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Embryo growth and seed germination requirements in underdeveloped embryos of *Elwendia caroides* and *E. wolfii* (Apiaceae)

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Abstract. *Elwendia wolfii* and *E. caroides* are two herbaceous perennials that were initially classified in the *Bunium* genus and later transferred to the *Elwendia* genus. They are a rich source of bioactive and antioxidant compounds and have the potential to become sources of oil-bearing crops. The aim of this study was to investigate requirements for embryo growth, dormancy break and seed germination in these two species. The effects of gibberellic acid (GA_3) and dry storage were also examined to determine the type of dormancy. The pericarps and seed coats of both species were water-permeable, and the embryos were small and differentiated but underdeveloped, with initial embryo lengths of 0.28 mm in *E. wolfii* and 0.96 mm in *E. caroides*, respectively. These underdeveloped embryos were either di (>98%) or tricotyledonous (<2%) and required cold temperatures to grow, indicating that these seeds had morphological dormancy (MD). The critical length required for germination was 3.44 mm for *E. wolfii* and 4.17 mm for *E. caroides*. However, seeds of both species reached less than 50% final germination if subjected only to cold incubation. Higher final germination was possible if seeds were pre-treated with dry cold storage (-22 ºC) or exposed to GA_3, indicating the existence of physiological dormancy (PD) in part of the seed population. Therefore, both species had non-deep complex morphophysiological dormancy (MPD). This study demonstrates that *E. caroides* and *E. wolfii* share the same germination requirements, suggesting a common ecological strategy in their seed germination process.

Keywords. *Bunium*, linear embryo, dormancy break, under-developed embryo, cold stratification, cold storage, Apiaceae, morphological dormancy, morphophysiological dormancy, post dispersal embryo growth.

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Introduction

Plant clades that diversified in the Mediterranean biodiversity hotspot often have biogeographic links to the Irano-Turanian region (Médail, 2010; Blondel *et al.*, 2010). Floristic relationships between the Irano-Turanian and Mediterranean regions have been recognized from old times (Blondel *et al.*, 2010). The Irano-Turanian region is characterized by high levels of endemicity and has a very high "species irradiation" (i.e. penetration of its floristic elements into neighboring regions). It has long been regarded as the source of many taxa found in neighboring regions, most notably the Mediterranean region (Akhani, 2007; Blondel *et al.*, 2010). The Irano-Turanian region displays seasonal climatic patterns similar to the Mediterranean region, but with several important differences: (i) often lower annual precipitation, (ii) lower winter temperatures, (iii) slightly higher summer temperatures, thus a distinctly higher continentality, and (iv) often a longer dry season lasting for 5 to 7 months.

Geophilic plants in the Irano-Turanian region have a short period of development during spring and early summer and survive unfavorable seasons due to the presence of tuber-like underground storage organs (Degtjareva *et al.*, 2013). One key adaptation is dormancy, which allows plants to conserve energy and protect themselves during unfavorable conditions. It is an essential survival strategy for these plants to endure the prolonged periods of drought and low temperatures (Shefferson *et al.*, 2005). Two primary forms of dormancy observed in these plants are seed dormancy and bud dormancy. Seed dormancy is crucial for the successful germination and establishment of plants. Seed dormancy plays a critical role in regulating germination to the time when environmental conditions are favorable for seedling survival and for maturation of the plant (Baskin & Baskin, 2014). Dormancy break

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during the season that is not favorable for seedling establishment means that seeds become nondormant at the beginning of the favorable season. Therefore, seedlings/juveniles will have the maximum period of time for growth/establishment before the onset of the next period of unfavorable conditions (Baskin & Baskin, 2014). Seed dormancy is established during embryo maturation, the final stage of seed development, and can be relieved through a period of dry storage called after-ripening, through moist chilling (cold stratification), warm stratification, chemicals or through seed coat scarification (Nelson *et al.*, 2017). After-ripening (AR) is a time- and environment- regulated process occurring in the dry seed, which increases its germination potential (Carrera *et al.*, 2008).

The genus *Bunium* L. (Apioideae, Apiaceae), currently comprises 48–50 species. Unlike other geophilic Apiaceae mainly restricted to Middle Asia, *Bunium* has a broader distribution in Asia, Europe, N and NW Africa and as an adventive in North America (Degtjareva *et al.*, 2013). The main center of *Bunium* diversity is the Mediterranean Basin, however *Bunium* species also grow in the mountains of the Irano-Turanian region. Recently, *Bunium* has been divided into two separate genera *Elwendia* (*Bunium*-I) and *Bunium* (*Bunium*-II) (Degtjareva *et al.*, 2013). Among *Bunium* species, most scientific studies have probed into the biological activity of *B. persicum* (*E. persica*), such as its antifungal, antibacterial, antiviral, and antioxidant properties, among others (Adelifar & Rezanejad, 2021). However, recent research has shed light on the high content of essential oils in other *Bunium* species such as *B. caroides* (*E. caroides*) and *B. wolfii* (*E. wolfii*), showcasing notable antimicrobial and antioxidant activities. These findings suggest potential applications of these species in the food and pharmaceutical industries (Adelifar & Rezanejad, 2021; Rezaee, 2021). The seed oil of these species contains a significant proportion of petroselinic acid, a valuable and distinctive acid found in certain Apiaceae (Bouchra *et al.*, 2017). While *E. caroides* is distributed across various regions of Iran, Turkey, Iraq, Syria, and Transcaucasia, *E. wolfii* is exclusively endemic to Iran (Sefidkon, 2014). Studies on *E. persica* (*B. persicum*) have shown that seed dormancy poses a significant challenge for commercial cultivation (Bonyanpour & Khosh-Khui, 2001; Saeidnejad *et al.*, 2013).

