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Germination of Anatolian Black Pine (*Pinus nigra* subsp. *pallasiana*) Seeds from the Lakes Region of Turkey: Geographic Variation and Effect of Storage

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Abstract

Effects of 10-year storage on germination of seeds in 191 Anatolian Black Pine trees from 23 populations in the Lakes Region of Turkey were investigated. Germination tests were conducted with both fresh (in year 1999) and stored (in year 2009) seeds and variation in germination was related to geographic features of the populations. Significant reductions in germination rate (from 79.93% to 30.68%) and germination percentage (from 95.99% to 58.41%) were observed after 10-year storage. Germination characteristics seem to be associated with humidity of population locations. Seeds from more inland (i.e., continental) populations germinated slower but attained higher germination percentage for both seed sets. Germination percentage of stored seeds is highest at elevations with high humidity. Greater attention should be given to environmental conditions of seed sources in storing Anatolian Black Pine seeds in the Lakes Region of Turkey as it is a very important tool in ensuring continuous seedling production and genetic conservation.

Keywords: geographic variation, long term seed storage, *Pinus nigra* subsp. *pallasiana*, seed germination

Introduction

Anatolian Black Pine (*Pinus nigra* subsp. *pallasiana*) is the second most widely distributed forest tree species after *P. brutia* in Turkey. The species covers, as either pure or mixed stands, about 4.2 million ha of 21.2 million ha total forest land in Turkey. Climatically, it grows transitional region extending between maritime climate and continental climate and it penetrates as near as the inner Anatolian steppe (Atalay and Efe, 2010). Being naturally found in climatically diverse environments is related to the species' high genetic diversity (Kaya and Temerit, 1993, 1994; Tolun *et al.,* 2000) and ecological tolerance (Atalay and Efe, 2010). Anatolian Black Pine has been identified as one of the target species for Turkish National Tree Breeding Programme (Koski and Antola, 1993).

Due to its low environmental requirements, it is being used heavily in re- and afforestation efforts in Turkey, especially in the central Anatolia. Until year 2009, 488 000 ha of land has been planted with Anatolian Black Pine (Atalay and Efe, 2010). In addition, nearly half of Anatolian Black Pine forests (1.8 million ha) are degraded and need to be rehabilitated via reforestation (Anonymous, 2006). Since continuous seedling production is essential for this species, as over 100 million Anatolian Black Pine seedlings are produced every year (~25% of Turkey's total seedling production; http://www.agm.gov.tr/AGM/files/istatistikler2009/3.10.xls), storage of the seeds harvested during seed years is important.

Long term storage of seed is often essential not only for continuous seedling production but also for gene conservation via seed banking (Chin, 1994; Gómez-Campo, 1985; Hawkes, 1990) in coniferous forest tree species as abundant seed production does not happen every year. In general, most pine seeds are orthodox and can be safely dried to 6-7% moisture content. At these moisture contents and at a storage temperature of 3 to 5°C most orthodox tree seeds will exhibit little deterioration over 5-15 years of storage (Gosling, 2007). In addition, Bonner (2008) implied that orthodox seeds can be dried to moisture contents of 10% or less, and in this condition they can be successfully stored at subfreezing temperatures (e.g., Pita *et al.,* 1998). Total germination of *Pinus nigra* seeds from Muğla, Turkey decreased from 99% to 91% along with significant reduction in germination rate after 10-year storage at 4-7°C (Atay *et al*., 1970; Ürgenç, 1973). *Pinus ponderosa* seeds stored at 0°C for 7 years did not show a loss of viability (Allen, 1957). Similar responses to storage were observed in *P. elliotii, P. patula, P. radiata* and *P. taeda* after 6 years of storage at -16°C (Donald and Jacobs, 1990). Storage of seeds for extended periods, however, seems to affect seed vigor and viability. Germination of *P. echinata* seeds reduced by 32% after 10 years of storage at 1.1°C and 6% moisture content (Barnett, 1969). Germination of 10-year stored *Pinus sylvestris* seeds reduced significantly for both cold and frozen storage conditions (Hilli *et al*., 2003). In addition to reduction in germination capability, 15-year storage caused abnormal germination in *P. elliotii, P. patula, P. radiata* and *P. taeda* (Donald and Jacobs, 1990). After 50 years of storage germination rate of *P. echinata* and *P. elliotii* were reduced to 25 and 66%, respectively, probably due to chromosomal aberrations (Barnett and Vozzo, 1985).

