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# Seed characteristics of *Flueggea anatolica*, an endangered forest shrub species

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**Abstract:** The Kadıncık shrub (*Flueggea anatolica*) is one of the endemic forest shrub species in Turkey. In this study, the morphological and physiological characteristics of *F. anatolica* seeds from three provenances (Tarsus, Kozan, Andırın) were investigated. The average 1000-seed weight was 4.9 g. There were significant differences between seeds from the different provenances in terms of their morphological characteristics, with the seeds from the Kozan provenance having the smallest seeds in terms of weight, thickness, and width. The seeds germinated at similarly high rates (90 ± 2%) at 16°C, 20°C, 24°C, and 28°C. However, the germinations occurred earlier at 24°C and 28°C, with 24°C appearing to be the most appropriate germination temperature. The results indicated that *F. anatolica* seeds have physiological dormancy, requiring approximately 10 weeks of prechilling for dormancy removal and germination. A 4-week warm-incubation period at 24°C prior to the prechilling treatment had only a slight effect on the prechilling duration time required to remove dormancy. Seeds stored in the refrigerator (+4°C) with a 2.7% or 4.0% moisture content conserved their high initial germination rates after one year of storage. The average germination rate of the prechilled seeds redried to an 8% moisture content and stored for one year at 4°C dropped from 92.7% to 63.6%. Basing on the results of this study, the suggested moisture content and storage temperature for storing *F. anatolica* seeds are 3–8% and 4°C or less, respectively.

**Additional key words**: *Flueggea anatolica,* seed, dormancy, prechilling

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## Introduction

The Kadıncık shrub (*Flueggea anatolica* Gemici), a member of the Phyllanthaceae family *sensu stricto* or Euphorbiaceae family *senso lato* (Barker and Van Welzen 2010; Govaerts et al. 2000; Hoffman et al. 2006; Hoffman 2007), is a critically endangered forest species in Turkey (Ekim et al. 2000; Özhatay et al. 2003; Serdar 2008). The species was discovered in recent decades (Gemici 1992; Gemici et al. 1996) and is one of the woody plants endemic to Turkey. *Flueggea* species are mostly found in relict populations (Webster 1984), and *F. anatolica* is described as a Tertiary relict plant (Gemici and Leblebici 1995).

There exist currently only three small distributions of the species (Ok 2006; Ok and Avşar 2007) with provenances in Tarsus, Kozan, and Andırın. In very limited areas, *F. anatolica* individuals arise primarily from sprouts, regeneration from seeds being rare. The seed of a plant is a symbol of its sustaina-

bility and diversity (Bradford and Cohn 1998), and for both *in situ* and *ex situ* conservation, an exploration of the seed characteristics of the species is needed.

Temperature is one of the most influential factors affecting the germination behavior of seeds (Bewley et al. 2013; Schmidt 2000). The temperature requirements of seeds for germination vary according to species, variety, and occasionally, provenance. The optimum germination temperature is also related to the ecology of the species location (Fenner and Thompson 2005). The favorable temperature for seeds is the temperature that provides the highest percentage of germination within the shortest period (Copeland and McDonald 2001).

Seed dormancy is extremely prevalent in temperate regions of the world (Suszka et al. 1996), with the depth of dormancy varying greatly and dependent on the species, provenance location, and individuals within the provenance (Smith et al. 2002; Desai 2004). The dormancy phenomenon of *Flueggea* species was unknown. Studies of seed storage, which is particularly important for endemic plant species such as *F. anatolica*, have also been increasing in recent decades. Before storing seeds, the storage characteristics of the species should be investigated.

The main objectives of this first detailed study on *F. anatolica* seeds were (1) to find out the morphological traits of the seeds; and (2) to explore the dormancy phenomenon, the pretreatment requirements, the effect of temperature on germination, and the storage characteristics of seeds. The interpopulation variation of seeds was also explored.