Species in the Apiaceae family typically possess seeds with underdeveloped embryos that are not fully developed at the time of dispersal (Martin, 1946). These seeds exhibit dormancy, which can be classified as morphological dormancy (MD) or morphophysiological dormancy (MPD) (Nikolaeva, 1977; Baskin & Baskin, 2014). In species with MD, favorable conditions of moisture and temperature lead directly to post-dispersal embryo growth and ultimately to germination (Baskin & Baskin, 2004). Dormancy in species with MPD is controlled by an additional physiological dormancy block (PD), preventing embryo growth and germination at times unfavorable for seedling establishment. Nine levels of MPD have been identified based on the response to gibberellic acid (GA3), requirements for cold and/or warm stratification to overcome dormancy, temperature requirements for embryo growth and timing of root and shoot emergence (Baskin & Baskin, 2014). So far, five of those types of MPD have been reported in Apiaceae including deep complex MPD, non-deep complex MPD, deep simple MPD, non-deep simple MPD and intermediate complex MPD (Baskin & Baskin, 2014). The key to understanding the germination of seeds having MPD is to identify which kind of environmental conditions overcome dormancy and promote embryo growth. In some species, both dormancy break and embryo growth are stimulated by the same environmental conditions; while in other species, the two processes require different conditions (Baskin & Baskin, 2014; Zhang *et al.*, 2018). Depending on the species, dormancy break and embryo growth might require (1) cold (0−10 °C) followed by warm stratification (>15 °C), (2) cold followed by warm and then cold stratification, (3) cold stratification only, (4) warm followed by cold stratification, and (5) warm stratification only (Baskin & Baskin 2014; Galíndez *et al.*, 2018).

Researchers have often employed cold stratification (0–5 °C), either alone or in combination with plant growth regulators, to overcome seed dormancy and promote germination in *Elwendia persica*. Emamipoor & Maziah (2014) reported that moist chilling at 2–5 C is the best treatment for seed dormancy break in *E. persica* with 54.7% germination, and moist chilling plus 6.3 µmoll-1 TDZ and 100 µmoll-1 GA3 increased the germination percentage to above 90%. However, Bonyanpour & Khosh-Khui (2001) reported that hormonal treatments had no effect on seed dormancy break in *E. persica* and only cold incubation at 3–5 °C for 46 days promoted seed germination, but reaching less than 25% final germination (Bonyanpour & Khosh-Khui, 2001).

To the best of our knowledge, there is currently no available information on the germination process of *E. caroides* and *E. wolfii*, highlighting the need for further research to understand the factors involved in their seed germination. Thus, the purpose of the current study was specifically to determine: (1) If embryos of *E. caroides* and *E. wolfii* are fully developed or under developed at the time of dispersal; (2) If embryos are underdeveloped, which one of two types of dormancy (MPD and MD) they have and if MPD, which one of the nine levels; (3) Does dry storage increase the germination response compared to a control treatment, indicating the presence of a physiological dormancy component?; (4) Are dormancy break and germination promoted by cold and/or warm stratification?; (5) Does GA3 promote dormancy break and germination, and is there any interaction between cold stratification and GA3 to break dormancy and improve germination?

Materials and methods

Fully matured mericarps (hereafter seeds) of *Elwendia caroides* and *E. wolfii* were collected in June 2021 from Shahr-eKord(32°24’10.767”,51°22’37.143”,1721masl)

and Sarduiyeh (28°9'53.6'', 57°18'36.14'', 2635 m asl), respectively. Seeds were collected at the point of natural dispersal, when they were dry on the mother plants. The experiments on them started 14 days after they were collected and during this period they were kept at room temperature.

Seed viability test

Seeds from each species (100 seeds per species) were placed in Petri dishes with filter paper moistened with distilled water. After water imbibition, the seeds were gently pinched using forceps under a stereo microscope to assess seed viability. This allowed us to observe whether the seeds contained firm, white embryos (indicating viability) or soft, light brown embryos (indicating non-viability). Additionally, a tetrazolium test was conducted to validate the viability of white embryos and confirm the non-viability of brown ones. For each of the species, 4 replications of 25 seeds were used and their embryos were carefully extracted from the seeds and placed in incubation solutions containing 0.1% tetrazolium chloride in phosphate buffer saline, following the method described by Hartmann & Kester (1983). The incubation period lasted for 12 hours, during which the embryos were kept in the dark at room temperature. The presence of a red coloration across the entire embryo was regarded as an indication of viability, suggesting that the seeds were capable of germination. Those that did not stain, were considered as non-viable.

