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Resurrecting a locally extinct species from Jordan: Pre-germination treatments affect seed dormancy, germination dynamics and seedling survival in *Rosa pulverulenta*.

Mohammad M. Al-Gharaibeh [a,*], Halim Adil Bakhit [a], Shifaa Masadeh [a], Dávid Nagy [b], Christoph Rosche [b,c]

[a] Department of Plant Production, Faculty of Agriculture, Jordan University of Science and Technology, P.O. Box 3030, Irbid 22110, Jordan
[b] Institute of Biology/Geobotany and Botanical Garden, Martin Luther University Halle-Wittenberg, Halle, Germany
[c] German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig, Halle, Germany

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ABSTRACT

Habitat loss, fragmentation, and climate change present significant threats to global plant biodiversity. Small and isolated populations residing in threatened habitats at the edge of their natural distribution are particularly vulnerable to local extinction. *Rosa pulverulenta* M.Bieb., had its southernmost distribution in the Mediterranean basin in Jordan, but this remnant population recently extinct in the wild. Our goal to resurrect this species is hindered by the limited availability of seeds from the extinct population and a high degree of combinational dormancy in *R. pulverulenta*. To preserve as many genotypes as possible for eventual reintroduction, we aimed to identify the most effective seed treatment for breaking seed dormancy, and enhancing germination, and seedling establishment. Seeds were collected one season before extinction. We applied seven pre-germination treatments. The pre-treated seeds were then germinated at three temperature regimes (4/4 °C, 20/15 °C, and 25/20 °C), simulating winter and spring germination conditions. Warm-cold stratification, integral to all pre-treatments, proved effective in dormancy release, significantly enhancing germination. The highest germination (68 %) occurred at 4 °C, particularly in seeds treated with warm-cold stratification alone. Conversely, minimal germination was observed at higher temperatures (20/15 °C and 25/20 °C), suggesting potential induction of secondary dormancy. Furthermore, warm-cold stratification, especially when followed by microbial fertilizer, positively impacted seedling survival. Our study provides insights into effectively resurrecting the southernmost boundary of *R. pulverulenta* distribution in the Mediterranean basin, thereby preventing the global decline in the species' extent of occurrence. Strategically releasing seed dormancy and enhancing germination has important implications on scientific efforts reversing the declining trend of biodiversity in the Anthropocene. Additionally, seed collection from populations on the brink of extinction for resurrection is crucially required as the best method to preserve their genetic diversity. Therefore, this work offers a valuable framework that can be adapted to similar conservation efforts for other endangered plant species facing comparable threats. We emphasize the importance of further investigating how climate

* Correspondence to: Department of Plant Production, Faculty of Agriculture, Jordan University of Science and Technology, P.O.Box 3030, Irbid 22110, Jordan.
E-mail address: mfagharaibeh@just.edu.jo (M.M. Al-Gharaibeh).

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change can negatively affect seed dormancy, germination, and plant regeneration, ultimately leading to the local extinction of threatened populations.

1. Introduction

Habitat loss, climatic fluctuations and fragmentation lead to the formation of small and isolated populations (Al-Gharaibeh et al., 2017a; Rosche et al., 2018a; Wilkinson et al., 2018). Isolated plant populations, particularly those located at the edge of their natural distribution range, face an elevated risk of decline and eventual local extinction (Boakes et al., 2018; Farnsworth and Ogurcak, 2008; Hampe and Petit, 2005; Rosche et al., 2022). In dry ecosystems, the local extinction of single species can have more rapid and pronounced consequences compared to an equivalent loss in mesic ecosystems (Alrababah et al., 2007). These dry ecosystems are presently much exposed to climatic variations, temperature fluctuations, and changes in rainfall patterns (Zeng et al., 2021), which can dramatically affect the sexual recruitment of threatened plant populations. Recruitment includes the stages of seed dormancy release, germination, and subsequent seedling survival. Failure to recruit new individuals in any of these processes can dramatically affect population persistence hastening the local extinction of threatened species (Rosche et al., 2018b, Gao et al., 2021). Thus, conservation efforts should prioritize the early stages of a species' life history (Schemske et al., 1994; Winkler et al., 2024), particularly focusing on how seed dormancy, germination and seedling establishment respond to changing environments (Pavlik and Manning, 1993; Robinson et al., 2018).

Dormancy can prevent seed germination under unfavorable conditions, making it an important ecological and physiological adaptation that aids wild plants to survive under harsh environments, especially in the context of climate warming (Stevens et al., 2020, Zhang et al., 2022). However, dormant seeds may take several seasons to release dormancy in nature, and during this dormancy period, seeds often lost their viability or are removed from soil seed bank due to biotic and abiotic factors (Kildisheva et al., 2020; Ma et al., 2020). This poses a challenge for the rapid natural regeneration of endangered wild populations (Cho et al., 2018; Gao et al., 2021). Under climate change, seed dormancy patterns may be particularly important, as they are influenced by shifts in seasonal environments (Klupczyńska and Pawłowski, 2021), where precipitation and temperature are crucial drivers for seed dormancy (initiation, break) of both woody and herbaceous plants (Walck et al., 2011; Zhang et al., 2022). Changes in habitat environmental conditions experienced by mother plants during seed maturation (maternal effects) exert a significant influence on plant phenotype and seed traits, leading to shifts in germination time, percentages, and phenology (Al-Gharaibeh et al., 2017b; Baskin and Baskin, 2014; Chen et al., 2022; Longás et al., 2021).

