DETERMINING THE FACTORS AFFECTING REPRODUCTION OF AN ENDEMIC BLACK PINE VARIETY [*PINUS NIGRA* SUBSP. *PALLASIANA* VAR. *YALTIRIKIANA*] IN TURKEY

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Abstract. *Pinus nigra* subsp. *pallasiana* var. *yaltirikiana* has a very restricted spread, and is an endemic black pine variety in Turkey. This plant has a poor generative reproduction performance. It is unclear which factors are responsible for this low reproduction capacity. Therefore, objective of this study was to investigate pollen and seed characteristics of the plant. For this purpose, pollen samples were collected in the pollination period, and seed samples were collected in the cone ripening period in the natural distribution site of this plant in Black Sea Region. Pollen samples were examined whether or not they have normal shape and size. Seed samples were tested for germination percentage and seed size and soundness were also examined. Results showed that pollen samples seemed to have normal shape and size, and seeds have a germination percentage of 71. This study indicated that the conservation of *Pinus nigra* subsp. *pallasiana* var. *yaltirikiana* through in-situ and ex-situ strategies and the establishment of a seed orchard in a different area by taking grafts are important in terms of ensuring continuity of limited number individuals.
Keywords: *Pinus nigra* subsp. *pallasiana, yaltirikiana, germination, seed, pollen*

Introduction

Pinus nigra J.F.Arnold subsp. *pallasiana* (Lamb.) Holmboe, which spreads across Balkans, Southern Carpathians, Crimea, Cyprus and Syria, constitutes forests in mountainsides of Black Sea, Marmara, Aegean and Central Anatolia regions and Toros Mountains of Turkey, and rarely seen at sea level (Coode and Cullen, 1965; Akkemik et al., 2011; Kandemir and Mataracı, 2018). In accordance with the recent studies, it is accepted that there are three varieties including var. *fastigiata* Businský, var. *yaltirikiana* Alptekin and var. *pallasiana* (f. *pallasiana* and f. *seneriana* (Saatcioglu) Kandemir & Mataraci) in Turkey, and that its taxa except *pallasiana* f. *pallasiana* are endemic (Kandemir and Mataracı, 2018).

Pinus nigra J.F.Arnold subsp. *pallasiana* (Lamb.) Holmboe var. *yaltirikiana* Alptekin is distinguishable from other varieties by the fact that its cones are bigger (at least 8 cm) and leaves are longer (at least 14 cm) (Alptekin, 1987; Yaltırık, 1988; Kandemir and Mataracı, 2018; Oral and Mataraci, 2018). Alptekin (1987), who named the variety, indicated the cone sizes of *Pinus nigra* subsp. *pallasiana* var. *yaltirikiana* as 7.8 cm (max.11.19) × 3.71 cm (max.4.4).

In Oral and Mataraci (2018)'s article, in which they identified a new epitype (ISTO 37320) instead of a holotype composed of only a cone, the average female cone size was 9.16 (7.69-10.23) x 3.94 (3.55-4.31) cm, male cone size was 1.58 (1.28-2.16) x 0.54 (0.48-0.62) cm; leave size was 17.21 (13.80-20.20) x 0.18 (0.16-0.22) cm and sheath length was 0.95 (0.67-1.16) cm. In addition, it was especially emphasized that the cone and leaf length should be evaluated concomitantly for the diagnosis of taxon.

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It was known that this variety was in 1985 in Karabuk-Yenice and Sinop-Boyabat (Alptekin, 1987). Oral and Mataracı (2018), during a field visit to these locations in 2017, stated that they survived owing to the fact that its individuals in Boyabat were taken under preservation. It is suggested by them that its danger catagory due to fewness of both individuals and seeds was "Critically endangered" (CR) (Ekim et al., 2000). During the field visit to Yenice, it never seen *Pinus nigra* subsp. *pallasiana* var. *yaltirikiana* even though all locations given in herbarium records were visited. There were black pines with shorter cones (5.66-8.50 cm) in these locations.

