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In Vitro Germination of the Mediterranean Xerophytes *Thymelaea hirsuta* and *Thymelaea tartonraira* ssp. *tartonraira* as Affected by Scarification, Temperature, Photoperiod and Storage

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Abstract

With the aim of developing an efficient propagation method for the exploitation of *Thymelaea hirsuta* and *T. artonraira* ssp. *tartonraira* in the xeriscaping and pharmaceutical industry, the effects of the following were examined on the in vitro germination of their seeds: (i) pretreatment (mechanical and chemical scarification or immersion in hot water; (ii) incubation temperature (5–30 °C); (iii) incubation light conditions (16 h photoperiod or continuous darkness); (iv) storage period at room temperature and darkness (up to 24 months). Seeds collected for two years from the same wild plants in Greece were surface-sterilized with a 15% commercial bleach solution for 15 min after the abovementioned treatments and placed for germination in Petri dishes containing a half-strength MS medium in growth chambers. The rate and final percentage of germination were recorded. For both species, scarification after immersion in concentrated H_2SO_4, preferably for 20 min, was necessary for seed germination, which indicates coat dormancy. Higher germination percentages were observed at temperatures of 10–20 °C, under continuous darkness for *T. hirsuta* (79–100%) and regardless of photoperiod for *T. tartonraira* (73–90%). Long storage reduced germination of only *T. tartonraira* (54–68% at optimum temperatures, 23 months after harvest), while *T. hirsuta* seeds stored for 5 months germinated at significantly lower percentages (40% maximum) compared to seeds stored for 9–24 months, revealing a dry after-ripening process. Seeds of both species harvested at different years showed stable behavior in terms of germination. For both species, an effective seed propagation protocol suitable for their exploitation as ornamental and landscape plants was developed.

Keywords: sexual propagation; scarification pretreatment; sulfuric acid; incubation temperature; light conditions; storage period; seed coat dormancy; xerophytic ornamentals

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1. Introduction

Thymelaea (Mill, F. Thymelaeaceae) is a large genus including around 30 species of evergreen xerophyllous shrubs [1,2], which are mostly native to the Mediterranean region [2,3]. The species *Thymelaea hirsuta* (L.) Endl. and *Thymelaea tartonraira* ssp. *tartonraira* (L.) All. are small evergreen shrubs resistant to heat and drought, which grow well in poor stony soils. Thus, they are considered suitable for xeriscaping, in urban and peri-urban green spaces, as well as in landscape restoration and archaeological sites, in arid and semi-arid regions with Mediterranean climate [4–6]. Sustainable landscaping is now a requirement and can be ensured by the use of native species, as well as by the consideration of their ecological requirements and their response to abiotic and biotic stress factors [7].

Thymelaea hirsuta (Figure 1A) is a dioecious and sometimes monoecious, perennial evergreen shrub, up to 1.0 m, with white-downy stems and shiny green small, thick and scale-like leaves (Figure 1B), densely overlapping along the stem. The plants have very small yellowish flowers, born in clusters (Figure 1C) from October to May [8,9]. Its prolonged inflorescence period, including over winter, makes it a valuable bee-friendly plant, and insects play a major role in its pollination process [10]. The system of sexual tetramorphism was revealed in its natural populations, which combines characteristics of subdioecy (subandroecious and subgynoecious individuals) and heterodichogamy (protogynous and protandrous individuals), as an evolutionary pathway from heterodichogamy to dioecy [11]. The increased genetic diversity of *T. hirsuta* populations and the richness of the plant in total lipids and phenols lead to increased plant adaptability and resilience to various harsh environmental conditions [12]. Moreover, *T. hirsuta* is an important medicinal plant, that has been used as traditional medicine in several countries of northern Africa [2,13,14], as a decoction that is promising for use in pharmaceutical industries [15] in the treatment of diabetes [14,15], infertility [15], skin infections [16], etc. Its extracts possess high levels of anti-oxidant activity [13,16,17], showing potential anticancer activities as well [13,14]. It could also be a potential source for biodiesel production [18].



Figure 1. Characteristic plant (**A**), leaves (**B**) and flowers (**C**) of *Thymelaea hirsuta*; typical plant (**D**), leaves (**E**) and flowers (**F**) of *Thymelaea tartonraira* ssp. *tartonraira*.

Thymelaea tartonraira ssp. *tartonraira* (L.) All. (Figure 1D) is a much-branched, silvery-grayish, evergreen dioecious shrub, up to 0.60 m, with numerous silkily hairy leaves (Figure 1E) and yellowish flowers, borne in small clusters of 2–5 at the base of the upper leaves (Figure 1F) from February to April [8,9]. Leaf extracts possess medicinal properties and are proposed for use in the treatment of diabetes [19], as antioxidant and specific antihyperglycemic agents [20] and against leishmaniasis [21].

The development of an efficient propagation method would facilitate their sustainable exploitation, since their natural populations are gradually declining [22,23]. However,

rooting of stem cuttings, which is a popular propagation method in horticultural practice, proved unsuccessful for these two species [23,24]. Regarding micropropagation attempts, although satisfactory in vitro culture establishment and shoot multiplication have been successful for both species when using node explants excised from in vitro grown young seedlings [4–6,25], microshoot rooting was ineffective [23] due to the microshoots failing to root [4,6,25].

Since vegetative reproduction proved difficult to implement, as described above, sexual breeding seems to be the only way to propagate these species and regenerate their natural population, with a potential use in conservation strategies [23]. However, their sexual propagation presents difficulties as a result of the following: (i) seasonal variation in fruit and seed production [23]; (ii) plants' dimorphism that greatly differentiates fruit productivity of each plant type, i.e., high fruit vs. low fruit production in females/protandrous plants and in males/protogynous plants, respectively [11]; (iii) extremely low seed germination ability [23,24]. Immersion of the seeds in concentrated H_2SO_4 for 10–20 min, combined with GA_3 pretreatment, increased their germination up to 45% [26], which indicates coat seed dormancy. Furthermore, research is required in order to increase its effectiveness by testing techniques to break seed dormancy and increase the efficiency of seed germination. Propagation by seed is an easy method for commercial nurseries to use, especially when it concerns native species, as it is also suitable for the production of plants intended for landscape regeneration or restoration due to genetic variability, which is desirable in the reintroduction of native species in the natural landscape [27]. Nevertheless, seeds of native species can vary widely in seed quality factors, such as seed size, purity, dormancy and germination vigor [28]. Propagation by seed can also be used to select desirable genotypes in breeding programs, which is of high importance for the studied *Thymelaea* species since they are both promising medicinal plants.

Dormancy and germination are two closely linked physiological stages of seeds, which have a great impact on adaptation and survival of seed plants. They are influenced by a variety of internal and external environmental factors and are also important for agricultural production [29–31]. Diverse endogenous hormones, such as abscisic acid (ABA) and gibberellins (GAs), are known to act antagonistically to regulate seed dormancy, while recent findings demonstrate that also auxin is critical for inducing and maintaining seed dormancy [29]. The determinant role of ABA in seed dormancy and the requirement for GAs for germination has been identified by genetic analysis [32]. Among the environmental factors, light and temperature are the most critical, as they regulate the ABA/GA biogenesis and signaling pathways, and affect the pathways of auxin and other hormones, such as ethylene, jasmonic and salicylic acid during seed germination, acting as a signal to determine whether seeds stay dormant or start to germinate [29,33–35]. Other factors, such as nitrogen availability, water content and bacteria in soil, also affect seed dormancy and germination [31,33].

Seed structures that surround the embryo can affect seed dormancy and germination, while compounds produced by the embryo itself or imported from the mother plant may also affect its growth potential [32]. Therefore, seed dormancy can be caused by hard seed coats, underdeveloped or primitive embryos and inhibitory compounds. In natural conditions, dormancy in seeds is broken by soil and microbial activity, soil melting and freezing, forest fires and the consumption of seeds by animals [36]. Seeds can also be released from their dormancy by scarification, which can be carried out mechanically, either by using sandpaper or a sharp instrument, to chip or pierce the seed coat, or chemically by immersion in sulfuric acid, followed by others like stratification, light and heat treatment, dipping in hot water, leaching, etc.; the most appropriate method varies according to the plant species [36,37].