Even if seeds lose dormancy, consideration of the environmental requirements of germination is necessary to determine limitations to the regeneration of wild populations. This is because their local environment may mismatch the species-specific germination requirements (Gao et al., 2021). In Mediterranean ecosystems, particularly in arid and semi-arid regions, temperature emerges as the primary driver of germination when sufficient water availability is ensured (Al-Gharaibeh et al., 2017b; Hardegree, 2006). In some plant species the range of temperatures that can trigger seed germination are very narrow (Van der Walt and Witkowski, 2017; Walck et al., 2011), and temperature beyond this range inhibits seed germination (Al-Gharaibeh et al., 2017b; Donohue et al., 2010; Huang et al., 2018). Additionally, the availability of water is another crucial germination requirement, which can limit seed germination, seedling survival, and establishment during early growth stages (Al-Gharaibeh et al., 2017b; Carvajal et al., 2014).

Due to ongoing climate change, plants of Mediterranean Basin experienced changes in seasonal environmental conditions including a decrease in amount of precipitation, change in rainfall regime, and a pronounced warming with increase in extreme temperature events frequency (Mattana et al., 2022). Such changes hinder the regeneration of plants from seeds, especially for small and isolated populations at the edges of species ranges (Giménez-Benavides et al., 2018). These changes affect seed dormancy (Huang et al., 2018), reducing germinability (Rosche et al., 2018a), and subsequently impact recruitment and survival (Hampe and Petit, 2005; Klupczyńska and Pawłowski, 2021; Pavlik and Manning, 1993). Consideration and understanding of climate change's impact on dormancy and germination traits in in-situ restoration planning often results in successful plant establishment and contributing to population size increment (Kildisheva et al., 2020).

When in-situ natural regeneration of a threatened population is limited, ex-situ cultivation becomes an effective measure to rescue declining populations and represents the first step in resurrecting extinct populations (Chen et al., 2022). Although a necessary complement to in-situ conservation, the ex-situ cultivation of wild plants can be challenging and critically relies on successful germination (Enßlin et al., 2011). Optimizing the release of seed dormancy, germination, and seedling establishment becomes crucial when seeds are scarce and limited for species resurrection. These stages are foundational for successful species recovery efforts, as they lay the groundwork for subsequent phases like reintroduction and reproduction in the wild. This underscores the importance of finding the best seed treatment to preserve as many genotypes as possible for eventual reintroduction following successful ex-situ cultivation.

In the recent years, *Rosa pulverulenta* M.Bieb. occurred in Jordan at only one site. This remnant population represented the southernmost distribution limit of this species in the Mediterranean basin (Al-Gharaibeh in Greuter and Raus, 2011). The population went extinct in 2022. Fortunately, seeds were collected one year prior to the local extinction, providing an opportunity for urgent intervention to reverse the local extinction of *R. pulverulanta* from Jordan, and thus conserving potentially important parts of its global genetic diversity. However, the achenes (seeds) of the *Rosa* genus exhibit various types of primary dormancy (Stoian-Dod et al., 2023). The hard achene endocarp prevents water absorption (physical dormancy), restricting embryo expansion and radicle emergence (mechanical dormancy). In addition, chemical dormancy inhibit the seed germination due to the high levels of abscisic acid (ABA) present in the testa and pericarp of *Rose* achenes (Bo et al., 1995). Although the embryos are fully formed without morphological

dormancy, physiological dormancy persists due to after-ripening requirements. Furthermore, exposure to temperatures of 20°C or higher can induce secondary dormancy in *Rosa* seeds (Nadella et al., 2003). Consequently, combinational dormancy (a combination of different dormancy types), is common in wild *Rosa* seeds (Pawłowski et al., 2020), leading to very poor natural regeneration due to seed dormancy and germination requirements (Gudin et al., 1990; Kazaz et al., 2010; Younis et al., 2007).

Given the challenges of dormancy release and the fact that Jordan has witnessed high fluctuations in annual precipitation and strong trends indicating an increase in annual minimum temperature in recent decades (Hamdi et al., 2009), we hypothesize that the specific requirements for dormancy release and germination, especially temperature, are not supported in *R. pulverulenta* natural habitat. This is potentially due to climate change, thereby negatively impacting the recruitment and persistence of this population. The objectives of this study are aimed at assessing the: (1) initial viability and dormancy of collected seeds; (2) effects of different pre-germination treatments on breaking dormancy and seedling survival; (3) effects of temperature on germination of treated seeds; and (4) survival of seedlings. These objectives are crucial for identifying effective pretreatments to release dormancy in *R. pulverulenta* seeds, maximizing germination, and producing seedlings for initial *ex-situ* resurrection efforts, which are essential for subsequent in-situ conservation measures.

2. Materials and methods

2.1. Site characterization

Rosa pulverelanta was newly recorded in 2010 from Queen Alia Forest, Jhayeer, Province of Shaubak, Jordan (30°32'55.1"N, 35°31'18.3"E. 1495 m asl). The highlands of the southern part of Jordan, including Shaubak provenance, are representing the southernmost Mediterranean refugium in the Levant (Danin, 1999). The site is characterized by semi-arid Mediterranean bioclimate with mean annual precipitation of 298 mm, mean annual temperature 15.5°C, and drought index 37.93 (Al-Eisawi, 1996; Al-Gharaibeh et al., 2017a). Climatological data for 44-year period (1970–2014; data from Shaubak weather station, 5 km from the forest), show a significant decrease in both annual rainfall amounts and number of rain events, with high interannual fluctuation (Fig. 1A & 1B). At the same time the mean daily annual maximum temperature during seed maturation months (September-October) and mean daily annual minimum temperature prior seed germination months (December-January) significantly increased (Fig. 1C & 1D). Furthermore, the condition of the forest woodland, characterized by the presence of very old, degraded, and threatened stands of *Pistacia atlantica* and *Juniperus phoenicea*, underscores the impact of drought on such Mediterranean ecosystems. Remarkably, the occurrences of many Mediterranean relict plant species from this region were recorded as southern-most limit of their natural distribution range (Albach and Al-Gharaibeh, 2015). Additionally, some species, including endemics and newly discovered ones not previously documented in scientific literature such as *Romulea petraea* and *Rubia danaensis*, are exclusively found within this region.