## Materials and Methods

Seeds were collected from trees from three small disjunct populations in southern Turkey with provenances in Tarsus, Kozan (September 22, 2009), and Andırın (October 6, 2009) (Table 1). *F. anatolica* fruits have an active ballistic dispersal mechanism, and the seeds are scattered during the fruit drying phase (Fig. 1). The seeds were therefore air-dried in the laboratory in perforated bags to an moisture content (MC) of approximately 8%. The MC of the seeds was determined by the low-temperature oven method for 17 hours at 104 ± 1°C (International Seed Testing Association 1996) and was expressed as the percentage of the fresh weight of the seed. Fresh seeds were also collected from Tarsus (August 1, 2009) and Andırın (September 3, 2009) to determine the maturation of early-collected seeds.

### Morphological characteristics of *F. anatolica* seeds

One hundred seeds from each provenance were randomly selected, and for each seed, three traits (length, width, and thickness) were measured with a sensitivity of 0.01 mm.

### Dormancy level and pretreatment requirements

The preliminary germination tests demonstrated that *F. anatolica* seeds are characterized by physiological dormancy. Therefore, to determine the presence

Table 1. Seed material used in the study

| Provenances (Populations) | Seed collection | Latitude | Longitude | Altitude [m] | Annual rainfall [mm] | Mean temperature [°C] | 1000-seed weight* [g] |
|---|---|---|---|---|---|---|---|
| Tarsus | September 22, 2009 | 37°05’ | 34°47’ | 300 | 787.8 | 16.3 | 5.93* |
| Kozan | September 22, 2009 | 37°31’ | 35°52’ | 380 | 941.7 | 18.0 | 3.75 |
| Andırın | October 6, 2009 | 37°32’ | 36°18’ | 970 | 1477.2 | 14.2 | 4.83 |

* Air dry weight, about 8% moisture content.

Fig. 1. The development of *F. anatolica* fruits (Ok 2006)

Table 2. Pretreatments of the *F. anatolica* seeds before the germination tests

| | |
|---|---|
| 1 | 8-week prechilling at 4°C |
| 2 | 10-week prechilling at 4°C |
| 3 | 12-week prechilling at 4°C |
| 4 | 4-week warm incubation at 24°C + 5-week prechilling at 4°C |
| 5 | 4-week warm incubation at 24°C + 7-week prechilling at 4°C |
| 6 | 4-week warm incubation at 24°C + 9-week prechilling at 4°C |

of dormancy, dormancy depth, and pretreatment requirements for germination, the seeds from each of the three provenances (Tarsus, Kozan, and Andırın) were pretreated with six different pretreatment regimes (Table 2). In the last three pretreatments, the seeds were incubated at 24°C for 4 weeks before subjecting them to the prechilling treatments (4°C). During both incubations (24°C and 4°C), the seeds were placed on moist double-layered filter paper in Petri dishes (maximum MC of *F. anatolica* is about 27%). To retain the moisture during the pretreatments, distilled water was added by spraying when necessary.

## Seed storage

Seeds of three provenances (Table 1) were stored under three conditions: (1) dormant seeds with a 4.0% MC, (2) dormant seeds with a 2.7% MC, and (3) nondormant seeds with an 8.0% MC, with the storage in sealed containers at 4°C in the dark for one year. A desiccator was used to reduce the MC of the seeds to 2.7% and 4.0%. To obtain the nondormant seeds, the seeds were prechilled for 10 weeks and then dried again to return them to an 8.0% MC. The germination tests were conducted before and after storage at 24°C.

## Maturation of seeds

The early-collected seeds harvested from Tarsus (August 1, 2009) and Andırın (September 3, 2009) were used in germination tests at 24°C after 10 weeks of prechilling.

## Germination temperature

To determine the effect of temperature on the germination behavior, seeds from the three provenances were prechilled in Petri dishes on moist double-layered filter paper for 10 weeks in the refrigerator (4°C) with the MC of the seeds maintained at the maximum level (27%). To maintain the seed MC at 25–27% MC, distilled water was sprayed on the seeds once every two weeks. After the prechilling treatments, the seeds were removed for germination testing under four different temperature regimes with constant temperatures of 16°C, 20°C, 24°C, and 28°C.