Temperature and light conditions are the most important environmental factors in seed germination, affecting both the germination rate and speed [38]. The timing of germination can also be controlled by temperature through the range over which seeds may germinate. In natural seed populations, environmental temperature regulates their dormancy status, allowing the seeds to avoid the establishment of seedlings under harsh environmental conditions [39]. The storage period and conditions also affect the germination process [40], as during the post-harvest phase of after-ripening, important physiological and biochemical changes are caused in seeds that ultimately break dormancy and enable germination through the modification of their internal environment [41]. After-ripening primarily occurs in dried seeds under dry storage conditions and determines germination potential [41].

In the present work, the effects of breaking dormancy pretreatment, incubation conditions (temperature and photoperiod) and storage period on the in vitro germination of *T. hirsuta* and *T. tartonraira* ssp. *tartonraira* seeds were examined, aiming to develop an efficient propagation method that would facilitate their wider use in commercial floriculture and landscaping, as well as in the pharmaceutical industry. Seeds were collected for two years from the same wild plants in Greece near Athens to check the stability of germination between different harvest years, and several experiments were conducted in which various factors were tested, i.e.: (i) pretreatment (mechanical scarification with sandpaper for 2 or 4 min, immersion in concentrated sulfuric acid (H_2SO_4) for 15 or 20 min and immersion in hot water (100 °C) for 1 or 5 min; (ii) incubation temperature (5, 10, 15, 20, 25 or 30 °C); (iii) incubation light conditions (16 h photoperiod, continuous darkness); (iv) storage period of seeds at room temperature and darkness (5, 9, 12, 18 or 24 months).

2. Materials and Methods

2.1. Seed Harvesting and Its Viability

Seeds of *T. hirsuta* were harvested in late May 2013 and 2015 in the Ilioupoli area (37°95′03.07″ N, 23°75′39.26″ E, altitude 170 m), a suburban municipality southeast of central Athens, at the foot of Mount Hymettus, Greece, while seeds of *T. tartonraira* ssp. *tartonraira* were harvested in late May 2014 and 2015 a little higher up in the foothills of Mount Hymettus (37°92′68.44″ N, 23°76′68.52″ E, altitude 396 m). Since both species are dioecious, only female plants with high seed production were carefully chosen, and both seed collections for each species were performed from the same wild plants. The seed material was stored in glass vessels at room temperature (about T = 21 °C and darkness), after having been left to dry for 15 d in an open-air shaded place. In order to be used for the experiments, the dried floral debris were removed from seeds by rubbing them together by hand (Figure 2A). The weight of 100 seeds was estimated after removing the floral debris.

Seed viability of *T. hirsuta* was determined 2 and 24 months after harvesting using seeds collected in May 2013. Seed viability of *T. tartonraira* was determined 2 months after harvesting using seeds collected in May 2014. Seeds were submitted to 2,3,5-triphenyltetrazolium chloride (TZ) staining (1.0%), at 20 °C, in the dark, for 24 h. For each test, a total of 100 seeds and 4 vessels containing 25 mL of TZ-solution each were used (25 seeds/Magenta^{TM} glass vessel). The seeds remained in the solution for 24 h, at 20 °C, in the dark. Then, the coloration of the embryo was observed using a portable QS.20200-P digital microscope (Euromex Microscopen, Arnhem, The Netherlands). The embryo of viable seeds was colored red. Embryos were considered non-viable when they had less than 1/2 cotyledon colored red or non-colored hypocotyl [42].

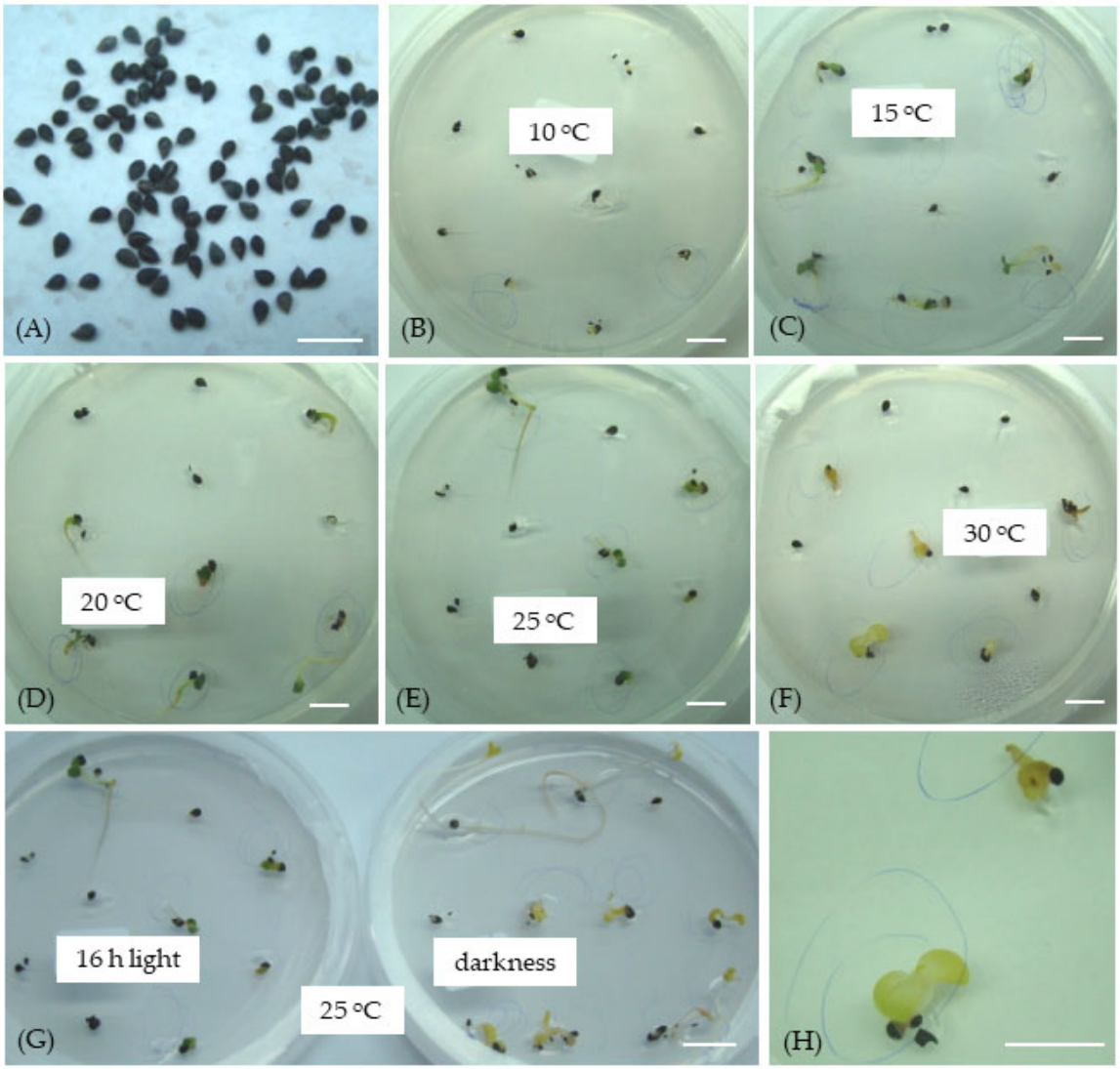


Figure 2. Seeds of *Thymelaea hirsuta* after removal of dried floral debris (**A**); typical germination of seeds scarified by concentrated H_2SO_4 for 20 min and incubated under 16 h photoperiod for 30 days at 10 °C (**B**), 15 °C (**C**), 20 °C (**D**), 25 °C (**E**) and 30 °C (**F**), as well as incubated at 25 °C, under 16 h photoperiod or continuous darkness (**G**); abnormal radicle development in seedlings germinated at 30 °C (**H**). Size bar = 1.0 cm.

2.2. Seed Surface Sterilization Method

In all of the germination experiments of both species, a common surface sterilization method of seeds was applied, which had previously been successfully used in other Mediterranean species [43,44]. This stage took place after the application of various pretreatments for breaking seed dormancy and before seeds' placement in Petri dishes for germination.

Surface sterilization of seeds was performed by immersion in a 15% *v*/*v* water solution of commercial bleach (4.5% *w*/*v* sodium hypochloride) for 15 min, in which 1–2 drops of Tween 20 (polyxyethylenesorbitan monolaurate, Merck KGaA, Darmstadt, Germany) were added. Following this, three 3 min rinses with sterile distilled water were performed.