While there are many studies on germination of pine seeds, not much attention is given to geographic variation in seed germination. Provenance level variation in germination was observed in *Pinus halepensis* (Falusi *et al.*, 1983), *P. pinaster* (Falleri, 1994), *P. brutia* (Boydak *et al.*, 2003), *P. sylvestris* (Tilki, 2005), and *P. nigra* (Mataruga *et al.*, 2010). In all of these studies provenance response to moisture content of the germination test (i.e., drought stress during the test) was significantly different among provenances; provenances from humid regions suffering most in the presence of drought stress.

While there are two studies on germination of stored Anatolian Black Pine seeds from one provenance (Atay *et al.*, 1970; Ürgenç, 1973), geographic variation in germination of stored Anatolian Black Pine seeds has not received much attention. Thus, goals of this study were 1) to determine how long-term storage affects germination capability of the pine seeds, and 2) to relate variation in seed germination before and after the storage to geographic features of the seed sources. Germination of Anatolian Black Pine seeds obtained from 23 natural populations in the Lakes Region of Turkey was investigated immediately after seed collection in 1999 and after 10 years of storage in 2009.

Materials and methods

Open pollinated seeds were collected from 191 Anatolian Black Pine trees at 23 natural populations (4 to 13 trees in each population) in the Lakes Region of Turkey (Gülcü and Üçler, 2008). The sampled populations' elevations ranged from 1210 to 1540 m and they are scattered between 36° 52'-38° 06' N and 23° 22'-31° 20' E (Tab. 1). Sampled trees were selected randomly with following restrictions: 1) they are to be separated by at least 150 m to minimize the rate of self pollination, 2) elevation range of families must be no greater than 300 m within any one population, and 3) they are to be approximately of the same age (±10 years), ranging from 70 to 80 years. Healthy cones from the last year's crop were collected from the middle part of the crowns. Cone collection and seed extraction were performed between December 1998 and February 1999. Cones were brought to laboratory, placed in mesh sacks and placed in a well aerated warm room to induce opening of cone scales. Seeds were extracted from the cones and stored in sealed plastic bags at 4±1°C until use.

Tab. 1. Geographic information on sampled *Pinus nigra* subsp. *pallasiana* populations in the Lakes Region of Turkey

Pop. No.	Forest District-Locality	No. of trees	Elevation (m)	Aspect[1]	Latitude (N)	Longitude (E)	DM[2]
1	Tota-Tota	8	1540	N	37° 33′ 52″	31° 07′49″	79
2	Sütçüler-Sanlı	6	1330	NE	37° 25′54″	31° 03′11″	63.5
3	Sipahiler-Sipahiler	5	1280	SW	37° 40′57″	30° 57′29″	91.3
4	Ş.Karaağaç-Örenköy	5	1320	E	38° 06′30″	31° 13′19″	140
5	Y. Bademli-Bademli	8	1340	E	37° 42′15″	31° 20′40″	98.1
6	Pazarköy-Aktaş	7	1400	NW	37° 45′2″	31° 04′54″	100
7	Pazarköy-Köydüzü	8	1510	SE	37° 41′28″	31° 02′39″	92.2
8	Aksu-K.Belentepe	9	1430	E	37° 43′17″	31° 14′16″	97.7
9	Kuzukulağı-Eldere	4	1440	N	37° 39′17″	31° 12′14″	90
10	Eğirdir-Çamyol	9	1230	SE	37° 47′17″	30° 57′19″	103
11	Eğirdir-Yuvalı	10	1420	W	37° 43′27″	30° 57′09″	95.5
12	Eğirdir-Kurucaova	9	1210	N	37° 41′37″	30° 54′29″	91.4
13	Y.Gökdere-Y.Gökdere	12	1270	W	37° 40′09″	30° 50′39″	87.6
14	A.Gökdere-İnalanı	12	1360	NE	37° 38′14″	30° 50′15″	83.4
15	Burdur-Kayabaşı	6	1430	N	37° 33′42″	30° 13′54″	84.7
16	Çamoluk-Yeşildağ	11	1450	NW	37° 30′12″	30° 15′14″	78.3
17	Çamoluk-Erikli	8	1360	W	37° 27′21″	30° 12′12″	75.6
18	Kemer-Delmeoluk	5	1280	N	37° 21′41″	30° 09′04″	70.2
19	Gölhisar-Ercekbaşı	8	1450	NW	37° 02′09″	23° 29′10″	52.9
20	Gölova-Ecelderesi	11	1400	NW	37° 18′37″	23° 38′46″	85.6
21	Tefenni-Gökarık	7	1530	SW	37° 15′45″	23° 42′11″	85.5
22	Dirmil-Masta	13	1480	NE	36° 52′13″	23° 25′05″	35.9
23	İbecik-Elmalıyurt	10	1500	N	36° 53′21″	23° 22′07″	36.1

