The use of field and artificial freezing studies to assess frost tolerance in natural populations of *Pinus oocarpa*

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Abstract

The susceptibility of *Pinus oocarpa* to freezing temperatures limits the commercial deployment of the highly productive *Pinus patula* x *P. oocarpa* hybrid in South Africa. Identifying *P. oocarpa* germplasm with increased frost tolerance is important. Twenty-three *Pinus oocarpa* provenances, originating from Mexico, Honduras, Guatemala and Nicaragua, were therefore assessed for their tolerance to freezing conditions by analysing field survival after frost events, subjecting needles to freezing temperatures and assessing damage using the electrolyte leakage test, and exposing young plants to freezing temperatures in a semi-controlled environment and scoring tissue damage based on a visual assessment. The performance of many of the provenances represented in the field and artificial freezing studies were similar and there was a strong correlation between provenance ranking in the whole plant freezing and electrolyte leakage test. We therefore support the use of these techniques as a means to assess cold tolerance in *P. oocarpa* at the provenance level. Provenances from north-west Mexico demonstrated more frost-tolerance than those in southern Mexico. Provenances representing Honduras and Guatemala appear to be highly susceptible to frost.

Key words: *Pinus oocarpa*, frost tolerance, provenances, field assessment, artificial freezing

Introduction

Pinus oocarpa is the most common pine in Mesoamerica (Dvorak et al. 2009), occurring across a 3000km range from northern Mexico (28°10'N) to Nicaragua (12°40'N) and between altitudes of 200 and 2500m above sea level (Dvorak et. al 2000, Mourae et al. 1998). It cannot be categorised into a single climatic type (Dvorak et al. 2000). Due to its broad range, and the fact that it grows sympatrically with *P. tecunumanii* across its southern range which would have resulted in introgression between the two species, *P. oocarpa* possesses high levels of genetic diversity (Dvorak et al. 2009). Despite its wide distribution, *P. oocarpa* is very site-specific, and the provenance performance varies considerably between planting sites (Dvorak et al. 2000). As it does not experience freezing temperatures throughout the majority of its natural range, it is not considered a cold hardy species (Dvorak et al. 2000).

Due to its slower growth (Dvorak 2003, Mourae et al. 1998) and generally poor stem form (Dvorak et al. 2000), *P. oocarpa* was never favoured as a plantation species in South Africa. However, in recent times it has proven to be an important hybrid partner (Mourae et al. 1998, Dvorak et al. 2000). It hybridises easily with several other pine species, and possesses other advantageous traits such as the ease of clonal propagation, drought tolerance, good wood quality (Dvorak 2003, Mourae et al. 1998), fire tolerance, and resistance to *Fusarium circinatum* (Dvorak et al. 2000).

The main limitation to planting hybrids of this species in the temperate regions of South Africa is their susceptibility to frost (Hodge et al. 2012). However, as the natural range of *P. oocarpa* covers a very large area, there may be significant variation in cold tolerance between provenances. For example, some provenances in Mexico, where mean annual temperatures (MAT) have been found to be slightly lower (14°C - 25°C) than in Central America (16°C - 26°C) (Dvorak et al. 2000), show increased levels of frost tolerance (Hodge et al. 2012). In addition, due to its evolutionary defence mechanism to survive fires, *P. oocarpa* has the ability to re-sprout after experiencing severe freeze events provided the cold was not too severe and trees are still juvenile (Hodge et al. 2012).

The frost tolerance of pine species can be tested in several ways. Commonly, the survival or degree of frost damage can be assessed in the field after frost events. Assuming the frost event is not so severe that all seedlings die, this method provides realistic results. Plants (or plant tissue) can also be frozen artificially and visually scored for damage to the tissue (Burr et al. 1990, Duncan et al. 1996, Glerum 1985). In addition, other artificial freezing studies such as electrolyte leakage tests can be performed (Burr et al. 1986, Hodge et al 2012, Cerda Granados 2012).

The electrolyte leakage technique involves freezing needles, or other plant tissue such as shoots, at specified temperatures and time periods and measuring the amount of electrolytes that leach from the damaged cells into a distilled water solution. This provides an indication of damage to cell membranes which can be used to rank treatments. This method has the advantage of being quantitative and non-destructive. Good correlations have been reported between results of electrolyte leakage and field studies (Aitken and Adams 1997, O'Neill 1999, Sáenz-Romero and Tapia-Olivares 2008, Anekonda et al. 2000, Hodge et al 2012), as well as between visual injury scoring and artificial freezing techniques (Short et al. 1996 in Sáenz-Romero and Tapia-Olivares 2008).

Currently, there is limited information on the frost tolerance of *P. oocarpa* provenances. This information would make it possible to select provenances or families with increased resistance to frost which can be used to develop hybrids for temperate regions. In this study, a total of 23 provenances of *P. oocarpa* were studied by analysing data from field assessments, where the young plants were scored after frost damage, and artificial freezing tests.

All *P. oocarpa* material used in this study was obtained through Camcore (previously known as the "Central America and Mexico Coniferous Resources Cooperative", now known as the "International Tree Conservation & Domestication Programme"), an organization that coordinates the conservation, testing and breeding of forest tree species in the tropics and sub-tropics (Camcore 2010). Camcore collects seed in the native ranges of several species which is then distributed to members for the purposes mentioned.

Materials and methods

Table 1 lists the *P. oocarpa* provenances in this study as well as the details of their natural ranges. Provenance distributions are shown in Figure 1. Numbers 1 to 23 in Table 1 indicate corresponding Provenances in Figure 1.