Water imbibition test

The permeability of seed coverings (pericarp and seed coat) was determined by measuring water uptake under laboratory conditions (20–25 °C, RH 45–50%). Five replicates of 100 seeds from each species were taken, and their initial weight was recorded as initial weight (Mo). Subsequently, the dry seeds were soaked in a 1:10 ratio of seeds to distilled water. The weight of each replicate was recorded at 1-hour intervals for the first 8 hours and then every 10 hours for the next 28 hours. The percentage of water uptake was calculated using the equation: Water uptake (%) = [(Mt - Mo) / Mo] × 100; where water uptake represents the percent increase in fresh mass of seeds, Mo is the initial weight of seeds, and Mt is the weight of seeds after a given time (Meng *et al.*, 2017).

Morpho-anatomical studies

The width and length of 100 seeds from each species were measured using a vernier caliper. To determine the initial length of the embryos, the seeds were soaked in water until seed tissues became sufficiently soft to allow for the extraction of the embryos. Then the embryos were carefully removed from the seeds using a razor blade and their lengths measured with a vernier caliper under a stereo microscope. Additionally, the position of the embryo within the seed, the presence of endosperm, and the ratio of embryo length to seed length, referred to as the Embryo:Seed or E:S ratio, were also recorded.

General conditions of the germination experiments

Laboratory experiments of embryo growth and seed germination were conducted in temperature- and light controlled incubators, a refrigerator and a freezer. The incubators were set at 12 h / 12 h daily alternating thermoperiods of 5/10 ± 1 ºC, 15/23 ± 1 º C, 18/30 ± 1 ºC. The daily photoperiod in the incubators was 14 h, with light lasting from 1 h before the beginning of the high-temperature period to 1 h after the beginning of the low-temperature period. Cool-white fluorescent tubes, producing a photosynthetic photon flux density 42 µmol m^{-2} s^{-1} with 400–700 nm, were used as the light source in incubators and the refrigerator. The refrigerator was set at a constant temperature of 5 ± 1 ºC, and the freezer was set at constant temperature of -22 ± 1 ºC. The refrigerator was equipped with a light and time clock and set at a constant temperature of 5 ± 1 ºC, while the freezer was set at constant temperature of -22 ± 1 ºC in continuous darkness.

During moist incubations, the Petri dishes were kept moist and all dishes were wrapped with plastic film to retard water loss. The critical E:S ratio (the E:S ratio at the time the seed coat splits but before radicle emergence) for germination was determined (Copete *et al.*, 2011). The criterion for germination was radicle tip emergence ≥ 1 mm. At the end of each experiment, ungerminated seeds were checked to determine whether seeds had a grayish brown and soft embryo (non-viable) or a white and firm embryo (viable) (Walk, 2002). A razor blade was used to excise the embryos of imbibed seeds. Tetrazolium tests confirmed that white embryos were viable and brown ones were not. To analyze germination data, the percentages were calculated based on the number of viable seeds. Percentage of germination = Number of germinated seeds / Number of total viable seeds ×100 (Kondo *et al.*, 2007).

Short-term effect of temperature on embryo growth and germination

The seeds were washed under running tap water overnight, and then their surface was sterilized with 70% ethanol for 1 min and 1% sodium hypochlorite solution for 10 min. Subsequently, the seeds were rinsed with deionized water and then dried. Seeds of *Elwendia wolfii* and *E. caroides* were incubated at temperatures of 5 ºC, 5/10ºC, 15/23 ºC and 18/30 ºC, light and dark 12 h/12 h for 30 days to assess their short-term embryo growth and germination in the absence of any pre-treatments.

Long-term effect of temperature on embryo growth and germination

To investigate the long-term effect of temperature on dormancy break, embryo growth and germination, 25 replicates of 50 seeds from each species were placed in 15 cm diameter plastic Petri dishes on two sheets of Whatman number 1 filter paper moistened with distilled water and were incubated at 5 ºC, 5/10 ºC, 15/23 ºC

and 18/30 °C for 20 weeks. Seeds were examined for germination and embryo growth every week. Germinated seeds were counted and removed from the dishes after each examination. Water was added as needed to the dishes.

Effect of dry storage pretreatment on embryo growth and germination

To investigate the effect of a dry storage pretreatment on dormancy break, 20 replicates of 50 seeds from each species were placed in 15 cm diameter plastic Petri dishes on two sheets of Whatman number 1 filter papers. Ten Petri dishes were cold-dry stored at -22 ºC (in the freezer) and the other 10 Petri dishes were stored at 23 ºC (in a room temperature ranging from 20 to 25 ºC, with an average of 23 ºC). Completely dry seeds were used to prevent damage at -22 ºC. After 6 months, the seeds were transferred to moist incubation at 5 ºC and 23 ºC for 12 weeks. Seed germination and embryo growth were monitored weekly during incubation. The seeds that were incubated at 5 ºC and 23 ºC without undergoing a cold or warm dry storage pretreatment were used as the control.