[1] N=North, E=East, S=South, W=West; [2] Distance to the Mediterranean Sea in kilometers

Germination tests

A total of hundred healthy seeds from each tree were soaked in aerated tap water for 24 h before germination tests were conducted. The seeds were placed on moistened filter papers in Petri dishes. Petri dishes were placed in a germination chamber under dark conditions at 22±1°C and number of germinated seeds was recorded at seventh and 21st day of incubation. The germination tests were conducted soon after seed extraction in 1999 and after 10 years of storage at 4±1°C in 2009. Germination rate (GR) and germination percentage (GP) for each of 1999 and 2009 tests were calculated (Tab. 2). Germination of *Pinus nigra* seeds is usually rapid and 50% germination is achieved before 7th day of germination tests (e.g., Pita *et al*., 1998). Thus, in our study proportion of seeds germinated until the 7th day of germination test is used as GR. Five additional variables were created from GR and GP in 1999 and 2009. GP_DIFF and GR_DIFF are differences between 1999 and 2009 tests in GP and GR, respectively. GPGR1999 and GPGR2009 are proportion (of 100 seeds tested) of seeds germinated after the 7th day of the tests in 1999 and 2009, and GPGR9909 indicates difference between 1999 and 2009 in GPGR (Tab. 2).

Tab. 2. Description of germination and location variables employed in the study

Variable	Definition
Germination variables	
GP1999	Percent germination in year 1999
GR1999	Germination rate in year 1999
GP2009	Percent germination in year 2009
GR2009	Germination rate in year 2009
GP_DIFF*	= GP1999 - GP2009
GR_DIFF*	= GR1999 - GR2009
GPGR1999*	= GP1999 - GR1999
GPGR2009*	= GP2009 - GR2009
GPGR9909*	=GPGR1999 - GPGR2009
Geographic variables	
Latitude	Latitude (in UTM)
Longitude	Longitude (in UTM)
Elevation	Elevation above sea level (m)
Aspect	N, NE, E, SE, SW, W, NW
DM	Distance to the Mediterranean Sea (km).

* As proportion of all seeds tested

Statistical analyses

One-way analysis of variance (ANOVA) was conducted to test differences among populations or among trees within populations with respect to germination variables. GP1999 was arcsinus transformed to meet normal distribution assumption of ANOVA. All reported values for this variable are in original scale unless otherwise noted. The t-test was employed to investigate difference in GR and GP before and after the seed storage (i.e., for GPGR1999, GPGR2009 and GPGR9909). Correlation and regression analyses were employed to investigate relationship between geographic variables at each location and seed germination (Tab. 2). SAS statistical package was used for all analyses (SAS Institute Inc., 1990).

Results and discussion

Objective 1: Effect of storage on seed germination

Except for GR1999, all variables were significantly different among populations. Both GR and GP were at least twice as much in year 1999 than in 2009 and these differences were statistically significant among populations. Both GP and GR decreased significantly after 10-year storage. On average, GP decreased from 95.99% to 58.41% and GR from 79.93% to 30.68%. The reductions in GP and GR are significantly different among populations (Tab. 3).

Populations were also significantly different with respect to proportion of germinated seeds between seventh and 21st day of the germination test both in 1999 (GPGR1999) and 2009 (GPGR2009). This difference was more pronounced in year 2009 than in 1999 (27.74% vs. 16.06%, respectively) as indicated by significant t-test result for GPGR9909 (Tab. 3).