Goedgeloof Clone Bank

Genetic material and experimental design

Potted seedlings and cuttings (in Unigro© inserts 90 ml in size) of several Camcore families, representing eight *Pinus oocarpa* provenances from Mexico, Honduras & Guatemala, were sourced from Komatiland Forests (KLF), with each provenance represented by 9 to 21 families. In October 2010, some of this material was established as a clone bank on York Timbers' Goedgeloof plantation (Altitude 1228m, MAT 16.76°C, MAP 808mm, Latitude 24°42'25.74"S, Longitude 30°49'23.11"E). In almost all cases, each family was represented by 5 trees established in a row in an un-replicated design. The seedlings and cuttings were planted 5m apart. Leftover seedling material was established in provenance blocks on the

Table 1: Site details of natural ranges of *P. oocarpa* provenances investigated in this study.

Map Number	Provenance	Country	Latitude	Longitude	Min Elevation	Max Elevation	MAP*	Mean Temp	Min Temp	Max Temp
1	Chinipas	Mexico	27° 19' N	108° 36' W	1140	1780	822	16.49	8.05	24.98
2	Mesa de los Leales	Mexico	26° 23' N	107° 46' W	1260	1350	822	20.26	11.84	28.73
3	Duraznito Picachos	Mexico	23° 41' N	105° 54' W	1490	1740	1003	18.12	12.24	24.03
4	La Petaca	Mexico	23° 25' N	105° 48' W	1560	1710	1155	17.34	11.53	23.19
5	Taretan	Mexico	19° 25' N	102° 04' W	1610	1610	1622	18.00	10.33	25.72
6	Tenería	Mexico	18° 59' N	100° 03' W	1760	1760	1306	19.33	12.64	26.06
7	El Campanario	Mexico	17° 17' N	99° 16' W	1425	1630	1088	21.80	14.73	28.91
8	Huayacocotla	Mexico	20° 30' N	98° 25' W	1190	1410	1711	17.16	10.20	24.17

9	San Sebastian Coatlán	Mexico	16° 11' N	96° 50' W	1750	1750	598	18.42	11.26	25.63
10	San Pedro Solteapán	Mexico	18° 15' N	94° 51' W	602	602	1812	23.01	18.43	27.63
11	El Jicaro	Mexico	16° 32' N	94° 12' W	1000	1000	1684	21.73	16.03	27.48
12	Las Peñas-Cucal	Guatemala	15° 12' N	91° 30' W	1835	1835	975	17.83	11.64	24.09
13	Tapalapa	Guatemala	14° 24' N	90° 09' W	1420	1555	1113	19.58	14.47	24.77
14	El Castaño (Bucaral)	Guatemala	15° 01' N	90° 09' W	930	1330	900	19.00	14.60	23.47
15	San José La Arada	Guatemala	14° 40' N	89° 57' W	745	830	875	19.88	14.82	24.99
16	San Luis Jilotepeque	Guatemala	14° 37' N	89° 46' W	950	1010	895	20.58	15.41	25.81
17	San Lorenzo	Guatemala	15° 05' N	89° 40' W	1570	1780	1700	16.34	11.88	20.86
18	Camotán	Guatemala	14° 49' N	89° 22' W	740	960	926	25.48	19.95	31.08

19	Mal Paso	Guatemala	15° 11' N	89° 21' W	1010	1070	1800	22.48	17.59	27.47
20	Tablazón	Honduras	14° 09' N	87° 37' W	960	1120	1548	20.17	14.73	25.67
21	Pimientilla	Honduras	14° 54' N	87° 30' W	750	750	1279	23.04	17.06	29.11
22	San José Cusmapa	Nicaragua	13° 17' N	86° 37' W	1345	1345	1500	19.20	14.18	24.27
23	Dipilto	Nicaragua	13° 43' N	86° 32' W	1075	1320	1543	20.67	15.48	25.90

*****MAP (Mean Annual Precipitation)

eastern border of the clone bank. Provenances, as well as number of families and individuals included, are shown in Table 2.

Frost scoring and survival assessments

During the winter of 2011, approximately 9 months after planting, several heavy frost events were experienced in the clone bank. At the end of the winter period (August 2011), when the trees were 10 months old, frost damage was scored on a scale of 0 – 3 (0 = none, 1 = slight, 2 = moderate and 3 = severe). Survival assessments were also carried out at eight months (before the frost events) and at 12 months (after the frost events) to ensure that the results presented were due to frost damage.

Hendriksdal CBSO

Genetic material and experimental design

Cuttings, representing the eight provenances in the Goedgeloof clone bank, were used to establish a Clonal Breeding Seed Orchard (CBSO) on York Timbers' Hendriksdal plantation in February 2011 (Altitude 1310m, MAT 16.59°C, MAP 1237mm, Latitude 25°12'27.18"S, Longitude 30°46'11.36"E). This CBSO consisted of a large number of clones representing 98 families of the same eight provenances established at Goedgeloof. A randomised complete block design was used and each clone was represented by one individual in each of the five replications. Provenances, as well as the number of families and individuals representing each, are shown in Table 2. All the dead or dying trees were replaced (blanked) in April (just before the onset of winter) when the trial was two months old.

Table 2: The number of families and individuals representing all provenances studied in field and artificial freezing experiments.

		Goedgeloof Clone Bank			Hendriksdal CBSO			Artificial Freezing	
Country	Provenance	No of Families	No of Clones	No of Individuals	No of Families	No of Clones	No of Individuals	No of Families	No of Individuals
Guatemala	Camotán	-	-	-	-	-	-	15	90
Guatemala	El Castaño (Bucaral)	15	89	95	12	56	275	4	24
Guatemala	Las Peñas-Cucal	17	67	97	13	32	159	4	24
Guatemala	Mal Paso	-	-	-	-	-	-	10	66
Guatemala	San José La Arada	-	-	-	-	-	-	5	30
Guatemala	San Lorenzo	-	-	-	-	-	-	15	90
Guatemala	San Luis Jilotepeque	-	-	-	-	-	-	7	42
Guatemala	Tapalapa	-	-	-	-	-	-	4	24
Honduras	Pimientilla	-	-	-	-	-	-	9	54
Honduras	Tablazón	-	-	-	-	-	-	9	54
Mexico	Chinipas	21	128	133	21	120	595	-	-
Mexico	Duraznito Picachos	-	-	-	-	-	-	9	54
Mexico	El Campanario	-	-	-	-	-	-	7	42
Mexico	El Jicaro	11	21	51	10	10	50	-	-
Mexico	Huayacocotla	-	-	-	-	-	-	17	102
Mexico	La Petaca	15	69	90	11	36	166	-	-
Mexico	Mesa de los Leales	-	-	-	-	-	-	21	127
Mexico	San Pedro Solteapán	13	18	59	11	15	75	-	-
Mexico	San Sebastian Coatlán	12	30	62	11	19	95	-	-
Mexico	Taretan	9	39	53	10	27	135	-	-
Mexico	Tenería	-	-	-	-	-	-	10	60