Effect of warm and cold stratification pretreatments on embryo growth and germination

To investigate the effect of warm and cold stratification pretreatments, 10 replicates of 50 seeds from each species were placed in 15 cm diameter plastic Petri dishes on two sheets of Whatman number 1 filter paper moistened with distilled water and were stratified at 5 ºC. After 20 weeks, the non-germinated seeds were transferred to higher temperatures at 15/23 ºC and 18/30 ºC. Similarly, other 10 Petri dishes were stratified at 23 ºC for two weeks and then transferred to low temperatures of 5 ºC and 5/10 ºC for germination. Other seeds were continuously kept at a stratification temperature of 5 °C and 23 ºC, which served as the control. The seeds were exposed to a 14-hour photoperiod both during stratification and incubation.

Effect of GA3 on embryo growth and germination

A total of 12 replicates of 50 seeds were distributed into 15 cm diameter plastic Petri dishes on two sheets of Whatman number 1 filter paper moistened with 10 ml of distilled water (control) and with concentrations of 10, 100 and 1000 mg/l of GA3 (dissolved in distilled water) and transferred to the incubator at 15/23 ºC and to the refrigerator at 5 ºC for 16 weeks. For each treatment at each temperature, three replicates were used. Filter papers were kept moist throughout the experiment with distilled water. Seed germination was monitored every week.

Statistical analyses

This study was based on a completely randomized design. One-way ANOVA was performed for the evaluation of pericarp permeability (water imbibition test). Embryo growth was also analyzed by one-way ANOVA, with post hoc Tukey's HSD test used to assess differences among treatments. Generalized Linear Models (GLM), with binomial error distribution and a logit link function were used to analyze germination percentages. ANOVA was conducted with SPSS Version 26 (IBM Corp., Armonk, N.Y), GLM with R version 3.4.1.

Results

Seed viability test

Embryoless seeds and completely empty seeds containing only a pericarp (lacking both embryo and endosperm) were categorized as non-viable seeds. Seed viability was 98 ± 1.4% for *E. caroides* and 97 ± 0.8% for *E. wolfii*.

Water imbibition test

The weight of 100 seeds was 0.27 mg for *E. caroides* and 0.14 mg for *E. wolfii*. Rapid water uptake was observed, resulting in a seed mass increase of 79.41% in *E. caroides* and 67.11% in *E. wolfii* after 1 hour. Subsequently, the rate of water uptake decreased over the following hours, reaching a maximum increase of 113.9% in *E. caroides* after 7 hours and 106.18% in *E. wolfii* after 6 hours. No further increase in water uptake was observed beyond this point (Figure S1, Supplementary Material).

Morpho-anatomical studies

The fruits of *E. caroides* and *E. wolfii* were schizocarps or cremocarps composed of two mericarps. The two mericarps were connected at the commissural suture and split along it when mature (Figure 1). The length and width of the seeds were 5.96 mm and 1.11 mm in *E. caroides*, and 4.04 mm and 0.86 mm in *E. wolfii*, respectively. Morpho-anatomical studies revealed that the mericarps (seeds) in both species had a thin pericarp and testa, a large endosperm, and a small embryo located at the stylopodium end. Both species had differentiated but underdeveloped linear embryos that needed to reach a critical length before germination. The embryos were surrounded by abundant endosperm. The underdeveloped embryos were di- or tri-cotyledonous, accounting for 98.6% and 1.4% in *E. wolfii* and 99% and 1% in *E. caroides*, respectively. No fusion between any parts of the cotyledons in tri-cotyledonous embryos was observed. Slight inequality of the cotyledons was observed in the tri-cotyledonous embryo, with the angle between two cotyledons being smaller compared to the others. The initial embryo length was 0.28 mm with an embryo to seed ratio (E:S ratio) of 0.07 in *E. wolfii*, and 0.96 mm with an E:S ratio of 0.16 in *E. caroides*.

Short-term effect of temperature on embryo growth and germination

The freshly collected seeds of *E. wolfii* and *E. caroides* were completely dormant because they did not germinate within 30 days at 5 ºC, 5/15 ºC, 15/23 ºC and 18/30 ºC.