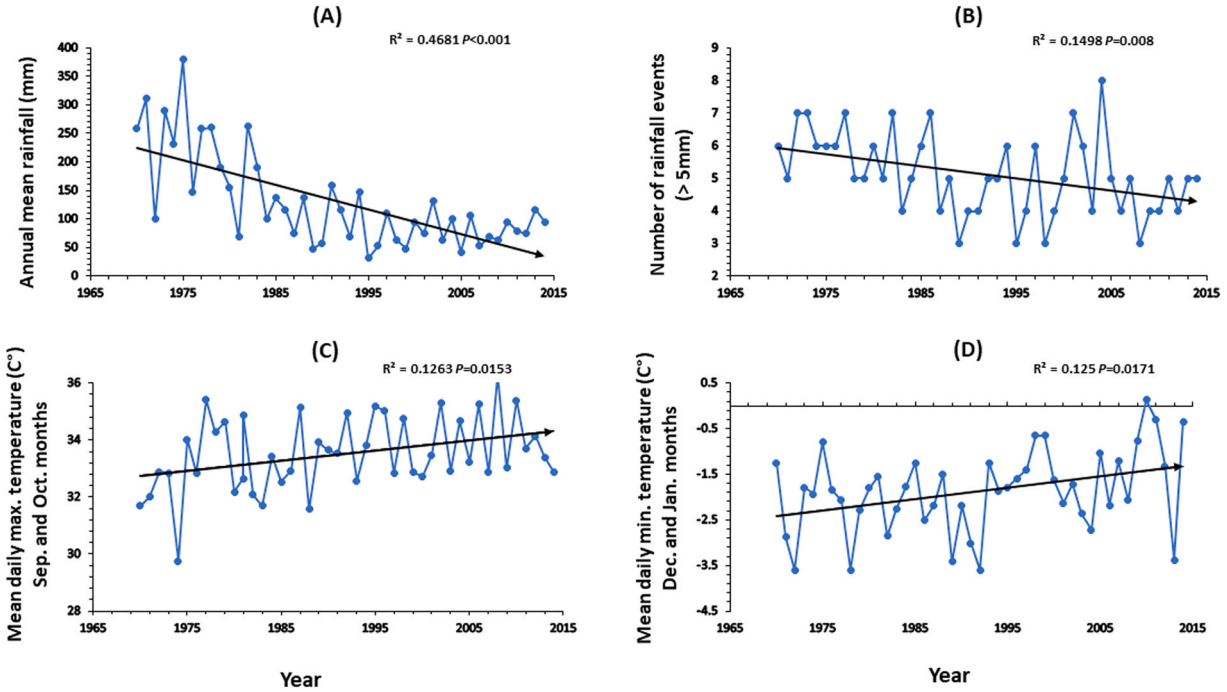

Fig. 1. Climatological data for a 44-year period (1970–2014) from the Shaubak weather station, 5 km from the forest. The data includes (A) Annual mean rainfall amount, (B), rainfall frequency, (C) mean daily maximum temperature for September and October, and (D) minimum temperature for December and January.

Unfortunately, the majority of these recorded plant species from this region are isolated populations, at their range edge, endangered and facing habitat degradation, as the area is severely suffered from drought, deforestation, overgrazing, and irrational activities of the local community (Al-Eisawi, 1996).

2.2. Study species

The *Rosa* genus stands out as one of the most economically significant and favorite ornamental plants, with the genetic resources of its wild relatives being highly sought after for cultivation (Haouala et al., 2013). *Rosa pulverulenta* (syn. *R. glutinosa* Sm., Rosaceae), the Pine Fragrant Rose or Cretan rose, is a dwarf shrub, with a compact, bushy habit and stems densely covered with prickles (Tohmé and Tohmé, 2014). The flowering period extends from late June to late August and the fruits ripen in the period from September to October. This species produces an aggregate of achene (a pericarp containing a single seed) inside an expanded hypanthium which called later a hip fruit. It is native to the Mediterranean basin and extending to the Caucasus (Tohmé and Tohmé, 2014). This species represents a broader distribution across approximately 30 Mediterranean and European countries, according to the Global Biodiversity Information Facility (https://europlusmed.org/).

In 2010, *R. pulverulenta* was newly recorded in Jordan, specifically from the Jhayeer, Shaubak provenance (Al-Gharaibeh in Greuter and Raus, 2011). Before 2010, the southernmost known occurrence in the Eastern Mediterranean region was Lebanon and Syria (Greuter and Raus, 2011). Therefore, the population of *R. pulverulanta* in Jordan is exceedingly isolated at the edge of its natural distribution (Fig. 2), increasing the likelihood of local extinction. However, the presence of *R. pulverulenta* in these refugia suggests that its current distribution gaps in the Eastern Mediterranean region may be influenced by historical climatic changes and the existence of cooler microclimates within its broader range. This population was very small in size, only four mature individuals separated from each other by 1 m were found in the whole area and similar habitat. Doubtless, this species is native and was common in the area in the past, as old local women were used to collect the hips (fruits) for making decorative necklaces (Greuter and Raus, 2011). This *Rosa* species is recognized as an important shrub due to its ability to tolerate drought stress (Özçelik et al., 2012). Consequently, it serves as a valuable genetic resource and stock for the commercial cultivation of ornamental roses in semi-arid gardens and ecosystems. In the *Rosa* genus, the successful germination of seeds and subsequent survival of seedlings play a vital role in their natural regeneration in the wild (Stoian-Dod et al., 2023). Throughout various monitoring visits, no natural recruitment of seedlings was observed since the recorded year (Al-Gharaibeh unpubl. observations), ultimately leading to its extinction from its natural habitat in 2022. The limited number of collected seeds and the extinction of this population restricted our ability to try innovative approaches. However, this limitation underscores the importance of finding scientifically supported solutions for effective resurrection and conservation measures.