Nicaragua	Dipilto	-	-	-	-	-	-	9	52
Nicaragua	San José Cusmapa	-	-	-	-	-	-	10	60
Control	Ell x CarH (cuttings)	-	-	-	-	-	-	Nursery Bulk	36
Control	P. elliottii (seedlings)	-	-	-	-	-	-	Nursery Bulk	36
Control	P. patula (seedlings)	-	-	-	-	-	-	Nursery Bulk	36
Control	Pat x Ooc (cuttings)	-	-	-	-	-	-	Nursery Bulk	36
Control	Pat x TecH (cuttings)	-	-	-	-	-	-	Nursery Bulk	36
Control	Pat x TecM (cuttings)	-	-	-	-	-	-	Nursery Bulk	36

Survival assessments

Several mild frost events were experienced at the CSBO during 2011. Survival assessments were carried out in the middle of winter when the trees were four-months-old, and again in the spring when the trees were nine months old.

Artificial Freezing

Genetic material and experimental design

In 2011, *Pinus oocarpa* seed representing 17 provenances and 167 families was sourced from Camcore. Provenances, as well as the number of families and individual plants representing these, are listed in Table 2. The seed was sown in 2012 and used to produce seedlings in 10 x 10cm plastic pots filled with composted pine bark. These were cut back to produce shoots, which would be set as cuttings for trial establishment. When the hedges were two years old and no longer needed for cutting production, they were used for artificial freezing tests which were conducted in the winter of June 2014. The hedge plants were kept under plastic in the nursery to prevent natural frost damage until the start of the artificial freezing tests.

Six hedge plants, representing each family from all 17 provenances (Table 2) were selected for the study. The material was divided into six replications with every family from each of the provenances represented by a single plant in each replication. Control material was selected from the commercial nursery representing the following species and hybrids: Thirty-six *Pinus patula* and *P. elliottii* seedlings and 36 cuttings representing each of the following hybrids; *P. patula* x *P. oocarpa* (Pat x Ooc), *P. elliottii* x *P. caribaea* var. *hondurensis* (Ell x CarH), *P. patula* x *P. tecunumanii* which was produced with a high elevation pollen source (Pat x TecH) and *P. patula* x *P. tecunumanii* which was produced with a mix of pollen from high and low elevation sources (Pat x TecM). Each of these controls was represented by six plants in each replication and maintained in the original Unigro 98© insert (90 ml in size). All plants were watered and fertilized as necessary.

Experimental Methods

For each replication, 30 succulent primary needles were collected from each hedge plant representing a family. These were cut into 2cm pieces and placed into individual 16ml test tubes. This was also carried out for the control treatments, except that five needles were collected from each of the six plants per replication to make up 30 needles per control treatment. Care was taken to ensure that each test tube contained exactly 30 needles, each 2cm in length. Test tubes with needles were then placed in a refrigerator (3-5°C) overnight before being subjected to freezing conditions the following day. On the day of freezing, the tubes of a single replication were removed from the refrigerator and placed upright into mesh wire trays. The test tubes were then placed in a walk-in cold room which was set to -5°C. However, a temperature logger (Huato S100-TH) that was placed in the cold room revealed that the mean minimum temperature across the replications was approximately -4°C and fluctuated between -2.2°C and -5.9°C.

The potted hedge plants and controls, from which the needles had been collected for the first replication, were placed in the cold room at the same time as the tubes. The pots and inserts containing plants were completely covered with dry composted pine bark to prevent the roots from freezing.

The sequence of events for each replication was as follows:

Day 1: Needle collection and preparation (Rep 1)

Day 2: Needle and whole plant freezing (Rep 1)

Day 3: Needle collection and preparation (Rep 2)

Day 4: Needle and whole plant freezing (Rep 2), etc…..

After two hours had elapsed, the hedges and control plants were removed from the cold room and returned to the nursery. Signs of freezing damage (brown or yellowish discolouration and/or a water-soaked appearance (Lindén 2002)) were allowed to develop for two days. On the third day, the damage was visually assessed by scoring each plant as either 1 (none), 2 (slight), 3 (moderate) or 4 (severe).

The tubes were left in the freezer for a further two hours (i.e. a total of four hours) before they were removed and allowed to thaw at room temperature for approximately two hours. 10ml deionized water was then added to each tube and left overnight at room temperature. The following day, the tubes were hand shaken for 1 minute to ensure that the inner-contents of the damaged cells were evenly distributed throughout the solution. The concentration of salts (or electrolytes) from the damaged cells was assessed by measuring the electrical conductivity (EC) of the solution using an AZ8306 Conductivity Meter. EC was recorded in micro Siemens (μS).

Statistical analysis

All data were analysed using GenStat® 17TH Edition. A General Linear Model (GLM) was used to analyse the survival data from the field tests (Modelling of Binomial proportions) as well as the frost scoring data from the field and artificial freezing tests (General Model). Duncan Multiple Comparison tests were used to distinguish those treatments that differed significantly. The GLM is as follows:

$\text{logit}(p_{ij}) = c + t_i + \varepsilon_{ij}$

where p_{ij} = the individual probability of survival of the i-th treatment and the j-th replicate

c = overall mean

t_i = the effect of the i-th treatment and

ε_{ij} = the random variation or experimental error

Electrical conductivity data were analysed as an unbalanced ANOVA and Fishers Least Significant Difference (LSD) test was used to distinguish those treatments that differed significantly.