Yaman and Saribas (1999) compared pollen sizes of *Pinus nigra* subsp. *pallasiana* var. *yaltirikiana* in Karabuk-Yenice, which is its holotype location, with pollens of the other endemic black pines, which were taken from natural areas. As a result of the study, it was stated that it should be compared with the pollen samples in Sinop-Boyabat due to the fact that frequency distribution of *Pinus nigra* subsp. *pallasiana* var. *yaltirikiana*'s pollen sizes (length-L and width-l) was not normal.

In this study, it is aimed at determining vitality of the seeds of *Pinus nigra* subsp. *pallasiana* var. *yaltirikiana* in order to ensure the sustainability of the existence of its in Sinop-Boyabat. Therefore, it has been investigated number of sound seeds and germination percentage, and the morphological characteristics of its pollen to determine whether it has especially a morphological development inhibitive to fertilization.

Materials and methods

Pollen and seed samples were collected from the trees growing only in Sinop-Boyabat, Karageriş Mountain, (41°18′49,91″K, 34°34′41,15″D) at elevation of 1239 m (*Figs.1 and 2*).

Figure 1. *Individuals of Pinus nigra* subsp. *pallasiana* var. *yaltirikiana in Boyabat-Kapaklıpınar*

Only four trees were present in the site with ages of approximately between 80-100 years and height of 10-12 m. Dominant vegetation in the site includes some tree species such as *Pinus nigra* J.F.Arnold subsp. *pallasiana* (Lamb.) Holmboe var. *pallasiana*, *Pinus sylvestris* L. var. *hamata* Steven f. *hamata*, *Juniperus communis* L. subsp. *saxatilis* Pall. in the overstorey cover, some schrub species such as *Cistus laurifolius* L., *Rubus canescens* DC. var. *canescens*, *Rosa canina* L., *Crataegus monogyna* Jacq. subsp. *monogyna*, *Mespilus germanica* L. and some herbaceous plants such as *Veronica chamaedrys* L, *Dactylis glomerata* L., *Poa annua* L. in the understorey cover.

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Mean annual precipitation is about 620 mm and most of it falls between September and June. The driest month is July, with 30 mm of rainfall. Most of the precipitation here falls in December, averaging 75 mm. Average annual temperature is around 12.9°C and changes between 4°C in January and 21.7°C in July (Climate-Data.org, 2019). The site has a deep soil (>80 cm) with sandy loam texture covered with mull type forest floor with 2 cm depth (GDF, 2011).

