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Can Testing 7000 Seeds Help to Understand the Dormancy Type of a *taxon*? A Case Study of *Linum mulleri*, an Endemic and Endangered Species of Sardinia (Italy)

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Abstract: *Linum mulleri* is an endemic *taxon* of southwestern Sardinia (Italy), categorised as Endangered (EN) on the IUCN Red List and included in Annexes II and IV of the Habitats Directive (92/43/EEC) as priority species for conservation. This study investigated the germination ecophysiology of *L. mulleri* and the possible presence of dormancy by using 7000 seeds, providing useful information for conservation strategies. The germination response of fresh seeds was evaluated under different temperatures, photoperiods, pre-treatments [cold stratification (C); warm stratification (W); W+C; C+W+C; dry after ripening (DAR)], and different gibberellic acid (GA_3) concentrations. *L. mulleri* germinates under controlled conditions, particularly at 15 and 20°C, while germination percentages (FGP) never exceeded 5% at 5 and 30°C. C and C+W+C induced secondary dormancy, delaying germination, whereas W, DAR and GA_3 stimulate it. Light and dark incubation showed no significant differences in FGP. W, DAR and 250 mg/L GA_3 effectively overcame physiological dormancy (PD), expanding the germination temperature range to below 10 and above 25°C. These responses suggested type 3 non-deep PD, as germination temperatures extended from moderate to both low and high temperatures. Analyzing 7000 seeds provided crucial information into dormancy and germination strategies, supporting both *ex situ* and *in situ* conservation efforts.

Keywords: *Linum*; endemic plant; seed germination; dormancy; pre-treatment

1. Introduction

Seed germination is a delicate event which determines the establishment of a plant, contributing to the persistence of the population [1]. Seed germination requirements are species-specific and they are also influenced by various factors such as temperature, humidity, and light [2]. In seasonal climates and wet soils, temperature is usually the main environmental factor influencing seed germination [3], and each species has a temperature range within which germination can occur [4]. Sometimes it may behave differently depending on whether the temperature is constant or alternating [5–7]. Whether germination under a wide range of conditions occurs more than four weeks, more probably the seeds have dormancy. Five main classes of seed dormancy are recognized, and, among them, Physiological Dormancy (PD) is the most worldwide common form [8]. PD seeds have a physiological inhibiting mechanism in the embryo that prevents radicle emergence [8,9] and, according to the conditions required to break it and promote germination, they are distinguished into three levels: non-deep (divided in types one to six depending on the temperature range of dormancy-break), intermediate and deep [10].

Linum L. is the most well-known among the 22 genus belonging to the Linaceae family, it is distributed in temperate regions, primarily in the northern hemisphere, although 14 species are

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known in the Cape Region of South Africa and a similar species in New Zealand [11]. It is estimated that there are about 230 species belonging to the *Linum* genus [12–14]. One of the distribution centres of the *Linum* genus is the Mediterranean area, with 75 species, along with India, where it likely originated in the northwestern region, then spreading to Ethiopia, the Fertile Crescent, and Russia [11,15]. In Italy, the *Linum* genus is represented by 27 *taxa* [16], among them 10 are native to Sardinia and only *Linum mulleri* Moris is reported as endemic of the island [17]. The species belonging to this genus mainly grow on rocks or well-drained limestone or sandy soils [11,15] and are divided into five subsections: *Linum* L., *Dasylinum* (Planch.) Juz., *Syllinum* Griseb., *Cathartolinum* Griseb., and *Linastrum* (Planch.) Bentham [18]. *Linum* species are annual or perennial herbs, generally erect with hard bark; the flowers can be blue, red, yellow, or white, borne on axillary or terminal racemes [15]. The genus has some economic relevance as it is cultivated for the production of seed oil and fibres, particularly with the species *L. usitatissimum* L. (common flax) [19]. Some studies have been conducted on the germination ecophysiology of *Linum* seeds, primarily focused on *L. usitatissimum*, whose seeds do not exhibit dormancy [20–22], while literature data on other *Linum* species appear to be scarce. It has generally been shown that *Linum* seeds can exhibit dormancy, and germination requires a period of cold stratification or post-maturation immediately after dispersal. For example, seeds of *L. perenne* L. were able to break dormancy after a period of cold stratification [23], *L. radiola* L. seeds required an after-ripening period [24], *L. olympicum* Boiss. was able to germinate both in light and dark conditions, with GA_3 and/or cold stratification [25], while *L. catharticum* L. seeds are light-dependent and require a period of cold treatment [26,27].

