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Full Length Research Paper

Using flotation in ethanol to separate filled and empty seeds of *Pinus nigra* ssp. *pallasiana*

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In this study, the separation of filled and empty seeds of Crimean pine [*Pinus nigra* Arn. ssp. *pallasiana* (Lamb.) Holmboe] by flotation in ethanol and the effect of this treatment on seed germination were investigated. Flotation tests in 96% ethanol by the density method and then germination tests were made on the seeds from three Crimean pine provenances of southern Turkey. In the flotation tests, 97.92% of the filled seeds sank in ethanol and 98.73% of the sunken seeds were filled seeds as the general mean values of all the provenances. Germination percentages of the flotation in ethanol and control treatments were 98.03 and 98.62%, and germination energies were 82.12 and 79.45%, respectively, in the general mean. While there was no statistically significant difference ($p > 0.05$) between the flotation and control treatments in terms of germination percentage for all three provenances, there was no significant difference ($p > 0.05$) for two provenances; however, there was a significant difference ($p < 0.05$) for one provenance in favour of the flotation treatment in terms of germination energy. The results of the study indicated that filled seeds of Crimean pine could be separated from empty seeds at a very high proportion by flotation in ethanol and this treatment did not affect negatively the germination percentage and germination energy of the seeds.

Key words: Crimean pine, filled seed, flotation method, ethanol, germination.

INTRODUCTION

Crimean pine (*Pinus nigra* Arn. ssp. *pallasiana* (Lamb.) Holmboe) is one of the main forest tree species of Turkey. It has a large natural distribution and geographical variations in Turkey (Alptekin, 1986) and its pure stands cover about an area of 2.5 million ha (Caliskan, 1998). Crimean pine is resistant to frost and drought and one of the foremost tree species in reforestation of dry regions (Saatcioglu, 1976). There are significant variations among Crimean pine clones in terms of cone and seed characteristics (Sivacioglu and Ayan, 2010). This species has been widely used in reforestation activities in Turkey and its seedlings have been produced at large quantities in forest nurseries. However, Crimean pine could produce empty seeds in its cones at considerable proportions (Atay, 1959); this has also necessitated some treatments for separation of filled and empty seeds before sowing in the nurseries.

The flotation method is one of the methods that can be used for the separation of filled and empty seeds. Many empty, broken, diseased or insect-damaged seeds in a seed lot can be removed by the flotation method (Bonner et al., 1994). In this respect, through a simple method, the proportion of filled seeds or in other words seed quality in a seed lot could be improved at considerable amounts by using the flotation method. Thus, more uniform seedling emergence and higher germination percentages could be achieved in seedling production activities. Indeed, it was determined that using this method, seed quality could be increased at a considerable extent in some tree species such as *Pinus brutia* Ten. (Sefik, 1965), *Pinus palustris* Mill. (McLemore, 1965), *Pinus banksiana* Lamb. (Brown,

Abbreviations: SPFS, Sinking proportion of filled seeds, **PFSSS**, proportion of filled seeds in sunken seeds; **LPFS**, loss proportion of filled seeds; **PISQ**, proportion of increase in seed quality; **FPES**, floating proportion of empty seeds; **FPIDS**, floating proportion of insect-damaged seeds.

1967), *Pinus glabra* Walt. (Barnett, 1970), *Cupressus sempervirens* L. var. *horizontalis* (Mill.) Gord. (Avsar, 2002a, b), *Juniperus excelsa* Bieb. (Gultekin et al., 2003) and *Casuarina equisetifolia* Forst (Sivakumar et al., 2007).

Various liquid media such as water (Sefik, 1965; Avsar, 2002b), n-pentane (McLemore, 1965), ether (Brown, 1967), ethanol (Barnett, 1970; Avsar, 2002a), sodium chloride solution (Gultekin et al., 2003) and petroleum ether (Sivakumar et al., 2007) could be used in separating filled and empty seeds by the flotation method. It is quite important not to damage the seed embryo and consequently seed germination ability during or after the flotation treatment by these liquids that have different densities. On the other hand, when Crimean pine seeds are left in water, the seeds generally float in water. In this respect, there are benefits in making studies on the use of flotation in a liquid with lower density than water such as ethanol.

In this study, the possibilities of using flotation in ethanol for the separation of filled and empty seeds of Crimean pine were investigated. For this, flotation tests were carried out in ethanol on the seeds from different Crimean pine provenances, germination tests were made to determine the effect of flotation in ethanol on seed germination and the data obtained were evaluated.

