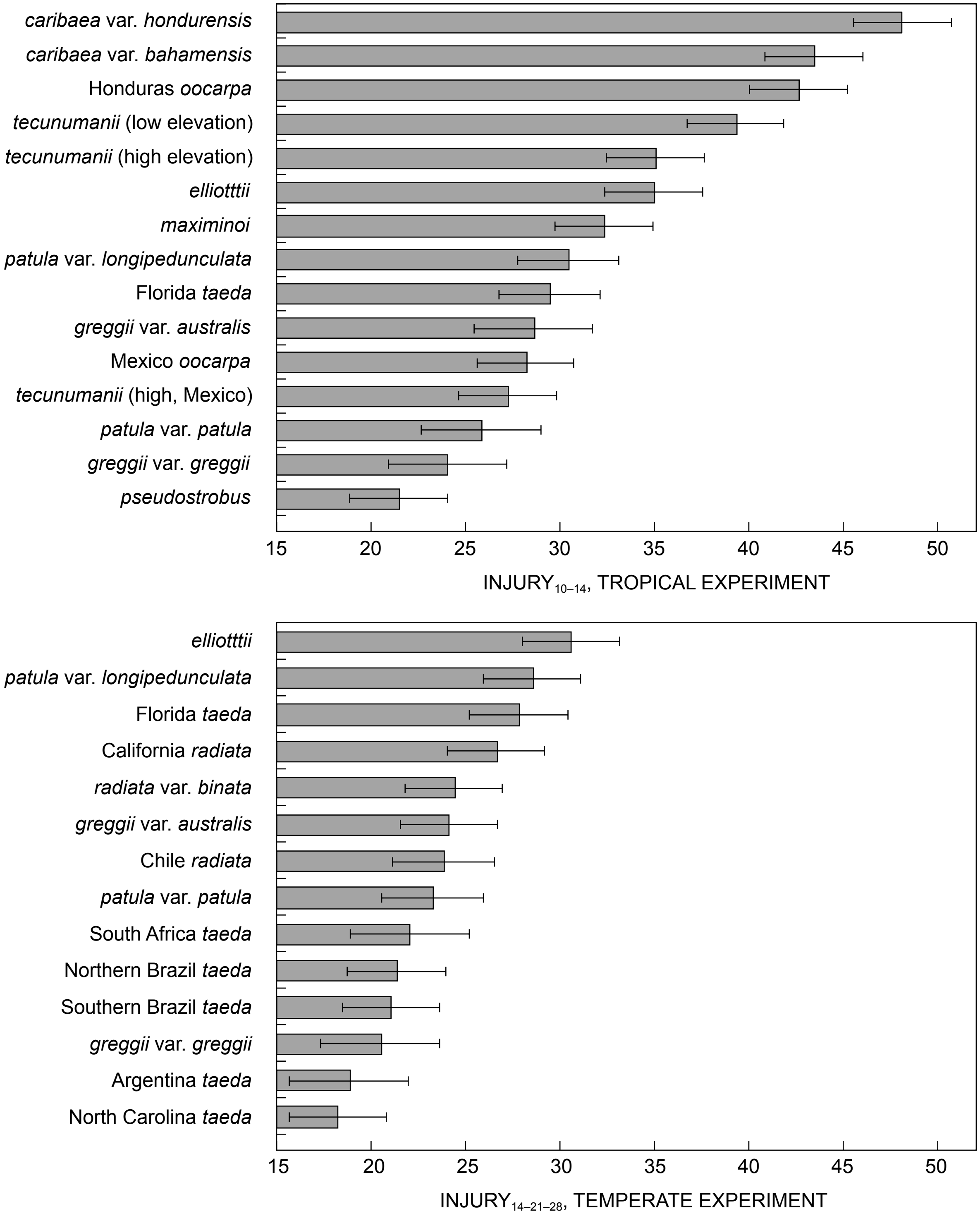

Figure 3: Injury index for each species in the Tropical experiment (top) and the Temperate experiment (bottom). Shaded bars represent least square means for species/variety across temperatures. The error bar respresents the SE

is assumed to measure the amount of membrane damage owing to the freezing treatments, but it is not completely clear if leakage is related to irreversible damage (from dead cells) or reversible damage (from injured but living cells) (Palta and Paul 1980, Johnson and Gagnon 1988). Murray et al. (1989) suggest that by measuring the rate of electrolyte leakage, shoots that are injured but alive can be accurately distinguished from shoots that have been killed by frost; a minimum of three conductivity measurements on the same samples would be needed to measure the rate.