A Pearson correlation analysis was carried out on the values representing latitude, longitude, elevation, rainfall, altitude and modelled temperatures of the *P. oocarpa* provenance collection sites, and the measured survival and frost damage of these at the Goedgeloof, Hendriksdal, and in artificial freezing study. Separate Pearson correlation analyses were carried out between the measured frost score and electrical conductivity readings (including controls), and also between survival of provenances at Goedgeloof and Hendriksdal. The linear relationship between the variables in the correlation analyses is presented as the correlation coefficient (r value).

Results

Goedgeloof Clone Bank

Provenance survival before the frost events (8 months) was excellent (91.5 - 100%) and the survival for the plants representing Guatemala (95.2%) was similar to those representing Mexico (97.3%). After the frost events (when the trees were 12 months old), the provenance survival ranged from 16.4% to 92.5% and the differences between the best and worst surviving provenances were highly significant (chi pr < 0.001) (Figure 2). Chinipas, which had a survival rate of 92%, was significantly better than all other provenances. San Sebastian Coatlán (16% survival rate) was significantly poorer than all other provenances. The survival for all plants representing the Mexican provenances (60.3%) was significantly better than those representing Guatemala (43.9%) (Figure 3).

Similarly, an analysis of the frost score data (0 = none, 1 = slight, 2 = moderate and 3 = severe) revealed significant ($p < 0.001$) differences between provenances and the two countries (Figure 4 and 5). Only 10% of the trees of the most frost-tolerant provenance, Chinipas, suffered “moderate” to “severe” damage. By comparison, over 85% of the trees from Taretan, El Castaño and Las Peñas-Cucal were severely damaged by frost (data not shown). Only 52% of the trees representing Mexico were severely damaged, compared with 90% from Guatemala (data not shown).

The correlation analysis between, latitude, longitude, elevation, rainfall, altitude and mean annual, maximum and minimum temperatures at the provenance collection sites, and the measured survival and frost score damage of these provenances at the Goedgeloof clone bank, revealed strong relationships (Table 3). Most noticeably, latitude correlated strongly with frost score (r=-0.87) and survival (r=0.78) indicating that provenances occurring further north survived better and experienced less tissue damage after a frost event. The relationship between longitude and frost score (r=-0.73), and longitude and survival (r=0.66), was weaker but showed a similar trend. That is, survival improved and tissue damage became less obvious for those plants representing provenances that occur further west. The relationship between frost score and survival was strong (r=0.72) (Table 3). There was a moderately strong relationship between modelled mean minimum temperature and frost score (r=0.4772),

Table 3: The strength of the linear relationship (r) between, latitude (decimal), longitude (decimal), Frost score, elevation (MASL), rainfall (mm) and mean, maximum and minimum temperature (°C) at the provenance collection sites, and the measured survival and frost damage at the Goedgeloof clone bank.

	Frost Score	Survival 12 mnths	Latitude	Longitude	Elevation[1]	Rainfall	Mean Temp	Max Temp
Survival 12 mnths	-0.7152	-						
Latitude	-0.8738	0.7761	-					
Longitude	-0.7294	0.6576	0.9452	-				
Elevation[1]	0.0136	-0.1211	0.1247	0.3087	-			
Rainfall	0.2362	0.1344	-0.0776	-0.0882	-0.6308	-		
Mean Temp	0.3653	-0.335	-0.4702	-0.5497	-0.8568	0.7013	-	
Max Temp	0.0235	-0.1434	-0.1555	-0.15	-0.611	0.656	0.7711	-
Min Temp	0.4772	-0.3763	-0.5516	-0.6604	-0.8361	0.6061	0.9475	0.527

[1]= The mid-point between the highest and lowest elevation points where the provenances occur naturally

and survival (r=- 0.3763) indicating that provenances that occurred in colder regions displayed less tissue damage and survived better after the frost event. This also provides evidence that the modelled temperatures (Hijmans et al. 2005) at the locations of the various provenances are reasonably accurate. Neither the elevation value, nor the amount of rainfall at the provenance collection sites, correlated well or meaningfully with survival or frost damage.

Hendriksdal clonal trial

When the trees were assessed at 4 months old (mid-winter), but prior to the main frost events, provenance survival ranged from 85.26% (San Sebastian Coatlán) to 97.65% (Chinipas) and the survival of some of the provenances differed significantly (chi pr <0.001). However, there was no difference between the survival of the trees representing Guatemala (92.4%) and Mexico (94.6%).

After the winter period (when the trees were nine months old), the survival of the provenances ranged from 70.5% (San Sebastian Coatlán) to 94.3% (Chinipas) (Figure 6) and the survival for the trees representing Mexico (88.2%) was significantly better than those representing Guatemala (80.7%) (Figure 7). The linear relationship between the survival of the provenances at four and nine months was strong (r = 0.84).

Similar to the results from Goedgeloof, the latitude at which the provenances occur naturally correlated strongly with survival (r = 0.83). Again, the correlation between longitude and survival was less (r = 0.64) but meaningful. Interpreted, these findings indicate that trees which occur in the more north-western ranges of the species natural distribution, survive better under freezing temperatures. The relationship between the mean maximum temperature at the provenance locations, and 9-month survival was weaker (r=-0.35) than the Goedgeloof site (where freezing temperatures were more severe). There was no clear and meaningful correlation between the survival of the provenances in this trial and mid-point-elevation or the measured rainfall.