## Germination tests

The germination tests were performed on double-layered filter paper in 12-cm diameter Petri dishes with 150 (3 dishes × 50 seeds) seeds at the four temperatures (16°C, 20°C, 24°C, and 28°C). When were observed fungal infections, the filter papers were replaced. The seeds were considered germinated when their radicles protruded approximately 3 mm and displayed geotropism. The Petri dishes were examined every two days, and the germinated seeds were counted and removed. The germination tests were terminated after 28 days.

## Germination parameters

During the germination tests, the percentage of seeds that germinated (GP) and the mean germination time (MGT) were calculated with the following formulas:

1. Where $GP(\%)$ is the percentage of seeds that germinated, $n_i$ is the number of germinated seeds at week $i$, and $N$ is the total number of incubated seeds per test; and
2. Where $MGT$ is the mean germination time, $t_i$ is the number of weeks since the beginning of the test, and $n_i$ is the number of germinated seeds recorded for week $t_i$.

## Statistical analyses

The data for the 1000-seed weight, seed length, thickness, and width, GP, and MGT were evaluated by analyses of variance. The treatment means were tested with Duncan multiple-range tests. Arcsine square root ($\sqrt{P}$) transformations were applied to the GP values to normalize the error distributions prior to the variance analyses.

# Results

## Morphological characteristics of *F. anatolica* seeds

*F. anatolica* seeds demonstrated significant variation in terms of their morphological traits (Table 3, Fig. 2). The average 1000-seed weight was 4.90 g, with the heaviest seeds found in the Tarsus provenance (5.93 g) and the lightest in the Kozan provenance (3.75 g). The average length, width, and thickness of the seeds were 2.57 mm, 1.93 mm, and 1.71 mm, respectively. In general, the seeds from the provenances were largest in Tarsus and smallest in Kozan, with those from Andırın falling intermediate between them.

Table 3. 1000-seed weight and dimensions of *F. anatolica* seeds

| | 1000-seed weight [g] | Length [mm] | Width [mm] | Thickness [mm] | 1000-seed weight of early-collected seeds [g] |
|---|---|---|---|---|---|
| Tarsus | 5.93a[1] | 2.69a | 2.07a | 1.88a | 5.87 |
| Kozan | 3.75c | 2.35b | 1.80c | 1.60b | – |
| Andırın | 4.83b | 2.67a | 1.92b | 1.65b | 4.52 |
| Average | 4.90 | 2.57 | 1.93 | 1.71 | |

[1] The values on the same column followed by the same small letters are not significantly different at P<0.01.


Fig. 2. *F. anatolica* seeds from the three different provenances

## Dormancy depth and prechilling requirements

Seeds in the control treatment (without any pretreatment) did not germinate. The average germination rates after 8, 10, and 12 weeks of prechilling were 67.6%, 92.7%, and 92.2%, respectively (Table 4). Prechilling the seeds for 10 or 12 weeks eliminated dormancy completely in seeds of all three provenances, and the seeds reached the highest germination rates under these conditions. A 4-week warm-incubation period prior to the prechilling treatments was partially effective at removing the *F. anatolica* seed dormancy. Treating seeds with a 4-week warm-incubation period followed by only 5 weeks of prechilling resulted in a higher germination rate than that of seeds only prechilled for 8 weeks (Fig. 3).

Table 4. Germination rates for *F. anatolica* seeds at 24°C after 6 pretreatment regimes.

| Provenance | Germination rate (%) | | | | | |
|---|---|---|---|---|---|---|
| | 8-w pc | 10-w pc | 12-w pc | 4-w wi + 5w pc | 4-w wi + 7w pc | 4-w wi + 9-w pc |
| Tarsus | 48.0c[1] | 95.3a | 94.0a | 70.0b | 89.3a | 91.3a |
| Kozan | 75.3b | 90.7a | 89.3a | 66.0c | 78.7b | 92.0a |
| Andırın | 79.3c | 92.0a | 93.3a | 60.7d | 82.0b | 84.0b |
| Average | 67.6c | 92.7a | 92.2a | 65.6c | 83.3b | 89.1a |

[1] The values on the same line followed by the same small letters are not significantly different at P<0.05.
Abbreviations: w – weeks; pc – prechilled (4°C); wi – warm incubation (24°C).