2.3. In Vitro Germination Experiments of T. hirsuta with Seeds Harvested in 2013

Aiming to investigate seed dormancy of *T. hirsuta* and incubation conditions (temperature and photoperiod) for in vitro germination, the following experiments were conducted with seeds collected in 2013:

A. Seeds, 5 months after harvest, were surface-sterilized and placed in Petri dishes for in vitro germination (ten seeds per dish) on a half-strength (1/2) MS medium [45] (Sigma-Aldrich, St. Louis, MO, USA), with sucrose (20 g L^{-1}) and agar (8 g L^{-1}), at temperatures of 5, 10, 15, 20 and 25 °C, and in a photoperiod of 16 h cool white fluorescent light (37.5 mmol·m^{-2}·s^{-1}) and 8 h darkness. The seeds, before surface sterilization, had received one of the following: (i) no pretreatment (control); (ii) they had been scarified with sandpaper (suitable for wooden surfaces) for 1 min; (iii) they had been immersed in concentrated H_2SO_4 (>95%, Fisher Scientific, Loughborough, UK) for 15 min. Sandpaper, which is suitable for iron surfaces, was also tested as a scarification material for the same period of time.

B. Seeds, 9 months after harvest, were subjected to various pretreatments: (i) control; (ii) scarification using sandpaper (suitable for wooden surfaces) for 2 or 4 min; (iii) immersion in H_2SO_4 for 15 or 20 min; (iv) immersion in boiling water (100 °C) for 1 or 5 min, before surface sterilization and incubation for in vitro germination in Petri dishes with a 1/2 MS medium, at 10 °C and 16 h photoperiod.

C. Seeds, 12 months after harvest, were either untreated (control) or immersed in H_2SO_4 for 15 min, subjected to the same surface sterilization and placed in Petri dishes with 1/2 MS medium at the temperatures 5, 10, 15, 20, 25 and 30 °C, under 16 h of light or continuous darkness. Seeds immersed in H_2SO_4 for 20 min were also incubated at the above temperatures, only under 16 h of light.

D. Seeds, 18 and 24 months after harvest, were immersed in H_2SO_4 for 20 min, surface sterilized and placed in Petri dishes with a 1/2 MS medium at temperatures of 5, 10, 15, 20, 25 and 30 °C, under 16 h of light or continuous darkness. In the experiments conducted 12 and 18 months after collection, it was observed that mainly seeds whose white endosperm had been partially or fully exposed after scarification germinated. Thus, in the experiment with 24-month-old seeds, after immersion in H_2SO_4, the coat of seeds (about 30% of the total), which had not broken but had become thin and brittle from the H_2SO_4 treatment, was carefully broken by pressing the seeds one by one with a scalpel into the container used for surface sterilization.

Five replicates of 20 seeds were used for each treatment. Germination observations were made every 3 days for a period of 1 month. The radicle should be longer than 1 mm in order for a seed to be considered germinated. In the experiments that took place 18 and 24 months after seed harvest, apart from seed germination percentage (%), the following were also estimated: (i) T_{50} that is the time needed for seeds to germinate at 50% of the final value and is calculated from the two germination values closest to median germination; (ii) time for full germination; (iii) percentage (%) of germinated seeds with well-developed root.

2.4. In Vitro Germination Experiments of T. tartonraira ssp. tartonraira with Seeds Harvested in 2014

Seeds of *T. tartonraira* harvested in May 2014 were limited. Therefore, only seed dormancy and incubation temperature were investigated with the seed lot of that year.

A. Two weeks after harvest, the seeds, after being surface-sterilized, were immersed in concentrated H_2SO_4 for 20 min and then placed in Petri dishes with 1/2 MS medium, initially at 10 °C for the first two weeks and then at 15 °C, under a 16 h photoperiod.

B. Four and twelve months after harvest, the seeds were immersed in concentrated H_2SO_4 for 20 min, surface-sterilized and placed in Petri dishes with a 1/2 MS medium at temperatures of 5, 10, 15, 20, 25 and 30 °C, under a 16 h photoperiod. In the 12-month-old seed experiment, those seeds whose coats had not broken after immersion in H_2SO_4 were carefully partially broken to reveal the white endosperm by pressing the seeds one by one with a scalpel into the container used for surface sterilization.

Observations were taken with the same frequency as in the case of *T. hirsuta,* and the same data were estimated.

2.5. Experiments on Germination of Both Thymelaea Species with Seeds Harvested in 2015

Due to the sufficient number of seeds collected for both *Thymelaea* species in May 2015, an experiment with the same treatments for both species could be carried out simultaneously, based on the results of the 2013 and 2014 experiments. This experiment was also intended to provide information on the stability of germination results between different years of seed harvest. So, the effect of three factors, i.e., chemical scarification, incubation temperature and light conditions, could be examined simultaneously in a three-factor experiment, which was repeated twice for each species, i.e., 9 and 23 months after seed harvest.

Seeds of *T. hirsuta* and *T. tartonraira*, 9 and 23 months after harvest, either received no pretreatment (control) or they were immersed in concentrated H_2SO_4 for 20 min. Those seeds whose coat had not broken after immersion in H_2SO_4 were carefully partially broken to reveal the white endosperm by pressing the seeds one by one with a scalpel into the container used for surface sterilization. The empty seeds were discarded in this way as well. Based on the results of tetrazolium test for seed viability, in order to have 100 viable seeds for each treatment, about 150 seeds were used to be scarified. Then, the seeds were surface-sterilized and placed for incubation in Petri dishes with 1/2 MS medium at temperatures 10, 15, 20 and 25 °C, under a 16 h photoperiod or continuous darkness. The temperatures 5 and 30 °C were excluded in these experiments, based on previous results. Five replicates of 20 seeds were used for each treatment. Germination observations were taken every 3 days for a period of 1 month. The radicle should be longer than 1 mm in order for a seed to be considered germinated. At the end of the one-month incubation period, the following were estimated: (i) germination percentage (%); (ii) germination percentage (%) with well-developed seedling (with normal leaf and root development); (iii) T_{50}.

2.6. In Vitro Culture Conditions

In all experiments, seeds were placed on the same substrate for germination, which was 1/2 MS, with the addition of 20 g L^{-1} sucrose, and solidified with 8 g·L^{-1} agar. Its pH was adjusted to 5.7 before the addition of the agar and autoclaving at 121 °C for 20 min. The incubation of seeds took place in Petri dishes (d = 9 cm) with a 10 mL substrate, where ten seeds were placed. The dishes were sealed on the side with parafilm (roll 5 cm × 15 m, Bemis, Sheboygan Falls, WI, USA). The seed dishes were placed in incubation chambers, with a 16 h photoperiod/8 h dark, at 37.5 mmol·m^{-2}·s^{-1} provided by cool-white fluorescent lamps. The seed dishes that received continuous darkness treatment were kept in the same incubation chambers but were wrapped in aluminum foil to create dark conditions.

2.7. Statistical Analysis

In all experiments, a completely randomized design was used. One-, two- or three-way analysis of variance (ANOVA) were used to test the significance of the results, and the comparison of the treatment means was carried out by Student's *t* test at $p \leq 0.05$ (JMP 13.0 software, SAS Institute Inc., Cary, NC, USA, 2013). Standard errors (SEs) were also calculated. Statistical analysis of the data on percentage was performed after arcsine transformation.

3. Results

3.1. In Vitro Germination of T. hirsuta Seeds Harvested in 2013

The weight of 100 seeds of *T. hirsuta* was 0.275 g, and the tetrazolium test showed that in fresh (2 months after collection) and stored for 24 months seeds the percentage of seed viability did not differ, since 74.0% and 72.0% of the seeds, respectively, were found viable.

In order to test the need for scarification pretreatment of *T. hirsuta* seeds and the effect of incubation temperature on germination, seeds, 5 months after their harvest, were incubated under a 16 h photoperiod and at temperatures of 5 to 25 °C, without any scarification pretreatment, after mechanical scarification with sandpaper for 1 min or with immersion in concentrated H_2SO_4 for 15 min. Non-scarified seeds germinated at percentages lower than 5% and only seeds that had been immersed in H_2SO_4 germinated at much higher percentages, reaching 40% at 10 °C, while at 15 to 25 °C, germination was 8 to 18% (Figure 3A). At 5 °C, there was no germination (Figure 3A).