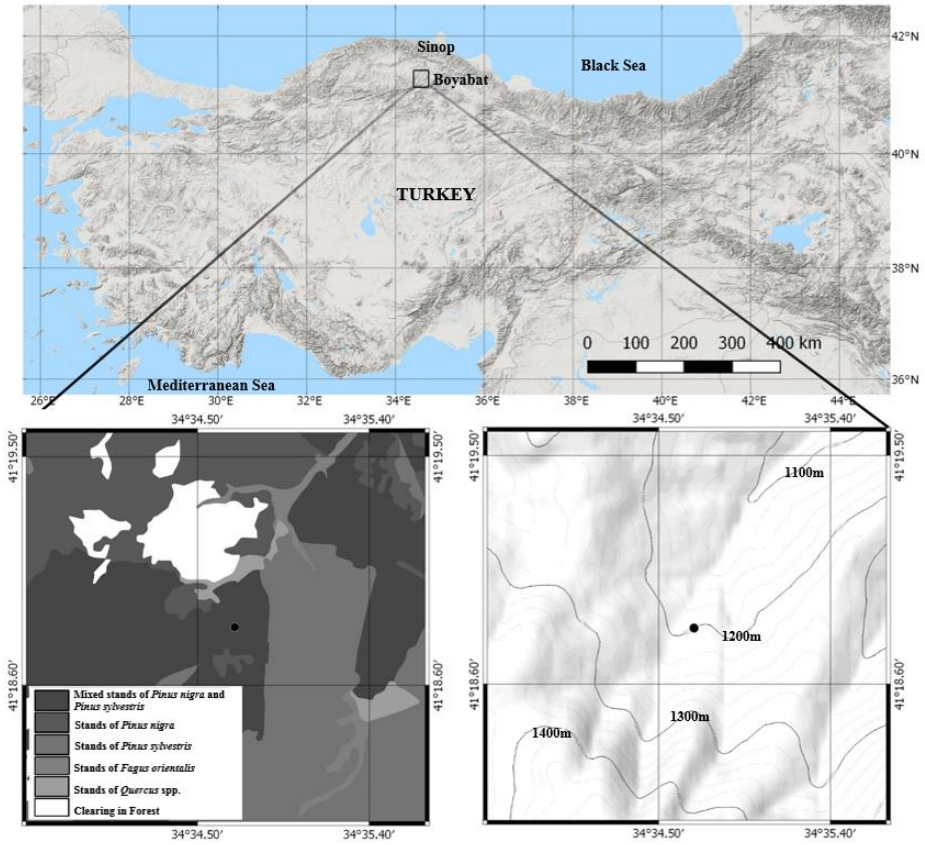

Figure 2. *The location of the sample collection area (●) and the different types of habitats in its surrounding*

Pollen samples were taken from each of four trees at the height of 3 m above soil surface in the pollination period in May 2018 and brought to Palynology Laboratory of Forest Botany Department at Istanbul University Cerrahpasa (I.U.C.) Faculty of Forestry. Then, pollen samples preparations were prepared separately for each individual tree according to the Wodehouse method (Wodehouse, 1959) and the measurements were carried out after waiting for 2 months for pollens in preparations to reach a normal form and sizes (Aytug, 1960). Then, they were examined. The measurements of pollen grains sizes in the preparations were done in the Leica DM750 light microscope (LM) as computer assisted, and 10x ocular, x40, x100 immersion objective were used. Under the light microscope, on equatorial axis of the pollen samples, length (β) and height (P) of saccus, distance from pollen to the outermost end of the saccus (p), height (h) of pollen body; on polar axis of theirs, length (L) and width (l) of pollen, length (B) and width (b) of saccus, distance from pollen to the outermost end of the saccus (be) were measured (*Fig. 3*) (Aytug, 1967).

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At least 50 measurements were performed for each pollen feature in each of the 4 preparations, then the averages were taken. A part of these pollens was kept in the fridge in +4°C to examine with Scanning Electron Microscope (SEM). The size of the pollen, and ornamentation were studied by taking photos with both SEM and LM.

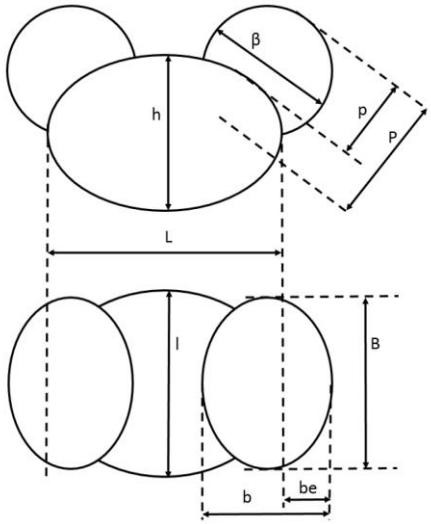

Figure 3. *Pine pollen sizes (upper: polar axis, β: length of saccus, P: height of saccus, p: distance from pollen to the outermost end of the saccus, h: height of pollen body; lower: equatorial axis, L: length of pollen, l: width of pollen, B: length of saccus, b: width of saccus, be: distance from pollen to the outermost end of the saccus)*

On December 20, 2018, cone samples were collected in order to determine the viability of the seeds in 2 years old cones of *Pinus nigra* subsp. *pallasiana* var. *yaltirikiana*. A total of 17 cones from four trees were collected from any side of canopy cover at the height of 3-6 m above soil surface. In order not to affect sustainability of its presence in the location, the number of cones was limited by also taking into account the fact that it already has a small number of individuals. The cones, which were brought to I.U.C. Faculty of Forestry, Department of Silviculture, Seed Technology and Silviculture Laboratory, were kept in drying-oven at a temperature of 46°C for 24 hours so that seeds could be taken out (Genc, 2004). Only seeds in 13 cones were counted as there were no seeds in 4 cones; seed length, width and thickness, and wing length and width were measured; and the seeds were weighed to determine their weights.