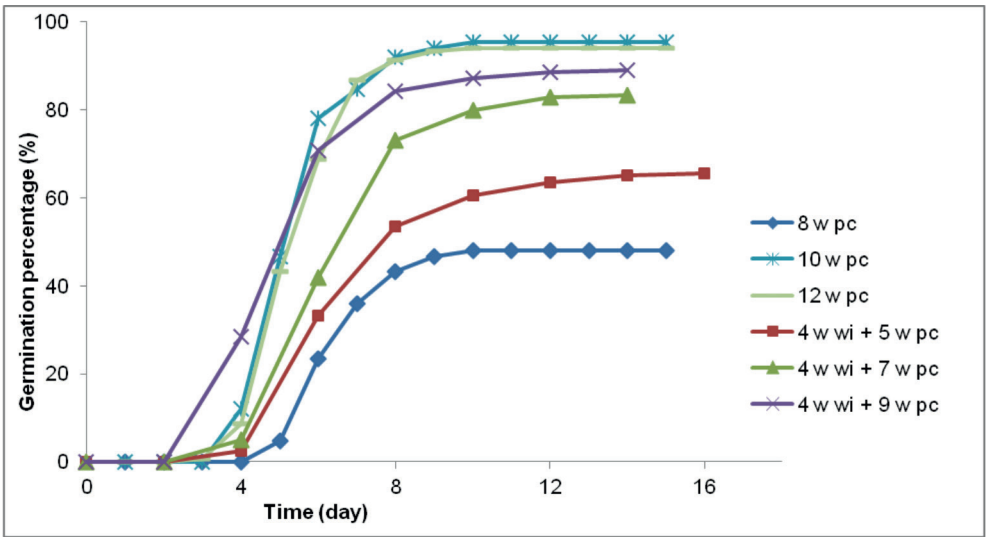


Fig. 3. Average germination rates for *F. anatolica* seeds after different pretreatments
Abbreviations: w, weeks; pc, prechilled at 4°C; wi, warm incubation at 24°C

Table 5. Mean germination times of *F. anatolica* seeds after 6 pretreatment regimes

| Provenance | Mean germination time (days) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 8-w pc | 10-w pc | 12-w pc | 4w wi + 5-w pc | 4-w wi + 7-w pc | 4-w wi + 9-w pc | Average |
| Tarsus | 9.6d[1] | 7.5b | 7.6b | 8.5c | 7.7b | 6.8a | 8.0C[2] |
| Kozan | 8.1c | 6.1a | 6.3a | 7.1b | 7.7b | 6.0a | 6.9B |
| Andırın | 7.0b | 4.9a | 4.8a | 6.9b | 6.2b | 5.0a | 5.8A |
| Average | 8.2c | 6.1a | 6.3a | 7.5bc | 7.2b | 5.9a | |

[1] The values on the same line followed by the same small letters are not significantly different at P<0.01.
[2] The values on the same column followed by the same capital letters are not significantly different at P<0.01.
Abbreviations: w – weeks; pc – prechilled (4°C); wi – warm incubation (24°C).

Table 6. Germination rates of *F. anatolica* seeds at different temperatures after prechilling (4°C) the seeds for 10 weeks

| Provenance | Germination temperatures (%) | | | | Average |
|---|---|---|---|---|---|
| | 16°C | 20°C | 24°C | 28°C | |
| Tarsus | 88.0a[1] | 90.0a | 94.0a | 92.7a | 92.2A[2] |
| Kozan | 89.3a | 92.0a | 90.0a | 92.7a | 91.6A |
| Andırın | 89.3a | 91.3a | 90.7a | 89.3a | 90.4A |
| Average | 88.9a | 91.1a | 91.6a | 91.6a | |

[1] The values on the same line followed by the same small letters are not significantly different at P<0.01.
[2] The values on the same column followed by the same capital letters are not significantly different at P<0.01.