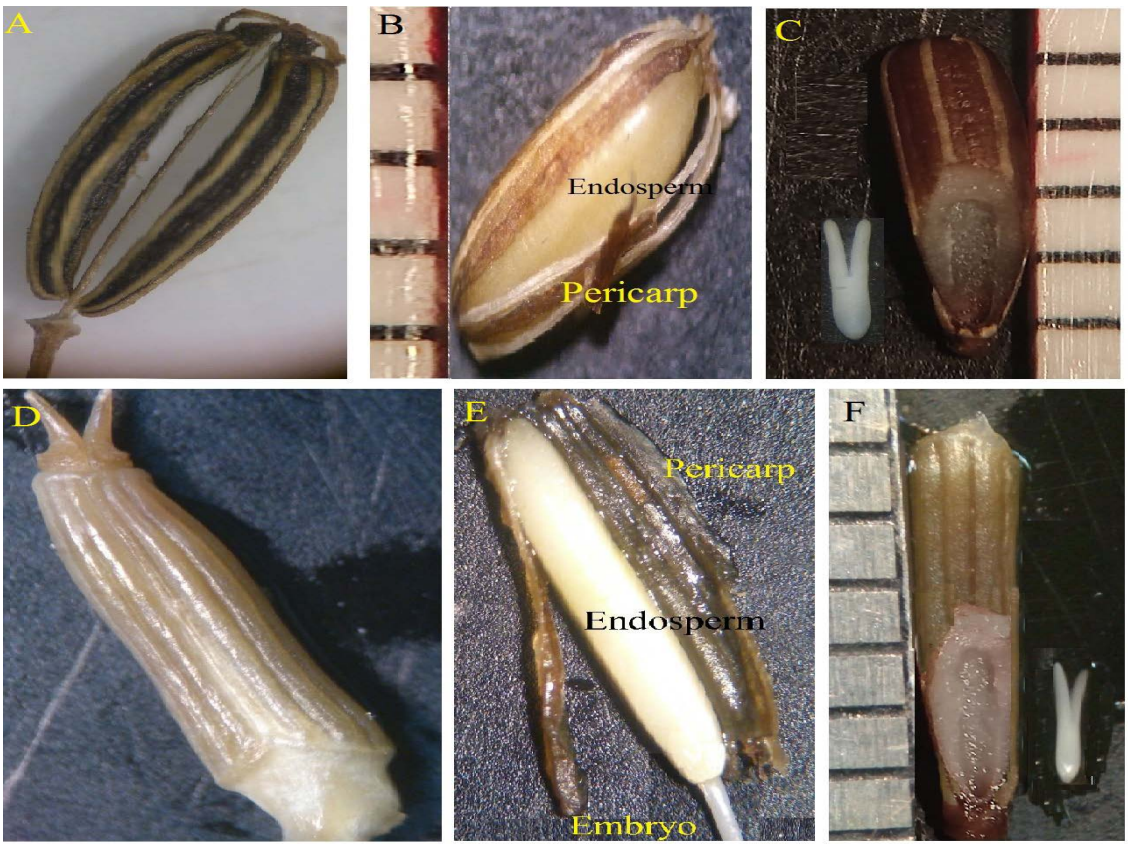

Figure 1. Seed morphology and anatomy of *Elwendia wolfi* (A–C) and *E. caroides* (D–F). A&D, A schizocarp composed by two mericarps connected at the commissural suture by a bifid carpophore; B&E, The structure of a seed (mericarp), including seed coverings (pericarp and testa), a large endosperm and an embryo enclosed by the endosperm; C&F, The position of embryo in the seed.

Table 1. Results of one-way ANOVAs showing the effects of Incubation temperature (5 ºC, 5/10 ºC, 15/23 ºC and 18/30 ºC) on embryo growth of *Elwendia caroides* and *E. wolfii* seeds.

	Elwendia wolfii					*Elwendia caroides*				
Component	Sum of Squares	df	Mean Square	F-value	P-value	Sum of Squares	df	Mean Square	F-value	P-value
Intercept	85	1	85	29899	P < 0.001	179	1	179	56265	P < 0.001
Temperature	55	3	18	6537	P < 0.001	53	3	17	5568	P < 0.001
Error	0.1	36	0			0.1	36	0		
Total	140	40				233	40			

Long-term effect of temperature on embryo growth and germination

Embryo growth and germination only occurred at low temperatures (5 ºC and 5/10 ºC), while higher temperatures (15/23 ºC and 18/30 ºC) inhibited embryo growth and, consequently, germination (Table 1, 2; Figure S2 & S3 in Supplementary Material). Incubation at 5 ºC promoted embryo growth to 2.5 mm and 3.21 mm with a germination percentage of 29.4 ± 4.2% and 39.54 ± 4.4% in *E. wolfii* and *E. caroides*, respectively. The germination percentage at 5/10 ºC was significantly higher than at 5 ºC. Embryo growth during moist chilling started shortly after water imbibition. Most ungerminated seeds were viable. Ungerminated seeds showed no or little embryo growth during moist chilling and hence did not reach to the critical length required for germination. Only a very small number of seeds (x≤3) were lost during the incubation at low temperature and they were not included in calculating the final germination percentage. The critical embryo length required for germination was 3.44 mm (c. 12-fold initial length) for *E. wolfii* and 4.17 mm (c. 3.5-fold initial length) for *E. caroides*.