406 seeds were divided into four groups and placed on filter paper saturated with deionized water in 4 petri dishes at 20°C (±0.5°C) for germination test. The seeds were considered to be germinated when radicles showed geotropism. Seed germinations were monitored for 28 days. As the seeds germinated they removed from the petri dishes. Ungerminated seeds were checked with cutting test for soundness. Germinated seeds were planted into pots (Boydak and Çalışkan, 2014). Besides, it was ensured that they continued development in order to determine the number of cotyledon. The seeds germinated within first week of the study. Planted into pots in the nursery and monitored for seedling survival for 60 days.

In order to determine whether or not a change exists in chromosome number, mitotic chromosomes were prepared from root tips and pre-treated with α-bromo-naphthalene (ABN) at +4°C for 24 h. Roots were fixed for a minimum of 2 h in absolute ethanol:

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glacial acetic acid, (3:1,v/v), hydrolysed at 60°C in 1 N HCl for 16 min and stained in Feulgen reagent. Finally, root tips were squashed in 1% aceto-orcein. Chromosomes were counted in the groups of in the preparation prepared from the crushed root tips (Altınordu et al., 2014).

Main statistical parameters were estimated and presented in the result section (Akalp, 2016).

Results and Discussion

The results of measurements made for pollen sizes are given in *Table 1* by comparison with the results of literature studies. According to the results of observations and measurements made in pollen preparations showed that pollens of *Pinus nigra* subsp. *pallasiana* var. *yaltirikiana* were not defective and that there was no obstacle for seed formation by fertilization.

Table 1. *Mean ($\bar{x}$ ±SD) measurements of pollen dimensions of Pinus nigra* subsp. *pallasiana varieties*

Measured parts (µm)		Present study	Results of previous (literature) studies			
		var. *yaltirikiana* (Boyabat-Sinop)	**var. *yaltirikiana* * (Yenice-Karabük)**	**var. *fastigiata* * (Kütahya)**	**var. *pallasiana* f. *şeneriana* * (Bolu)**	**var. *pallasiana* f. *pallasiana* ** (Muğla)**
Polar view	**L**	46.67±3.00***	46.78±6.79***	51.97±8.44***	55.13±3,68***	57.04±4.21***
	l	37.32±3.62	43.57±5.86	46.20±6.53	47.03±3.63	44.96±3.12
	B	35.18±2.09	38.37±3.60	39.45±5.19	41.17±2.23	39.80±3.01
	b	26.95±1.84	28.96±3.71	29.11±3.42	30.41±2.23	30.20±2.38
	be	10.23±1.61	10.73±2.68	10.97±2.45	11.28±2.27	10.28±2.28
Equatorial view	**h**	32.27±2.32	37.38±3.91	40.04±5.41	41.65±2.90	39.84±3.32
	β	29.46±1.68	32.74±2.32	32.39±5.21	33.56±2.83	31.48±3.21
	P	24.36±1.36	28.15±2.78	27.54±4.19	28.22±3.42	25.76±2.19
	p	16.31±1.26	14.21± 3.59	15.80±2.75	15.03±1.82	17.20±2.04
Ratios	**L/l**	1.25	1.07	1.12	1.17	1.27
	L/h	1.45	1.25	1.30	1.32	1.43
	B/b	1.31	1.32	1.36	1.35	1.32
	be/b	0.38	0.37	0.38	0.37	0.34
	β/P	1.21	1.16	1.18	1.19	1.22
	p/P	0.67	0.50	0.57	0.53	0.67

* Yaman and Sarıbaş (1999), ** Aytuğ (1967), ***SD (standard deviation)

Figure 4 shows polar and equatorial view of *Pinus nigra* subsp. *pallasiana* var. *yaltirikiana* pollens under LM and their exine surfaces under SEM. As in the other varieties (Yaman and Sarıbas, 1999), bisaccate pollens are inaperture; recesses and protrusions are apparent; pollen ornamentation is verrucate, and its structure is tectatae; saccus ornamentation is regular; it consists of small closed islets and canals (*Fig. 4*).