In this work, we focused our attention on studying the germination ecophysiology of *Linum mulleri* Moris, an exclusively endemic plant of southwestern Sardinia, particularly in the Iglesiente biogeographic subsector. This *taxon* is in an unfavourable conservation status due to the fragility of the habitat in which it grows, the small size, and the isolation of its populations. Additionally, the environmental restoration activities of abandoned mining sites pose a further risk to some areas within the range of *L. mulleri* [28]. The high risk of extinction has led to *L. mulleri* being listed as Endangered (EN) on the IUCN Red List [29]. The *taxon* has been included in Annex II and IV of the Habitats Directive (92/43/EEC) as a priority species for conservation. *Linum mulleri* is included among the species studied in the LIFE SEEDFORCE—LIFE20 NAT/IT/001468 (Using SEED banks to restore and reinFORCE the endangered native plants of Italy) project, which aims to improve the conservation status of 29 plants species listed in Annex II of the Habitats Directive (92/43/EEC).

Plant conservation requires a thorough understanding of the plant life cycle, for these reasons, determining the germination characteristics of the seeds of these species under study is essential to contribute to this purpose. Currently, to our knowledge, there is no information available in the literature regarding the ecophysiology of germination of *L. mulleri*. Based on existing literature for the genus *Linum* [24–27], we hypothesized that the seeds of *L. mulleri* might also exhibit dormancy. To explore this hypothesis, the objectives of this study were: (I) evaluate the germination response of seeds to different temperatures and photoperiods, (II) evaluate the germination response of seeds to different pre-treatments (warm and/or cold stratification, dry after ripened), and (III) investigate the germination response of seeds to different concentrations of gibberellic acid (GA_3). To purpose these objectives, controlled laboratory experiments were conducted; understanding the germination behaviour, whether dormancy is present and, if so, determining its class and type, are useful for implementing more effective management and conservation measures for this species.

2. Results

2.1. Germination DURING PRE-TREATMENT

During the pre-treatments of W, C, W+C, and C+W+C, germination occurred. According to GLM results, the type of pre-treatment applied showed statistically significant ($p < 0.05$) in seed germination. Seeds exposed to W and W+C recorded germination percentage that reached up to 59% (W up to 58.72% and W+C up to 59.14%). In both pre-treatments, germination occurred during the first 90 days; in the case of W+C, during W cycle the germination reached 60%, while after 90 days

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(i.e. during the C cycle) the increase was very limited, bringing the final germination (after 180 days) to 60.75% (Figure 1). Seeds under C recorded a very low germination percentage, even below 1% (0.14%); whereas seeds subjected to C+W+C recorded germination percentages of 0% for the first 90 days (first C cycle), from the 90th to the 180th day the percentage reached 6.87% (W cycle), while the final germination percentage (after 270 days, second C cycle) was 11.14% (Figure 1).

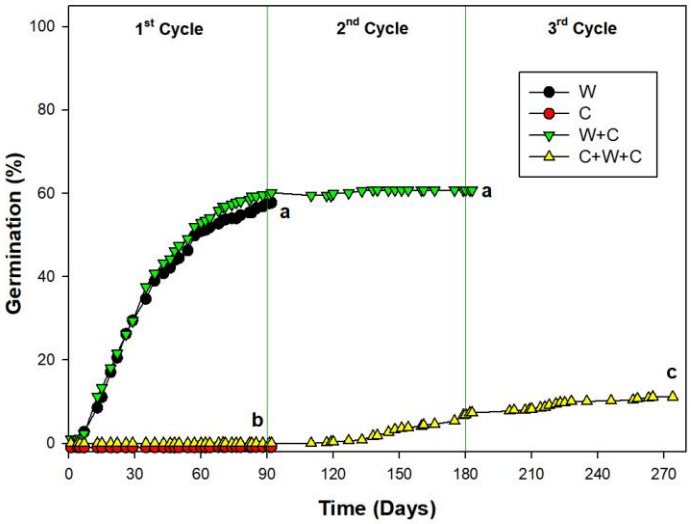

Figure 1. Germination trends during pre-treatments. I, II, and III correspond to the three applied pretreatment cycles on *Linum mulleri* seeds, with I = 90 days for W and C, II = 180 days for W+C, and III = 270 days for C+W+C. Data are the mean of four replicates (±SD). The GLMs were carried out on the germination, and values with the same letter are not statistically different at $p > 0.05$ by post hoc pairwise *t*-test comparisons.