2.3. Seed collection

All mature hips from the four individuals were collected in October 2021. Despite the conditions of *R. pulverulanta* individuals being weak and unhealthy, heavy fruit production was observed during the collection trip. At the laboratory, seeds (achenes) were extracted from hips and cleaned from hip residuals. After extraction, seeds were soaked in water for 4 hours to remove empty and immature seeds. The sank down seeds were collected and dried in the lab (shadow and open-air) for 3 days and kept in one bag (bulk sample) at room temperature until the beginning of the treatments. Storing seeds at room temperature, although it may impact dormancy less

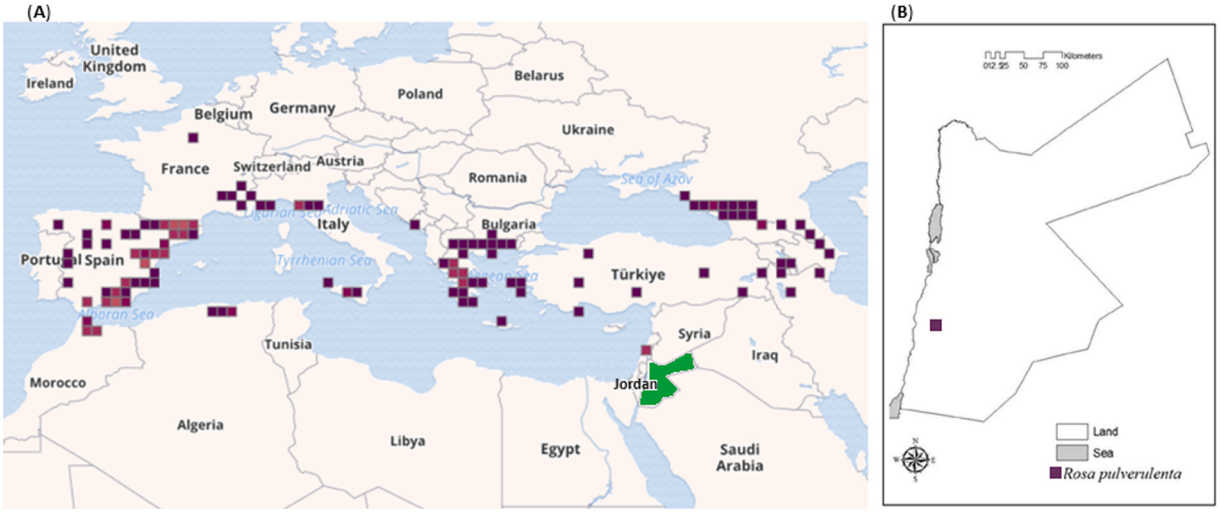

Fig. 2. Natural distribution maps of *Rosa pulverulanta* in the Mediterranean countries (A) and in Jordan (B). Source of maps (A): GBF (https://www.gbif.org/), (B) Jordan Plant Red List Volume II (Taifour, 2017).

than colder conditions such as a fridge, provides a closer approximation of natural habitat conditions, potentially leading to more precise dormancy assessments.

2.4. Initial seeds viability and dormancy (pre-treatments)

To evaluate seed viability after one year of collection, 50 seeds were soaked in acidic solution (0.1 ml of HCl 36 % diluted by 500 ml distilled water) for 24 hours to soften their endocarp. Soaked seeds were removed, washed and their endocarp were split in half by scalpel along the axis. Split embryos were removed from endocarps and then incubated in petri dishes with 0.01 TTC solution and covered by Aluminum foil for 24 hours at room temperature (ISTA, 1999). After 24 hours, the stained seeds were counted as viable, while seeds that did not stain red or were empty were considered non-viable. Seed viability was calculated using the following formula:

Seed viability percentage %= Number of seeds stained red/Number of total seeds (50) × 100%

To assess seed dormancy, 100 seeds were randomly chosen from the seed bulk and soaked in 1 % sodium hypochlorite for one minute to sterilize them, then rinsed by distilled water for 40 seconds. Seeds were distributed across four 90-mm petri dishes (each 25 seeds) and placed on filter paper moistened with 10 ml distilled water. Petri dishes with lids were incubated at two different temperature/light regimes (Biobase, model: BJPX-A1000CI) which we chose in an effort to mimic the germination conditions that the *Rosa* seeds experienced during the germination season: 1) early spring (day/night: 20 °C /15 °C, 10 h light/14 h dark) and 2) late spring (day/night: 25 °C /20 °C, 12 h light/12 h dark). Seed germination (i.e., when the radicle protruded to at least 2 mm length) was checked and recorded every two days. After 30 days, the germination tests were terminated as no seeds had germinated or exhibited signs of germination, such as endocarp rupturing, within this period. Following termination, another viability test was promptly conducted using the same procedure. Germinated seeds were counted as viable and non-dormant seeds and dormancy percentage was calculated according to the following formula:

Seed dormancy percentage %= Number of Non-germinated seeds stained red / (Number of total germinated seeds + Number of Non-germinated seeds stained red) × 100 %

2.5. Dormancy release treatments

Various physical-mechanical, chemical, and biological treatments were experimented to release dormancy and improve germination percentage across *Rosa* genus seeds (Table 1). Consequently, single or combined pre-germination treatments were tailored and applied to address both exogenous and endogenous dormancy in our study species. Therefore, as listed in Table 1, seeds were subjected

Table 1
Summary of the main pre-germination treatments used to stimulate *Rosa* seed germination.