It is seen that sizes of pollens of all varieties of *Pinus nigra* subsp. *pallasiana* (*Table 1*) were similar; however, pollens of *Pinus nigra* subsp. *pallasiana* var. *yaltirikiana*, which were collected from both locations, were smaller than the pollens of other varieties. When *Pinus nigra* subsp. *pallasiana* var. *yaltirikiana* pollens of two locations (Boyabat and Yenice) were compared, it was found that the width (l), height (h) and saccus sizes (β, P, B, b, be) of pollens in Sinop were lower, while distance from pollen to the outermost end of the saccus (p) was higher. In contrast to pollens of

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Yenice (Yaman and Sarıbas, 1999), the frequency distributions of pollen dimensions of samples collected from Boyabat were normal. When L/l and L/h ratios were compared, it can be said that pollens in Boyabat is slightly flattened in equatorial view.

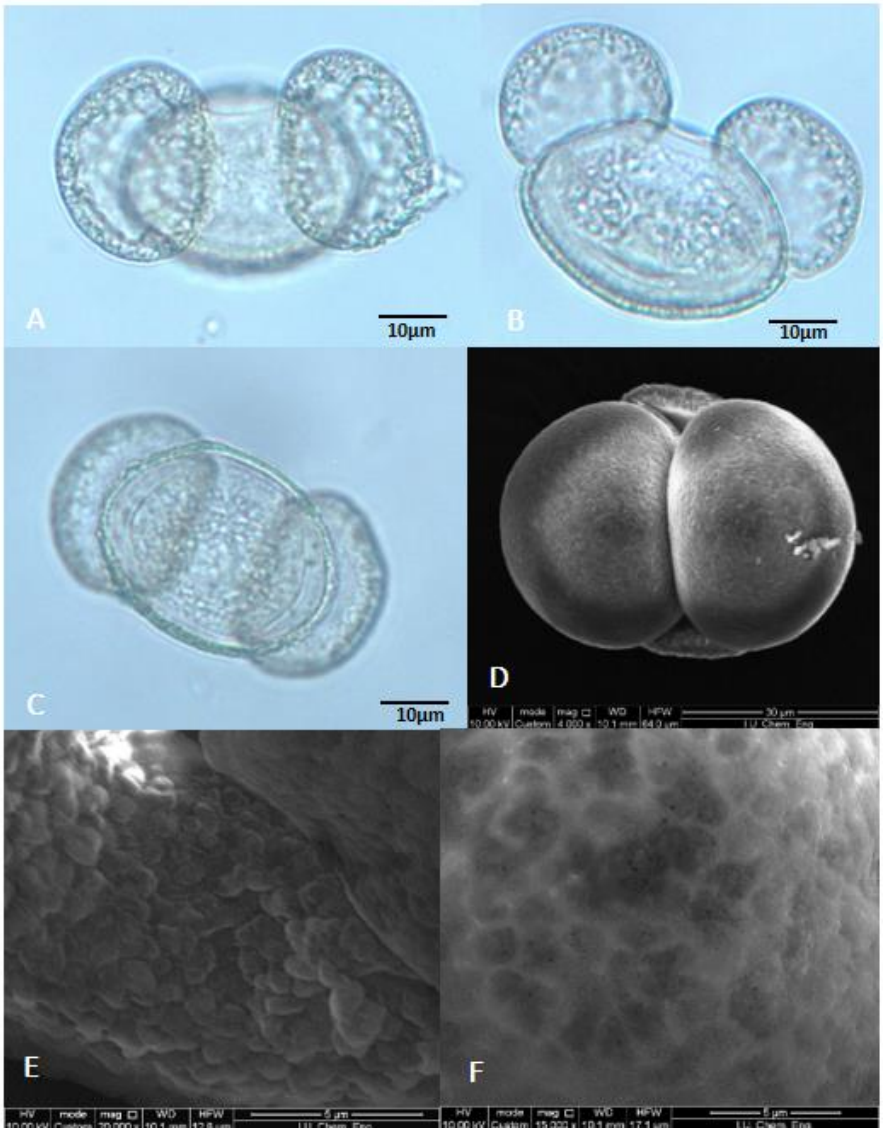

Figure 4. *Pollens of Pinus nigra* subsp. *pallasiana* var. *yaltirikiana. A-C (Light microscope-LM): A-Polar (Distal), B-Equatorial, C-Polar (Proximal) views; D-F (SEM): D-Dry pollen grain, E-Exine surface (proximal pole), F-Exine surface (saccus)*