2.2. Effect of Photoperiod, Treatment and Pre-Treatments on Seed Germination

The incubation temperature (T), the treatment and pre-treatment (Tr), and the interaction of these two factors (T * Tr) had a significant effect on FGP ($p < 0.001$) (Table 1).

Table 1. GLM results of Final Germination Percentage (FGP) in *Linum mulleri* seeds depending on the following factors: Treatment and Pre-Treatment (CTR, C, W, W+C, C+W+C, DAR, Dark), incubation Temperature (5, 10, 15, 20, 25, 30 and 25/10°C), and their interactions (Tr * T).

	Df	Sum Sq	Mean Sq	F	*p* value
Treatment and Pre-Treatment (Tr)	6	4.026	0.6711	36.916	< 0.001 ***
Temperature (T)	6	11.714	1.952	107.399	< 0.001 ***
Tr * T	36	3.243	0.090	4.956	< 0.001 ***

The post-hoc analysis indicated that there were no statistically significant differences in the germination response of seeds incubated in dark conditions compared to all other treatment and pre-treatments applied (Table 3). The W and DAR were statistically significantly different in FGP compared to C and C+W+C, while W+C was statistically significantly different only compared to DAR (Table 2). The incubation seeds under CTR conditions, however, reveal statistical differences compared to C+W+C (Table 2).

Table 2. Pairwise comparisons on different treatment applied. Pairwise comparisons using *t*-test with pooled statistical differences for final germination percentage for the treatment and pre-treatments (CTR, C, W, W+C, C+W+C, DAR, Dark) applied in *Linum mulleri* seeds.

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	C	C+W+C	CTR	DAR	Dark	W
C+W+C	1					
CTR	0.069	< 0.001 ***				
DAR	< 0.05 *	< 0.001 ***	1			
Dark	1	0.253	0.737	0.317		
W	< 0.05 *	< 0.001 ***	1	1	0.974	
W+C	1	1	0.074	< 0.05 *	1	0.106

Table 3. GLM results of Final Germination Percentage (FGP) in *Linum mulleri* seeds depending on the following factors: Concentration (0, 250, 500, 1000 mg/L of GA_3), incubation Temperature (5, 10, 15, 20, 25, 30 and 25/10°C), and their interactions (Co * T).

	Df	Sum Sq	Mean Sq	F	*p* value
Concentration (Co)	3	1.154	0.384	19.127	< 0.001 ***
Temperature (T)	6	4.498	0.749	37.275	< 0.001 ***
Co * T	18	1.037	0.057	2.866	< 0.001 ***

At a temperature of 5°C, the highest FGP (5%) was achieved in seeds that had previously undergone a W+C period; for all other treatment and pre-treatments, the FGP was equal to or slightly above 0% (Figure 2). At 10°C, the W recorded the highest FGP (89.3%), while seeds previously incubated at C showed low germination capacity, not exceeding 5%, along with seeds that underwent W+C (around 5%). Seeds incubated in light and dark achieved FGP of 40.62% and 33.37%, respectively. The DAR had an FGP of 58.65% (Figure 2). At 15 and 20°C, the highest germination percentages were recorded, with FGP exceeding 40%, regardless of the treatment or pre-treatment considered. The lowest FGP (42.23%) was recorded for seeds that had previously undergone to C+W+C at 15°C. The highest FGP (95.82%) was recorded for seeds that had previously undergone DAR and were then incubated at 20°C. At 25°C, the highest FGP was recorded for seeds that had previously undergone to DAR, with a percentage of 61.82%, while the lowest FGP was recorded for seeds that had previously undergone the C+W+C (12.25%) (Figure 2). At 30°C, as at 5°C, the FGPs were consistently low, with percentages not exceeding 35%, regardless of the treatment and pre-treatment considered (Figure 2). The alternating temperature of 25/10°C for seeds incubated in light recorded a FGP of 80.57%, while, as also seen at other temperatures, seeds previously incubated at C+W+C treatment had a lower FGP compared to all other treatment and pre-treatments (1.2%) (Figure 2).