Effect of dry storage pretreatment on embryo growth and germination

Dry storage pretreatment reduced the length of the cold period required for dormancy break and seed germination in both species. The highest germination percentage,

Table 2. Results of Generalized Linear Models (GLMs) for the effect of incubation temperature, dry storage pretreatment, warm and cold pretreatment and GA_3 concentrations on seed germination of *Elwendia wolfii* and *E. caroides*. P-value: $p < 0.001$

	Incubation temperature		Dry storage pretreatment		Warm and cold stratification pretreatment		GA_3 concentrations	
Species	Df	Chisq	Df	Chisq	Df	Chisq	Df	Chisq
Elwendia wolfii	18	414	19	673	20	827	91	8
Elwendia caroides	19	511	19	1205	21	1212	685	8

along with the maximum embryo length (93 ± 1.7% with 3.4 mm for *E. wolfii* and 92.1 ± 2.8% with 4.15 mm for *E. caroides*), was achieved by cold-dry storage (-22 °C) followed by incubation at 5 ºC. Embryos in seeds subjected to this combination of treatments had faster development (Table 2, 3; Figure S4 in Supplementary Material). Warm-dry storage followed by incubation at low temperatures resulted in a germination percentage of 46.82 ± 3.6% and embryo length of 2.86 mm for *E. wolfii* and 53.46 ± 4.3% with 3.54 mm for *E. caroides.*

Effect of warm and cold stratification pretreatments on embryo growth and germination

No embryo growth or seed germination was observed in seeds stratified at 23 ºC (Table 2, Figure S6 in Supplementary Material). The effect of warm stratification at 23 ºC followed by incubation at low temperatures of 5 ºC and 5/10 ºC was significantly higher than just cold stratification at 5 ºC. During cold stratification at 5 ºC, less than 50% of the seeds in both species germinated. No embryo growth or germination occurred when ungerminated seeds were transferred from 5 to 15/23 ºC and 18/30 ºC. Long-term incubation of seeds at moist conditions with high temperatures was accompanied by seed deterioration, and the embryos underwent a transition from a white and firm appearance to a brown and soft state.

Effect of GA3 on embryo growth and germination

In both species, none of the tested GA_3 concentrations led to seed germination at a temperature of 15/23 °C and the embryos gradually lost their viability. At 5 ºC, significant differences were observed among different concentrations of GA_3. GA_3 at 100 mg/l in combination with incubation at 5 ºC proved to be more effective in promoting germination compared to the control (i.e. seeds incubated at 5 ºC). Seeds incubated with GA_3 at 100 mg/l showed the highest germination percentage (74.4 + 3% for *E. wolfii* and 84.3 + 4% for *E. caroides*), followed by seed incubated with 10 mg/l (Table 2, Figure S7 in Supplementary Material). The combination of GA_3 at 1000 mg/l with cold temperature (5 ºC) resulted in a lower germination percentage compared to the control.

Discussion

This study showed that *Elwendia wolfii* and *E. caroides* embryos were underdeveloped and needed moist chilling to grow. In part of the seed populations of both species (< 50%), embryo growth occurred immediately after incubation at temperatures ≤ 10 ºC, indicating that these seeds had no physiological block preventing seed germination, thus the seeds only exhibited morphological dormancy (MD). These seeds required several weeks of moist chilling to reach the critical length for germination. In the rest of the seed population, embryo growth or germination did not occur even after a prolonged incubation at high or low temperatures, indicating the presence of physiological dormancy (PD). Overall, these results confirm that both *E. wolfii* and *E. caroides* have morphophysiological seed dormancy (MPD) (Baskin & Baskin, 2004).

Table 3. Results of two-way ANOVAs showing the effects of dry storage pretreatment (at 23 ºC and -22 ºC) and incubation temperature (at 5 ºC and 23 ºC) on embryo growth of *Elwendia caroides* and *E. wolfii* seeds.

	Elwendia wolfii					*Elwendia caroides*				
Component	Sum of Squares	df	Mean Square	F-value	P-value	Sum of Squares	df	Mean Square	F-value	P-value
Intercept	153	1	153	40275	P < 0.001	317	1	317	168032	P < 0.001
Dry storage	2	2	1.02	268	P < 0.001	2.26	2	1.13	598	P < 0.001
Temperature	104	1	104	27374	P < 0.001	106	1	106	56573	P < 0.001
Error	0.2	54	0.0			0.1	54	0.0		
Total	262	60				429	60			

MPD can be divided into two categories, simple and complex. High temperatures (≥ 15 ºC) are required for embryo growth in seeds with simple MPD, whereas low temperatures (0–10 ºC) are required for embryo growth in seeds with complex MPD (Nikolaeva, 1977; Baskin & 2014). Since *E. wolfii* and *E. caroides* required low temperatures to stimulate embryo growth, they must have complex MPD. To further delineate the level of MPD present in these two *Elwendia* species, we need to know if the application of GA_3 does break dormancy. Non-deep complex MPD seeds