Treatment	Dormancy	Description	Germination %	Species	Reference
Stratification	Exo./ Endogenous	Dry storage 68 w + cold stratification 16–24 w	72–79 %	*R. multibracteata*	Zhou et al. (2009a)
Stratification	Exo./ Endogenous	Warm stratification 11 w + cold stratification 20 w	18.8 % 13.8 % 13.5 %	*R. canina* *R. pulverelanta* *R. dumalis*	Alp et al. (2009)
Stratification	Exo./ Endogenous	Cold (5°C) for 10–12 weeks Warm (25°C) for 10–12 weeks	8–30 % 3.6–7.3 %	*R. rubiginosa*	Haouala et al. (2013)
Scarification	Exogenous	H2SO4 50 % (5 and 10 mn)	30 %	*R. rugosa*	Lee et al. (2010)
		H2SO4 50 % (1 h)	70.2 %	*R. soulieana*	Zhou et al. (2009a)
		H2SO4 50 % (2 h)	75.3 %		
		H2SO4 50 % (3 h)	55 %		
		H2SO4 50 % (30 s)	41 %	*Rosa* 'Gruss an Teplitz'	Younis et al. (2007)
		H2SO4 50 % (60 s)	42.7 %		
		H2SO4 50 % (90 s)	17 %		
		H2SO4 50 % (30, 45, and 60 mn)	0 %	*R. rubiginosa*	Haouala et al. (2013)
		H2SO4 (2, 4, 6 h)	0 %	*R. multibracteata*	Zhou et al. (2009a)
Microorganism	Exogenous	Inoculation natural microflora	3 %	*R. corymbifera*	Morpeth and Hall (2000)
		Inoculation in *Klebsiella oxytoca* C1036	50 %	*R. rugosa*	Lee et al. (2010)
GA3	Endogenous	200 ppm GA3 for 6–24 h in greenhouse	0 %	*R. canina*	Hoşafçi et al. (2005)
		400 ppm GA3 for 6–24 h in greenhouse	5.3–0 %		
		2000 ppm GA3 for 6–24 h in greenhouse	0.3–2.3 %		
		2000 ppm GA3 for 6 h in cold greenhouse	5.3 %	*R. canina*	Alp et al. (2010)
		1000 ppm GA3 for 6 h in cold greenhouse	4 %		
		500 ppm GA3 for 6 h in cold greenhouse	3.8 %		
Microorganism + Stratification	Exo./ Endogenous	Microbial fertilizers+ Warm stratification 4 w + cold stratification 150 d	84 %	*R. damascena*	Kazaz et al. (2010)
Stratification+ GA3	Exo./ Endogenous	4 °C + 200 ppm GA3 for 6–24 h in field	11–17 %	*R. canina*	Hoşafçi et al. (2005)
		4 °C + 400 ppm GA3 for 6–24 h in field	13–22 %		
		4 °C + 2000 ppm GA3 for 6–24 h in field	25–36 %		

to the following pre-germination treatments: (T1) warm-cold stratification, (T2) warm-cold stratification followed by soaking in gibberellic acid, (T3) warm-cold stratification followed by activated microbial fertilizer (EM-1), (T4 & T5) sulfuric acid followed by cold stratification, (T6) gibberellic acid and (T7) control. The details of pre-germination treatments are summarized in Table 2.

A total of 2100 mature intact seeds were selected randomly, and 12 replications (25 seeds in each replicate) were used for each of the seven treatments (T1-T7). For all treatments required stratification (T1-T5), seeds were mixed with moistened Peat moss and sand (stratification media) with a ratio of 1:1 (v\v) and then placed in zip-lock plastic bags. These bags were stored in a climate incubator at 25 °C for 4 weeks then transferred to refrigerator at 4 °C for 24 weeks for treatments required warm-cold stratification or directly in refrigerator for treatments required only cold stratification. However, the starting date of stratification, either warm-cold or cold only, was scheduled to ensure that the whole duration ends at the same time. Stratified seeds and media were checked, for germination and moisture, on a weekly basis for warm stratification period and twice a week for cold stratification. The total number of germinated seeds from each treatment during the stratification period was added to the total germination count recorded on the first day of the germination test (4 °C, 24 h dark). At the end of the stratification period, all seeds were washed by distilled water and subsequently seeds of T2, T6 and T3 were treated with gibberellic acid and microbial fertilizers, respectively, to synchronize the starting day of all germination tests.

2.6. Germination tests

Due to the limited number of available seeds, germination experiments were conducted under only three different temperature/light regimes. These regimes were selected to identify the optimal climatic conditions for achieving the highest germination percentage and to assess the effects of temperature stress on germination. Pre-germination treated seeds were placed on filter paper moistened with 10 ml distilled water in 90-mm petri dishes. Four replicates from each of the seven pre-germination treatments were germinated at following temperature/light regimes: (25/20 °C, 12 h light/12 h dark), (20/15 °C, 10 h light/14 h dark) and (4 °C, 24 h dark). Germination was checked every two days and germinated seeds were transferred into pots filled with moistened peat moss and raised in the greenhouse. After each germination check, the position of each petri dish inside the climate incubators and refrigerator was randomly changed. The tests were terminated after 60 days to ensure comprehensive documentation of germination rates and patterns, especially considering the intermittent germination behavior observed until day 50.