It is shown that results of the measurements made on 406 seeds and 234 seed wings of *Pinus nigra* subsp. *pallasiana* var. *yaltirikiana* in *Table 2*. The number of seeds in the first three cones were very low. The sizes of the seeds were 7.10 x 3.83 x 2.40 mm; while the seed-wing sizes were 34.88 x 8.26 mm. In literature, the seed sizes of *Pinus nigra* subsp. *pallasiana* was given as 6.5 (4-9) x 3.71 (3-4) mm; the wing sizes of it as 24.73 x 7.7 mm (Alptekin, 1987; Kandemir and Mataracı, 2018). Accordingly, wing of *Pinus nigra* subsp. *pallasiana* var. *yaltirikiana* seed is longer.

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Atay (1959) stated that average germination capability of black pine seeds was 91.3%, average germination energy was 62%, and 1000 seed weight was 22.5 g. As compared to results of previous studies about seeds of *Pinus nigra* subsp. *pallasiana* var. *yaltirikiana*, mean germination percentage was 71% on 28th day and the average germination energy was 47%, and 1000 seed weight was around 21.14 g (*Table 3*). Although the germination percentage is considered as the percentage of seeds germinated within the first 7 days, the values at days 4, 7 and 10 can also be used in some cases that require precision (Boydak and Çalışkan, 2014).

Table 2. *Some characteristics of the seeds of Pinus nigra* subsp. *pallasiana* var. *yaltirikiana*

	Number of sound seed in a cone	1000 seed weight (g)	Seed (n=406)			Seed wing (n=234)	
			Length (mm)	Width (mm)	Thickness (mm)	Seed (n=406)	Width (mm)
Mean	18	21.14	7.10	3.83	2.40	34.88	8.26
Range	0-35	11.4-35.1	4.15-8.69	2.59-4.79	0.96-3.42	25.79-41.07	5.29-10.42

Table 3. *Percentages of sound seed, germination, germination rates and number of cotyledon for each cone samples of Pinus nigra* subsp. *pallasiana* var. *yaltirikiana*

	All seeds	Sound seeds	Empty seeds	Germination seeds (for all seeds)	Germination rate (for sound seeds)					Cotyledon
					4	7	10	14	28	
Number	406	198	208	140	70	93	109	126	140	8.3 (6-10)
%	-	49	51	35	35	47	55	63	71	-

As seen from *Table 3*, almost half of the seeds were sound with a germination percentage of 71 (*Fig. 5*).

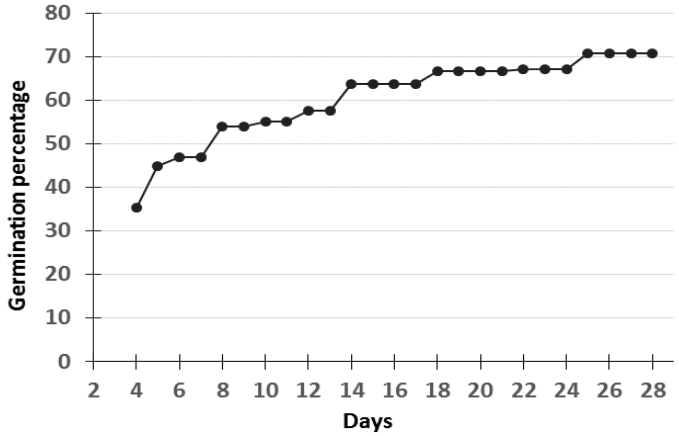

Figure 5. *Germination of Pinus nigra* subsp. *pallasiana* var. *yaltirikiana seeds under 20°C*

As compared to results of previous studies sound seed production and germination of *Pinus nigra* subsp. *pallasiana* var. *yaltirikiana* was lower when sound seeds were considered. Moreover, if all seeds including unsound ones were considered, germination percentage dropped to 35%, and 49.3% of the germination took place in the four-day period in germination chamber.