It is common to calculate an LT_{50}, the temperature at which 50% of maximum conductivity is observed (e.g. Burr et al. 1990, Grossnickle 1992, Aldrete et al. 2008). In this study, RC values and injury index values were almost always lower than 50%, making it impossible to calculate LT_{50} values. The low RC and injury values observed in this study are not uncommon, however. Sutinen et al. (1992) report that severely frost-injured mid-winter samples of *P. nigra* and *P. resinosa* needles had a maximum injury index of 30%. Similarly, in experiments with *P. strobus* and *P. wallachiana*, Lu et al. (2007) reported RC values ranging from 7% to 25%. In both of these studies, the authors were able to use these lower injury and RC values to quantify seasonal variation in cold hardiness of those species.

A complicating factor in conducting artificial freeze tests is deciding upon the exact conditions of pretreatment or acclimation, and then the exact conditions of the freeze treatment (e.g. rate of cooling, duration of time at minimum temperature, and time after freezing before measuring initial conductivity). A myriad of different environmental scenarios could be used, but in this study the goal was to reflect actual growing conditions in the field in subtropical regions of the Southern Hemisphere, and then to subject the samples to a rapid and severe freeze to simulate an overnight frost event. The freezing treatments lasted 5 h, with 3 h at the minimum temperature. It is possible that a longer duration at the minimum temperature would have produced more damage and higher RC and injury values.

Supercooling is defined as the cooling of a solution below the freezing point of the solution prior to ice formation. Typically, in plants the degree of supercooling is quite small due to the ubiquity of nucleators that can trigger ice formation, which then rapidly spreads throughout the plant (Levitt 1980). The use of cut needle segments in this study should have provided numerous nucleation points for ice formation even at lower temperatures. However, it is possible that some degree of supercooling prior to ice formation may have limited frost damage in the mildest freezing treatments.

Survival results in field tests after freezing temperatures certainly provide a measure of cold hardiness, but survival can also be affected by a number of variables including pre-freeze weather conditions, the size and health of the seedlings, the timing and severity of the freeze, and associated vegetation cover in the trial. A perfect correlation between any laboratory results and field results is impossible, but the species ranks in this study agreed quite well with known variation among species in field cold hardiness. This suggests that the conditions and methodology used are in fact measuring some aspect of cold hardiness important in real-life field conditions.

Species intolerant to freezing temperatures

The Caribbean pine varieties, *Pinus oocarpa* from Central America and low-elevation sources of *P. tecunumanii* from Honduras and Nicaragua have no to little (biologically meaningful) frost tolerance based on the results of this study (Figure 3) and our field observations (Table 6), and one should not expect anything different based on the climates from which they originate (Table 1). *Pinus caribaea* var. *bahamensis* from the Bahama Islands, where extreme minimums rarely reach 4–7 °C, occasionally shows better survival in field studies after a light frost than its tropical cousin *P. caribaea* var. *hondurensis* from Central America, but this might be because the island variety produces secondary needles quickly, possibly offering some protection from the cold. Climent et al. (2009) has found that primary needles were significantly more sensitive to freezing than secondary needles in some pine species. We have heard foresters claim that seedlings from seed collections taken from trees of *P. caribaea* var. *hondurensis* in plantations that have survived (or avoided) periodic freezing temperatures have more cold resilience than Caribbean pine introductions from tropical climates. We have also seen two-year-old *P. oocarpa* resprout in a plantation after a killing frost that occurred in the Jaguariaiva, PR, Brazil region (24° S). The sprouting ability of *P. oocarpa* is an evolutionary defense mechanism to survive fires and the species will apparently respond in the same manner to freezes if the cold temperatures are not too severe and the trees are still juvenile. Nevertheless, *P. oocarpa* from Central America is not frost tolerant and should be managed as a frost-sensitive species.