MATERIALS AND METHODS

Seed material

In this study, seeds of three different Crimean pine provenances from southern Turkey were used. While seeds of Goksun-Buyukcamurlu and Pos-Sogukoluk provenances were obtained from newly collected seeds by the forest enterprises in 2005, seeds of Andirin-Akifiye provenance were obtained from seeds dry stored at 4°C for about 1 year in cold store of Serinyol Forest Nursery. 1000-seed weights were found according to the International Seed Testing Association (ISTA) rules (Anonymous, 1985) and on the seeds estimated to be filled. 1000-seed weights of the seeds from Goksun-Buyukcamurlu, Andirin-Akifiye and Pos-Sogukoluk provenances were 27.72, 37.77 and 29.37 g, respectively. The mean 1000-seed weight of Crimean pine seeds is 22.50 g (Atay, 1959).

Flotation tests

Flotation tests were carried out according to the density method (Simak, 1973). A 600-ml beaker was filled up to its half with 96% ethanol (density = 0.79 g/cm^3), 50-seed randomly selected from normal-looking seeds of Crimean pine was left in the beaker. After about 1 min, floating and sunken seeds were removed separately from the beaker; then each seed was recorded as filled, empty or insect-damaged by the cutting test. These tests were repeated four times for each provenance. Thus, flotation tests were carried out on a total of 600 seeds as 3-provenance x 50-seed x 4-replication.

Success of flotation tests was determined with evaluation of two different proportions. One of them was the sinking proportion of filled seeds (SPFS) and the other was the proportion of filled seeds in sunken seeds (PFSSS) (Jones et al., 2002). The test in which these two proportions were higher, were evaluated as more successful. In addition, the loss proportion of filled seeds (LPFS), proportion of increase in seed quality (PISQ), floating proportion of empty seeds (FPES) and floating proportion of insect-damaged seeds (FPIDS) were also calculated separately for each provenance. In order to find the proportion of increase in seed quality, the initial proportion of filled seeds was subtracted from the proportion of filled seeds obtained after flotation test and the obtained value was divided by the initial proportion of filled seeds.

Statistical analysis was performed to determine whether there was a statistically significant difference in terms of the sinking proportion of filled seeds and the proportion of filled seeds in sunken seeds among Crimean pine provenances. While the Kruskal-Wallis H-test, a non-parametric test, was applied for the sinking proportion of filled seeds; one way analysis of variance (ANOVA) was applied after the data were arcsin(p)^{½} transformed and then Duncan's multiple range test (Kalipsiz, 1981) to determine different groups was made for the proportion of filled seeds in sunken seeds.

Germination tests

A 600-ml beaker was half-filled with ethanol, 300 normal-looking seeds of Crimean pine were randomly selected and immersed in the beaker. After about 1 min, floating and sunken seeds were removed separately from the beaker; the removal of sunken seeds and washing them with water lasted for about 1 min. Thus, it can be said that the seeds contacted with ethanol for a total of 2 min. Then, sunken seeds were washed thoroughly with tap water for few minute to move ethanol from the surface of the seeds and four random samples of 50 seeds each from them were taken for germination test. These treatments were separately repeated for each provenance.

Germination tests were made to determine germination percentage and germination energy of the seeds floated in ethanol and control seeds, to which no flotation was applied. The seeds were placed on filter paper in glass Petri dishes (diameter 9 cm) and four replicates of 50 randomly selected seeds were used for each treatment (flotation and control). The tests were conducted under laboratory conditions (25 ± 1°C and normal light) and according to the completely randomized design; the seeds were moistened adequately during the tests. When the radicle extended at least 2 mm, the seeds were considered as germinated. Germinations were checked daily, the test period was 21-day (Anonymous, 1985). Germination percentage was found by the proportion of the number of normally germinated seeds to the number of filled seeds. Germination energy was evaluated by germination percentage of 7th-day (Atay, 1959; Saatcioglu, 1971). The seeds were regarded as abnormally germinated when the radicle did not extend, but cotyledons emerged from seed. Germination tests were carried out on a total of 1200 seeds as 2-treatment x 3-provenance x 50-seed x 4-replicate.

Statistical analysis was performed to determine whether there was a statistically significant difference in terms of germination percentage and germination energy between the flotation in ethanol and control treatments for each Crimean pine provenance. The Mann-Whitney U test, a non-parametric test for germination percentage and t-test (Kalipsiz, 1981) after arcsin(p)$^{1/2}$ transformation of the data for germination energy were applied.