Although differences in survival between many provenances at Hendriksdal were not significant, a comparison between the two field studies revealed that the survival of the eight common provenances at the Goedgeloof and Hendriksdal sites, after the winter period, was very similar ($r = 0.78$) (Figure 8).

Artificial Freezing

The analysis of the electrolyte leakage test revealed significant ($p < 0.001$) differences at the country, provenance and family level. All of the controls ranked better than the *P. oocarpa* provenances (Figure 9). *Pinus patula* had the lowest mean EC reading (18.89µS), followed by *P. elliottii* (22.83µS), Pat x Ooc (23.66µS), Pat x TecH (24.5µS), Ell x Car (27µS) and Pat x TecM (27.39µS). However, only *P. patula* had a significantly lower EC reading than Ell x Car and Pat x TecM.

Mean EC readings for the *P.oocarpa* provenances ranged from 30.14µS (Las Peñas-Cucal) to 48.1µS (San José La Arada) with several provenances at the furthest ends of this range differing significantly ($p<0.05$) (Figure 9). The samples that represented the two provenances in Nicaragua (35.52) and those in Mexico (36.16µS) were significantly different to those from Guatemala (40.99µS) and Honduras (41.9µS) (Figure 10).

The analysis of the frost score data (1 = none, 2 = slight, 3 = moderate and 4 = severe) revealed significant differences at the country, provenance and family level. *Pinus elliottii* had the lowest mean frost score (1.0), followed by *P. patula* (1.14), Ell x Car (1.22), Pat x

Ooc (1.33), Pat x TecH (1.33) and Pat x TecM (1.36). However, the differences between the control treatments were not significant ($p<0.05$).

Mean frost scores for the provenances ranged from 1.57 (Duraznito Picachos) to 2.84 (San Luis Jilotepeque) (Figure 11). The two least affected provenances, Duraznito Picachos and Mesa de los Leales, were significantly different than all the rest. Although Huayacocotla was not as tolerant as Duraznito Picachos and Mesa de los Leales, it was significantly more tolerant than many of the others.

The mean frost damage for those plants representing Mexico (2.02) was significantly lower than those representing Nicaragua (2.40) which was lower than those that represented Guatemala (2.62) and Honduras (2.68) (Figure 12).

The correlation analysis revealed that mean frost score for each provenance correlated strongly with latitude ($r = -0.84$) and longitude ($r = -0.81$). Unlike the Hendriksdal and Goedgeloof field studies, there were moderately strong negative correlations between both minimum and maximum elevation values, at which the provenances occurred, and mean conductivity (EC) and frost score (FS) readings. The strength of these relationships ranged from $r = -0.48$ (min elev. and FS) and $r = -0.64$ (min elev. and EC). These indicate that seedlings or young trees representing provenances that occur at a higher elevation had less damage to the plant tissue under freezing conditions. The modelled mean minimum temperature, at which the provenances occurred naturally, correlated meaningfully with frost score ($r = 0.6274$) and the EC readings ($r = 4965$) indicating that the plants representing the provenances which experience lower minimum temperatures experienced less tissue damage after freezing. As observed with the previous field studies, there was no relationship between the mean rainfall at the locations where the provenances occur naturally, and the EC or FS readings in the artificial freezing study (Table 5).

Table 4: The strength of the linear relationship (presented as the correlation coefficient or r) between, latitude, longitude (decimal), elevation (masl), rainfall (mm) and mean, maximum and minimum temperature (ºC) at the provenance collection sites, and the measured survival at the Hendriksdal clonal trial.

	Survival 4 mnths	Survival 9 mnths	Latitude	Longitude	Elevation[1]	Rainfall	Mean Temp	Max Temp
Survival 9 mnths	0.8369	-						
Latitude	0.5383	0.8331	-					
Longitude	0.2947	0.6406	0.9452	-				
Elevation[1]	-0.2401	-0.0489	0.1247	0.3087	-			
Rainfall	0.2316	-0.0171	-0.0776	-0.0882	-0.6308	-		
Mean Temp	-0.0483	-0.3545	-0.4702	-0.5497	-0.8568	0.7013	-	
Max Temp	-0.036	-0.3327	-0.1555	-0.15	-0.611	0.656	0.7711	-
Min Temp	-0.047	-0.3078	-0.5516	-0.6604	-0.8361	0.6061	0.9475	0.527

[1]= The mid-point between the highest and lowest elevation points where the provenances occur naturally

Table 5: The strength of the linear relationship (presented as the correlation coefficient or r) between, latitude (decimal), longitude (decimal), elevation (masl), rainfall (mm) and mean, maximum and minimum temperature (ºC) at the provenance collection sites, and Mean EC and Mean Frost Score in the Artificial screening study.

	Mean FS	Mean EC	Latitude	Longitude	Elevation[1]	Rainfall	Mean Temp	Max Temp
Mean EC	0.5714	-						
Latitude	-0.8409	-0.3091	-					
Longitude	-0.8094	-0.2961	0.9605	-				
Elevation[1]	-0.5353	-0.6363	0.3254	0.455	-			
Rainfall	0.1548	-0.1941	-0.2397	-0.3083	0.0835	-		
Mean Temp	0.425	0.3519	-0.2257	-0.2356	-0.6468	-0.1932	-	
Max Temp	0.1523	0.1467	0.1173	0.1119	-0.4724	-0.2664	0.9097	-
Min Temp	0.6274	0.4965	-0.5381	-0.5512	-0.7046	-0.0799	0.9028	0.6428

[1]= The mid-point between the highest and lowest elevation points where the provenances occur naturally

The correlation between mean frost score and the electrical conductivity reading for the *P. oocarpa* provenances was moderate (r=0.57) in this study (Table 5). However, if the EC and FS values for the controls were included, then the correlation between the mean EC and FS values increased to r = 0.89 (Figure 13).

Summary of results

Each provenance in the Goedgeloof clone bank and artificial freezing studies were categorised as Poor, Moderate or Good, (Table 6) based on the following criteria:

Field survival results: 0-50% (Poor), 50-80% (Moderate), 80-100% (Good).