require a warm followed by a cold stratification before they can germinate, and embryo growth takes place during the cold stratification period. Seeds with intermediate complex MPD only need cold stratification to overcome physiological dormancy and promote embryo growth. However, gibberellic acid can substitute for cold stratification in promoting germination (Nikolaeva, 1969; Baskin & Baskin, 2014). On the other hand, seeds with deep complex MPD need cold stratification to overcome physiological dormancy and facilitate embryo growth, but gibberellic acid does not enhance germination (Nikolaeva, 1969; Baskin & Baskin, 2014). Dry storage, warm stratification pretreatment and GA_3 treatment followed by incubation at cold temperature (5 ºC) was required for dormancy break in our two study species. Therefore, they had non-deep complex MPD. In seeds with non-deep complex MPD, embryo growth is delayed until physiological dormancy loss is completed during dry storage (Hawkins *et al.*, 2010; Baskin & Baskin, 2014). This study showed that the seed populations of *E. wolfii* and *E. caroides* have a combination of morphological dormancy and non-deep complex morphophysiological dormancy. Hidayati *et al*. (2000) revealed that approximately half of the freshly matured seeds of *Lonicera morrowii* and *L. maackii* had MD, and the remaining seeds had MPD. *Lonicera morrowii* seeds with MPD required 6 weeks of warm stratification to germinate, and those of *L. maackii* with MPD required 12 weeks of cold stratification to germinate (Hidayati *et al.*, 2000).

The results of this study differ from the findings of Soltani *et al.* (2019) regarding the Apiaceae *Cuminum cyminum* (cumin). After breaking physiological dormancy during dry storage, embryo growth in cumin seeds occurred at both cold and warm incubation temperatures (Soltani *et al.*, 2019), however, in seeds of *Elwendia caroides* and *E. wolfii*, embryo growth only took place at low incubation temperatures. This study is however in accordance with studies of seed dormancy break in the more closely related *E. persica* (*Bunium persicum*) which showed that cold stratification (5 ºC) is the most effective treatment to overcome seed dormancy, but cold stratification alone results in low germination percentage between 25–65% (Bonyanpour & Khosh-Khui, 2001; Saeidnejad *et al.*, 2013; Emamipoor & Maziah, 2014). Some studies have reported that cold stratification plus plant growth regulators increase the germination percentage more than cold stratification alone in *E. persica*. In this study, the highest germination percentage, without adding growth regulators, was observed in seeds cold stored at -22 ºC and subsequently transferred to 5 ºC. One of the effects of low temperatures is the breakdown of endosperm reserves (conversion of stored proteins into soluble nitrogen compounds) and their availability for embryo growth and development (Stokes, 1953).

In the present study, dry storage (warm and cold) followed by cold incubation increased the germination percentage and decreased the length of the cold incubation period required for seed germination. However, physiological dormancy was broken in more seeds during cold dry storage compared to warm dry storage. A short period of dry storage could not increase germination in *Cuminum cyminum* seeds, but seeds came out of physiological dormancy after a longer period (20 months) of dry storage. Dry storage leads to reduced viability and germination in some species, but in other species, dormant seeds acquire the capacity to germinate through a period of dry storage called after-ripening (AR), a biological process that occurs at 5–15% content moisture when most metabolic processes cease (Nelson *et al.*, 2017). Seed responses to dry storage vary among different species and some species exhibits two different responses to two types of dry storage (cold and warm). Drying *Vaccinium membranaceum* seeds for 7 days reduced germination from 73 to 59%, but this dormancy was lost during cold dry storage of the seeds (Shafii & Barney, 2001). It is possible to slow the rate of physiological changes in many seeds by storing them dry at low temperatures, but in some species, changes in germination responses occur while seeds are stored dry at low temperatures. Germination percentages increased following dry storage of *Amaranthus bouchonii* (4 ºC), *Delphinium cultorum* (2 ºC); *Lepidium virginicum* (-18 ºC), and *Dactylis glomerata* (-75 ºC) (Baskin & Baskin, 2014). Seeds of some forbs germinate following freezing at -18 ºC (Wesche *et al.*, 2006). During almost 40 years of dry storage of Brassicaceae seeds in sealed vials at -5 and -10 ºC, dormancy break, dormancy induction or no change in dormancy state occurred, depending on the species (Perez-Garcia *et al.*, 2007). Understanding how dormancy loss through after-ripening occurs in a dry and metabolically quiescent seed is a pending question for plant science (Nelson *et al.*, 2017). Transcriptome studies have observed differential accumulation of stored dry seed mRNAs with after-ripening of multiple species (Chitnis *et al.*, 2014; Meimoun *et al.*, 2014).