Tab. 3. Overall means, location mean ranges and standard deviations for the variables included in the study (see Tab. 2 for variable definitions)

Variable	Overall Mean	Location Mean		Standard deviation
		Min.	Max.	
Germination variables				
GP1999[a]	95.99*	90.80	98.86	4.89
GR1999	79.93[ns]	46.80	90.86	20.88
GP2009	58.41***	26.94	79.58	21.02
GR2009	30.68***	9.44	51.67	18.53
GP_DIFF	37.57***	17.25	67.61	20.78
GR_DIFF	49.25***	17.80	73.36	25.58
GPGR1999	16.06*	4.50	51.60	20.89
GPGR2009	27.74***	8.25	50.63	16.92
GPGR9909	-11.68***	-33.25	5.10	22.10
Geographic variables				
Elevation	1392.72	1210	1540	93.76
DM	81.17	35.9	140	21.86

[ns] Non-significant; * P-value < 0.05 for H0: location means are equal as a result of ANOVA; *** P-value <0.001 for H0: location means are equal as a result of ANOVA; *** P-value < 0.001 for H0: mean of paired differences between 1999 and 2009=0 for the variables written in bold as a result of t-test; [a] Arcsinus transformed to meet the assumptions of ANOVA. The values are in original scale

Objective 2: Geographic variation in seed germination

Trends in GR with respect to location variables were similar in both test years; seeds coming from western part of the studied populations at higher elevations near the Mediterranean Sea germinated faster, although the effect of elevation is not significant for 2009 test (Tab. 4).

Tab. 4. Pearson correlations between population mean of each seed germination variable and geographic variables. See Tab. 2 for variable definitions

	Latitude	Longitude	Elevation	DM
GP1999	0.48*	0.57*	-0.11	0.51*
GR1999	-0.36	-0.52*	0.44*	-0.60*
GP2009	-0.12	0.12	-0.03	0.19
GR2009	-0.61*	-0.45*	0.22	-0.36
GP_DIFF	0.21	-0.03	0.01	-0.11
GR_DIFF	0.27	0.03	0.12	-0.09
GPGR1999	0.43*	0.60*	-0.43*	0.66*
GPGR2009	0.46*	0.59*	-0.24	0.58
GPGR9909	-0.10	-0.08	-0.14	-0.02

* P-value < 0.05

Contrary to GR, more inland and eastern locations had greater GP in year 1999. In year 2009, however, there was no significant relationship between geographic variables and GP (Tab. 4). Proportion of germinated seeds between the seventh and 21st days of the germination test (GPGR) showed similar patterns in both test years; it increases as the populations move eastward and inland at lower elevations (Tab. 4). There is no significant relationship between geographic variables and differences in germination rate (GR_DIFF), total germination (GP_DIFF) and proportion of germinated seeds after the seventh day of the tests (GPGR9909) between the two test years (Tab. 4).

Significant differences were observed among aspects of seed sources for GP2009, GR2009, GP_DIFF, GR_DIFF and GPGR2009. Highest GR and GP were observed in seeds coming from populations facing west. In general, high GR is associated with high GP except for seeds from east aspect, where seeds were slow to germinate but attained second highest total germination (Fig. 1a). Reduction in GR and GP was highest in seeds coming from populations facing southeast (Fig. 1b). The slowest germination was observed in seeds coming from populations facing east and southeast as over 60% of all germination occurred after the 7th day of the test (Fig. 1c).

Fresh pine seeds, in general, are easy to germinate and provide uniform germination (Gosling, 2007; Krugman and Jenkinson, 2008). Findings of current study are in ac-