When homogeneity of variances was not met, although appropriate transformations of the data obtained were made, the non-parametric tests mentioned above were applied. In all statistical analyses, a confidence level of p = 0.05 was used for statistical significance and the analyses were carried out by using Statistical Package for the Social Sciences (SPSS) 11.5 package.

Table 1. The results of flotation tests in ethanol by Crimean pine provenances.

Provenance	Floating				Sunken			
	Filled	Empty	Insect-damaged	Total	Filled	Empty	Insect-damaged	Total
Goksun-Buyukcamurlu	2.00	10.50	0.25	12.75	36.00	-	1.25	37.25
Andirin-Akifiye	-	0.75	-	0.75	49.25	-	-	49.25
Pos-Sogukoluk	0.50	17.00	0.50	18.00	31.75	-	0.25	32.00
General Mean	0.83	9.42	0.25	10.50	39.00	-	0.50	39.50

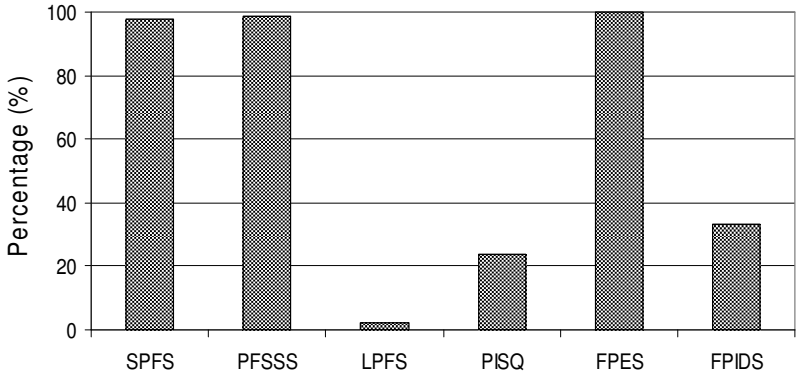

Figure 1. Some proportions obtained in flotation tests in ethanol (as the general mean values).

RESULTS

Flotation tests

The results of flotation tests in ethanol are presented as mean values in Table 1. In Goksun-Buyukcamurlu provenance, 94.74% of the filled seeds sank and 96.64% of the sunken seeds were filled seeds. In this provenance, with a loss of 5.26% in the filled seeds, an increase of 27.16% was achieved in seed quality by increase of the proportion of filled seeds from 76.00 to 96.64%. In addition, all of the empty seeds and 16.67% of the insect-damaged seeds floated.

In Andirin-Akifiye provenance, all of the filled seeds sank and all of the sunken seeds were filled seeds. In this provenance, with a loss of 0% in the filled seeds, an increase of 1.52% was achieved in seed quality by increase of the proportion of filled seeds from 98.50 to 100.00%. In addition, all of the empty seeds floated and insect-damaged seeds were not encountered in the tests.

In Pos-Sogukoluk provenance, 98.45% of the filled seeds sank and 99.22% of the sunken seeds were filled seeds. In this provenance, with a loss of 1.55% in the filled seeds, an increase of 53.83% was achieved in seed quality by increase of the proportion of filled seeds from 64.50 to 99.22%. Moreover, all of the empty seeds and 66.67% of the insect-damaged seeds floated.

According to the general mean values of three provenances of Crimean pine, 97.92% of the filled seeds sank and 98.73% of the sunken seeds were filled seeds. Thus, with a loss of 2.08% in the filled seeds, an increase of 23.94% was achieved in seed quality by increase of the proportion of filled seeds from 79.66 to 98.73%. Besides, all of the empty seeds and 33.33% of the insect-damaged seeds floated in ethanol (Figure 1).

The Kruskal-Wallis H-test showed that there was a statistically significant difference among Crimean pine provenances in terms of the sinking proportion of filled seeds ($\chi^2 = 6.611$; $p = 0.037$). According to the results of one way ANOVA, there was a nearly statistically significant difference among Crimean pine provenances in terms of the proportion of filled seeds in sunken seeds ($f = 4.173$; $p = 0.052$); and the provenances were divided into two different groups (group I: Goksun-Buyukcamurlu and Pos-Sogukoluk; group II: Pos-Sogukoluk and Andirin-Akifiye) by Duncan's multiple range test.

Germination tests

Germination percentages of the flotation and control treatments were determined to be respectively, 98.98 and

Table 2. Germination percentages by the treatments and days in Crimean pine provenances.