In order to further test the most appropriate seed pretreatment, 9 months after harvest, seeds received various pretreatments and were incubated at 10 °C under a 16 h photoperiod. Only pretreatment with H_2SO_4 for 15 or 20 min induced increased germination (74 and 64%, respectively), in contrast to scarification with sandpaper for 2 or 4 min, immersion in boiling water for 1 or 5 min or the absence of any pretreatment, where germination was less than 4% (Figure 3B). Sandpaper, either for wooden or iron surfaces, was not suitable as a scarification material because it destroyed the seeds by crushing them. The germination of 9-month-old seeds was significantly increased compared to 5-month-old seeds (Figure 3A,B).

Further, the effect of photoperiod on germination was tested. Thus, seeds 12 months after harvest, which received no pretreatment or were immersed in H_2SO_4 for 15 min, were incubated at 5 to 30 °C under a 16 h photoperiod or continuous darkness. Under a 16 h photoperiod, seeds, as in previous experiments, showed the highest germination (60%) at 10 °C, while at 15–30 °C, germination ranged from 5 to 30% (Figure 3C). However, under continuous darkness, high germination (58–68%) was observed in a wider range of temperatures, from 10 to 20 °C. At 5 °C, no seeds germinated, while at 25 and 30 °C, seedlings were dehydrated and weak and had reduced growth. Non-scarified seeds did not germinate regardless of the light conditions (Figure 3C).

Scarification with H_2SO_4 was tested further as for the immersion time, with 12-month-old seeds, under a 16 h photoperiod and 5–30 °C incubation temperatures (Figure 3D). It was shown that 20 versus 15 min of immersion caused slightly higher germination at higher temperatures (25 and 30 °C) and faster completion of germination under all temperatures (Table 1A). Faster germination was also caused by increasing the incubation temperature (Table 1A).

Based on the above results, in the following experiments with 18- and 24-month-old seeds, scarification was performed by a 20 min immersion in H_2SO_4. Seeds, 18 months after harvest, showed similar germination responses to incubation temperatures and photoperiods compared to 9- and 12-month-old seeds (Figures 3 and 4A,B). The increase in temperature and incubation under darkness decreased T_{50} and time to full germination (Table 1A–C).

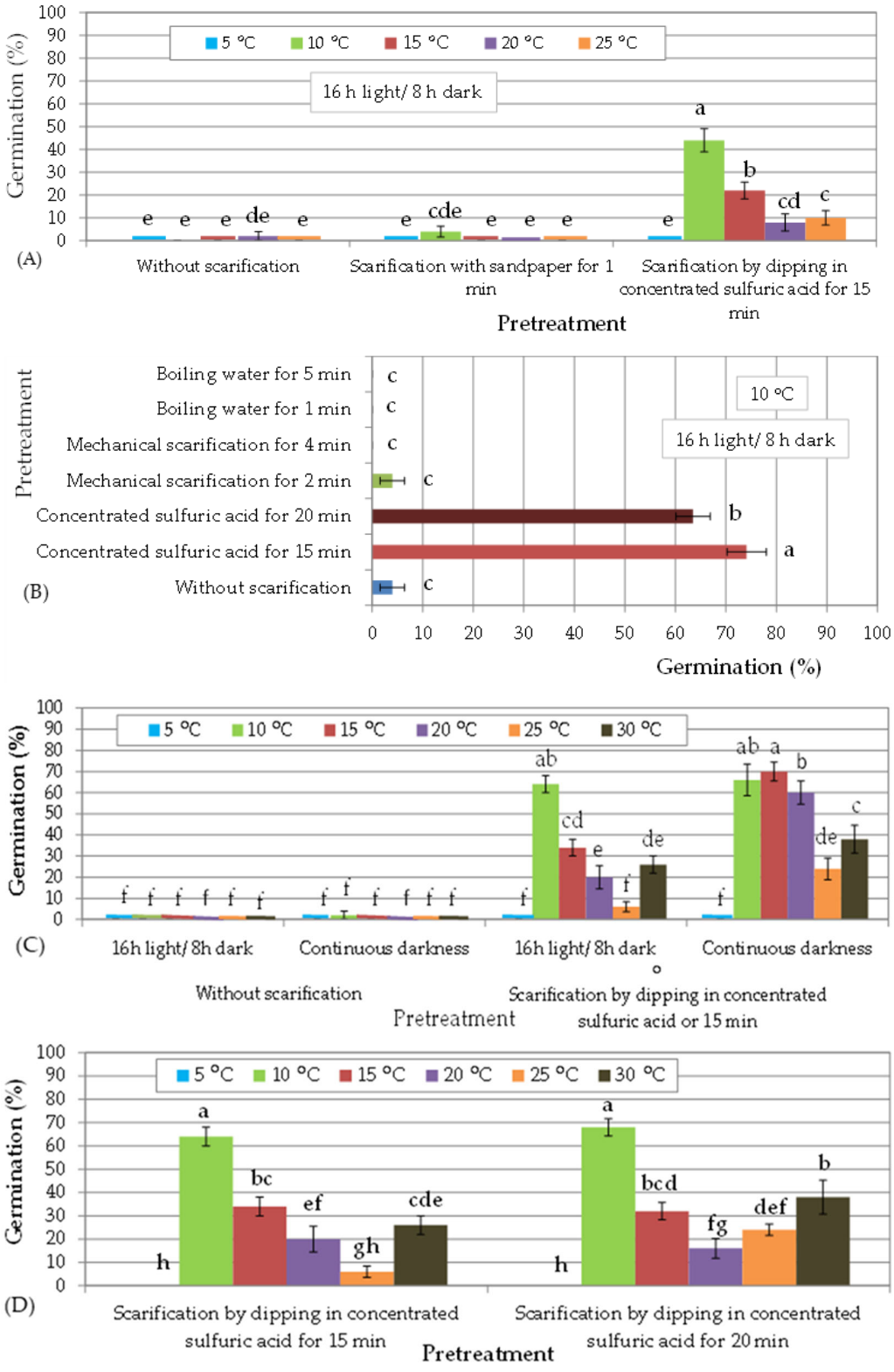


Figure 3. Effect of seed pretreatment, as well as of incubation temperature and photoperiod on in vitro germination of *Thymelaea hirsuta* seeds harvested in 2013, 5 (**A**), 9 (**B**) and 12 (**C**,**D**) months after harvesting and storage at room temperature. Mean values (±SE, n = 5 repetitions of 20 seeds) in each figure followed by the same lower-case letter do not differ significantly at $p \leq 0.05$ by Student's t test. ANOVA for (**A**): two-way ANOVA Fscarification × temperature **, Fone-way ANOVA **; ANOVA for (**B**): Fone-way ANOVA **; ANOVA for (**C**): three-way ANOVA Fscarification × temperature × light **, Fone-way ANOVA **; ANOVA for (**D**): two-way ANOVA Fscarification × temperature **, Fone-way ANOVA **; **: significant at $p \leq 0.01$.

Table 1. Effect of incubation temperature and pretreatment or photoperiod on T_{50} (time for 50% germination), time for full germination and percentage of germinated seeds with well-developed roots, during in vitro germination of *Thymelaea hirsuta* seeds, harvested in 2013, 12 (**A**), 18 (**B**) and 24 (**C**) months after harvesting and storage at room temperature. Seeds had been scarified by sulfuric acid for 20 min, except for (**A**), where immersion for 15 min was also applied.

(**A**) Seed storage duration: 12 months												
Pretreatment	Sulfuric acid for 15 min						Sulfuric acid for 20 min					
Temperature (°C)	5	10	15	20	25	30	5	10	15	20	25	30
T_{50} (d)	-	21	15	18	9	12	-	21	15	9	9	12
Time (d) for full germination	-	30	27	24	27	27	-	27	21	12	21	18
(**B**) Seed storage duration: 18 months												
Photoperiod	16 h light/8 h dark						Continuous darkness					
Temperature (°C)	5	10	15	20	25	30	5	10	15	20	25	30
T_{50} (d)	-	21	12	9	9	6	-	21	12	6	6	6
Time (d) for full germination	-	27	27	24	21	18	-	27	24	21	12	15
(**C**) Seed storage duration: 24 months												
Photoperiod	16 h light/8 h dark						Continuous darkness					
Temperature (°C)	5	10	15	20	25	30	5	10	15	20	25	30
T_{50} (d)	-	19	11	7	7	7	-	15	7	7	7	7
Time (d) for full germination	-	28	28	25	22	25	-	22	22	15	25	25
Germinated seeds with well-developed root (%)	-	92.9	88.5	76.2	22.7	0.0	-	97.3	91.4	83.3	50.0	31.8

-: no data available, as no seeds germinated at 5 °C.