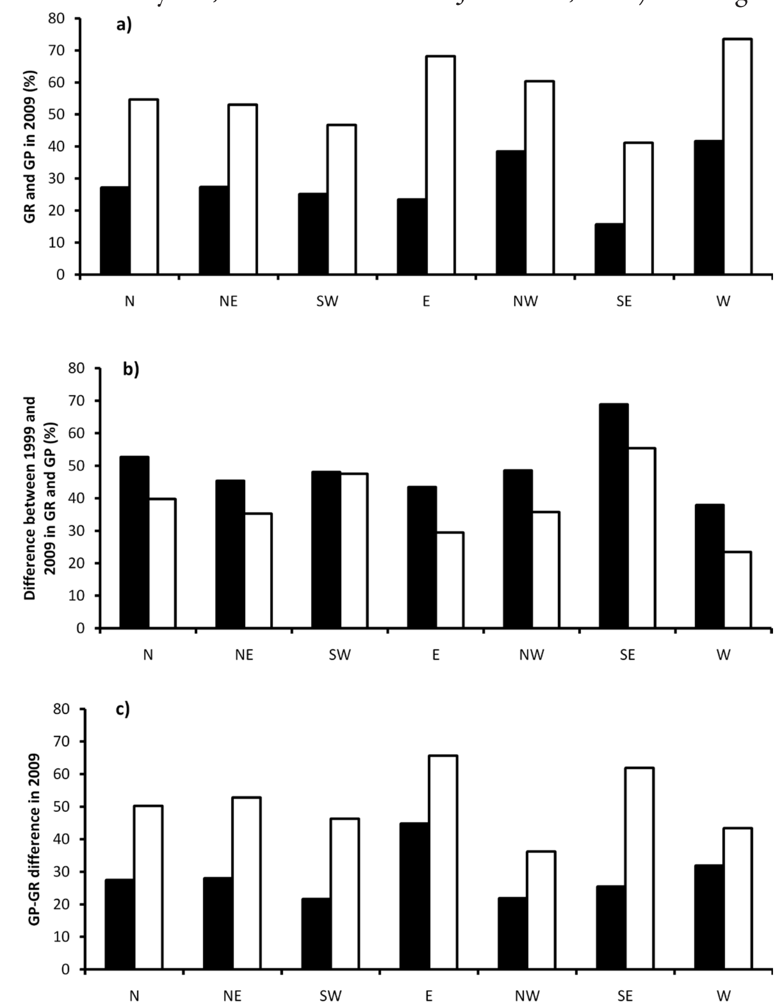

Fig. 1. Germination rate (%, black bars) and germination percentage (%, white bars) in 2009 test (a), difference between 1999 and 2009 tests for GR (%, black bars) and GP (%, white bars) (b), and difference between GP and GR in 2009 test as proportion of all tested seeds (black bars) and of total germinated seeds (white bars) (c) for each aspect of populations

cordance with the earlier studies. In the germination test conducted immediately after seed collection (the 1999 test) almost all of the seeds from 23 populations germinated (population mean range: 90.80 - 98.86%). Thus, populations under investigation differed significantly, albeit slightly, for GP in this test. Greater variation was observed for GR than that of GP in this test (population mean range: 46.80 - 90.86%), but within population variation was so high that no statistically significant differences were observed among populations (Tab. 3).

Pinus nigra seeds are orthodox, meaning that they can be stored safely for long periods at 3-5°C once their moisture content is reduced to 6-8% (Gosling, 2007). While *Pinus ponderosa* (Allen, 1957), *P. elliotii, P. patula, P. radiata* and *P. taeda* (Donald and Jacobs, 1990) seeds maintained their germination capabilities after six- to seven-year storage, Barnett (1969) reported 32% reduction in germination of *P. echinata* seeds stored for 10 years. In our study, both GR and GP decreased significantly after the storage. Effect of storage was more pronounced in GR than in GP. In the 2009 test, mean GR decreased by about 62% (to 30.68%) and mean GP by about 39% (to 58.41%). On population mean basis, GP was as low as 26.94% (Tab. 3). Ürgenç (1973) reached to a similar result with 10-year stored Anatolian Black Pine seeds where GR suffered more from long term storage than GP.

Effect of seed source location attributes on GR and GP were found to be similar in both fresh (1999 test) and stored (2009 test) seeds (Tab. 4). As the seed source moves inland, hence distances from the Mediterranean Sea, germination speed (GR) decreases (Fig. 2) but total germination (GP) increases (Fig. 3). Elevation of the seed sources, on the other hand, has a reverse effect on germination speed; it increases as seed source moves to higher elevations (Fig. 4). Germination percentage of fresh seeds seems to be not affected by seed source elevation in 1999 test, but total germination increases until 1400-1450 m above sea level then decreases in 2009 test (Fig. 5).

