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Overcoming dormancy of *Pinus pinceana* seeds

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Summary

After several unsuccessful attempts to germinate seed of *Pinus pinceana* Gordon, seeds from 12 populations in three regions of Mexico were subjected to four treatments (whole seeds, de-coated seeds, de-coated seeds placed on half of their seed coats and cracked seed coat in the micropylar area) to determine the type of dormancy and promote germination in *P. pinceana* seeds. The germination percentage ranged from 9–86% for whole and de-coated seed, respectively. The speed of germination ranged from a peak value of 0.53 for whole seed to 8.75 for de-coated seed. It was concluded that mechanical restriction imposed by the seed coat and chemical inhibition were the major factors preventing germination. Variation in germination parameters was found to be under strong genetic control. Family variance ranged from 16.5% to 23.7% of the total variance for germination percentage and peak value, respectively.

Introduction

Fourteen pinyon pine species occur in semi-arid regions of Mexico. Eight of those species, including *Pinus pinceana* Gordon, are endemic (Perry, 1991) and are included on the list of rare and endangered taxa (published in the NOM-059-ECOL-2001 by the Mexican government, Secretaria del Medio Ambiente y Recursos Naturales, 2002). *Pinus pinceana* grows in widely dispersed and isolated populations, often in association with *P. cembroides* Zucc., in areas where rainfall is less than 400 mm, with an 8-month dry season and 60% of the annual rain falling in summer (Perry, 1991). These species have an important role in semiarid woodland ecosystems, providing food and habitat for wildlife (Perry, 1991). Local people use the edible pinyon "nuts," and harvest trees or branches for firewood and construction.

Conservation and restoration of *P. pinceana* for ecological and local community sustainability requires knowledge of cultivation procedures, including germination of dormant seed. Under natural conditions, the seeds are dispersed by birds and rodents at the beginning of the dry season and they become dormant soon after they are shed from the tree. Seed dormancy is an adaptive strategy that allows the seeds to retain

viability during adverse environmental conditions by ensuring that germination occurs at an appropriate time for successful establishment of seedlings (Bewley, 1997; Foley and Fennimore, 1998).

There are a number of different forms of dormancy found in forest tree seeds. Physical dormancy, caused by tissues surrounding the embryo (e.g., seed coat) (Baskin and Baskin 2001), is common in conifers (Stone, 1957; Barnett, 1976; Downie and Bewley, 1996). These tissues may inhibit germination by any or all of the following: constituting a physical barrier for water and oxygen uptake, mechanically preventing embryo growth; emitting inhibitors such as phenolic compounds and abscisic acid; and blocking the leaching of inhibitors from the embryo (Rolston, 1978; Corbineau and Come, 1995; Baskin and Baskin, 2001). Although other forms of dormancy, caused by physiological or morphological factors are not common in *Pinus* species, they have been reported in *P. cembra* L., *P. koraiensis* Sieb. *et* Zucc. and *P. sibirica* Du Tour seeds (Krugman and Jenkinson, 1974).

Basic knowledge concerning dormancy in *P. pinceana* seeds and how to handle and produce seedlings, which is necessary in order to restore and conserve the natural populations of this species, is lacking. Thus, the objectives of this study were:

1) To determine the type of dormancy involved in *P. pinceana* seeds.
2) To determine the best treatment for overcoming dormancy in *P. pinceana* seeds.
3) To examine genetic variation in germination among populations.

Materials and methods

Seed Collection

In this study, a population of *P. pinceana* is defined as an inter-mating group of individuals, which may include scattered trees as well as more cohesive groups, that occurs in a given area and is isolated from others by geographic barriers, such as mountains or distance. *Pinus pinceana* seeds were collected in October 2002 from 12 populations in three regions of the Sierra Madre Oriental, covering the species' range (table 1). Cones were collected from 30 trees in each population. Most cones were already open on the tree when they were harvested and some seeds had fallen. The sampled trees were scattered throughout the populations and each tree-seed lot was given a permanent identification number.

Seed Extraction

Seeds were extracted by tapping the cones on a hard surface and manually removing the debris. Seed from each tree was considered a single seed lot. Empty seeds were separated from filled seeds by flotation in 70% ethanol for 1 min. Filled seeds (that sank) were removed and rinsed with de-ionized water, then air dried at room temperature for 4 days.

Water content of filled seeds was determined after the 4-day drying period using 34 random samples of seeds (five seeds per sample) (Kolotelo *et al.*, 2001). After storage for 15 months, water content was determined. Seed water content determinations were not done for all populations as only limited seed was available.

Table 1. Geographic location of 12 populations of *P. pinceana* Gordon.

Populations	State	Latitude (N)	Longitude (W)	Altitude (m)
Northern region				
SJ Carbonerillas	Zacatecas	24°28'15"	101° 26'58"	2300
Lomas Oregano	Zacatecas	24°30'14"	101° 27'45"	2305
Santa Elena	Coahuila	25°01'47"	101° 24'39"	2077
Palmas Altas	Coahuila	25°07'62"	101° 26'56"	2090
El Cinco	Coahuila	25°10'35"	101° 41'14"	2250
El Recreo	Coahuila	25°17'44"	101° 00'03"	2238
Central region				
Matehualilla	San Luis Potosi	22°42'34"	100° 27'49"	2020
La Trinidad	San Luis Potosi	22°40'02"	100° 28'20"	1945
Southern region				
Maguey Verde	Queretaro	21°05'18"	99° 41'48"	2176
El Tepozan	Queretaro	20°54'23"	99° 39'28"	2188
El Arenalito	Hidalgo	20°39'41"	99° 02'49"	1882
San Cristobal	Hidalgo	20°37'43"	98° 58'41"	1915

Evaluation of Seed Germination and Viability

a) Initial germination test

Five seed lots (50 seeds per lot) were chosen at random from the 30 single tree collections made from each population in each region and these were randomly mixed to form a bulk seed collection for each population. These bulked seeds were placed on Kimpak moistened with 250 mL de-ionized water in Petawawa germination boxes (Wang and Ackerman, 1983). Seed was germinated in a Conviron (Control Environmental Limited, Winnipeg Manitoba) (25°C/15°C, day/night temperature, 8 hours light with a constant relative humidity (RH) of 80%). There were 10 replications with five seeds per replication from each population. For all germination tests, seed was considered germinated when the radicle was at least 5 mm long.