2.7. Terminal seeds viability and dormancy (post-treatments)

To evaluate seed viability and dormancy after treatments (pre-germination and germination tests), all ungerminated seeds were checked again for viability according to the following formula:

Seed viability percentage %= (Number of Non-germinated seeds stained red+ total germinated seeds)/Number of total seeds (100) × 100%

and dormancy percentages according to the following formula:

Seed dormancy percentage %= Number of Non-germinated seeds stained red / (Number of total germinated seeds + Number of Non-germinated seeds stained red) × 100%

2.8. Seedlings survival

All germinated seeds were transferred into 1 liter (13 ×11 cm) labeled pots filled with moistened peat moss, each one seed. To reveal if there is an effect of dormancy release treatments later on seedling establishment (survival), seedlings were raised in the greenhouse under identical conditions (pot size, growing media, and amount of irrigation and sunlight exposure) and mortality was documented per each pre-germination treatment. Survival percentages were calculated according to the following formula:

Survival %= number of survived seedlings/total number of emerged seedlings × 100%

Table 2
Summary of pre-germination treatments.

Treatment	Description
T1	Stored in climate chamber 25 °C (4 weeks) then transferred to refrigerator 4 °C (24 weeks)
T2	Warm-Cold stratification as in T1 followed by soaking in GA3 (200 ppm) 6 h
T3	Warm-Cold stratification as in T1 followed by soaking in microbial fertilizer EM−1 (25 min)
T4	Scarified by H_2SO_4 (50 %, 60 sec) followed by cold stratification at 4 °C (24 weeks)
T5	Scarified by H_2SO_4 (50 %, 75 sec) followed by cold stratification at 4 °C (24 weeks)
T6	Soaked in GA3 (200 ppm) for 6 h
T7	Control (no treatment)

2.9. Statistical analysis

All statistical analyses were performed with R version 4.2.3 (R Core Team, 2023). To analyze the germination data, we used mixed-effects Cox models with the R package coxme 2.2–16 (Therneau, 2012). The Cox models, i.e., time-to-event analyses, are a powerful method for analyzing germination data because they meet more statistical assumptions than non-linear regression models (McNair et al., 2012; Onofri et al., 2010; Romano and Stevanato, 2020). Treatment (pre-germination treatments) was applied as fixed factor, while the seeds within Petri dish was applied as random effect. Explanatory variables for our first model was the applied pre-germination treatments on the complete dataset. A second model were run on the subset of T1 treatment were the explanatory variable was the temperature/light condition. To visualize germination probability patterns, we used Kaplan-Meier curves (Kaplan and Meier, 1992) with the "survfit" function of the R-package "survival" ver. 3.5.3 (Therneau, 2022).

Regarding seedlings survival, we used a generalized linear model to test whether establishment of seedlings from initially sown seeds (binomial) depended on the treatment the seeds experienced before their germination assessment. A Tukey's post-hoc test was used for comparison between groups.

3. Results

3.1. Initial seeds viability and dormancy (pre-treatments)

The seed viability test revealed that not all sank down achenes (seed enveloped by endocarp) had fully mature seeds, which means that some of the tested achenes were heavy enough to sink down but contain immature or empty seeds. However, all fully matured seeds were observed to be viable, and the total seed viability was 76 % after one year of collection. Despite this high viability, no seeds germinated because dormancy in *R. pulverulanta* seeds was 100 % under both temperature/light regimes (Table 3).

Treatments: Early spring: germination conditions (temperature and light) that prevail during the early spring season (day/night: 20 /15 °C, 10 h light/14 h dark) and Late spring season (day/night: 25/20 °C, 12 h light/12 h dark). The asterisk indicates the total number of non-viable seeds including dead, immature and empty seeds.

3.2. Dormancy release treatments and germination tests

Twelve seeds germinated before the end of the cold stratification period, which lasted for 24 weeks. The first germinated seed was recorded after 21 days (third week) of cold stratification and was from the warm-cold treated seeds. Three days later, another germinated seed from warm-cold/ microbial fertilizer treatment was documented. Two days before the end of the cold stratification period 6, 3, and 1 seed were germinated from the treatments warm-cold, warm-cold/ GA_3, and warm- cold/ microbial fertilizer, respectively.

After transferring the seeds into the germination chambers, germination at 4 °C, 24 h dark was continued until day 50, while at higher temperature regimes (25/20 °C, 12 h light/12 h dark) and (20/15 °C, 10 h light/14 h dark) germination occurred only through the first six days and stopped completely afterwards (Fig. 3, Table 4). Under constant temperature 4 °C, 24 h dark, only seeds from the treatments T1, T2, T3, T4 germinated whereas seeds from other treatments T5, T6, T7 did not germinate at all. In contrast, germination was only observed in seeds from treatments T1, T2, and T3 at 25/20 °C, as well as in treatment T3 at 20/15 °C. However, germination percentages under fluctuating temperatures were markedly lower compared to constant temperature conditions and were nearly zero at 20/15 °C.

We then assessed the likelihood of germination with time-to-event analyses, which represent the probability of a germination event occurring on consecutive days across different treatments. Due to the negligible germination observed under the 20/15 °C and 25/20 °C temperature regimes, our statistical analysis focused solely on the data obtained from the 4 °C temperature regime. Under 4 °C, germination patterns varied significantly among pre-germination treatments (χ^2=22.04; $p<0.001$). In particular, the cumulative germination events visualized in Kaplan-Meier curves (Fig. 4) and estimated through cox-regression models (Table 5) were highest for seeds from T1 having a germination probability of 68 % at 4 °C. Seeds of T2 also showed a high germination probability (67 %). which was higher than that of the seeds from treatment T3 (49 %) and T4 (28 %) and observed only at cold/dark regimes (4 °C, 24 h dark). As such, only seeds from two treatments (T2 and T1) achieved 50 % germination, with T2 reaching this threshold on day 18 and T1 accomplishing it two days later.

For the best pre-germination treatment (T1), we also compared germination across the different temperature/light regimes (χ^2=14.25; $p<0.001$). The germination probability was significantly higher at 4 °C, 24 h dark regime than 25/20 °C while there was no

Table 3
Dormancy and viability of *R. pulverulanta* seeds after one year of collection.