Prechilling duration also affected the time required to germinate. In general, the longer the prechilling period, the earlier the germination occurred. The average MGT was roughly between 6 and 8 days. The population factor also had an effect on the average MGT (Table 5). The average time required for seed germination was least for the seeds from Andırın, most for those from Tarsus, with those from Kozan requiring a time intermediate between the other two provenances.

## Effect of temperature on germination

The GPs were similar at all of the tested temperatures (16°C, 20°C, 24°C, and 28°C), with seeds of different provenances demonstrating similar germination rates at the four different temperatures (Table 6). In contrast, the MGT distinctly differed at the various temperatures. The overall mean GPs of the seeds from Tarsus, Kozan, and Andırın were 92.2%, 91.6%, and 90.4%, respectively. Germination was accelerated by an increase in the germination temperature (Table 7), occurring earliest at 24°C and 28°C. The optimum temperature appeared to be approximately 24°C, where the GP was high and the MGT was shorter than that of the seeds germinated at 16°C or 20°C. Importantly, *F. anatolica* seeds did not germinate during the 4°C prechilling period.

## Maturation of *F. anatolica* seeds

The early-collected *F. anatolica* seeds, harvested in 2009 on August 1st (Tarsus) and September 3rd (Andırın), generally demonstrated high germination rates after 10 weeks of prechilling. For the seeds from the Tarsus provenance, there were no significant differences in GP between the August 1st and September 22nd collections (Fig. 4). In contrast, the GP for the seeds collected in the Andırın provenance, which represents the seed source at the highest altitude for the species, was significantly higher for fully mature seeds collected on October 6th than for the early-collected seeds on September 1st. The MGT was similar between the early-collected and mature seeds.

## Storage of *F. anatolica* seeds

Seeds from the three provenances stored with a 2.7% or 4.0% MC at 4°C retained their initial germination rates after one year of storage. The average

Table 7. Mean time until germination of *F. anatolica* seeds incubated at different temperatures after prechilling (4°C) the seeds for 10 weeks

| Provenances | Mean germination time (days) | | | | Average |
|---|---|---|---|---|---|
| | 16°C | 20°C | 24°C | 28°C | |
| Tarsus | 13.20c[1] | 7.99b | 5.90a | 6.24a | 8.33B[2] |
| Kozan | 12.41c | 7.69b | 5.37a | 5.25a | 7.68AB |
| Andırın | 11.73c | 6.95b | 4.80a | 4.80a | 7.07A |
| Average | 12.45c | 7.54b | 5.36a | 5.43a | |

[1] The values on the same line followed by the same small letters are not significantly different at P<0.01.
[2] The values on the same column followed by the same capital letters are not significantly different at P<0.01.

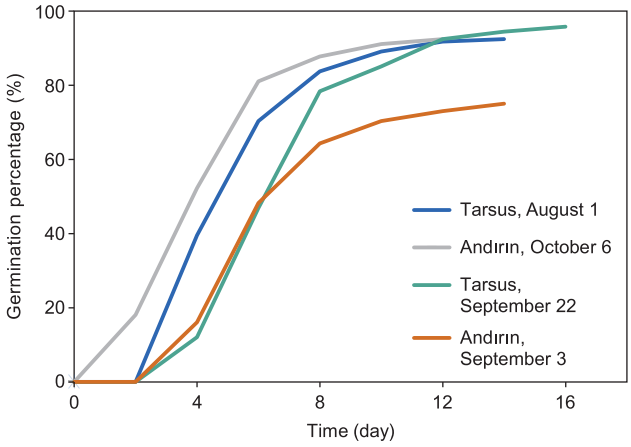


Fig. 4. Germination percentages of *Flueggea anatolica* Gemici seeds collected on different dates after prechilling (4°C) for 10 weeks