b) Assessment of the influence of soaking and temperature on seed germination

Four hundred seeds from 15 randomly chosen seed lots from each of six populations (SJ Carbonerillas, Palmas Altas, Matehualilla, La Trinidad, Maguey Verde and El Tepozan), representing the three geographical regions, were used to determine whether the duration of soaking and alternating temperatures influence the germination of *P. pinceana* seeds. To assess the effect of soaking on germination, seeds were imbibed in beakers containing 600 mL of de-ionized water at room temperature for 72 hours. Seed lots were divided equally between soaked and control treatments, but control seeds were not soaked. Seeds were placed on Kimpak moistened with 250 mL de-ionized water in Petawawa germination boxes. Seeds, arranged in eight replications with 50 seeds per replication for each population, were exposed to either 1) 15°C for 16 hours of darkness and 25°C for 8 hours of light, RH 80% or 2) 20°C for 16 hours of darkness and 30°C for 8 hours of light, RH 80%.

c) Influence of light, fungicide and cold treatment on seed germination

Fungus infestation was common and appeared to originate from the seed coats during the germination tests described above; therefore, seeds were treated with fungicide to determine whether the incidence of fungus affected germination. Seeds from the six populations noted above were used to test the effects of a fungicide treatment and light on seed germination. Seeds from 15 randomly chosen seed lots from each of the six populations were mixed. One-hundred seeds were placed in each of two types of germination boxes, 12 clear (light treatment) and 12 black, and 50 seeds were covered with peat moss in each box. Fifty of the seeds in each box were considered a replication for either the seeds covered with peat moss or the control seeds. Half the seeds were sprayed with a 0.8% Captan solution. All seeds in the 24 germination boxes were initially placed in the cold room at 3°C for 33 days, then germinated as previously described at 25°C for 8 hours of light, RH 80%.

d) Tetrazolium seed testing

Due to the lack of germination of seed from the same seedlots in the previous germination tests, a test was conducted to determine if the embryos in stored seed were viable. Seed collected from the following populations—SJ Carbonerillas, Santa Elena, Matehualilla, La Trinidad, Maguey Verde and San Cristobal—were used in the tetrazolium test to determine embryo viability. Initially, seeds were soaked for 24 hours at room temperature on Kimpak, dampened with 125 mL de-ionized water, in Petawawa germination boxes that contained an additional 125 mL of de-ionized water. Then seed coats and megagametophytes were removed using a scalpel. Embryos were placed on moist Kimpak to ensure that they did not dry out before tetrazolium testing. One hundred and sixty embryos were used for each population and embryos were arranged in 10 replications with 16 embryos per replication. Testing was carried out by immersing one embryo per well in a 96 Well Cell Culture plate (Corning Incorporated Life Sciences, Nagog Park Acton, MA, USA) with a 1% tetrazolium solution. Plates containing embryos were incubated at 30°C for 18 hours in the dark according to ISTA (2003). An embryo was scored as viable when the whole embryo turned red or non-viable when either the cotyledons, root or hypocotyl were colorless.

Assessment of the Role of the Seed Coat in Inhibiting Germination

a) Determination of seed coat thickness and force required to crack seed

Seed coat thickness and force required to crack *P. pinceana* seeds were determined using two different sets of seeds from 12 populations. Three hundred seeds from each of the 12 populations were used to determine the seed coat thickness. The seed coat was cut using a scalpel and thickness was determined by taking the average of measurements (in millimeters) at both the micropylar end and at the middle of the seed coat using a digital caliper. Determination of the force required to crack *P. pinceana* seeds was based on 60 seeds from each of the 12 populations. Each seed was placed between the two steel bars of a press used to test wood strength (Wood Science and Technology Laboratory at the University of New Brunswick). The force required to crack a seed, which is considered a measurement of seed coat hardness, was recorded in Newtons (N) by a computer connected to the press.

b) Germination test

Seeds from all 12 populations were included in a germination test (table 1) in which they were subjected to four treatments: (i) whole seed (control), (ii) de-coated seeds, (iii) de-coated seeds placed on half of their seed coats and (iv) seed coat cracked in the micropylar areas. Four replications of 25 seeds from each population were used in each treatment.

Seeds were sterilized in 2.56% sodium hypochlorite solution for 10 minutes and rinsed three times with de-ionized water to reduce fungus infestation. For the de-coated seeds, seeds were soaked for an hour in de-ionized water before the seed coats were removed using scalpel and tweezers, under sterile conditions. All seeds were germinated in a Conviron at 30°C/20°C, day/night temperature, 8 hours light with a constant relative humidity (RH) of 80%.

Germination was scored daily from day 5 to 20. The peak value (PV) was calculated according to the following equation (Kolotelo *et al.,* 2001):

PV= MAX (cumulative of Σ germination percentage/days).

The PV is a measurement of germination speed and mathematically it is the maximum proportion of cumulative germination percentage over days (Kolotelo *et al.,* 2001).

c) Distribution of genetic variation

To determine the distribution of the genetic variation among regions, populations and families, seeds from one tree were considered to be a half-sib family. De-coated seeds from 15 half-sib families from each of the 12 populations were included in this germination trial with five replications (five seeds per replication) per half-sib family. Whole seeds were sterilized as previously described before the seed coats were removed. De-coated seeds were placed in rows on dampened Kimpak in Petawawa clear germination boxes to keep the identity of each half-sib family. The environmental conditions in the Conviron were the same as those in the last experiment. Germination was scored daily from 4 (when germination was first evident) to 20 days.

Germination percentage, PV and germination value (GV) were calculated at the end of the experiment. The GV was the product of the PV and the mean daily germination (MDG, the total germination divided by the number of days in the experiment) (Czabator, 1962; Kolotelo *et al.,* 2001). Both PV and GV are estimates of seed vigor (Czabator, 1962; Kolotelo *et al.,* 2001).