Species with limited frost tolerance

High-elevation populations of *P. tecunumanii* from Central America and Chiapas, Mexico, exhibit varying degrees of

Table 6: Comparison between field survival results and laboratory injury values from artificial freezing experiments.

Test	Location	Country	Species	*N*	Survival (%)	Injury[1]
02E	Commonage	South Africa	*P. patula* var. *patula*	346	92	23.2
			P. patula var. *longipedunculata*	519	40	28.5
02C1	Rush Valley	South Africa	*P. greggii* var. *greggii*	598	96	20.4
			P. greggii var. *australis*	97	81	24.0
			P. patula var. *patula*	33	69	23.2
02C2	Bendoran	South Africa	*P. greggii* var. *greggii*	502	85	20.4
			P. greggii var. *australis*	55	76	24.0
			P. patula var. *patula*	35	73	23.2
01A	Tacuarembó	Uruguay	*P. tecunumanii* (high elevation, Chiapas)	196	34	27.2
			P. tecunumanii (high elevation, Guatemala)	196	12	35.0
			P. tecunumanii (low elevation, Nicaragua)	196	5	39.3
			P. maximinoi (Guatemala)	196	4	32.3
			P. caribaea var. *hondurensis*	147	1	48.1

[1] For the South Africa tests, Injury is the species LS mean from the Temperate experiment (average injury across −14, −21 and −28 °C); for the Uruguay test, Injury is the species LS mean from the Tropical experiment (average injury across −10 and −14 °C)

cold hardiness. Populations of the species from the San Cristobal de las Casas Plateau in Chiapas, Mexico showed the greatest frost tolerance in both this study and field results. The plateau is located at approximately 2 200 m elevations and cold temperatures during winter in the region probably approach a minimum of −7 °C. Plantation growers wanting to develop a frost-tolerant strain of *P. tecunumanii* should begin by making selections within this group of populations. However, breeders should realise that these populations only exhibit average to below-average growth relative to other high-elevation *P. tecunumanii* populations (Hodge and Dvorak 1999), and there was only modest survival of 34% of the 'plateau sources' in Uruguay after an extended freeze event (Table 4).

Central American sources of high-elevation *P. tecunumanii* have shown more resilience than low-elevation sources to cold temperatures, but not nearly to the level of high-elevation sources from Chiapas. When both Central American high-elevation and low-elevations sources were grown in a containerised nursery in South Africa and subjected to several nights of −2 to −3 °C temperatures for several hours, the low-elevation populations turned brown after the event and high-elevation populations remained alive and green (G Mitchell, pers. comm., 2009). The relative difference in cold hardiness of high-elevation and low-elevation Central American sources is also reflected in the results of this study (Figure 3, Table 6). However, we suggest that foresters should still use caution when planting high-elevation Central American sources of *P. tecunumanii* in a frost-prone area; they should always be planted at the beginning of the growing (rainy) season to maximise their shoot height before the next winter's frost, and be established on the tops of hills or slopes to avoid frost pockets. In subtropical regions, many of these hilltop/slope areas are now being planted to *Eucalyptus grandis* or *E. grandis* hybrids.

Pinus maximinoi grows sympatrically with *P. tecunumanii* through much of its geographic range in southern Mexico and Central America. Casual observations in natural stands would suggest that the two species possess the same basic tolerance to freezing temperatures when planted as exotics. The results of this study suggest that *P. maximinoi* is slightly more cold hardy than high-elevation *P. tecunumanii* from Central America (Figure 3), whereas field trials suggest it is less cold hardy (e.g. Table 6). Even though provenance variation in cold hardiness might exist in *P. maximinoi*, especially if one considers the potentially more frost-tolerant sources from western Mexico, the species should be handled as described for Central American provenances of *P. tecunumanii* when establishing plantations.