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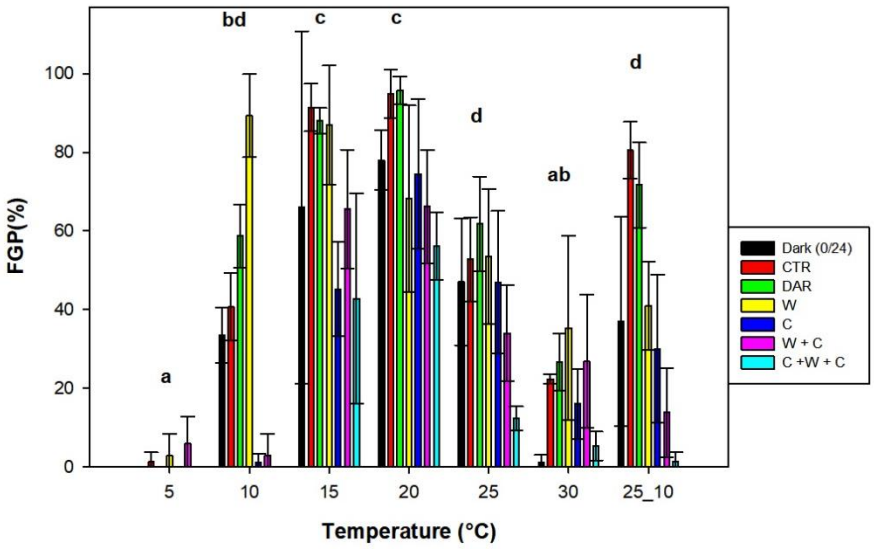

Figure 2. Final Germination Percentage (FGP) of *Linum mulleri* seeds under different treatment and pre-treatment (CTR, C, W, W+C, C+W+C, DAR and Dark) and incubated at constant (5, 10, 15, 20, 25, 30°C) and alternating (25/10°C) temperatures. Data are the mean of four replicates (±SD). GLMs were carried out on the germination, and values with the same letter are not statistically different at $p > 0.05$ by post hoc pairwise *t*-test comparisons.

2.3. Effect of Gibberellic Acid (GA_3) on Seed Germination

The concentration (Co), the incubation temperature (T), and the interaction of these two factors (Co * T) showed significant effect on FGP ($p < 0.001$) (Table 3).

At temperatures of 15, 20, and 25/10°C, the concentrations behave in the same way, recording germination percentages above 60% (Figure 3). At the temperature of 10°C, the concentration of 0 mg/L of GA_3 recorded the lowest germination percentage (40.6%), while other concentrations were always above 50%, in particular, the concentration of 250 mg/L of GA_3 recorded FGP of 92.39%, 500 mg/L of GA_3 recorded FGP of 87.65%, and 1000 mg/L of GA_3 recorded FGP of 57.59% (Figure 3). The temperatures of 5 and 30°C resulted in lower FGP. In particular, the FGP at 5°C reached 54.4% at a concentration of 250 mg/L of GA_3, while at a concentration of 0 mg/L of GA_3, FGP did not exceed 2%. At 30°C, again, the concentration of 0 mg/L of GA_3 recorded the lowest FGP (22.3%), while 250 mg/L of GA_3 recorded the highest FGP (66.67%) (Figure 3).

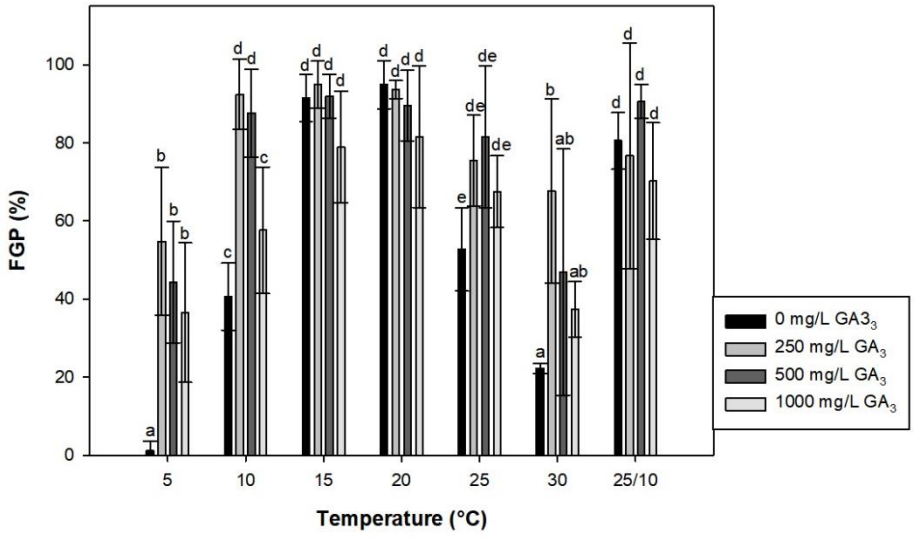

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Figure 3. Effect of different concentration of GA_3 (0, 250, 500, 1000 mg/L) on seed germination of *Linum mulleri* incubated at constant (5, 10, 15, 20, 25 and 30°C) and alternating (25/10°C) temperatures. The data represent the mean of four replicates (±SD). Values with the same letter are not statistically different at $p > 0.05$ by post hoc pairwise *t*-test comparisons.