Statistical Analysis

No percentage variables included in this study nor their arcsine transformation met the assumption of normality so they were transformed to their ranks before the analysis of variance was conducted using GLM-SAS/PC for Windows version 8.2 (SAS Institute Inc., 2001)

The analyses of variance were based on the following models.

Embryo viability:

[1] $Y_{ij} = \mu + P_i + \xi_{ij}$

where Y_{ij} = individual observation; μ = the overall mean; P_i = the population effect; and ξ_{ij} = Error.

Seed coat thickness and the force required to crack seed:

[2] $Y_{ijk} = \mu + R_i + P_{j(i)} + \xi_{ijk}$

where R_i = the region effect; and $P_{j(i)}$ = the population-within-region effect.

Germination percentage and peak value:

[3] $Y_{ijkl} = \mu + T_i + R_j + T_i*R_j + P_{k(j)} + T_i*P_{k(j)} + \xi_{ijkl}$

where T_i = the treatment effect; T_i*R_j = the treatment X region interaction effect; and $T_i*P_{k(j)}$ = the treatment X the population-within-region interaction effect.

Distribution of variance among family, population and region:

[4] $Y_{ijkl} = \mu + R_i + P_{j(i)} + F_{k(j\,i)} + \xi_{ijkl}$

where $F_{k(j\,i)}$ = the family-within-population-within-region effect.

For all of the analyses of variance, treatment and region were considered as fixed effects whereas population and family effects were considered to be random. Tukey's studentized range test was applied for comparisons of treatment, region and population means. The variance components were estimated using VARCOMP-SAS/PC for Windows version 8.2 (SAS Institute Inc., 2001) using the REML method which considered all effects in the model as random.

Individual heritability (h^2_i) was estimated using the following equation in which a conservative coefficient of relationship was considered (Squillace, 1974):

[5] $h^2_i = 3\sigma^2_{f(p\,r)}/[\sigma^2_e + \sigma^2_r + \sigma^2_{p(r)} + \sigma^2_{f(p\,r)}]$

where σ^2_e = plot variance; σ^2_r = region variance; $\sigma^2_{p(r)}$ = population-within-region variance; and $\sigma^2_{f(r\,p)}$ = family-within-population variance.

The standard error for heritability ($SE_{(h^2)}$) was estimated according to the procedure described by Falconer (1989).

[6] $SE_{(h^2)} = \sigma_{h^2}/(nN - 1)^{1/2}$

[7] $\sigma^2_{h^2} = 9\{2[1 + (n - 1)(h^2_i/3)]^2[1 - (h^2_i/3)]^2/[n(n - 1)(N - 1)]\}$

where σ_{h^2} = Standard deviation of the h^2_i; $\sigma^2_{h^2}$ = Variance of the h^2_i; N = Number of families; and n = Number of individuals per family.

Results

Water content

The mean initial water content of the seeds was 6% with a standard error (S.E.) of 0.05 and it varied from 6 to 7%. Immediately after water content determination, seeds were stored at 4°C in sealed plastic bags. After storage for 15 months, the initial water content of the seeds had a mean of 7% (S.E. = 0.13) and ranged from 6 to 8% (table 2). Therefore, the mean seed water content increased 1% after 15 months in storage.

Table 2. Seed water content of *P. pinceana* after seeds were stored for 15 months in plastic bags at 4°C.

Populations (Region)	Seed water content
S. J. Carbonerillas (North)	7.07±0.11†
Palmas Altas (North)	7.35±0.06
Matehualilla (Central)	7.79±0.05
Maguey Verde (South)	7.56±0.04
El Tepozan (South)	6.40±0.28
Mean	7.22±0.13

† Standard error

Germination Trials

Standard germination procedures for *Pinus* spp. yielded very low germination, averaging 11% over all populations and ranging from 0 for seed from the southern population at El Arenalito to 32% for Lomas Oregano in the north (table 3). Regions averaged 16% for seed from the north, 8% for the central region and 7% for seed from the south. Soaking and alternating temperature did not improve germination. The overall mean germination percentage was 5% varying from 3 to 9% in seeds from Palmas Altas and La Trinidad, respectively (table 4). Neither light nor covering the seeds with peat moss had a positive effect on germination and, although the fungicide prevented fungal growth on the seeds, there was no improvement in germination even when the seeds were also stratified for 33 days. The average percentage germination in these treatments was 1%.

Table 3. Germination percentage of *P. pinceana* seeds from 12 populations in the natural range of this species.

Populations	No. trees	Germination percentage
Northern region	30	15±3†
SJ Carbonerillas	5	24±11
Lomas Oregano	5	32±9
Santa Elena	5	10±6
Palmas Altas	5	12±7
El Cinco	5	6±6
El Recreo	5	8±6
Central region	10	8±5
Matehualilla	5	10±8
La Trinidad	5	6±6
Southern region	20	7±3
Maguey Verde	5	18±8
El Tepozan	5	6±4
El Arenalito	5	0±0
San Cristobal	5	4±4
Mean	60‡	11

†Standard error; ‡Total number of trees

Table 4. Mean germination percentage of *P. pinceana* seeds tested in two soaking treatments and two alternative temperatures.

Populations	No. trees	No soaking		Soaking		Mean
		15-25°C	20-30°C	15-25°C	20-30°C	
Northern Region						
SJ Carbonerillas	15	4±2†	12±1	1±1	2±1	5
Palmas Altas	15	2±1	0	6±3	4±2	3
Central Region						
Matehualilla	15	1±1	8±1	3±2	0	3
La Trinidad	15	1±1	7±1	1±1	26±4	9
Southern Region						
Maguey Verde	15	0	8±2	0	6±3	4
El Tepozan	15	2±1	3±2	8±3	4±2	4
Mean		2	6	3	7	
Overall mean	90‡	4		5		5

†Standard error; ‡Total number of trees

Tetrazolium staining indicated that the number of viable embryos was very high and did not differ significantly among populations (table 5). The average overall percentage of viable embryos was 98% and ranged from 97% to 99% for San Cristobal and Matehualilla, respectively (table 5). In addition, 90 excised embryos were placed in nutrient media and they were found to germinate quickly, forming healthy seedlings (results not shown). These results suggest that most embryos had the potential to germinate and that the low seed germination may result from seed dormancy.