Provenance	Treatment	Days			
		7	10	14	21
Goksun-Buyukcamurlu	Flotation	93.37	97.46	97.98	98.98
	Control	91.98	97.33	98.92	100.00
Andirin-Akifiye	Flotation	57.16	86.98	96.10	97.19
	Control	53.76	85.33	94.85	96.89
Pos-Sogukoluk	Flotation	95.83	97.92	97.92	97.92
	Control	92.60	97.86	98.97	98.97
General Mean	Flotation	82.12	94.12	97.33	98.03
	Control	79.45	93.51	97.58	98.62

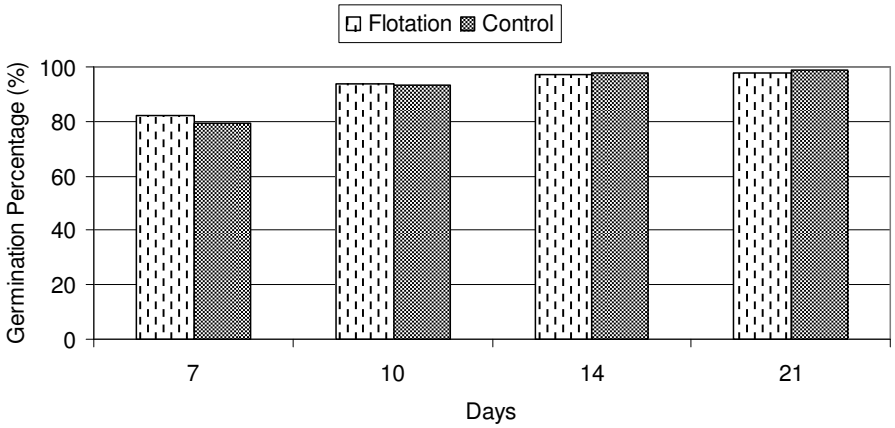

Figure 2. Germination percentages of the flotation and control treatments by days (as the general mean values).

100.00% for Goksun-Buyukcamurlu provenance, 97.19 and 96.89% for Andirin-Akifiye provenance, 97.92 and 98.97% for Pos-Sogukoluk provenance. According to the general mean values of three provenances of Crimean pine, germination percentages of these treatments were 98.03 and 98.62%, respectively (Table 2, Figure 2).

The results of the Mann-Whitney U test showed that there was no statistically significant difference between the flotation and control treatments in terms of germination percentage in Goksun-Buyukcamurlu ($z = -1.512$; $p = 0.131$), Andirin-Akifiye ($z = -0.289$; $p = 0.773$) and Pos-Sogukoluk ($z = -1.348$; $p = 0.178$) provenances.

Germination energies of the flotation and control treatments were found to be respectively, 93.37 and 91.98% for Goksun-Buyukcamurlu provenance, 57.16 and 53.76% for Andirin-Akifiye provenance, 95.83 and 92.60% for Pos-Sogukoluk provenance. According to the general mean values of three provenances of Crimean pine, germination energies of these treatments were 82.12 and 79.45%, respectively (Table 2, Figure 2).

The results of t-test showed that there was no statistically significant difference between the flotation and control treatments in terms of germination energy in Goksun-Buyukcamurlu ($t = 0.588$; $p = 0.578$) and Andirin-Akifiye ($t = 0.365$; $p = 0.728$) provenances; but there was a significant difference in favour of the flotation treatment in Pos-Sogukoluk provenance ($t = 2.623$; $p = 0.039$).

DISCUSSION

At the end of flotation tests, it was determined that 97.92% of the filled seeds sank in ethanol and 98.73% of the sunken seeds were filled seeds, as the general mean values. Thus, in spite of a loss of 2.08% in the filled seeds, an increase of 23.94% was achieved in seed quality. In addition, all of the empty seeds and 33.33% of the insect-damaged seeds floated in ethanol. Namely, empty seeds were completely separated in ethanol, unlike insect-damaged seeds of which only a certain part was separated. These results indicate that filled and empty seeds of Crimean pine could be separated from

each other at a very high proportion by flotation in 96% ethanol. It was reported that filled seeds are separated from empty seeds by flotation in 95% ethanol in *P. nigra* Arnold (Karschon, 1961) and in *P. glabra* Walt. (Barnett, 1970) as well. But flotation in 95% ethanol did not give good results in *Larix laricina* (Du Roi) K. Koch (Eavy and Houseweart, 1987).