2.4. Rate and Widening of Germination Temperature

The seeds treated with CTR, W and DAR reached T_{50} in less time compared to the other pre-treatments (C, W+C and C+W+C) at all tested temperature (Table 4). At temperatures of 15 and 20°C, a notable lower T_{50} values were recorded compared to the other temperatures and pre-treatments tested. At 15°C, W showed the fastest germination (T_{50} = 5 days) compared to CTR and DAR (T_{50} = 9 ± 1 and 8 ± 1, respectively). At 20°C, DAR was the fastest with T_{50} of 8 ± 2 days, compared to 8 ± 4 for CTR and 14 ± 16 days for W. Even at 10°C, W and DAR achieved 50% of germination, a result not achieved with the other pre-treatments and treatments applied to the seeds. The values obtained were 7 ± 2 for W and 12 ± 1 days for DAR (Table 4).

Table 4. The germination rate (T_{50}) expressed in days across each tested temperature of all treatment and pre-treatments (CTR, W, C, W+C, C+W+C, DAR, 250, 500, 1000 mg/L of GA_3) where 50% of germination has been achieved.

Treatment	Temperature						
	5	**10**	**15**	**20**	**25**	**30**	**25/10**
CTR	-	-	9±1	8±4	56±44	-	37±5
W	-	7±2	5±0	14±16	100±21	117±3	-
C	-	-	-	55±14	125±71	-	-
W+C	-	-	72±42	63±5	-	-	-
C+W+C	-	-	31±2	60±8	-	-	-
DAR	-	12±1	8±1	8±2	42±12	-	30±9
GA_3 250 mg/L	-	16±1	8±1	6±0	15±5	32±6	14±3
GA_3 500 mg/L	65±24	15±1	9±1	8±2	19±5	36±1	17±1
GA_3 1000 mg/L	-	16±2	11±1	8±1	16±2	-	18±11

Regarding the GA_3 treatment, the 250 mg/L concentration recorded the lowest T_{50} values at all tested temperatures compared to the other concentrations. The only exception occurred at 10°C, where 500 mg/L concentration showed a T_{50} of 15 ± 1 compared to 16 ± 1 days for the 250 mg/L concentration (Table 4).

Figure 4 shows the correlation among germination percentages and incubation temperatures, considering the treatments and pre-treatments for which germination equal to or greater than 50% were observed (CTR, DAR, W, and 250 mg/L of GA_3), using a Gaussian curve. The results highlighted, in these treatments, an extension of the temperatures at which 50% germination could be achieved. In CTR, temperatures extended from a minimum of approximately 12°C up to a maximum of 26°C. In the DAR pre-treatment, the range expands from about 11°C to 27°C, while in the W pre-treatment, the range extends from about 8 °C to 25.5 °C. Seeds sown with 250 mg/L of GA_3 showed the greatest extension of temperature range, with a range from approximately a minimum of 5°C to a maximum of 32°C.

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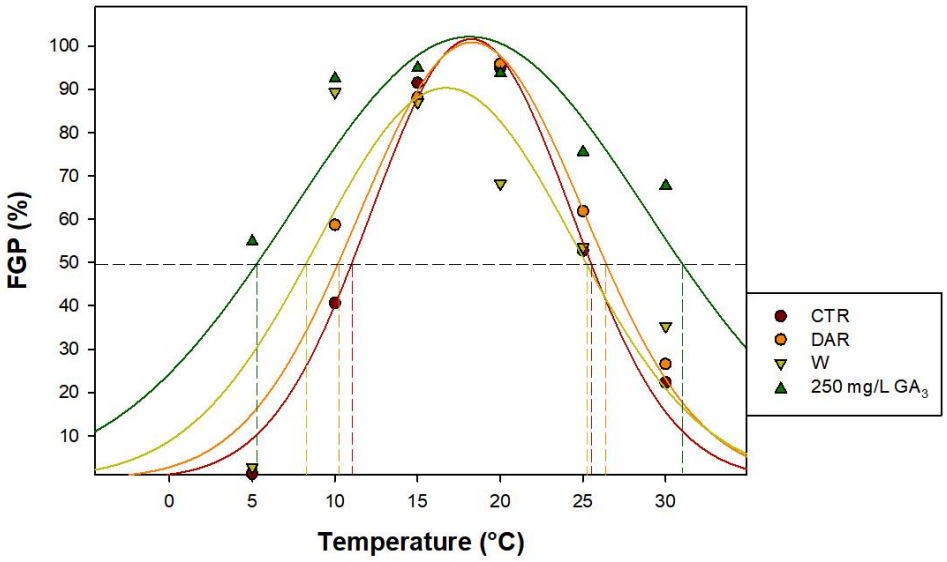