Table 5. Viability percentage of *P. pinceana* embryos from six populations.

Populations	Stained Embryos	Unstained (%)			
	(%)	Cotyledons	Radicle	Hypocotyl	Embryos
Northern Region					
SJ Carbonerillas	99	1	1	0	0
Santa Elena	97	1	1	1	0
Central Region					
Matehualilla	99	0	1	0	0
La Trinidad	99	1	0	0	0
Southern Region					
Maguey Verde	99	0	0	0	1
San Cristobal.	97	0	3	1	1
Mean	98	1	1	0	0
Mean overall	98		2		0

Assessment of the Role of the Seed Coat in Inhibiting Germination

a) Seed coat thickness and force required to crack seed

Significant differences were detected in both seed coat thickness and force required to crack seeds between populations, but not among regions, using Tukey's studentized range test (table 6). The average seed coat thickness was 1091 μm and ranged from 1043 to 1163 μm for the northern and southern regions, respectively. Seeds from the southern region had significantly thicker seed coats than those from the other two regions.

Seed coat thickness ranged among populations from 982 to 1257 μm for seeds from El Cinco and El Tepozan, respectively. The mean seed coat thickness for seeds from three of the four populations within the southern region were significantly different from each other according to the Tukey's studentized range test (table 6) and these three populations in the southern region had significantly thicker seed coats, on average, than any of the other populations, although seeds from Maguey Verde had thinner seed coats than three of the northern populations. The only two populations included in this study from the central region, Matehualilla and La Trinidad, had seed coat thickness measurements that were clustered in one group by Tukey's studentized range test. Seed coat thickness for seeds from populations within the northern region varied between 982 and 1121 μm for seeds from El Cinco and SJ Carbonerillas, respectively.

Table 6. Mean thickness of the seed coat and force required to crack *P. pinceana* seeds.

Populations	No. trees	Thickness (μm)	Force (Newton)
Northern region	90	1043A	292A
SJ Carbonerilla	15	1121c	285de
Lomas Oregano	15	989f	297bcd
Santa Elena	15	1060de	290cd
Palmas Altas	15	1094cd	321bc
El Cinco	15	982f	254e
El Recreo	15	1007f	303bcd
Central region	30	1093A	320A
Matehualilla	15	1085cde	329b
La Trinidad	15	1101c	312bcd
Southern region	60	1163A	327A
Maguey Verde	15	1051e	304bcd
El Tepozan	15	1257a	370a
El Arenalito	15	1187b	301bcd
San Cristobal	15	1160b	332b
Overall mean	180‡	1091	309

† Within columns, means with the same letter are not significantly different among regions (upper-case letters) and within regions (lower letters) according to Tukey's studentized range test (0.05); ‡Total number of trees

The overall mean force required to crack *P. pinceana* seeds was 309 N and ranged between 292 and 327 N for seeds from northern and southern regions, respectively. The mean force required to crack seeds among regions was not significantly different (table 6). The mean ranged among populations from 254 to 370 N for seeds from El Cinco and El Tepozan, respectively, and significant differences were found among populations in both northern and southern regions. The force required to crack the seed was considered a measure of hardness, indicating that seeds from El Tepozan are the hardest. The correlation between seed coat thickness and amount of force required to crack the seed was $r = 0.73$ ($P < 0.01$), based on population average values, pooling data from the same six families, so about 50% of the variation in the force required to crack the seed can be explained by seed coat thickness differences at the population level.

Both seed coat thickness and force required to crack the seed showed an inverse relationship with germination of intact seed at the population level (r = -0.44 and -0.24, respectively), but correlations at the population mean level were not statistically significant. Germination percentages for intact seed were very low and dropped over time, possibly reflecting continued hardening of the seed coat as a result of gradual loss of moisture. Whole seed was included in subsequent germination trials as a control, but conclusions could not be drawn from differences among populations because of the very low germination values and rank changes, in some cases, from one trial to the next over time.

b) Germination test: whole, cracked, de-coated and de-coated seed placed on half their seed coats

Germination percentages of whole seeds from the three regions were lower than those observed in the initial test (carried out 16 months earlier) and varied from 0% to 25% for seeds from Matehualilla and El Cinco, respectively (table 7).

There were significant differences among treatments and populations within regions ($\alpha < 0.01$), but not among regions. The mean germination percentage over all populations varied between 9% and 86% for whole seeds and de-coated seeds, respectively, whereas de-coated seeds placed on half their seed coats and seeds cracked in the micropylar area were intermediate, exhibiting 78% and 59% germination, respectively (table 7).

Within each treatment, the mean germination percentages for populations and regions were clustered in different groups by Tukey's studentized range test (table 7). Germination percentages for de-coated seeds, from the three regions, were not significantly different, with means varying from 82% to 90% for southern and northern regions, respectively. However, germination percentages for de-coated seeds from the 12 populations did not differ significantly, although they varied from 77% for San Cristobal to 96% for El Recreo.

The other treatments were more variable among regions and populations than de-coated seeds. The mean germination percentage for de-coated seeds placed on half of their seed coats from the three regions did not differ significantly. The mean germination percentage for de-coated seeds placed on half of their seed coats ranged among regions from 71% for the southern region to 88% for central regions. The 12 populations were clustered in four groups. The mean germination percentage for this treatment varied among populations from 54% for SJ Carbonerillas to 97% for La Trinidad.

Table 7. Germination percentage of *P. pinceana* seeds from 12 populations in three regions of Mexico (WSC= whole seeds; DCS= de-coated seeds; DHS= de-coated seeds placed on half of their seed coats; CSC = cracked seed coat in the micropylar area).