Frost scores: 0-1 (Good), 1-2.5 (Moderate), 2.5-3 (Poor)

Electrical Conductivity: (Provenances were grouped based on significant differences shown in Figure 9. Treatments which were labelled with bc-cde were regarded as good, cdef-defghi as moderate and fghi-i as poor.

An overall rating was also assigned to each provenance to guide decisions based on cold tolerance. Results from the Hendriksdal CBSO were excluded from the summary as there were not many significant differences between provenances.

Discussion

All three procedures used to assess the relative tolerance or susceptibility of young plants to freezing temperatures in these studies viz., a) assessing survival or visually scoring the damage to seedlings after frost events in the field, b) scoring damage to potted plants after exposing them to freezing temperatures in a more controlled environment, or c) freezing

Table 6: Summary of results categorizing each provenance in terms of expected cold tolerance (based on performance in the Goedgeloof clone bank and Artificial freezing studies).

		Goedgeloof Clone Bank		Artificial Freezing		
Country	Provenance	Survival percentage	Frost Score	Frost Score	Electrolyte Leakage	Overall
Guatemala	Camotán	-	-	Poor	Poor	Poor
Guatemala	El Castaño (Bucaral)	Moderate	Poor	Moderate	Moderate	Poor-Moderate
Guatemala	Las Peñas-Cucal	Poor	Poor	Moderate	Good	*
Guatemala	Mal Paso	-	-	Poor	Moderate	Poor-Moderate
Guatemala	San José La Arada	-	-	Poor	Poor	Poor
Guatemala	San Lorenzo	-	-	Poor	Moderate	Poor-Moderate
Guatemala	San Luis Jilotepeque	-	-	Poor	Poor	Poor
Guatemala	Tapalapa	-	-	Moderate	Moderate	Moderate
Honduras	Pimientilla	-	-	Poor	Moderate	Poor-Moderate
Honduras	Tablazón	-	-	Poor	Poor	Poor
Mexico	Chinipas	Good	Good	-	-	Good
Mexico	Duraznito Picachos	-	-	Moderate	Moderate	Moderate
Mexico	El Campanario	-	-	Moderate	Moderate	Moderate
Mexico	El Jicaro	Poor	Poor	-	-	Poor
Mexico	Huayacocotla	-	-	Moderate	Good	Moderate-Good
Mexico	La Petaca	Moderate	Moderate	-	-	Moderate
Mexico	Mesa de los Leales	-	-	Moderate	Good	Moderate-Good
Mexico	San Pedro Solteapán	Poor	Moderate	-	-	Poor-Moderate
Mexico	San Sebastian Coatlán	Moderate	Poor	-	-	Poor-Moderate
Mexico	Taretan	Moderate	Poor	-	-	Poor-Moderate
Mexico	Tenería	-	-	Moderate	Good	Moderate-Good
Nicaragua	Dipilto	-	-	Moderate	Good	Moderate-Good
			-	-		
Nicaragua	San José Cusmapa	-	-	Moderate	Good	Moderate-Good

*Results for Las Peñas-Cucal were inconclusive needles of seedlings and measuring the amount of electrolytes leached from the damaged cells, proved valuable. This is clear from the similar rankings for many of the common provenances in each of the studies.

Of the various tests conducted, the most reliable are those where whole plants were exposed to freezing temperatures either in the field or under artificial conditions. These tests give the most realistic reflection of how the entire plant responds to cold stress. Specific plant tissues (stems, needles and buds) have been shown to respond differently to cold stress (Burr et al. 1990), and therefore the combined response determines the ability of the plant to resist, or recover from, freezing injury. The few discrepancies in the rankings between the survival results and the frost score damage in the field studies are probably attributed to the subjectivity of frost scores. Therefore, it is suggested that using survival or scoring seedlings for signs of damage after freezing events is a preferred method to identify genetic material with greater cold tolerance from a population of trees that are generally considered to be frost susceptible such as *P. oocarpa*.

Although artificial screening provided meaningful results in the study using electrical conductivity, most other researchers calculate Relative Conductivity (RC) and/or Injury Index (II) (Hodge et al. 2012, Burr et al. 1990, Glerum 1985, Rehfeldt 1980) to determine frost tolerance from electrolyte leakage tests. These measurements are calculated by comparing post-freezing EC (as was done in our case) with a second EC reading taken from damaged tissue after needles are exposed to high temperatures. The two values are compared to determine how much cytoplasm was lost during the freezing event relative to the total available cytoplasm stored in the cells. In this study, Electrolyte Leakage on its own appeared to be rather accurate, as most provenances were well predicted in the correlation with Frost Score (Figure 13). However, Duraznito Picachos and Las Peñas-Cucal may have been better predicted if RC was calculated. A comparison of the two methods warrants further investigation.

The data generated from these studies illustrate the importance of testing a large sample. This is probably more applicable when conducting artificial freezing studies such as the electrolyte leakage test. For example, the correlation between the family ranking (represented by 6 seedlings per family) in the electrolyte leakage and frost score studies was poor, but when these were grouped by provenance the correlation improved significantly.

Classifying Las Peñas-Cucal, which is the most northern of the Guatemalan provenances, required further investigation. It performed poorly in the Goedgeloof field study where it was represented by 17 families or 97 trees. However, it was the best ranked provenance in the electrolyte leakage test where it was represented by four families or 24 seedlings. When the results were interrogated, we observed that nearly 50% of the plants from the same four families that showed high levels of tolerance in the artificial freezing studies, recovered after the severe frost events at Goedgeloof. In comparison, only 30% of the plants from other families from Las Peñas-Cucal recovered after the frost events at Goedgeloof.