Figure 4. Gaussian curves representing the effect of CTR, W, DAR and 250 mg/L of GA_3 on the germination dynamics of *Linum mulleri* seeds. Points correspond to actual data and solid lines indicate the fitted lines from gaussian regressions. Vertical dotted lines indicate the minimum temperature at which 50% germination can be achieved in the different treatments. The data represent the mean of four replicates.

3. Materials and Methods

3.1. Study Species

Linum mulleri is a perennial suffrutescent plant, which flowers between May and June and bears fruit between June and July. The fruit is a capsula globose, and seeds are elliptical, flat [28]. *L. mulleri* is a Sardinian endemic that grows only in the Iglesiente biogeographic subsector, distributed in only three main localities: Miniere di San Giovanni di Bindua, Miniere di Monteponi and Monte Marganai [45]. It is a xerophilous species that grows in glareicolous and garrigue environments, on poor or embryonic soils and in the cracks of rock walls, it is found mainly on metamorphic substrates, on limestones and mining dumps characterized by high concentrations of heavy metals, and sometimes it behaves like a pioneer species, colonizing mine tailings landfills [28]. It is a characteristic taxon of the *Polygalo sardoae-Linetum mulleri* community, rich in endemics and found near mines on steep rocky slopes composed of Paleozoic metalliferous limestones [46].

3.2. Seed Lot Collection and Preparation

Fruits and seeds were collected during the time of natural dispersal in late June 2022 from Miniere di San Giovanni di Bindua locality (39.306746° N, 8.489168° E), municipality of Iglesias. The collected seed lot was stored at controlled conditions (20°C and 40% relative humidity) for two weeks at Sardinian Germplasm Bank (BG-SAR) of the University of Cagliari before the germination tests [47]. Seeds were cleaned manually with the removal by hand all foreign matter.

3.3. Controlled Laboratory Experiments

3.3.1. Germinations Tests

The tests were conducted using 7,000 seeds. The collection of such a large quantity of seeds was possible thanks to the remarkable capacity of the plant to produce them, which generates thousands of seeds every year. Such abundance allows us to collect from 10 to 30% of available mature seeds without compromising the natural population as suggested by Bacchetta et al. [48,49], ensuring at the same time the long-term conservation of different seed lots at the Sardinian Germplasm Bank (BG-SAR) of the University of Cagliari [47]. This approach ensures that our experiments were conducted in a sustainable way, minimising any negative impact on the natural population and the conservation of the species. To investigate the ecophysiology of germination of *L. mulleri* four replicates of 25 seeds

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were sown on the surface of 1% agar water in 60 mm diameter plastic Petri dishes incubated in growth chambers (Sanyo MLR-351/350; SANYO Electric, Osaka, Japan) with white fluorescent lamps (FL40SS.W/37 70–10 µmol m-2 s-1, Sanyo, Osaka, Japan), at constant (5, 10, 15, 20, 25 and 30°C) and alternating temperatures (25/10°C), in light (12 h light/12 h dark) conditions (hereafter Control, CTR). In the alternating temperature regime, the 12 h light period coincided with the elevated temperature period. Furthermore, the following pre-treatments were applied: (i) cold stratification (C); (ii) warm stratification (W); (iii) warm stratification followed by cold stratification (W+C); (iv) cold stratification followed by warm stratification and another cold stratification periods (C+W+C); (v) dry after ripened (DAR) (see details in Table 5). After which the seeds from each pre-treatment were incubated in the light (12 h light/12 h dark) at the temperature regimes mentioned above.

Table 5. Experimental designed for *Linum mulleri*. After the C, W, W+C, C+W+C, DAR pre-treatments, the seeds were incubated at all tested temperature (5, 10, 15, 20, 25, 30°C and 25/10°C).