Populations	No. trees	Germination percentage (%)			
		WSC	DCS	DHS	CSC
Northern region	90	11	90A†	80A	67A
SJ Carbonerilla	15	4	92a	54d	70abc
Lomas Oregano	15	2	86a	83abc	44cd
Santa Elena	15	18	83a	86ab	84a
Palmas Altas	15	5	93a	82abcd	69ab
El Cinco	15	25	89a	84abc	69ab
El Recreo	15	14	96a	93ab	66abc
Central region	30	1	84A	88A	62A
Matehualilla	15	0	84a	78cd	54bcd
La Trinidad	15	1	84a	97a	70ab
Southern region	60	10	82A	71B	46A
Maguey Verde	15	5	79a	86ab	51bcd
El Tepozan	15	24	84a	80abcd	46bcd
El Arenalito	15	4	87a	60cd	60abc
San Cristobal	15	5	77a	56d	28d
Overall mean	180‡	9d	86a	78b	59c

† Within columns, means with the same letter are not significantly different among regions (upper-case letters) and within regions (small-case letters) according to Tukey's studentized range test (0.05). Within the last row, overall means with same letter are not significantly different among treatments (lower-case letters) according to Tukey's studentized range test (0.05); ‡Total number of trees.

Mean germination percentages, by region, for seeds cracked in their micropylar areas varied from 46% to 67% for the southern and northern regions, respectively. The populations were clustered in four groups (table 7) and mean germination percentages varied from 28% to 84% for seeds from San Cristobal and Santa Elena, respectively.

The peak values were significantly different ($\alpha < 0.01$) among all four treatments and populations within regions, but were not significantly different among regions (table 8). The mean peak value (speed of germination) of de-coated seeds was the highest, at 8.75, followed by de-coated seeds placed on half of their seed coats, with a peak value of 6.00 (figures 1 and 2; table 8).

It was found that 69% of de-coated seeds germinated after 8 days, 64% of de-coated seeds placed on half of their seed coat germinated after 11 days and 54% of seeds that were cracked in the micropylar area germinated after 16 days (figures 1 and 2). Peak values for whole seeds were very low, ranging from 0.03 to 0.73 for the central and northern regions, respectively. De-coated seeds from the central region germinated faster than those from the other two regions. Population peak values for de-coated seeds were clustered in three groups and varied from 6.42 for Palmas Altas to 11.00 for Maguey Verde.

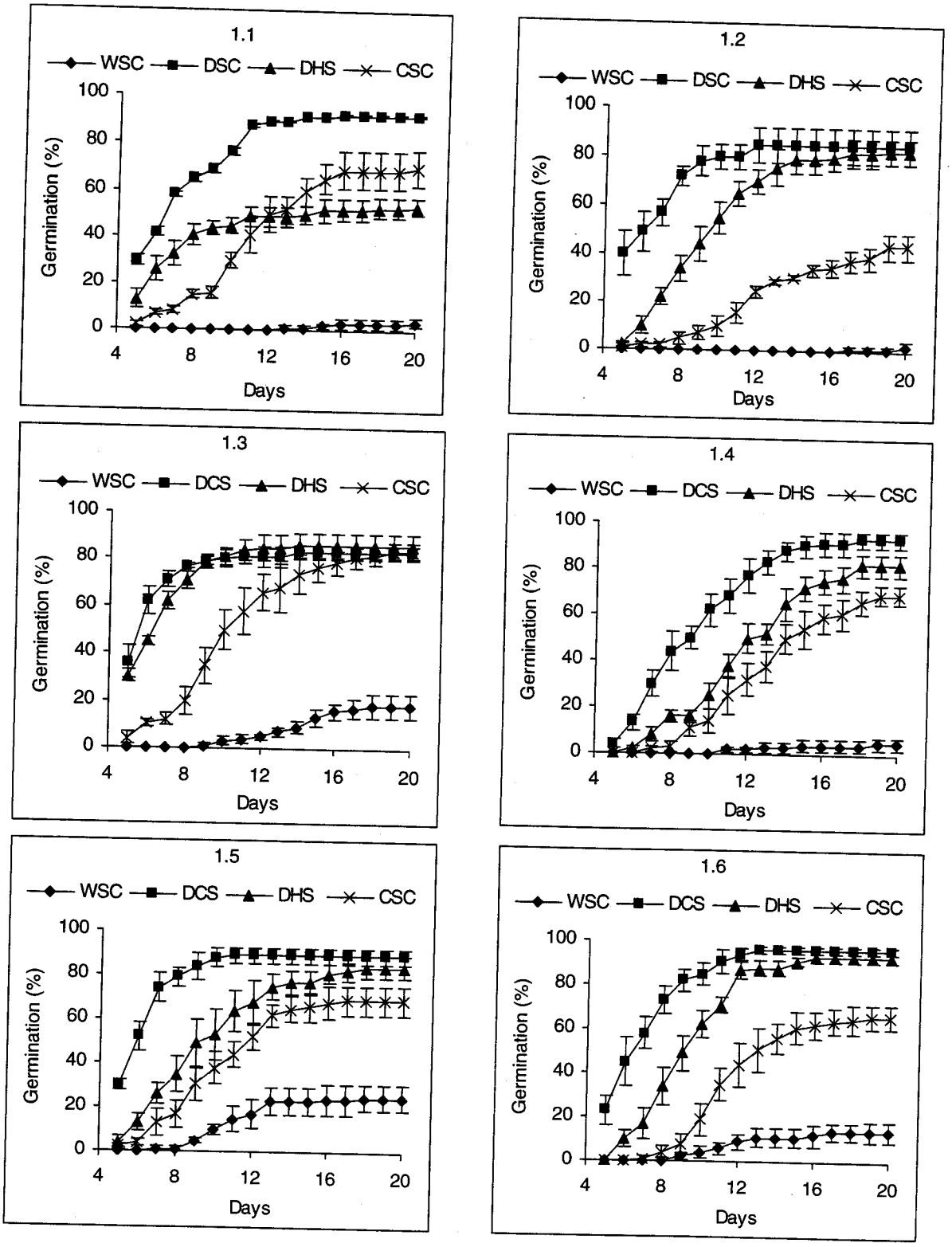

Figure 1. Cumulative germination of *P. pinceana* seeds from 1.1) SJ Carbonerilla, 1.2) Lomas Oregano, 1.3) Santa Elena, 1.4) Palmas Altas, 1.5) El Cinco and 1.6) El Recreo from the northern region subjected to four treatments (DSC= de-coated seed (square), DHS= de-coated seeds placed on half of their seed coat (triangle), CSC= cracked at the micropylar area (X) and WSC= whole seed (diamond)).