T/L treatment	Rep.	No. of total Seeds	No. of non-germinated. Seeds	No. of non-germinated seeds stained red	No. of Nonviable seeds *	Dormancy %
Early spring	1	25	25	19	6	100 %
	2	25	25	21	4	100 %
Late spring	1	25	25	22	3	100 %
	2	25	25	19	6	100 %

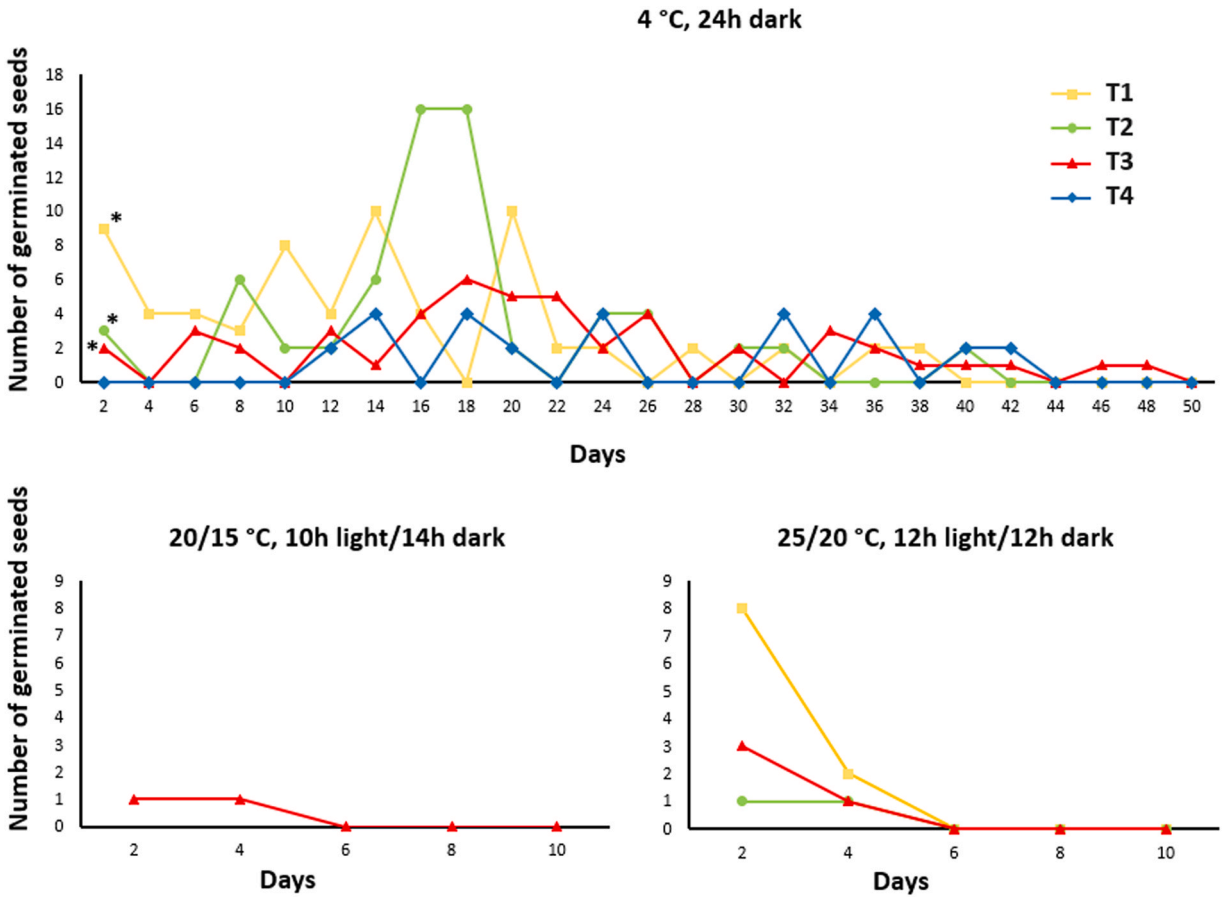

Fig. 3. Graphical representation of germination over time at three different germination temperature/light regimes. The asterisk indicates the total germinated seeds at the first check day including seeds germinated during the warm-cold stratification. Treatments with zero germination are excluded. Treatments: T1, warm-cold stratification; T2, warm-cold stratification +gibberellic acid; T3, warm-cold stratification +microbial fertilizer; T4, sulfuric acid 60 s+cold stratification. T5-T7 are not shown because there was zero germination.

germination at all at 20/15 °C (Fig. 4B, Table 4). The other pre-germination treatments behaved similarly, i.e., they showed the best germination under 4°C, if there was germination at all.

3.3. Terminal seeds viability and dormancy (post-treatments)

Terminal viability declined in seeds exposed to pre-germination treatments involving warm-cold stratification (i.e., T1, T2, and T3, see Table 4). However, the lowest terminal viability was detected in those seeds scarified by H_2SO_4 (T4 and T5). Conversely, seeds that did not undergo any type of stratification, specifically those treated with Gibberellic acid (T6) and the control group (T7), maintained terminal viability at an average level comparable to the initial viability.

Regarding terminal dormancy, the pre-germination treatments involving Warm-cold stratification (T1-T3) have noticeable effect on releasing dormancy (Table 4). This effect was more pronounced in seeds that germinated at temperature regime 4 °C, while it was low at higher germination temperatures. Additionally, terminal dormancy in seeds that were sacrificed by H_2SO_4 for 60 seconds (T4) also decreased, but only at the temperature regime of 4 °C. Meanwhile, dormancy percentage remained unchanged in seeds treated with H_2SO_4 for 90 seconds (T5), Gibberellic acid (T6), and control (T7).