Seed germination is of great importance in the life cycle of plants, and being timed to a favorable period is critical for seedling emergence and establishment (Baskin & Baskin, 2014). The Irano-Turanian climate is characterized by cool, wet winters and hot, dry summers, making spring the most favorable season for seedling establishment (Djamali *et al.*, 2012). *Elwendia* seeds are mature at late spring or early summer and they disperse in late spring to early autumn. Dispersal may last for several months, with mature mericarps retained on dead, upright stems. From an ecological perspective, seeds of *Elwendia* species are prevented from germinating after dispersal in summer since they are dormant and/or temperatures are too high for their requirements. Warm-dry conditions during summer and early autumn may be enough to overcome physiological dormancy in seeds with non-deep complex MPD, and growth of the embryo does not start until temperatures have dropped in late autumn or winter. Thus, the mechanism for dormancy break and germination in *Elwendia* seeds allows for an exact timing of seedling emergence. The requirement for cold incubation temperatures prevents germination in summer or autumn and germination during winter leads to establishment during the moist season. The requirement for a short cold period for embryo growth in seeds with non-deep MPD guarantee that at least some seeds will germinate in spring if the seeds are dispersed to (a) areas with a lack of normal levels of precipitation resulting in a

relatively short cold wet period during winter or (b) lower elevations with relatively short winters.

The embryos in both species were linear underdeveloped at dispersal. Different types of embryos are found in Apiaceae including linear (most frequent), spatulate and rudimentary. The most advanced type of embryo is spatulate followed by the linear and then rudimentary (Baskin & Baskin, 2014). The number of cotyledons is a taxonomically important character. A dicotyledonous embryo represents the ancestral state of Apiaceae (Kljuykov, 2020). For the first time, the occurrence of tricotyly in *Elwendia caroides* and *E. wolfii* was reported in the present study. One of the three cotyledons was frequently slightly larger than the other two. The angle between the two smaller cotyledons was smaller than the angle between each of these cotyledons and the larger cotyledon. This observation could indicate their origin through fission. The fact that one of the three cotyledons is often noticeably larger than the other two is linked to their origin, where the smaller pair of seed leaves results from the complete fission of one cotyledon, a phenomenon known as schizocotyly (van Cotthem, 1979). Schizocotyly is a heritable anomaly observed in many dicots (Holtorp, 1994). Palmer (1975) suggested that tricotyly is inherited, and the variation in expression is regulated by the environment

Conclusions

As far as we know, this is the first report describing the type of dormancy in these two species. This study showed *Elwendia wolfii* and *E. caroides* share similar germination requirements. Cold-dry storage at -22 ºC followed by cold incubation (< 10ºC) result in the maximum germination percentage without the need for employing plant growth regulators. The information gained from this study will enable horticulturalists and seed ecologists to reduce the time to obtain *E. wolfii* and *E. caroides* seedlings, and thus, provide a useful reference for the agriculture industry, for species conservation and for understanding the ecophysiology of post-dispersal embryo growth.

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Conflict of interest

None.

Authorship contribution

He.R.: Conceptualization, Methodology, Formal analysis and investigation, Writing - original draft preparation; F.R: Conceptualization, Methodology, Formal analysis and investigation, Writing - review and editing, Funding acquisition, Resources, Supervision; S.A.A.: Conceptualization, Methodology, Formal analysis and investigation, Resources, Supervision; G.R.S.-S.: Conceptualization, Methodology, Formal analysis and investigation; Hi.R.: Conceptualization, Methodology, Formal analysis and investigation,

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Supplementary Material

S1. Water imbibition by *Elwendia wolfii* and *E. caroides* seeds**.** The curve shows seed mass increases (mean ± SE) by intact seeds incubated on a moist substrate at room temperature (23 °C) for 28 h.

S2. Embryo growth (mean ± SE) of *Elwendia wolfii* and *E. caroides*. Effect of incubation temperature at 5 °C, 5/10 °C, 15/23 °C and 18/30 °C on embryo growth.

S3. Seed germination (mean ± SE) of *Elwendia wolfii* and *E. caroides*. Effect of incubation temperature at 5 °C, 5/10 °C, 15/23 °C and 18/30 °C on seed germination. Seed germination was measured in weekly intervals.

S4. Embryo growth (mean ± SE) of *Elwendia wolfii* and *E. caroides*. Effect of dry storage pretreatment (at temperatures of 23 °C and -22 °C) and incubation temperature (at temperatures of 5 °C and 23 °C) on embryo growth of *E. caroides* and *E. wolfii* seeds.

S5. Seed germination (mean ± SE) of *Elwendia wolfii* and *E. caroides*. Effect of dry storage pretreatment (at 23 °C and -22°C) and wet incubation temperature (at 5°C and 23°C) on seed germination of *E. caroides* and *E. wolfii* seeds**.** Seed germination was measured in weekly intervals.

S6. Seed germination (mean ± SE) of Elwendia wolfii and E. caroides. Effect of cold and warm pretreatment (at 5 °C and 23 °C) and incubation temperature (at 5 °C, 5/10 °C, 15/23 °C and 18/30 °C) on seed germination of E. caroides and E. wolfii seeds**.** Seed germination was measured in weekly intervals.

S7. The effect of GA_3 in combination with cold incubation temperature on seed germination (mean ± SE) of *Elwendia caroides* and *E. wolfii*. The seeds of each species were incubated in different concentrations of GA_3 (10, 100 and 1000 mg l^{-1}), and then transferred to cold temperature at 5 °C. Seed germination was measured in weekly intervals.