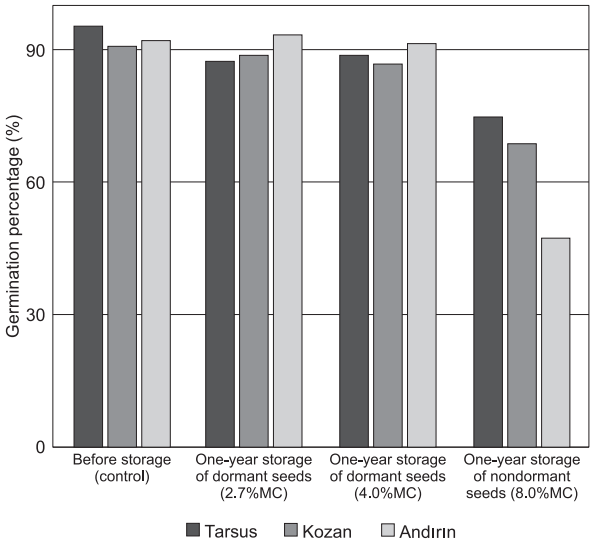


Fig. 5. Germination percentages of dormant and nondormant *F. anatolica* seeds after storage lasting one year at 4°C
MC: Moisture content

GPs for the seeds prior to storage and after storage for one year with 2.7% or 4.0% MC were 92.7%, 89.8%, and 88.9%, respectively (Fig. 5). In contrast, the GP of the nondormant seeds significantly declined after one year of storage in comparison to the initial germination rate, with a decline of 29.1% (92.7% to 63.6%) in the average germination rate after storage. The loss of viability in the nondormant seeds after storage was relatively higher for seeds from the Andırın provenance than that for seeds from the other two provenances. The mean germination time was similar for the dormant seeds, while the nondormant seeds germinated significantly more slowly.

## Discussion

The Kadıncık shrub *(F. anatolica)*, a recently discovered endemic woody species, is distributed in three small populations in southern Turkey (Ok and Avşar 2009). This first detailed study of seed characteristics of *F. anatolica* revealed the morphological characteristics, the depth of dormancy, the effects of temperature on the germination behavior, and the storage traits. Although the germination tests were terminated after 28 days, the experiments appeared to show that the test period of two weeks was a sufficient germination time for the *F. anatolica* seeds.

Based on the metric traits of *F. anatolica* seeds, the three provenances differed significantly (Table 3). Both the seed dimensions and seed weights varied, particularly between provenances. This result is consistent with the hypothesis that the habitat variation of a species is one of the factors that shapes seed morphology (Fenner and Thompson 2005).

*F. anatolica* seeds are generally bigger than those of other *Flueggea* species (Thomson 2006; Royal Botanic Garden Kew 2014), and the 1000-seed weight of *F. anatolica* (4.90 g) was heavier than that of *F. spirei* Beille (2.58 g) and *F. virosa* (Willd.) Voigt (3.45 g) (Royal Botanic Garden Kew 2014). Seed size plays a vital role in the ecology of a plant species, and genetic traits and environmental factors are the major determinants of seed size and shape (Leishman et al. 2000; Flores 2002). The seed mass is also highly dependent on environmental conditions during seed development (Michaels et al. 1988; Castro et al. 2006). In this study, significant variations were observed within and among the provenances in terms of seed size. Similar variations have been recorded in many other tree species including *Fagus sylvatica* L. (Thomsen and Kjaer 2002), *Pinus sylvestris* L. (Boydak 1975), *Cedrus libani* A.Rich. (Odabaşı 1967), *Pinus brutia* Ten. (Şefik 1964), *F. ornus* subsp. *cilicica* (Lingelsh.) Yalt. (Yilmaz and Tonguç 2009), *Fagus orientalis* Lipsky (Yilmaz 2005), *Sorbus* spp. L. (Bednorz et al. 2006), and *Juniperus communis* L. (Garciâ et al. 2000).

The depth of dormancy generally varies depending on the species, species location, or individuals at the same site (Schmidt 2000; Bewley et al. 2013). Periods of prechilling lasting eight weeks were clearly insufficient for the complete elimination of dormancy of the *F. anatolica* seeds, while they exhibited their full germination potential after 10 and 12 weeks of prechilling. The seeds from the three provenances had similar depths of dormancy and high germination rates after 10 weeks of prechilling. A 4-week warm incubation period was only slightly effective at removing dormancy. There was no evidence regarding the influence of the seed weight on germination rates, because the seeds from all provenances

demonstrated similarly high germination percentages after full removal of dormancy. The experiments suggest that 10 weeks of prechilling is sufficient for *F. anatolica* seeds to escape dormancy, and that the 4-week warm-incubation period was not necessary.