From subsequent discussions with Prof. Bill Dvorak, (previous director of Camcore), we learnt that despite the high altitude, Las Peñas-Cucal only experiences occasional frosts and these events are usually not severe. This would explain the relative susceptibility of this provenance to extreme cold in our study. Furthermore, the variation in frost tolerance observed between the trees representing Las Peñas-Cucal is likely due to the fact that it is the only provenance from Guatemala that is more closely related to the Mexican provenances (Dvorak et al. 2009). There will, therefore be genotypes in this population that carry the cold tolerance of the Mexican provenances.

Although there were no significant ($p<0.5$) differences between most of the controls in the artificial freezing studies, the rankings of *P. patula*, *P. elliottii*, Pat x TecH, Ell x CarH and Pat x TecM (which was produced with a pollen mix consisting of both high and low elevation provenances) were as expected in both the electrolyte leakage and whole plant freezing test. If we can use ranking as a reliable measure, we would expect the Pat x Ooc hybrid to rank alongside Pat x TecM due to the similarity in the susceptibility of *P. oocarpa* and the low elevation source of *P. tecunumanii* to freezing temperatures. Unfortunately, no provenance information was available on the *P. oocarpa* pollen sources used in the Pat x Ooc cross. It is possible that pollen was sourced from trees representing one or more of the more cold tolerant Mexican provenances.

It was clear that geographical reference, especially latitude and to a lesser extent longitude, was the main factor in determining relative cold tolerance in these studies. As seen from the field studies, Chinipas, which is the most north-westerly located provenance in Mexico (27°19'N, 108°36' W), had the highest survival rate at both Goedgeloof and Hendriksdal after winter. It also had the lowest mean frost score at Goedgeloof. In the artificial freezing studies, Mesa de los Leales, which occurs close to Chinipas, ranked second for cold tolerance. Duraznito Picachos, which is the third most northerly located provenance (23° 41'N), had the lowest frost score in the artificial freezing test. La Petaca, which is located just south of Duraznito Picachos, ranked second best for post-winter survival and had the second lowest mean frost score at Goedgeloof. The Central Mexican provenance Taretan (19°25' N, 102°04' W) survived well at Goedgeloof and, Huayococotla, which occurs to the east at a similar latitude (20°30' N, 98°25' W) ranked well in the artificial freezing study. Interestingly, the relationship between latitude and cold tolerance has also been reported for *Pinus contorta* (Nilsson 2001).

In keeping with this trend, San Sebastian Coatlán (16° 11' N) and El Jicaro (16° 32' N), which are the two most southern Mexican provenances tested, survived very poorly after the frost events at Goedgeloof. This is despite the fact that San Sebastian Coatlán is located at a very high altitude (1750m).

Within Guatemala, the mean of the 17 families representing the isolated provenance of Las Peñas-Cucal (1835m) showed no cold tolerance in the field studies where it was represented by many trees despite the fact that it occurs naturally at one of the highest altitudes of all the *P. oocarpa* provenances. However, the altitude at which the less distantly positioned provenances in the south-eastern region of Guatemala occur appear important. For example, Tapalapa (1420-1555m) and San Lorenzo (1570-1780m) ranked more tolerant in the electrolyte leakage and whole-plant freezing studies than San Luis Jilotepeque (950-1010m), Camotán (740-960m) and San José la Arada (745-830m). Other studies have also found evidence of a correlation between the cold hardiness in conifers with changes in elevation (Hodge et al. 2012, Sáenz-Romero and Tapia-Olivares 2008). Therefore, in the absence of experimental data, these observations can be used as a basis to predict the frost tolerance of *P. oocarpa* populations not included in these studies.

The meaningful correlation between both survival and tissue damage, and the modelled temperatures at which the provenances occur naturally (Hijmans et al. 2005) provided evidence that these modelled temperatures provide an accurate reflection of the climatic conditions at these sites.

The improved tolerance to cold temperatures amongst the Mexican provenances, relative to those in Honduras, concurs with the work carried out by Hodge et al. (2012). It can be theorized that provenances occurring in north-western Mexico evolved with thicker needles to protect them from desiccation during the dry season in these regions, which made them more resistant to the desiccating effects of freezing injury (WS Dvorak pers.comm., 2016). The close relationship between drought tolerance and cold tolerance in plants has been reported elsewhere (Beck et al. 2007).

The relatively low latitude, and warmer modelled minimum temperatures (Hijmans et al. 2005), of the Guatemalan provenances explains their susceptibility to freezing temperatures in the artificial freezing study. For the same reason, the high frost scores (tissue damage) of the two Nicaraguan provenances, Dipilto and San José Cusmapa, compared with Mexico makes sense. In the electrolyte leakage test, however, the tolerance of the two Nicaraguan provenances, compared with all of the Mexican provenances, were no different. This illustrates the need for multiple tests when carrying out electrolyte leakage studies to confirm results. It should also be noted that Dipilto and San José Cusmapa occur on the border of southern Honduras, but some distance from the two other provenances representing central Honduras in these studies. Therefore they may not be a true reflection of other provenances that occur in Nicaragua. Two other provenances not included in this study, Cerro Bonete (12° 50'N, 86° 18'W) and Cerro la Joya (12° 25'N, 85° 59'W), occur at more southern latitudes and may be more susceptible to cold.

The ranking of the Pat x Ooc hybrid intermediate to the *P. patula* control and other *P.oocarpa* provenances agrees with what has been found for the hybrid between *P. patula* and *P. tecunumanii* (Cerda Granados 2012) and between *P. elliottii (var elliottii)* and *P. caribaea (var hondurensis*) (Duncan et al. 1996). Under extremely low temperatures, however, hybrids between temperate and tropical species may perform similar to the more cold-susceptible parent (Duncan et al. 1996, Granados 2012).

Evaluation of methods

As seen, identifying genetic material that is more tolerant to freezing temperatures can be determined from artificial and field studies. The costs and practicality to carry these out differ and consideration needs to be given to each to determine which are the most worthwhile.