Even though Central American sources of *P. oocarpa* have no frost tolerance, it appears that populations from the extreme of the species' geographic range in northwestern Mexico can tolerate frosting events of minimal severity based on our EL results. The two populations from north-western Mexico included in this study were Chiapas and Mesa de los Leales located at 27° N latitude and 1 300–1 500 m elevation in Chihuahua State (see Dvorak et al. 2009 for more details about these sources). Although increased cold hardiness with increasing latitude seems intuitive, most *P. oocarpa* in north-western Mexico occur in protected canyons facing moderating waters of the Pacific Ocean. Cluster analysis of climatic data from 50 locations where *P. oocarpa* naturally occurs from north-western Mexico to central Nicaragua indicated a continuum rather than well-defined climatic groups (Camcore unpublished data). Sampling points at the ends of the continuum provide the cold hardiness differences shown within *P. oocarpa* in Figure 3.

Pinus patula var. *longipedunculata* from southern Oaxaca included in this study exhibited only limited cold hardiness, similar to high-elevation sources of *P. tecunumanii* from Central America. This variety's cold susceptibility relative to *P. patula* var. *patula* is also apparent in the field trial planted at Commonage, South Africa (Table 6). The practical implication of the finding is that var. *longipedunculata* needs to be planted in areas similar to those to *P. tecunumanii*, not *P. patula* if frosts are a consideration. It should be noted that great differences in cold tolerance have been found within var. *longipedunculata* based on Camcore field studies. Populations called var. *longipedunculata* from northern Oaxaca, such as Santa Maria Papalo and Ixtlán (see Dvorak et al. 2000, 2001), are as cold hardy as other *P. patula* var. *patula* populations in the Sierra Madre Oriental. Populations from southern Oaxaca (such as those included in this study) and Guerrero in the Sierra Madre del Sur appear to be much more susceptible to subfreezing temperatures.

Species with moderate cold tolerance

Both *P. patula* var. *patula* and *P. greggii* var. *australis* occur sympatrically over parts of their natural range and exhibit moderate cold tolerance. Although information on temperature extremes are scarce, Vela Gálvez (1980) and Eguiluz-Piedra (1978) report minimum temperatures of −9 and −14 °C, respectively, occurring in the natural range of *P. patula*. Based on our field observations in natural stands in Mexico, we have assumed that *P. patula* should be slightly more cold tolerant (but less drought tolerant) than *P. greggii* var. *australis* because it occurs at higher elevations. Aldrete et al. (2008) found the minimum cold tolerance level of this *P. greggii* variety to be −12 °C.

The results of this study indicate that *P. patula* is slightly more frost tolerant than *P. greggii* var. *australis*. Our field results indicate that *P. greggii* var. *australis* often survives as well as or better than *P. patula* on cold sites in South Africa (see Table 6). However, what makes the field survival results difficult to interpret for *P. greggii* var. *australis* is that cold tolerance is often confounded with drought tolerance. The cold sites in South Africa in Table 6 also are somewhat dry (between 800 to 900 mm annual precipitation); thus, the more drought-tolerant *P. greggii* trees might have been bigger and in better health when the freeze events occurred. Our recommendations for these two species is to plant *P. patula* on moderately temperate, misty sites and to establish *P. greggii* on moderately cold sites prone to seasonal droughts.

Pinus radiata is also moderately cold hardy, but less so than *P. greggii* var. *australis*. Temperatures in natural populations of *P. radiata* in California rarely fall to −6 to −7 °C (Dvorak 1991), but there is little information available about the cold tolerance of island populations (Rogers 2002). Our results suggest that the Guadalupe Island population is more cold hardy than California mainland populations that occur at lower elevations. Whether the differences in cold hardiness between the mainland and island populations are meaningful is being studied in field tests (Burdon et al. 1998). The Chilean landrace was found to be the most cold hardy of the group tested, apparently adapting to local climatic conditions. The majority of plantations of *P. radiata* established around the world primarily had their origin in California (Burdon et al. 1998, Espinei et al. 1995).