The separation of filled and empty seeds of Crimean pine could be made by the Steigsichter apparatus, which makes a mean error of 3% (Atay, 1959). When the mean error rates in the present study were considered as 2.08% for the sinking proportion of filled seeds and 1.27% for the proportion of filled seeds in sunken seeds, it was seen that the error rate in flotation in ethanol was lower than the error rate in this apparatus. In Crimean pine, the separation of filled and empty seeds may also be in question by light or dark coloured seeds with a certain error rate. Indeed, Atay (1959) determined that 83.50% of the light coloured seeds were empty seeds and 79.90% of the dark coloured seeds were filled seeds in Crimean pine, as the mean values. It is obvious that the error rate of a separation to be made by the colour of the seeds will be relatively high. Furthermore, it is required to consider that while both the Steigsichter apparatus and a separation by seed colour could be generally used in laboratory studies, flotation in ethanol could be used in both laboratory and nursery studies.

There was a statistically significant difference ($p \leq 0.05$) among Crimean pine provenances in terms of both the sinking proportion of filled seeds and the proportion of filled seeds in sunken seeds. This shows that these relative values obtained after flotation in ethanol could vary to a certain extent by the provenances, as well. However, these values were essentially very high in all three provenances. Similarly, Jones et al. (2002) found considerable variability among seed collections in response to separation in 95% ethanol in *Betula occidentalis* (Hook.).

The proportions of increase achieved in seed quality at the end of flotation tests differed widely by the provenances. Indeed, the highest proportion of increase in seed quality was achieved in Pos-Sogukoluk provenance (53.83%), followed by Goksun-Buyukcamurlu (27.16%) and Andirin-Akifiye (1.52%) provenances. As seen, this proportion was the highest in Pos-Sogukoluk provenance, in which the proportion of empty seeds was the highest, and was lowest in Andirin-Akifiye provenance, in which the proportion of empty seeds was the lowest. Namely, if the proportion of empty seeds in a Crimean pine seed lot is much higher, the proportion of increase achieved in seed quality at the end of flotation in ethanol would also be so high. Avsar (2002a) also achieved an increase of 17.40% in seed quality (36.71% by the method in the present study) with a loss of 2.11% in filled seeds at the end of flotation in 50% ethanol in *C. sempervirens* L. var. *horizontalis* (Mill.) Gord.

In germination tests, as the general mean values, germination percentages of the flotation and control treatments were 98.03 and 98.62%; and germination energies for these two treatments were 82.12 and 79.45%, respectively. Atay (1959) reported that the mean germination percentage and germination energy of Crimean pine seeds were 91.30 and 62.00%, respectively. Thus, in the present study, relatively higher proportions of germination were obtained for each of the two treatments than the reported values. Because Crimean pine seeds do not exhibit seed dormancy (Saatcioglu, 1971), the seeds germinated at a very high proportions under normal conditions. Additionally, Crimean pine seeds can keep their high germination capacity at the end of eight-year cold storage period at 5 - 7°C (Atay et al., 1970).

It was determined that while there was no statistically significant difference ($p > 0.05$) between the flotation and control treatments in terms of germination percentage in all three provenances of Crimean pine; there was no significant difference ($p > 0.05$) in the two provenances, but there was a significant difference ($p < 0.05$) in favour of the flotation treatment in the one provenance (Pos-Sogukoluk) in terms of germination energy. Moreover, the values of germination percentage and germination energy of the flotation and control treatments were quite close to each other in all three provenances. Thus, it can be said that flotation in ethanol did not mainly have a negative impact on the germination percentage and germination energy of Crimean pine seeds. Barnett (1970) also noted that viability of *P. glabra* Walt. seeds subjected to flotation in 95% ethanol did not decrease.

The contact of the seed coat with ethanol lasted for about 2 min in the study. It is understood that this period did not make a detrimental effect on the seed embryos and consequently germination percentage and germination energy of Crimean pine seeds. Schmidt (2000) stated that alcohols are poisonous to seed embryos; thus, potential damage is dependent on the liquid and the absorption rate, which depends on the seed coat structure and the period of exposure.

In the study, it was determined that germination energy of the flotation treatment was statistically higher than that of the control treatment in Pos-Sogukoluk provenance. This indicates that germination energy of the seeds from some provenances could increase at the end of flotation in ethanol and then washing thoroughly with tap water. Atay (1959) reported that soaking the seeds in water for 1 - 2 h at room temperature accelerated the seed germination of Crimean pine.

It is seen that flotation in 96% ethanol gave successful results with a very high proportion in separating filled and empty seeds and did not affect negatively the germination percentage and germination energy of the seeds in Crimean pine. In this respect, taking into account the conditions applied in the study, using flotation in ethanol could be recommended to separate filled and empty seeds in Crimean pine.

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