The study area is located in transition zone between maritime and continental climates and thus represents hu-

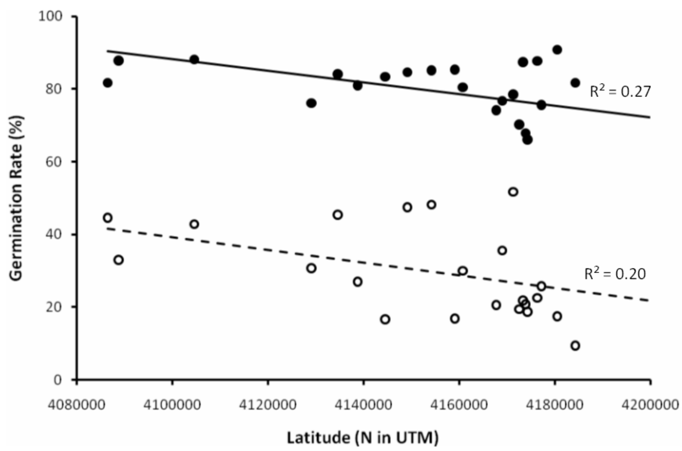

Fig. 2. Relationship between germination rate of Anatolian Black Pine seeds and latitude of the seed sources in 1999 (•) and in 2009 (○) tests

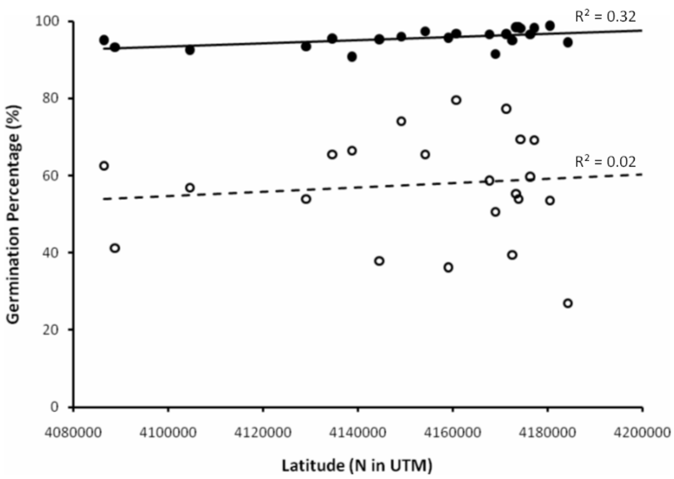

Fig. 3. Relationship between germination percentage of Anatolian Black Pine seeds and latitude of the seed sources in 1999 (•) and in 2009 (○) tests

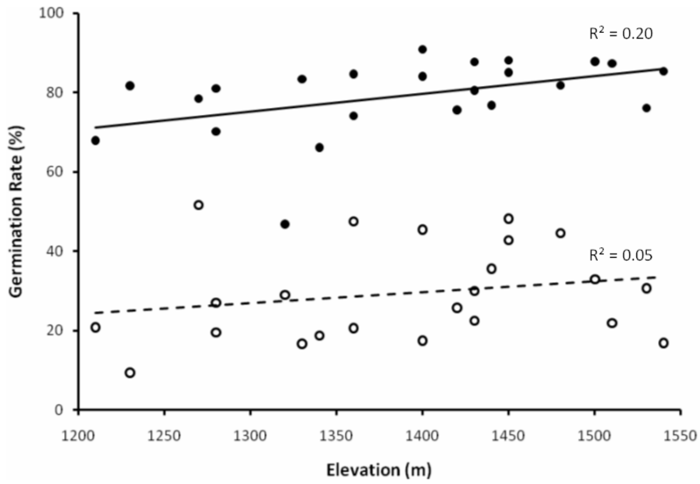

Fig. 4. Relationship between germination rate of Anatolian Black Pine seeds and elevation of the seed sources in 1999 (•) and in 2009 (○) tests