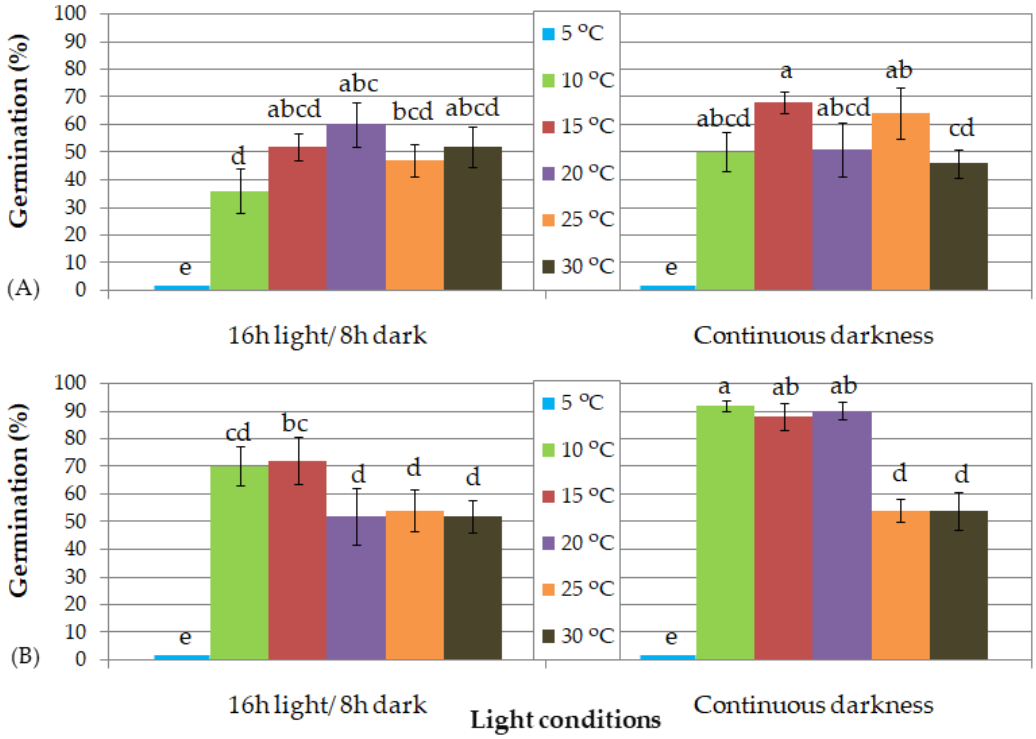


Figure 4. Effect of incubation temperature and photoperiod on in vitro germination of *Thymelaea hirsuta* seeds, harvested in 2013 and scarified by sulfuric acid for 20 min, 18 (**A**) and 24 (**B**) months after harvesting and storage at room temperature. Mean values (±SE, n = 5 repetitions of 20 seeds) in each figure followed by the same lower-case letter do not differ significantly at $p \leq 0.05$ by Student's t test. ANOVA for (**A**): two-way ANOVA Fphotoperiod × temperature ^{NS}, Fphotoperiod ^{NS}; Ftemperature **; Fone-way ANOVA **, ANOVA for (**B**): two-way ANOVA Fphotoperiod × temperature *; Fone-way ANOVA: **; NS or * or **, non-significant at $p \leq 0.05$ or significant at $p \leq 0.05$ or $p \leq 0.01$, respectively.

Twenty-four months after harvest, the highest seed germination rates (90%) of all experiments were observed, at 10 to 20 °C, under continuous darkness (Figure 4B). In this experiment, only seeds whose endosperm had been exposed were used, either after immersion in concentrated H_2SO_4 or after the additional breaking of the coat with a scalpel. In this temperature range, the superiority of incubation under darkness over germination under 16 h of light on seed germination percentage was obvious (Figure 4B). Besides, only seedlings that were germinated at temperatures from 10 to 20 °C had normally developed radicles in very high percentages. On the contrary, at 25 and 30 °C, in addition to the fact that the seedlings were dehydrated, the normal development of the radicle was hindered (Figure 2H), and this was more pronounced under conditions of 16 h light compared to continuous darkness (Table 1C, Figure 4B).

3.2. In Vitro Germination of T. tartonraira ssp. Tartonraira Seeds Harvested in 2014

The weight of 100 seeds of *T. tartonraira* was 0.265 g, and the tetrazolium test showed that 68.0% of the seeds were viable 2 months after collection.

T. tartonraira seeds, 2 weeks after harvest, germinated at a high percentage (78%) at 15 °C, under a 16 h photoperiod, after pretreatment with concentrated H_2SO_4 for 20 min.

Four and twelve months after harvest, only seeds whose endosperm had been exposed after pretreatment with H_2SO_4 for 20 min were used for germination experiments under a 16 h photoperiod at temperatures ranging from 5 to 30 °C. Both the storage period and incubation temperature affected germination (Figure 5). Twelve-month-old seeds germinated at higher percentages than four-month-old seeds, and their germination was high (75–85%) at a wider range of temperatures, specifically 15–25 °C. In 4-month-old seeds, there was an indication that they germinated at a higher percentage (68%) at 15 °C compared to germination at 10 and 20 °C (50%), while at 25 and 30 °C, germination was reduced (46 and 39%, respectively). At 5 °C, no seeds germinated (Figure 5).

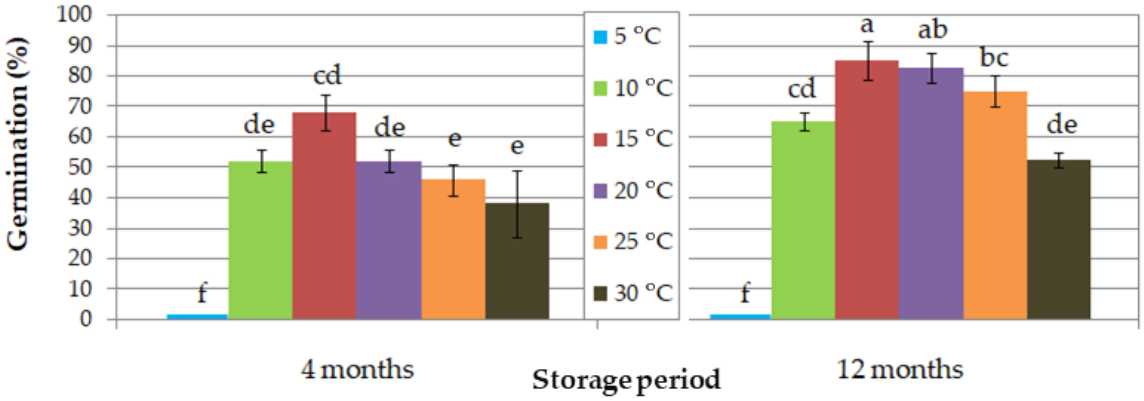


Figure 5. Effect of incubation temperature and storage period on in vitro germination of *Thymelaea tartonraira* ssp. *tartonraira* seeds harvested in 2014. Seeds were scarified by sulfuric acid for 20 min and incubated under a 16 h photoperiod. Mean values (±SE, n = 5 repetitions of 20 seeds) followed by the same lower-case letter do not differ significantly at $p \leq 0.05$ by Student's t test. Two-way ANOVA: Ftemperature × storage ^{NS}, Fstorage **, Ftemperature **; Fone-way ANOVA:**; NS or **: non-significant at $p \leq 0.05$ or significant at $p \leq 0.01$, respectively.

The increase in temperature decreased the time for T_{50} and the time for full germination in this species, as in *T. hirsuta*. A higher percentage of seedlings with well-developed roots was observed at 15 and 20 °C, followed by 10 °C (Table 2).

Table 2. Effect of incubation temperature on T_{50}, time for full germination and percentage of germinated seeds with well-developed roots, during in vitro germination of *Thymelaea tartonraira* ssp. *tartonraira* seeds, collected in 2014, scarified by sulfuric acid for 20 min and incubated under 16 h photoperiod, after 4 (A) and 12 (B) months of storage.

Storage Period	**(A) 4 Months**						**(B) 12 Months**					
Incubation temperature (°C)	5	10	15	20	25	30	5	10	15	20	25	30
T_{50}	-	18	10	8	6	6	-	15	7	5	5	5
Time (d) for full germination	-	32	22	22	14	32	-	28	25	25	25	11
Percentage (%) of germinated seeds with well-developed root	-	-	-	-	-	-	-	76.9	82.4	81.8	66.7	0.0

-: no data available, as no seeds germinated at 5 °C.