Treatment and pre-treatment	Description
CTR	Light condition (12 h light/12 h dark) and incubation at all tested temperature
C	3 months at 5°C
W	3 months at 25°C
W+C	3 months at 25°C, followed by 3 months at 5°C
C+W+C	3 months at 5°C, followed by 3 months at 25°C, followed by 3 months at 5°C
DAR	3 months at 25°C inside a sealed glass container with colour-changing silica gel at a ratio seed/silica gel of 1:1 which ensure the dry condition
0/24	24 hours of dark condition and incubation at all tested temperature
GA_3	0, 250, 500, 1000 mg/L GA_3 in germination medium and incubation at all tested temperature

In order to evaluate the effect of photoperiod on seed germination, four replicates of 25 seeds each were incubated in growth chambers at constant (5, 10, 15, 20, 25, 30°C) and alternating temperatures (25/10°C) in total darkness (0 h light/24 h dark) by wrapping dishes in two aluminium foils (Table 5).

Germination, defined as visible radicle emergence (>1 mm), was recorded three times per week. At the end of the germination tests (for a maximum of 120 days), when no additional germination had occurred for two weeks, a cut test was carried out to determine the firmness of the remaining seeds and the number of empty seeds; firm seeds were considered to be viable. All germination experiments were conducted starting at the same time in the laboratories of BG-SAR.

3.3.2. Effect of Gibberellic Acid (GA_3) on Seed Germination

In order to determinate the effect of GA_3 on seed germination, four replicates of 25 seeds were sown in 60 mm Plastic Petri dishes with 1% agar water substrate and GA_3 at different concentration (0, 250, 500 and 1000 mg/L GA_3) (Table 5) and incubated in the light (12 h light/12 h dark) , at constants (5, 10, 15, 20, 25 and 30°C) and under an alternating (25/10°C) temperatures regime. At the end of the test, the firmness of the remaining seeds was determined as previously detailed.

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3.4. Data Analysis

The final germination percentage (FGP) was calculated as the mean of the four replicates (± SD) on the basis of the total number of filled seeds (empty seeds were excluded). Additionally, the germination rate (T_{50}) was calculated as the time in days required to reach 50% of germination. Generalised Linear Models (GLMs) were applied to (i) evaluate the effect of pre-treatments, photoperiod and temperature on the FGP, (ii) comparison of germination rate and assess the effect of pre-treatment on T_{50}, (iii) evaluate the effect GA_3 on the FGP. Significant differences were then analysed with a post-hoc pairwise comparison *t*-test (with Bonferroni adjustment). The GLMs with a logit link function and quasi-binomial error structure was used to analyse germination percentages, while a log link function and quasi-Poisson error structure was used for analysing T50. The F-tests with an empirical scale parameter instead of chi-squared on the subsequent Analysis of Variance (ANOVA) were used to overcome the residual over dispersion [50]. All statistical analyses were performed using R v. 3.0.3 [51].

4. Discussion

This study is the first to investigate the ecophysiology of germination in *Linum mulleri* and demonstrates that this *taxon* is able to produce seeds capable of germinating under controlled conditions, in particular at 15 and 20°C without any treatments. The germination observed during pre-treatments, as in the W, C, W+C, and C+W+C phases, is attributed principally to the effect of high incubation temperature (i.e., 25°C). In fact, FGP achieved during moist warm stratification "W" and warm followed by cold stratification "W+C", with over 50% in both pre-treatments, differed by FPG obtained during cold stratification "C" and with the pre-treatments starting with a cold stratification "C+W+C" (less than 1% in both pre-treatments). The incubation temperature of seeds is a factor that significantly influences the germination phase, either inhibiting or promoting the physiological processes involved in radicle emergence [30,31]. Incubation temperature also significantly influenced seed germination after the application of treatments and pre-treatments, both in light and dark conditions. The lowest germination percentage, but the highest percentage of ungerminated and viable seeds, was recorded at 5°C and 30°C. This limitation in seed germination at too high or too low temperatures could represent an ecological advantage, as germination is prevented under unfavourable climatic conditions, allowing germination to commence when temperatures are milder [2,32]. Low germination percentage and rates were also observed in seeds that underwent a cold stratification process before being incubated at other incubation temperatures. In many species, cold and moist conditions act as a mechanism to delay seed germination until the end of winter when more favourable conditions arise [33,34]. C and C+W+C in seeds of *L. mulleri* seems to impose a secondary dormancy, delaying seed germination even at low temperatures after these cycles of pre-treatments, respect to W and DAR that stimulate germination also at lower temperatures. This response could be a sign of the presence of physiological dormancy "PD" [2]. This behaviour provides an ecological advantage for seeds, allowing germination to be completed at milder temperatures [2,6]. The highest FGP (more than 60%) was recorded at temperatures of 15 and 20°C. In a Mediterranean climate, such as the one where *L. mulleri* grows, it is common for seed germination to occur at temperatures between 15 and 20°C. This characteristic is identified as the "Mediterranean germination syndrome" [35,36], which ensures complete germination during early spring, or autumn rainy season, and avoids exposure of young plants to summer drought (e.g., [35–39]). The ability to germinate even at the alternating temperature of 25/10°C, both in treated and untreated seeds, might indicate that germination may occurs also in the superficial soil layers, where the influence of alternating temperatures is greater [5,40]. This suggests that this *taxon* grows in climatic areas with large differences in day and night temperatures. These results are consistent with several studies reporting a positive effect of fluctuating temperature regimes on seed germination percentage (e.g., [6,7,31,41]) and can partly explain the germination niche of a species, and thus its habitat requirements and distribution [7]. Contrary to what is reported in the literature for some *Linum* species [24,26,27], *L. mulleri* does not depend strictly on light for seed germination, as seed incubation