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140 seeds were germinated under laboratory conditions, and germinated seeds were planted into pots. 93 out of 140 germinated seeds were planted into pots seven days after the germination and monitored for seedling survival. As seen from *Figure 6*, more than half of the seedlings (56%) died and 44% survived for 60 days.

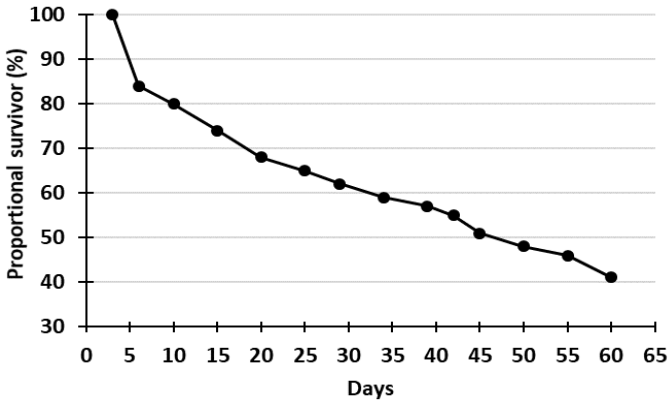

Figure 6. *Proportional survival of Pinus nigra* subsp. *pallasiana* var. *yaltirikiana seedlings*

The number of cotyledons was 8.3 (6-10). Likewise, Kaya and Temerit (1994) stated that the average number of cotyledons for 7 black pine populations they collected from Central Anatolia was 8.25.

In this study, the chromosome number of *Pinus nigra* subsp. *pallasiana* var. *yaltirikiana* was determined as 2n = 24, as known for black pines (Kaya et al., 1985; Salajova and Salaj, 1992; Hizume et al., 2002; Naydenov et al., 2003).

Ensuring the continuity of the limited number individuals of *Pinus nigra* subsp. *pallasiana* var. *yaltirikiana* in the natural distribution area is important in terms of genetic diversity and sustainability. These individuals should be taken under conservation by in-situ and ex-situ conservation strategies. As it is determined that seeds with the capacity to produce sound seeds and capability of germination can be obtained through current *Pinus nigra* subsp. *pallasiana* var. *yaltirikiana* individuals in the area, seedlings to be grown in the nursery by seeds with these characteristics can be planted around existing individuals to increase the number of theirs in this area.

In addition, seed orchard can be established in a different area by taking grafts from existing individuals, and sound seeds with the capability of germination can be obtained from these individuals. On the other hand, on the long run, observation must be performed in terms of purity of these seeds against the possibility of fertilizing female flowers of *Pinus nigra* subsp. *pallasiana* var. *yaltirikiana* individuals of the pollens of *Pinus nigra* subsp. *pallasiana* var. *pallasiana* individuals located in the same spread area.

Conclusions and Suggestions

Pinus nigra subsp. *pallasiana* var. *yaltirikiana* is an endemic variety in Turkey. The chromosome numbers of this taxon was found to be as 2n = 24. Its pollen dimensions were measured and the result showed that pollen seemed healthy with good shape. Therefore, we can conclude that there is nothing wrong with the pollens for reproduction of *Pinus nigra* subsp. *pallasiana* var. *yaltirikiana.* On the other hand, it

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seemed that this plant taxon has poor cone, germinable seed production with poor seedling survival. Because 49% of the seeds were sound, and 71% of them germinated within 28th days. Additionally, only 44% of seedlings survived and more than half of the seedlings (56%) died within 60 days. These meant that seedling survival of this taxon under nursery conditions was poor. Sound seed production and their germination percentage were lower compared to the results of previous studies. These findings can be considered as an indicator of poor reproduction of *Pinus nigra* subsp. *pallasiana* var. *yaltirikiana.* As it has a limited distribution, it is possible that this taxon which is in danger of survival in the near future, can be removed successfully from the list of danger categories in the future with the protection of the habitat and protection efforts for population growth. Some additional studies are needed for better understanding for seedling survival and growth under field conditions in the second step. It is necessary to maintain the existing stand of this tree firstly to ensure the sustainability of the taxon; and then to increase the presence in the natural distribution area with the individuals to be grown from the seeds.

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