3.3. In Vitro Germination of Both Thymelaea spp. with Seeds Harvested in 2015

To test the stability of the seed response of the two *Thymelaea* spp. to germination treatments, a factorial experiment was conducted for each species, with seeds collected in a subsequent year, where the factors of seed pretreatment (scarification with H_2SO_4 for 20 min or not), incubation temperature (from 10 to 25 °C), photoperiod (16 h of light or continuous darkness) and storage time (9 and 23 months) were examined.

3.3.1. *T. hirsuta* (2015 Harvest Year)

Regarding the effect of the four experimental factors on the in vitro germination of *T. hirsuta* seeds, four-way ANOVA showed a significant interaction of the experimental factors, as well as three-way ANOVA for each storage period and two-way ANOVA for each storage period and scarification. So, germination data were further analyzed by one-way ANOVA (Figure 6).

Storing the seeds for 9 and 23 months led to the following similar results:

Scarification pretreatment was necessary for seed germination, since non-scarified seeds did not germinate (except 2% of non-scarified 9-month-old seeds that germinated at 10 and 15 °C in the dark, Figure 6). It should be emphasized that of the scarified seeds, only those whose white endosperm was revealed were used for incubation.

Germination was also affected by the photoperiod. Seeds that were incubated under continuous darkness germinated at higher percentages (up to 100%) than those incubated under 16 h of light and 8 h of dark (Figure 5).

The effect of temperature differed depending on the photoperiod during incubation. When incubation took place under a 16 h photoperiod, higher germination percentages (53–76%) were observed at 10 °C than at 15–25 °C. When incubation took place under continuous darkness, higher germination percentages were recorded at 10–20 °C (71–100%) compared to 25 °C (50–84%) (Figure 6A,D). At the optimal temperature range for each light condition, almost all seedlings were well developed (Figure 6B,E). Increasing the temperature from 10 °C to 20 °C accelerated most seed germination and reduced time T_{50} by half (Figure 6C,F).

The storage period (up to 23 months) did not have any negative effects on germination capacity. On the contrary, even higher germination percentages were recorded in the optimum treatments (Figure 6).

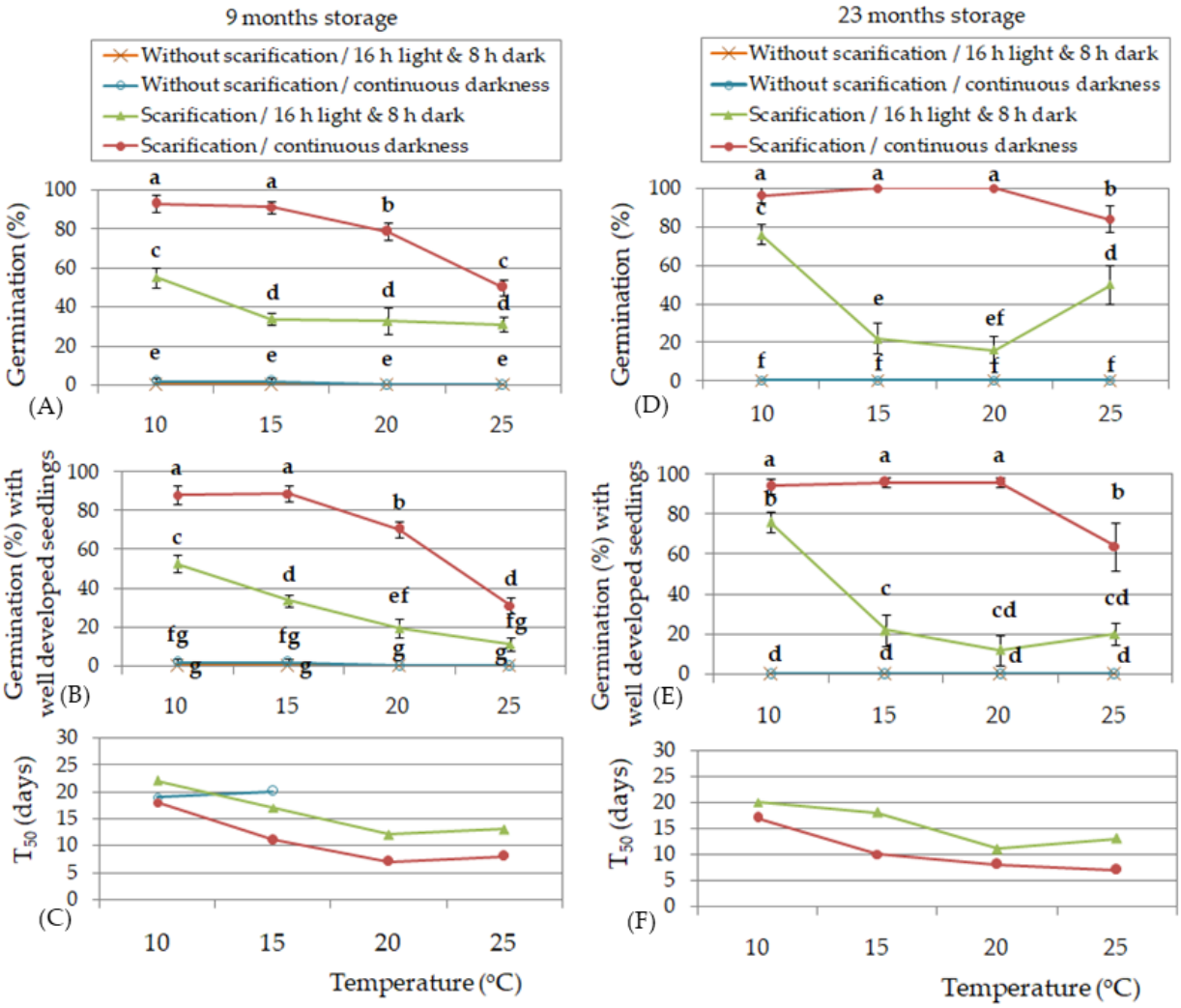


Figure 6. Effect of scarification, as well as of incubation photoperiod and temperature, on in vitro germination (%) (**A**,**D**), germination with well-developed seedlings (**B**,**E**) and T_{50} (**C**,**F**) of *T. hirsuta*, 9 and 23 months (9 M/23 M), respectively, after seed collection in 2015 and storage at room temperature. In Figures (**A**), (**B**), (**D**) and (**E**), *F*one-way ANOVA ** and mean values (±SE, $n = 5$ repetitions of 20 seeds) followed by the same lower-case letter do not differ significantly at $p \leq 0.05$ by Student's *t* test. **: significant at $p \leq 0.01$.

3.3.2. *T. tartonraira* ssp. *tartonraira* (2015 Harvest Year)

Similarly to *T. hirsuta*, in *T. tartonraira*, four-way ANOVA showed significant interactions of the experimental factors. However, nine months after seed harvest and storage, three-way ANOVA did not show any interactions of the experimental factors, i.e., scarification, incubation temperature and photoperiod, regarding the germination percentage and the germination percentage with well-developed seedlings. Only pretreatment with chemical scarification was found to have a significant effect, while incubation conditions did not.

Twenty-three months after seed harvest and storage, three-way ANOVA, regarding seed germination percentage, showed a significant interaction among the three factors (scarification, incubation temperature and photoperiod). With regards to germination percentage with well-developed seedlings, there was only interaction between scarification and photoperiod, while temperature had an insignificant effect. Further, two-way ANOVA used for the effect of incubation conditions for scarified seeds at two storage periods showed no significant effects as a result of temperature and photoperiod changes, except

in 23-month-old seeds, where continuous darkness significantly promoted germination in well-developed seedlings (Table 3).

Also, in this species, scarification pretreatment was necessary for seed germination, given that non-scarified seeds germinated at 0–2% compared to scarified seeds that germinated at 44–90% (Figure 7).