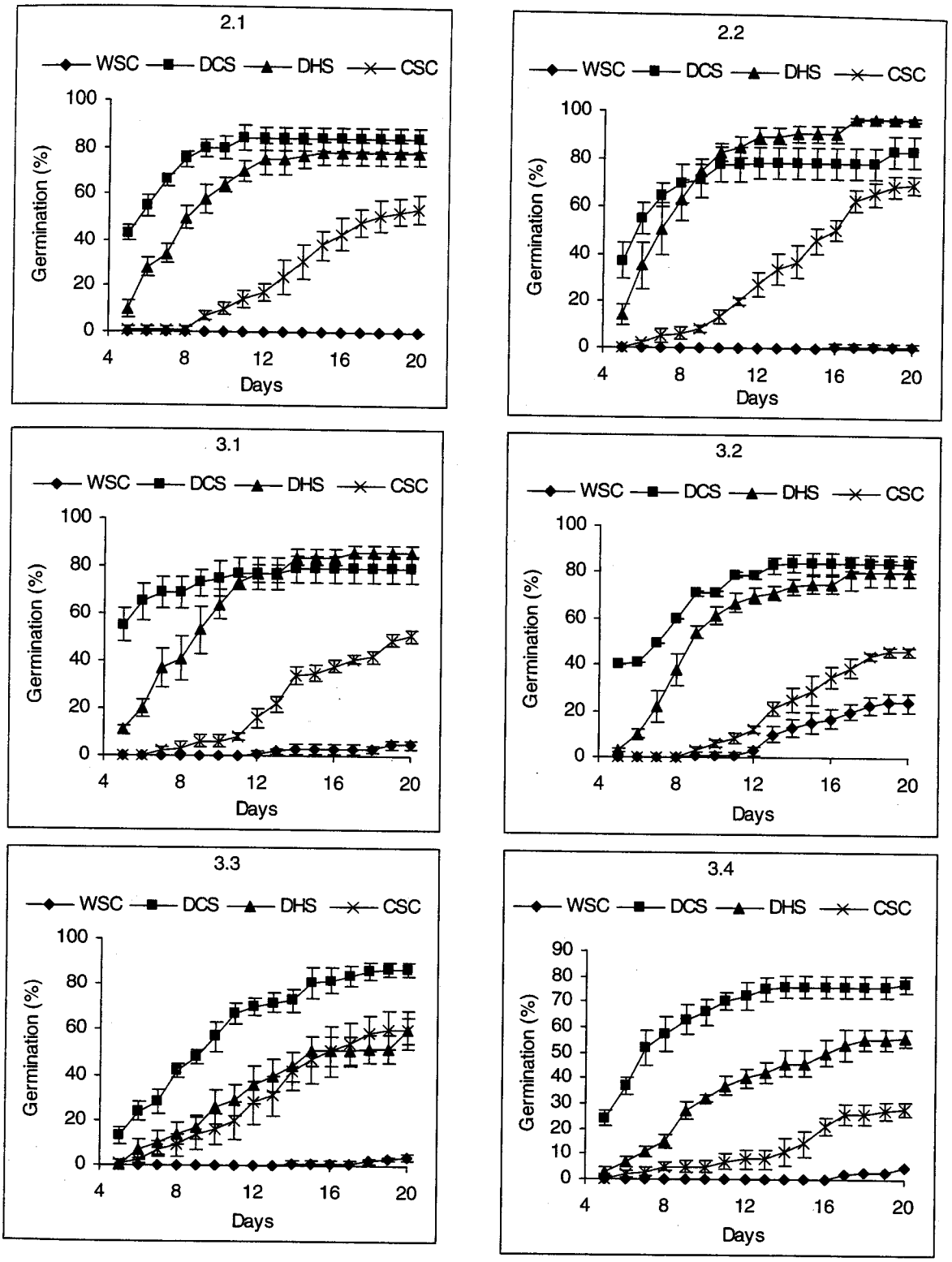

Figure 2. Cumulative germination of *P. pinceana* seeds from 2.1) Matehualilla and 2.2) La Trinidad from the central region; 3.1) Maguey Verde, 3.2) El Tepozan 3.3) El Arenalito and 3.4) San Cristobal from the southern region subjected to four treatments (DSC= de-coated seed (square), DHS= de-coated seeds placed on half of their seed coats (triangle), CSC= crack at the micropylar area (X) and WSC= whole seed (diamond)).

Table 8. Peak values of *P. pinceana* seeds from 12 populations in three regions of Mexico; (WSC= whole seeds; DCS= de-coated seeds; DHS= de-coated seeds placed on half of their seed coats; CSC = cracked seed coat in the micropylar area).

Populations	No. trees	Peak value			
		WSC	DCS	DHS	CSC
Northern region	90	0.72	8.98A†	6.29A	4.14A
SJ Carbonerilla	15	0.20	7.90abc	5.13def	4.33abc
Lomas Oregano	15	0.10	9.00abc	5.91cde	2.32de
Santa Elena	15	1.00	10.33a	8.88a	5.50a
Palmas Altas	15	0.27	6.42bc	4.80ef	3.69abcd
El Cinco	15	1.92	10.57a	5.77cdef	4.92ab
El Recreo	15	0.85	9.63abc	7.25abc	4.07abc
Central region	30	0.03	9.36A	7.39A	3.27A
Matehualilla	15	0.00	9.43ab	6.44bcde	2.83bcde
La Trinidad	15	0.06	9.29abc	8.33ab	3.71abcd
Southern region	60	0.50	8.10A	4.88A	2.45A
Maguey Verde	15	0.26	11.00a	6.64abcd	2.58cde
El Tepozan	15	1.28	7.89abc	6.10bcde	2.44cde
El Arenalito	15	0.20	6.09c	3.40f	3.28abcde
San Cristobal	15	0.25	7.43abc	3.36f	1.53e
Overall mean	180‡	0.53d	8.75a	6.00b	3.43c

† Means among regions were not significantly different at $p < 0.05$. Within columns, means with the same letter are not significantly different within regions (small-case letters). Within the last row, overall means with same letter are not significantly different among treatments (lower-case letters) according to Tukey's studentized range test (0.05). ‡Total number of trees.