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in light or dark conditions showed no significant differences in FGP. The ability of *L. mulleri* seeds to germinate both in light and darkness and at alternating temperatures highlights their great adaptability to different environmental conditions.

It is known that GA_3 stimulates germination, especially in species that exhibit dormancy [42,43]. For example, in *L. olympicum* [25], GA_3 at different concentrations (250, 500 and 1000 mg/L) was used as a substitute for cold stratification, proving particularly effective in breaking dormancy at a concentration of 1000 mg/L. In our study, GA_3 stimulated seed germination by widening the temperature range. At the temperature of 5°C, the 0 mg/L GA_3 concentration recorded a percentage of less than 2%, while at the 250 mg/L GA_3 it reached as high as 54.4%. The same occurred at the temperature of 30°C. Our study, in comparison to the existing literature (see [25]), highlights how different concentrations of GA_3 have different effects on germination and consequently underscores the importance of always testing different concentrations.

Based on the results obtained it is possible affirm that GA_3, with a preference for a concentration of 250 mg/L, is capable of breaking a form of PD in seeds of *L. mulleri*, expanding the range of temperatures at which this species is able to germinate, both for temperatures below 10°C and for those above 25°C. Freshly matured seeds with non-deep PD either can germinate over only a very narrow range of temperatures or cannot germinate at any temperature [2], and in the presence of non-deep PD, it is possible to break dormancy using warm or cold stratification, or by using GA_3 [8,44]. The W, DAR and GA_3 treatments in seeds of *L. mulleri* have proven to be effective in break dormancy also at lower and higher temperatures and consequently in widening the temperature range of germination, especially when the temperature drops below 15 and 20°C. Accordingly, the seeds of *L. mulleri* have a non-deep PD. In agreement with our results, *L. radiola* [24] requires an after-ripening period of 30°C for 28 days to break dormancy; in contrast, many authors have reported in the literature the positive effect of cold stratification on breaking seeds dormancy in other species belonging to the genus *Linum* (see [23,25–27]). Furthermore, since the temperature range at which *L. mulleri* seeds can germinate has widened from medium to both low and high temperatures, the seeds exhibit a type 3 non-deep PD [8].

5. Conclusions

The data presented in this study provide useful information for both *ex situ* and *in situ* conservation of this threatened and European protected species. Testing 7000 seeds may help to understand the dormancy type, in fact, this study allowed to detect that seeds of *Linum mulleri* have a type three non-deep PD and that this species showed the typical Mediterranean germination syndrome considering that the highest germination percentages were recorded at temperatures of 15°C and 20°C. Additionally, it was detected that W, DAR and GA_3 (in particular concentration of 250 mg/L) stimulated the germination at lower (5 and 10°C) and higher (25 and 30°C) temperatures. Information on its seed germination strategies is critical for optimizing the timing of and determining the success of *in situ* conservation efforts, such as translocation and environmental recovery. As other studies have shown (e.g., [52–54]), knowing how to germinate the seeds is vital for developing effective procedures and protocols for promoting *ex situ* conservation for rare and threatened species.

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