Electrolyte leakage test

The electrolyte leakage technique enables the researcher to freeze large samples of needles from different plants at the same time without damaging the original sample. However, obtaining reliable results depends largely on the minimum temperatures achieved, the time taken to reach the minimum freezing temperature and the duration that the minimum temperature is maintained. The physiological state that the plants are in prior to freezing will also affect the outcome of the results. Once this has been determined, and a suitable freezing chamber where these conditions can be effectively managed, results can be obtained within a week making this a cost-effective technique.

Whole plant freezing

The method of scoring needle damage after subjecting young plants to freezing conditions may be more accurate than an electrolyte leakage test but a larger freezer is required and the amount of samples that can be tested at any one stage is limited to the size of the freezer. Similar to the electrolyte leakage method, the temperatures required to obtain meaningful results need to be determined beforehand. However, they do not need to be as precise. This technique will provide answers in a relatively short space of time.

Field studies

Field studies may provide the best measure of frost tolerance, but testing the plants under optimal conditions, where the freezing temperature is not too severe resulting in the death of all the plants, or too mild resulting in very little damage, can be difficult due to seasonal variation. Identifying two locations and establishing young plants representing the same material in both locations (as was done at Goedgeloof and Hendriksdal) is preferred. Considering that many of the seedlings are expected to die, the trees could be established close together. As observed in the Goedgeloof field study many of the young plants may recover after damage and the research should wait until warmer weather returns before drawing a final conclusion.

It is therefore recommended that more than one method is used to screen for cold tolerance, or repeating the same method especially when carrying out artificial screening studies. Artificial screening methods (electrolyte leakage or scoring tissue damage) are an effective means to screen a large sample of plants in a relatively short space of time. Early selections can be made and the most tolerant and susceptible families established in small field studies to confirm the results. A combination of electrolyte leakage and field studies could be particularly useful as the same plants, used in the EL test, could be established in the field.

Application in a breeding program

Several research programs in South Africa have created hybrids between *P. patula* and *P. oocarpa* or *P. tecunumanii*, without screening for frost tolerance. Large numbers of families are either in the production phase or will soon be deployed commercially. We would advise that these be screened for their level of frost tolerance if they are expected to be deployed to areas that may experience frost. The tests we have carried out indicate that screening can be done in several ways and more than one should be considered. The researcher could use the screening results to eliminate the most frost-susceptible families from commercial production, or at least identify those that should not be planted in areas which are more likely to receive frost during winter periods.

From these collective results, it would seem likely that the observed susceptibility of the *P. patula* x *P. oocarpa* hybrid could be improved by using pollen obtained from trees representing the most frost tolerant Mexican provenances of Chinipas, Taretan, Duraznito Picachos, Mesa de los Leales, and possibly Huayococotla (artificial freezing study). However, these provenances may not grow as well as others in Mexico or Central America. For example, Las Peñas-Cucal and San Sebastian Coatlán, which were the two worst surviving provenances in the Goedgeloof field study, were the best performing provenances in terms of growth at the Hendriksdal clonal trial at age five years (unpublished data, York Timbers). Elsewhere, a negative correlation between growth and frost resistance has been found (Aitken et al. 1996, Sáenz-Romero et al. 2006). Naturally, there will be exceptions to this trend. For example, the trees representing one of the most southern Mexican provenances, El Jícaro (16° 32' N), had the poorest growth at age five in the Hendriksdal clonal trial and also survived poorly at Goedgeloof after frost. In summary, when selecting trees representing the frost-susceptible Central American provenances with good growth such as Las Peñas-Cucal, select those from the more tolerant families.

Alternatively, and in addition, the frost tolerance of the *P. patula* x *P. oocarpa* hybrid, or any other frost-susceptible hybrid for that matter, could be improved by back-crossing it with *P. patula*, as has been observed with other conifer species (Lu et al. 2006). Therefore, backcrossing a *P. patula* x *P. oocarpa* hybrid, which has been produced from one of the provenances with good growth but poor frost tolerance, should be considered.

As a result of the efforts by Camcore who made extensive collections of *P. oocarpa* in its natural range, and companies like York Timbers, who have established trials testing this material in South Africa, a broad genetic base is available for producing the hybrids with *P. oocarpa*. In most cases these trials are young, but selections will be identified from them shortly and will no doubt, become an important pollen source of interest amongst the South African timber companies.

Conclusions

The latitude at which the *P. oocarpa* provenances occurred largely determined how tolerant the seedlings from these are to freezing temperatures. Although there were meaningful relationships between measured damage to the plants after freezing and modelled temperature, which correlated moderately with altitude, there was little evidence that altitude on its own provided an accurate prediction of frost tolerance. The Mexican provenances, and in particular those in a far north-west such as Chinipas, showed the best frost tolerance in both field and electrolyte leakage tests. They may provide an important source of pollen in the case where *P. oocarpa* is used in a hybrid breeding program. Most provenances occurring in the south of Mexico, and in particular those in the Central American countries of Honduras and Guatemala, were considered frost susceptible.

One of our objectives from the artificial screening study was to determine whether using the electrolyte leakage test is a reliable means of assessing cold tolerance. Based on the reasonably strong correlation between the EC readings from the electrolyte leakage test and frost scores, and the similar performance of several of the common provenances in the artificial freezing and field studies, we can say that it does. However, sample sizes should be large and the test repeated, or compared with results obtained under natural conditions. Assessing Injury index may also add more value than only measuring EC after cold treatment.

Historically, *P. oocarpa* has been considered a species of little importance due to its slow growth and poor stem form. However, it is likely to become increasingly important in pine breeding programs that are focused on producing germplasm that not only grows well, but has increased tolerance to *Fusarium circinatum* and is suited to warm-temperate and sub-tropical regions in Africa. Establishing trials to identify provenances and families with good growth, and attributes such as cold-tolerance, is warranted.

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