The mean peak value for de-coated seeds on half seed coats varied between 4.88 and 7.39 for the southern and central regions, respectively. The mean peak value for seeds cracked in their micropylar areas varied between 2.45 and 4.14 for southern and northern regions, respectively. Populations were clustered in six groups for de-coated seeds on half seed coats and five groups for seeds cracked in their micropylar areas. Peak values varied between 3.36 and 8.88 for de-coated seeds on half-seed coats from San Cristobal and Santa Elena, respectively and from 1.53 to 5.50 for seeds that were cracked in the micropylar area from the same two populations.

c) Distribution of genetic variation

The germination percentage, peak and germination values were all significantly different among populations within regions and families within populations but they were not significantly different among regions (table 9). The variation in germination percentage among families within populations was 16.5%, whereas the variation among populations within regions and among regions was 2.3 and 3.7%, respectively, of the total variation. The variation in peak value among families within populations, populations within regions and regions was 23.7%, 6.9% and 7.4%, respectively, of the total variation (table 9).

Families within populations, populations within regions and regions accounted for 21.7%, 12.4% and 7.6%, respectively, of the total variation in germination value.

The individual heritability for germination percentage was 0.49, indicating that this germination parameter is under strong genetic control. Heritabilities for peak value and germination value were under stronger genetic control, at 0.71 and 0.65, respectively (table 9).

Table 9. Variance components and individual heritability (h^2_i) for germination in de-coated seeds of *P. pinceana*.

Germination parameter	Variance Components as % of the σ^2_T					h^2_i ±S.E.†
	σ^2_T	σ^2_e	σ^2_r	$\sigma^2_{p(r)}$	$\sigma^2_{f(r\,p)}$	
		(716)‡	(2)	(9)	(167)	
Germination percentage	36732	77.6	3.7ns	2.1*	16.5**	0.49±0.004
Peak value	68667	62.5	6.5ns	7.4**	23.7**	0.71±0.004
Germination value	70002	58.3	7.6ns	12.4**	21.7**	0.65±0.004

†S.E. = Standard error; ‡ = Degrees of freedom; * = significant at $\alpha < 0.05$, ** = significant at $\alpha < 0.01$ and ns = non-significant assessed using GLM (SAS);
σ^2_T = total variance = $\sigma^2_e + \sigma^2_r + \sigma^2_{p(r)} + \sigma^2_{f(r\,p)}$; σ^2_e = plot variance; σ^2_r = region variance; $\sigma^2_{p(r)}$ = population-within-region variance; $\sigma^2_{f(r\,p)}$ = family-within-population variance;

Discussion

Germination of intact *Pinus pinceana* seeds determined in the preliminary germination trials was very low. Embryos were viable as demonstrated by the tetrazolium test and by the results of tests removing or damaging the seed coats. Potential causes for poor germination of intact seed were deduced to be the seed coat thickness and hardness, which constitute a mechanical barrier to germination; the presence of one or more germination inhibitory compounds in either the papery membrane or in the seed coat itself; or both. *Pinus pinceana* seeds had thicker seed coats (averaging 1.1 mm) than seeds of most other Mexican piñon species. For example, seed coat thickness ranged from 500 to 1000 µm for *P. cembroides* seeds and between 100 and 400 µm for *P. remota* (Little) Bailey et Hawksworth seeds (Perry, 1991).

Mechanical constraints imposed by the seed coat have been shown to play an important role in delaying germination in *Pinus elliottii* Englem., *P. echinata* Mill., *P. sondereggeri* H.H. Chapm. and *P. taeda* L. (Barnett, 1972; 1976). The seed coats in *P. taeda* seeds restricted the swelling of the megagametophyte and embryo, so germination was inhibited because seeds could not imbibe the quantity of water necessary to induce germination (Barnet, 1976). Mechanical restriction and germination inhibitors also delayed germination in *Chamaecyparis nootkatensis* (D. Don) Spach seeds (Ren and Kermode, 1999).

Both the seed coat and the papery membrane have been shown to play significant roles in preventing germination in *P. taeda* seeds (Cooke *et al.*, 2002). The papery membrane also inhibits germination in *P. lambertiana* Dougl., *P. monticola* Dougl. and *P. sylvestris* L. seeds (Baron, 1978; Hoff, 1987; Tillman-Sutela and Kauppi, 1995).

The papery membranes and the nucellar caps were attached to the megagametophytes and embryos in seeds with seed coats removed and they appeared not to influence germination in *P. pinceana* seeds. Germination of seed with the seed coat removed on growth medium was high (93%). Thus, by a process of elimination, the seed coat was responsible for preventing germination.

The results of the germination experiments indicated that both mechanical restriction imposed by the seed coat and chemical germination inhibitors on the seed coat were factors in preventing germination. If germination failure of whole seeds was caused only by mechanical restriction, germination of de-coated seeds placed on their half seed coat would have been expected to be as high as that of de-coated seeds. The difference between the germination percentages for de-coated seeds, de-coated seeds placed on half seed coats and seeds cracked in micropylar areas could be produced by germination inhibitors in the seed coats. If germination inhibitors were the only cause, however, the seeds cracked in micropylar areas would be expected to germinate at the same low rate as whole seeds. The significantly lower germination in whole seeds provides evidence that seed coats also mechanically restrict germination in *P. pinceana* seeds.