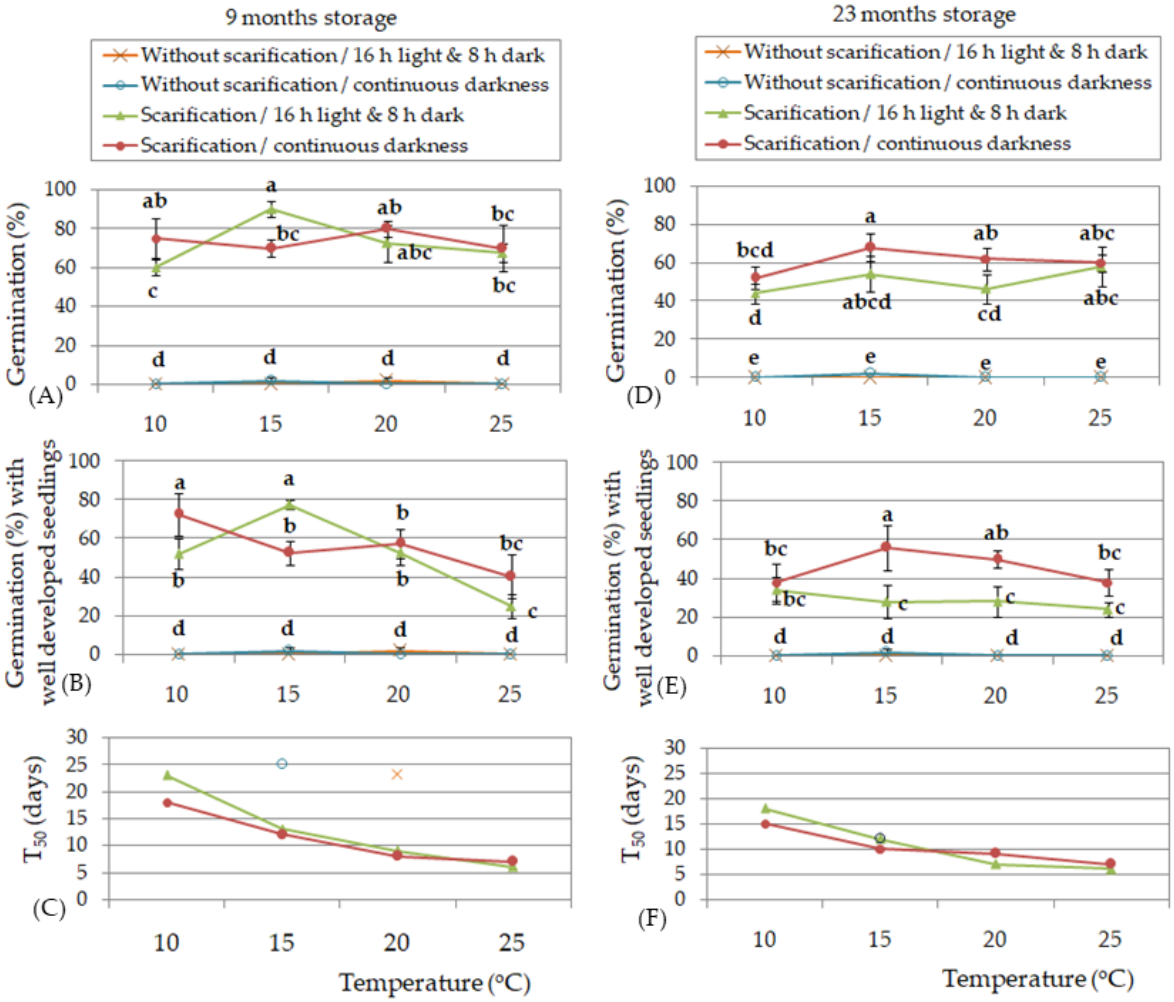


Figure 7. Effect of scarification, as well as of incubation temperature and photoperiod, on in vitro germination (%) (**A**,**D**), germination with well-developed seedlings (**B**,**E**) and T_{50} (**C**,**F**) of *T. tartonraira* ssp. *tartonraira*, 9 and 23 months (9 M/23 M), respectively, after seed harvest in 2015 and storage at room temperature. In Figures (**A**), (**B**), (**D**) and (**E**), *F*one-way ANOVA ** and mean values (±SE, $n = 5$ repetitions of 20 seeds) followed by the same lower-case letter do not differ significantly at $p \leq 0.05$ by Student's t test. **: significant at $p \leq 0.01$.

Nine months after seed collection and storage, scarified seeds of *T. tartonraira* generally germinated at satisfactory percentages (60–90%), irrespective of incubation conditions (Figure 7A), which were equally high to those recorded for *T. hirsuta* seeds stored for the same period (Figure 6A). The highest germination percentages were recorded by scarified seeds incubated under a 16 h photoperiod at 15–20 °C, as well as those that were incubated under continuous darkness at 10–25 °C (Figure 7A). However, regarding germination percentages with well-developed seedlings, this was highest for scarified seeds that were incubated under a 16 h photoperiod and 15 °C or under continuous darkness and 10 °C (Figure 7B).

Twenty-three months after seed harvest and storage, in scarified seeds, lower germination percentages (44–68%) were recorded compared to seed stored for 9 months

(60–90%) (Figure 7A,D), which were lower than those recorded for *T. hirsuta* seeds stored for 23 months (Figure 6D). Germination percentages of scarified seeds were more or less similar under all incubation conditions tested, with 15–20 °C under continuous darkness giving a better response, especially regarding germination percentage with well-developed seedlings (Figure 7D,E). In this species as well, increasing temperature from 10 °C to 25 °C reduced time for T_{50}. Furthermore, seeds stored for 23 months presented less time for T_{50} than seeds stored for 9 months, mainly at the lower temperatures (Figure 7C,F).

Table 3. Effect of experimental factors (2-way ANOVA), i.e., incubation temperature and photoperiod, in in vitro germination of *T. tartonraira* ssp. *tartonraira*, 9 and 23 months (9 M/23 M) after seed harvest in 2015 and storage at room temperature.

Experimental Factors		Germination (%)		Germination (%) with Well-Developed Seedlings	
		9 M	23 M	9 M	23 M
Light conditions	16 h light/8 h dark	72.5 a [¥]	50.6 a	51.7	28.6 b
	Continuous darkness	73.8 a	60.5 a	55.7	45.5 a
Temperature	10 °C	67.5 a	48.0 a	62.2	36.0 a
	15 °C	80.0 a	61.0 a	65.0	42.0 a
	20 °C	76.3 a	54.2 a	55.0	39.1 a
	25 °C	68.8 a	59.0 a	32.7	31.0 a
[§] *F*photoperiod		NS	NS	-	*
*F*temperature		NS	NS	-	NS
*F*photoperiod × temperature		NS	NS	*	NS

[¥] Mean values per experimental factor in each column followed by the same lower-case letter do not differ significantly at $p \leq 0.05$ by Student's *t* test. [§] NS or *, non-significant or significant at $p \leq 0.05$, respectively.

4. Discussion

The low germination percentages that have been reported for *T. hirsuta* [23,24], along with the ineffectiveness of other vegetative propagation techniques [4,6,23–25] and the necessity for the development of an efficient method for the sustainable exploitation of *T. hirsuta* and *T. tartonraira,* led to the investigation of the in vitro germination of their seeds in the present study. The first objective of our research was to investigate if the seeds of both species presented dormancy and to determine the cardinal temperatures for their germination, while the importance of photoperiod and seed age, as well as the year of harvest, were also tested.

Based on all the conducted experiments, seeds harvested in two different years showed the same response to germination treatments, revealing a stable behavior of seeds in terms of germination capacity that is not affected by environmental conditions during ripening, to the contrary of other maquis vegetation species that showed a large variation in germination between harvest years [46].