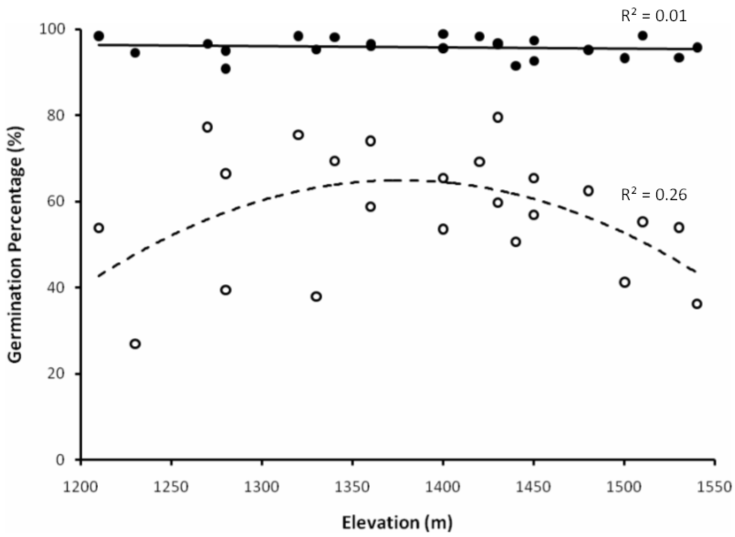

Fig. 5. Relationship between germination percentage of Anatolian Black Pine seeds and elevation of the seed sources in 1999 (•) and in 2009 (○) tests

mid, sub-humid and semiarid climate conditions (Atalay and Efe, 2010). Variation in germination speed with geography may be an evolutionary response to these climatic conditions. Seed germination characteristics are known to be controlled, at least partially, by genes (El-Kassaby *et al*., 1992; Whittington, 1973); therefore, local populations of a widely distributed species may develop adaptive variability in germination ecology (Quinn, 1977). Seeds in drier climates (i.e., more inland sources) evolved to increase chances of seedling survival by delaying germination until suitable moisture conditions are present for seedling establishment. Increase in total germination with increased continentality may be a corollary of this adaptation. Similarly, humidity and rainfall increases due to orographic rains on the mountains in the region with elevation, providing suitable environment for rapid germination. Total germination pattern of stored seeds with elevation is in accordance with the nature of orographic rainfalls; they increase until certain altitude and then decrease resulting in drier conditions at higher elevations. Weber and Sorensen (1992) reported a gradient in germination speed of *Pinus ponderosa* seeds with rainfall patterns in seed sources in central Oregon. In addition, provenance differences in seed germination have been explained by the conditions of the site of origin for several species (Boydak *et al*., 2003; Fraser, 1971; Gibson and Bachealard, 1987; Moore and Kidd, 1982; Wang *et al*., 2010). Mechanism for this adaptation may be via differences in water uptake characteristics of seeds. In a recent paper on *Pinus nigra* seeds from five provenances in the Balkans, Mataruga *et al*. (2010) have reported that *Pinus nigra* seeds coming from some of the harsher environments within each provenance took up lower amount of water than those coming from more suitable environments.

Improper storage conditions and biochemical and physiological changes in seeds have been shown to reduce germination capacity of conifer seeds. Among the storage conditions storage temperature and moisture content of the seeds are shown to be most influential. For most pine species storage at 4°C with 6-8% moisture content is recommended (Gosling, 2007). In our study, air dried seeds were stored at 4°C in sealed plastic bags, but their initial moisture contents were not measured. Thus, the effect of seed moisture content on reductions in germination rate and percentage cannot be excluded. Biochemical and physiological changes during the storage include oxidative damage (Tommasi *et al*., 2006), alterations in reserve substances (Simola, 1974), chromosomal dislocations (Roberts, 1972; Simak and Gustafsson, 1968), and leakage of substances from seed (Roberts, 1972). Seasonal periodicity has also been shown to affect germination of pine seeds, especially in seeds stored for longer periods (Barnett and Mamonov, 1989). To what extent reductions in germination occurred as a result of which of these factors in this study are beyond the scope of this paper and requires further attention.

One shortcoming of this study is that due to lack of enough number of seeds germination tests were conducted with only one replication. Our sample, on the other hand, includes seeds from 3-12 trees from the sampled populations. Therefore, although there was only one replication in each germination test, each population was represented by at least three different trees in the germination tests.

Conclusions

Long-term storage of Anatolian Black Pine seeds is essential not only for continuous seedling production, but also for genetic conservation in Turkey. Both germination rate and germination percentage of Anatolian Black Pine seeds decrease after 10-year storage and these reductions are found to be associated with environmental conditions of seed sources in the Lakes Region. It should be noted, however, moisture content of stored seeds has direct influence on their viability and in this study initial moisture contents of the stored seeds from different origins are not known. Thus, to what extent variation in initial moisture content of the seeds, if any, influenced germination patterns of stored Anatolian Black Pine seed sources requires further investigation.

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