A warm incubation is employed for seeds with morphological dormancy (Baskin and Baskin 2004). This study showed that *F. anatolica* seeds have mature seeds when the seeds ripen around September, having only a physiological dormancy that requires about 10 weeks of prechilling for germination. The seed coat of the seeds allows both moisture and air to enter, and thus they do not have physical dormancy.

The depth of seed dormancy is closely associated with the natural distribution of the species and the distribution range (Schmidt 2000; Fenner and Thompson 2005). In this study, the seeds from the three provenances demonstrated a similar depth of dormancy, likely because of their relatively close distribution in a limited area of the eastern Mediterranean part of Turkey.

Germination temperature is generally very effective on seed germination parameters (Bewley et al. 2013; Schmidt 2000). However, temperatures applied in these experiments only effected the time required for germination (Table 6). Among the tested temperatures, 24°C appeared to be most efffecttive, as germination occurred significantly later at temperatures below 20°C. The germination temperature of a species is closely linked to the species distribution range (Baskin and Baskin 2001; Yilmaz 2005). Since the *Flueggea* species are distributed in warm geographic regions, the seeds demonstrated good germination behavior at 24°C and 28°C. Unlike many temperate woody species, such as *Fagus* spp., *Quercus* spp., and *Acer* spp., the seeds did not germinate at 4°C.

The maturation time of seeds varies with the climate, altitude, habitat, and slope, with the seeds generally ripening when they reach their maximum dry weight (Kozlowski and Pallardy 1997; Ohto et al. 2008). In this study, the seeds collected early (August 1) from low altitude (Tarsus, 300 m) had a higher germination rate than seeds collected early (September 3) at high altitude (Andırın, 970 m). This infers that *F. anatolica* seeds can be harvested in August and September at low altitudes, but that seeds from high altitudes should be harvested at the end of September. Seed viability is at its highest level when the seed reaches physiological maturity and begins to decrease thereafter (Priestley 1986; Yilmaz and Dirik 2008). *F. anatolica* seeds must be harvested from trees before their dispersal, because the fruits have a ballistic system and cannot be collected from the ground due to their size. Owing to the small size of the seeds, *F. anatolica* trees generally bear an abundant number of seeds each year. After collection, the MC of the fruits should be reduced to 6–8% in perforated bags.

Seed lifespan is closely associated with the MC of the seeds and the storage temperature (Roberts 1973; Priestley 1986). Respiration and deterioration increase with rising MC and temperature, causing seed aging to occur more rapidly (Harrington 2012; Copeland and McDonald 2001). The results from this study indicate that the MC of *F. anatolica* seeds can be safely reduced to 2.7%. Therefore, for longer-term seed storage, the seeds of this endangered plant species should be stored in seed banks with a 3–6% MC. For short-term storage (1 year), the *F. anatolica* seeds can be stored at 4°C. When storing orthodox seeds for longer time periods, temperatures of –5°C to –15°C are more suitable (Suszka et al. 1996; Li et al. 2008).

The storage characteristics of prechilled nondormant seeds vary by plant species, and prechilled nondormant seeds can be stored when MC returns to a 6–8% level after the prechilling treatment (Edwards 1996; Chien et al. 2002; Yilmaz 2008). These experiments revealed that *F. anatolica* seeds with an 8% MC could be stored for at least one year in the refrigerator (4°C), albeit with approximately a one-third loss of viability.

This study also showed that *F. anatolica* seeds have a deep physiological dormancy, and the sowing time in forest nurseries in Turkey is critical. For seed propagation, the dormant seeds should be sown around the beginning of December, thus naturally eliminating the dormancy of the seeds during the winter. On the other hand, nondormant seeds could also be sown in the spring after approximately 10 weeks of prechilling. Dry seeds of the species should not be difficult to store in seed banks for the long period. Urgent programs and projects are needed for the *in situ* and *ex situ* conservation of this endangered forest shrub species.

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