Cold hardy species

Pinus greggii var. *greggii* has more frost tolerance than *P. elliottii* and is similar to *P. taeda*, based on our results. Aldrete et al. (2008) quantified its minimum temperature tolerance level as −18°C. At Rush Valley, in the northern section of the Eastern Cape province, South Africa, *P. greggii* var. *greggii* survives snow and cold better than *P. patula* or *P. greggii* var. *australis*; its field ranking is also consistent with our laboratory results (Table 6).

Although it was expected that *P. pseudostrobus* would be more frost tolerant than *P. maximinoi,* it was surprising that *P. pseudostrobus* had the lowest level of injury in the Tropical experiment, even lower than *P. greggii* var. *greggii*. *Pinus pseudostrobus* has not been as widely tested as *P. maximinoi* and the two varieties of *P. greggii* and *P. patula,* but these results suggest that further field testing on cool sites in South Africa and southern Latin America are warranted.

Pinus taeda, being a temperate species, exhibits great cold-hardiness and is particularly tolerant to rapid temperature fluctuations. Among the non-Florida sources of *P. taeda,* the differences among the LS means for injury were not statistically significant, although for the comparison of North Carolina *P. taeda* vs South African *P. taeda,* the *p*-value was just over 0.05 ($\text{injury}_{14-21-28} = 18.2\%$ vs 22.0%, $p = 0.0523$). Nevertheless, there is a clear trend among the *P. taeda* sources, with cold hardiness decreasing as the climate of the source becomes more tropical. Of the non-Florida sources of *P. taeda,* the sources from South Africa and Paraná, Brazil, were the least cold hardy. The recent origin of the South African land race used in this study is made up of selections from tropical Zimbabwe and local South Africa selections that came from various locations in the southern USA. The material from Paraná was from plantations in subtropical areas in the state as well as neighbouring São Paulo, where fewer than five to six light frosts are experienced in any one year. *Pinus taeda* from Lages, Santa Catarina, which experiences approximately 16 frosts each winter, had a slightly lower injury value in this study. The origin of the Santa Catarina land race appears to be mainly South Carolina coastal plain with some recent introductions from local land races in South Africa. Finally, we expected the Argentinean material collected in the Misiones to rank close to the Florida material in cold tolerance because its origin is central Florida. However, it grouped more closely to the North Carolina coastal plain sources. The Misiones region typically gets from 20 to 30 frosts per year.

Overall, the results with *P. taeda* suggest that one generation of selection for growth and adaptability in an exotic breeding program may indirectly result in changes in cold hardiness. Another possible explanation is that differences in temperature experienced by the mother tree may lead to epigenetic differences in the frost hardiness of the progeny. There is mounting evidence that higher temperatures during embryogenesis and seed development in *Picea abies* lead to a number of changes related to cold hardiness and adaptability, including later bud set and decreased autumn frost hardiness (Johnsen et al. 2005, Kvaalen and Johnsen 2008, Yakovlev et al. 2010). Whatever the mechanism, there is some evidence that tree populations have an ability to quickly adapt to changing temperature regimes, which should be welcome news to those concerned about how forest populations might adapt to global climatic change.

Outlook and future studies

The methods used in this study provide good rankings of the relative cold hardiness of different pine species, varieties and seed sources. Breeders should find this information useful to predict cold hardiness of new hybrid combinations. For example, the results of this study, and other artificial screening studies (Duncan et al. 1996), confirm that *P. elliottii* is more cold hardy than both varieties of *P. caribaea*, and indicate that *P. caribaea* var. *bahamensis* is more cold tolerant than *P. caribaea* var. *hondurensis.* Data from field studies of *P. elliottii* and *P. elliotti* × *P. caribaea* hybrids are in line with the artificial screening results: the hybrids have less cold tolerance than pure species *P. elliottii*, and var. *hondurensis* hybrids are the most susceptible (López-Upton et al. 1999).

The artificial screening method should also be useful for testing pine hybrids to determine if the hybrid more closely resembles the more-susceptible or more-tolerant parent species, and also for screening specific hybrid families for frost tolerance. Currently, studies are underway with *P. patula* × *P. tecunumanii* hybrids to address these objectives.

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