Seed coats may influence gas exchange in *P. pinceana* seeds because the germination percentages in seeds that were cracked on their micropylar areas were significantly different from the germination percentages in whole seeds. Although dry intact seed coats of *P. pinceana* seeds could be permeable for oxygen uptake, once water was imbibed by whole seeds and seed coats were not cracked by the swelling of the megagametophytes and embryos, water occupied the empty cavities in the covering seed tissues so that seed coats and water could be barriers to oxygen supply to *P. pinceana* embryos. Cracking the seed coat on the micropylar areas in *P. pinceana* seeds could increase the oxygen uptake of the embryos. The seed coat was found to be a barrier for gas exchange and oxygen uptake, preventing germination in *P. jeffreyi* Grev. & Balf., *P. strobus* L. and *P. taeda* (Stone, 1957; Kozlowski and Gentile, 1959; Barnett, 1976).

Germination in seeds with hard thick seed coats is enhanced by scarification (Bewley and Black, 1985). Cracking or removing the seed coat was the only treatment that promoted germination in *P. pinceana* seeds. Cracking or removing the seed coat also enhances germination in seeds of several other pines. For example, removing the seed coat improved germination in *P. strobus* seeds (Kozlowski and Gentile, 1959). Both stratification and removing the seed coat were effective treatments to overcome dormancy in *P. jeffreyi*, *P. taeda,* and *P. lambertiana* seeds (Stone, 1957; Carpita *et al.,* 1983; Baron, 1978). *Pinus lambertiana* seeds exhibiting seed coat-imposed dormancy took 10 to 14 days to complete germination when the seed coat was removed (Taylor and Wareing, 1979). Germination percentage for *P. monticola* in stratified seeds and de-coated seeds was 87%, whereas the germination percentage in whole seeds of this species was 7% (Hoff, 1987).

In nature, once dispersed, *P. pinceana* seeds are exposed to wide differences in daily diurnal temperatures. The alternating temperatures promote germination in *Bidens tripartitus* L. and *Nicotiana tabacum* L., which show coat-imposed dormancy (Bewley and Black, 1985). Although daily alternating temperatures may soften *P. pinceana* seed coats, germination was not promoted by alternating temperature in the present study. Moreover,

P. pinceana seeds are an important source of food for birds and rodents, which crack the seeds before they eat them. Birds and rodents undoubtedly drop some cracked seeds that may germinate when environmental conditions are suitable.

The difference in the peak value between de-coated seeds and de-coated seeds placed on their half seed coats could be caused by germination inhibitory compounds present in the seed coats. These results suggest that inhibitory compounds influenced the germination rate in *P. pinceana* seeds. The rate of germination in *P. pinceana* seeds cracked in the micropylar area was strongly influenced by the seed coat, indicating that inhibitors on the seed coat likely had a major influence on germination. The rate of germination is very important for seedling survival in semiarid regions where moisture is a limiting factor (Kigel, 1995; Allen and Meyer, 1998). Germination rate also has a practical significance in nursery culture as it influences the uniformity of seedling development and the variation in their size at the end of the nursery period (Kolotelo *et al.,* 2001). The germination value is a measurement of seed vigor (Kolotelo *et al.,* 2001), so seeds from the central region had the highest quality, as indicated by the peak and germination values of de-coated seeds.

Removing seed coats can be very tedious and the scalpel method is time consuming, limiting its practicality in nursery production of seedlings. Two methods were tested to crack the seeds using tools that are readily available in the areas where *P. pinceana* grows naturally: hand-held pliers and a vise. Germination was better when the seed coats were removed using pliers than with a vise (results not shown), because the individual attention given each seed results in less damage. However, removing the seed coats using a vise is faster than using pliers and the reduced germination percentage may be an acceptable loss in return for greater efficiency. On average, 43 seeds were cracked each time with the vise. Placing seeds on the vise and cracking them took approximately 7 min. In the same period of time, on average, only 20 seeds were cracked using pliers.

The germination traits were found to be highly heritable and this is typical of a number of conifers. Reported heritabilities for germination percentage in *Tsuga mertensiana* seeds varied between 0.35 and 0.73 (El-Kassaby and Edwards, 1998). Broad-sense heritabilities for germination percentage, peak value and germination value were found to be 0.92, 0.91 and 0.93, respectively, in *Pseudotsuga menziesii* (El-Kassaby *et al.,* 1993). Estimated broad-sense heritabilities for germination percentage, peak value and germination value were 0.79, 0.76 and 0.77, respectively, in *Picea sitchensis* (Chaisurisri *et al.,* 1992). The narrow-sense heritability for germination was reported at 0.72 in *Pinus leucodermis* (Giannini and Bellari, 1995).

It should be noted that the germination percentages for whole seeds from some populations varied in some of the experiments. For example, results presented in tables 3 and 7 were different. The data that were presented in table 3 were collected 16 months before the data in table 7. The environmental conditions were different (data presented in table 3 were collected from seeds germinated at 25°C/15°C day/night and data presented in table 7 were collected from seeds germinated at 30°C/20°C, day/night) and perhaps hardening of the seed coat also continued during the storage and influenced germination of intact seeds. However, germination was irregular among populations. Although the germination percentage decreased for whole seeds from SJ Carbonerillas and Lomas

Oregano included in table 3, germination percentage increased for whole seeds from Santa Elena, El Cinco and El Tepozan included in table 7. Each treatment that cracked or removed the seed coats improved the germination of *P. pinceana* seeds. Complete removal of the seed coat promoted the highest germination.

Conclusions and recommendations

The inability of *P. pinceana* seeds to germinate was caused by the seed coat. Germination inhibitors and, especially, mechanical restriction imposed by the seed coat were the major factors that prevented germination in *P. pinceana.* Removing or cracking the seed coat enhanced germination. Family within population within region was the highest source of variation, indicating that germination in *P. pinceana* is under strong genetic control. To understand the ecology of this species, it will be necessary to study how the *P. pinceana* seeds become dormant and the environmental factors that promote their germination in nature.

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