Pretreatment with chemical scarification was the most determinant factor for in vitro germination of both *Thymelaea* species, while the effect of incubation temperature, photoperiod and storage period depended on the species. Scarification is a primary technique to release seeds from their dormancy [36,37] in cases where seed dormancy is caused by hard seed coats [36]. Regarding the effect of various pretreatments on germination of both *Thymelaea* species, only scarification with H_2SO_4 for 15 or 20 min significantly increased germination percentages, confirming previous studies on *T. hirsuta* [26] and other Mediterranean shrubs, such as *Teucrium* sp. [43,47], *Anthyllis* sp. [44,48,49], *Salvia* spp. [50] and *Astragalus* sp. [51]. Immersion for 20 min was preferred due to the slightly greater germination percentages and the relatively faster germination. In experiments where only seeds whose white endosperm had been exposed were used, either after immersion in

concentrated H_2SO_4 or after further breaking the coat of chemically scarified seeds with a scalpel, even higher seed germination percentages were recorded. In this way, all seeds with atrophic endosperm were discarded as well. Although the further breaking of the coat of chemically scarified seeds with a scalpel is not applicable on a commercial scale, it showed how important removing the seed coat was for germination. On the other hand, non-scarified seeds showed 0.0 to 0.2% germination percentages in all treatments. These observations constitute a strong indication of a coat-imposed dormancy, which may be caused by the impermeable coat or the mechanical prevention of radicle extension or inhibitory substances that prevent embryos from germinating [32,52], even under ideal conditions [36]. The germination ability of seeds of other Thymeleaceae species, such as *Thymelaea velutina* [53], *Daphne* sp. [54] and *Pimelea arenaria* [55], was also considerably low because of dormancy. Dormancy provides seeds with advantages by maximizing seed dispersal and gradual germination, reducing competition for resources among seedlings and mother plants and preventing germination at the wrong season, even if there are short periods of favorable conditions [30]. Seed longevity and ecological adaptation is benefited by an impermeable coat, since it ensures recolonization of burnt area after fire and resistance of seeds to ingestion by animals and birds [56]. Seeds of other species of Thymeleacea family, such as *Pimelea trichostachya*, *P. simplex* and *P. elongata*, which are endemic to arid regions of Australia, under similar conditions to the Mediterranean region, have also been reported to be strongly dormant for years [57], whereas *Aquilaria malaccensis* [58] and *Gyrinops walla* [59], which grow in tropical regions of Southeastern Asia and Sri Lanka, respectively, possess recalcitrant seeds that have a short viability period, germinating efficiently directly after collection.

Moreover, mechanical scarification with sandpaper was not only ineffective in breaking seed dormancy, which has also been reported for *Teucrium capitatum* [43], but also crushed the seeds, destroying the embryo. On the other hand, in *Anthyllis hermanniae* [44], as well as in *Salvia fruticosa* and *S. officinalis* [50], both chemical and mechanical scarification were effective in increasing seed germination, through the facilitation of water entry and gas exchange. Neither pretreatment by immersion in hot water had any effect on the improvement of seed germination of *Thymelaea* sp., in accordance with what has been reported for *Teucrium* sp., in which no seed germinated after immersion in boiling water [43,47]. Although scarification methods are useful tools to soften hard seeds, in order to improve germination and enhance seedling establishment, their effectiveness varies depending on the duration of imposed treatments and the species, and detailed studies are required on each species [60]. For instance, in *Astragalus hamosus* and *Medicago orbicularis* [61], as well as other *Medicago* sp. [62], hand scarification with sandpaper was proved more effective than both chemical scarification and soaking in hot water, in accordance with the studied *Thymelaea* species.

A wide range of temperatures, from 5 to 30 °C, was tested in seed germination, revealing that temperatures from 10 to 20 °C were the most appropriate for *T. hirsuta* and those from 10 to 25 °C for *T. tartonraira*. At 5 °C, no seeds germinated, similarly to *T. capitatum* [43] and *A. hermanniae* [44], whereas at 30 °C, germination percentages were significantly lower, and seedlings were dehydrated and abnormal, since radicle development was hindered; this was the case for *T. capitatum* [43] as well. The increase in temperature from 10 °C to 20 °C accelerated seed germination, reducing time for T_{50}, as temperature is the most important environmental factor in seed germination, affecting the speed of germination, in addition to the germination percentage [38]. For other Mediterranean species, the optimum temperature to reach maximum germination has also been found to be in the range of 10–25 °C [43,44,46,63–65], although the effect of the incubation temperature on germination behavior depended strongly on species [63]. The percentage and rate of germination can be

affected by temperature through at least three separate physiological processes: (i) seed deterioration, which mainly depends on moisture content and temperature; (ii) seed dormancy, which is usually reinforced by high temperatures or broken by low temperatures in response to stratification; (iii) once seeds have lost dormancy, their rate of germination is affected positively by temperature until the optimum temperature and negatively at temperatures higher than the optimum one [66].

As regards the effect of photoperiod conditions, in *T. hirsuta*, seed germination was significantly favored by continuous darkness, since not only were higher germination percentages recorded compared to the 16 h photoperiod, but also, seeds germinated efficiently at a wider temperature range (10–20 °C) compared to 10 °C, which was the optimum temperature under a 16 h photoperiod. Such an intermediate response towards photoperiod was also found for some Lamiaceae of Crete [64,65], as seeds partially germinated in darkness, but their germination was significantly enhanced by light, while an absolute requirement of light for germination was found in *Origanum vulgare* subsp. *hirtum* [64]. In *T. tartonraira*, no photoperiodical requirement appeared. Similarly to *T. hirsuta*, germination of *T. velutina* was highest when seeds were left in the dark [53], whereas *T. capitatum* [43], *Anthyllis* sp. [44,49] and *Coridothymus capitatus* [64] responded as *T. tartonraira*, since the photoperiod had no significant effect on their germination. Light can control seed dormancy and seed germination through the regulation of hormone metabolism and signaling pathways [34]. Light may interact with temperature and hormones on the breaking of seed dormancy and germination through synergistic relationships and a variety of parallel, mutually interacting mechanisms [67].

Seed germination of the studied species also differed in response to storage periods. Germination of *T. hirsuta* was significantly affected by dry storage, as seeds stored for 9 months germinated at a higher percentage (75%) than those stored for 5 months (40%) and received the same pretreatment and incubation conditions, while *T. tartonraira* seeds stored for 12 months showed only a 15% increase in germination compared to seeds stored for 4 months. Thus, in the germination ecophysiology of *T. hirsuta*, the dry after-ripening seems to operate in a similar way to that observed for *Thymelaea velutina* [53] and other native Mediterranean species that disperse their seeds before the wet season as they face a long dry summer, such as *Dianthus cruentus* [68], *D. morrisianus* [69], *Satureja thymbra* [64], *Periploca angustifolia* [70], *Teucrium capitatum* [43] and *Clinopodium nepeta* [71].

Increasing the storage period up to two years reduced the germination capacity of only *T. tartonraira* seeds as opposed to *T. hirsuta* seeds, which germinated at even higher percentages at optimum temperatures 23 months after harvest and storage at room temperature. The preservation of germination ability or even higher germination percentages in older seeds compared to younger ones have been found for *T. velutina* [53], *T. capitatum* [43] and other Lamiaceae, possibly as a result of the volatization of essential oils present on their coat [64]. On the other hand, similarly to *T. tartonraira*, scarified seeds of *A. hermanniae* stored for 18 months showed slightly reduced germination percentage, as opposed to unscarified ones, which showed low germinability consistently throughout the storage period [44], while in *A. barba-jovis*, germination was also reduced by storing for more than 24 months but mostly of seeds that had not received scarification [49].

5. Conclusions

In both *T. hirsuta* and *T. tartonraira* ssp. *tartonraira*, the chemical scarification of the seeds by immersion in concentrated H_2SO_4 for 20 min was necessary for in vitro germination, since it resulted in a significant increase in germination percentages (up to 73–100% at optimum incubation conditions) compared to almost zero germination in non-scarified seeds, which indicates dormancy caused by hard coat.

In *T. hirsuta*, the highest germination percentages (79–100%) were observed after H_2SO_4 pretreatment and incubation at 10–20 °C, under continuous darkness.

In *T. tartonraira* ssp. *tartonraira*, no large variations were observed in germination percentages of scarified seeds (60–90%) regarding the tested temperature range, i.e., 10–25 °C, and photoperiod conditions, while increasing the seed storage time, up to almost two years, reduced their germination capacity (44–68%).

Higher germination percentages (73–100%) were observed at temperatures of 10–20 °C, under continuous darkness for *T. hirsuta* and regardless of photoperiod conditions for *T. tartonraira*.

The storage time (up to two years) reduced the germination only of *T. tartonraira* seeds, whereas in *T. hirsuta*, older seeds germinated at even higher percentages. *T. hirsuta* seeds stored for 5 months germinated at significantly lower percentages (40% maximum) compared to seeds stored for 9–24 months, revealing a dry after-ripening process.

To sum up, chemical scarification was the most determinant factor for the in vitro germination of *T. hirsuta* and *T. tartonraira* ssp. *tartonraira*, due to the successful breaking of seed coat dormancy, while the effect of incubation temperature, photoperiod and storage period on germination depended on the species. For both species, an effective seed propagation protocol suitable for their exploitation as ornamental and landscape plants was developed.

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