3.4. Seedlings survival

The survival of the emerged seeds differed significantly among pre-germination treatments (χ^2=17.17; $p<0.001$; Fig. 5; Table 6). warm-cold stratification is more effective as a pre-germination treatment particularly concerning seedling survival. Warm-cold stratification, especially when followed by microbial fertilizer, appears to enhance seedlings survival, while other treatments may have limited success under certain conditions, especially when compared to chemical scarification.

Table 4
Terminal germination, seed viability and dormancy (viability of non-germinated seeds evaluated after the germination test) in *R. pulverulanta* seeds across seven pre-germination treatments and at three different temperature regimes.

	4 °C, 24 h dark						20/15 °C, 10 h light/14 h dark						25/20 °C, 12 h light/12 h dark					
	G	**NG**	**VNG**	**NV**	**V%**	**D %**	**G**	**NG**	**VNG**	**NV**	**V%**	**D %**	**G**	**NG**	**VNG**	**NV**	**V%**	**D %**
T1	68	32	4	28	**72**	**5.6**	0	100	58	42	**58**	**100**	10	90	36	54	**46**	**78.3**
T2	67	33	3	30	**70**	**4.3**	0	100	56	44	**56**	**100**	2	98	50	48	**52**	**96.2**
T3	49	51	7	44	**56**	**12.5**	2	98	57	41	**59**	**96.6**	4	96	49	47	**53**	**92.5**
T4	28	72	14	58	**42**	**33.3**	0	100	41	59	**41**	**100**	0	100	43	57	**43**	**100**
T5	0	100	32	68	**32**	**100**	0	100	30	70	**30**	**100**	0	100	31	69	**31**	**100**
T6	0	100	70	30	**70**	**100**	0	100	73	27	**73**	**100**	0	100	74	26	**74**	**100**
T7	0	100	72	28	**72**	**100**	0	100	80	20	**80**	**100**	0	100	78	22	**78**	**100**

Abbreviations: G: germinated seeds; NG: non-germinated seeds; VNG: viable (stained red) non-germinated seeds; NV: Non-viable seeds; V%: viability percentage; D%: Dormancy percentage. Treatments: T1, warm-cold stratification; T2, warm-cold stratification +gibberellic acid; T3, warm-cold stratification +microbial fertilizer; T4, sulfuric acid 60 s+cold stratification; T5, sulfuric acid 75 s+cold stratification; T6, gibberellic acid; T7, control. Germinated seeds are counted as viable and non-dormant seeds.

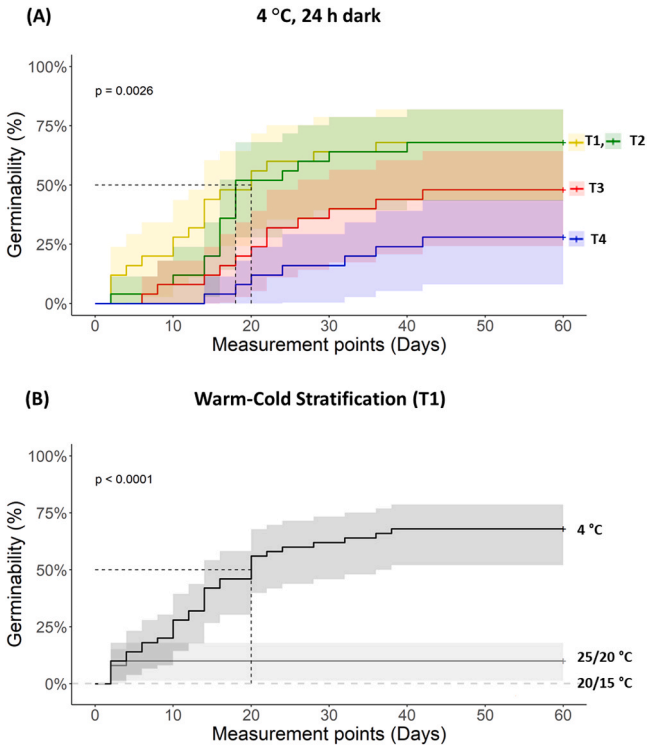

Fig. 4. Kaplan-Meier functions and their 95 % confidence intervals for the seven pre-germination treatments (T1-T7) at 4°C (A) and for the treatment T1 (showcasing the highest germination probability among the seven pre-germination treatments) across the three temperature regimes (B). Treatments: warm-cold stratification (T1), warm-cold stratification +gibberellic acid (T2), warm-cold stratification +microbial fertilizer (T3), sulfuric acid 60 s+cold stratification (T4). The dotted lines represent the day at which 50 % of the seeds germinated. The dashed lines represent the zero germination of T1 at temperature regime (20/15 °C). T5-T7 are not shown in (A) because there was zero germination.

Table 5
Summary table of the cox regression model. The z-value, of the Wald test, tests the hypothesis that $\beta i = 0$ against the alternative $\beta i \neq 0$. p-value represents the probability of obtaining a z-value larger in absolute value than the one obtained. Statistically significant p-values are displayed in bold. The exp (coefficient) represents the Hazard Ratio (HR). Treatment (T) was applied as fixed factor, while the individual seed ID was applied as random effect.

4 °C, 24 h dark					
Treatment	Coefficient	SE	z-value	p-value	HR (95 % CI)
T2	0.7730118	0.4210555	−0.61	0.54	0.7730118
T3	0.3775815	0.4491638	−2.17	**0.03**	0.3775815
T4	0.1739543	0.5106436	−3.43	**0.00061**	0.1739543

Treatments: warm-cold stratification (T1), warm-cold stratification +gibberellic acid (T2), warm-cold stratification +microbial fertilizer (T3), sulfuric acid 60 s+cold stratification (T4).

4. Discussion

4.1. Initial seeds viability and dormancy

While most of *R. pulverulenta* tested seeds are viable, dormancy prevents their germination. According to Stoian-Dod et al. (2023), most *Rosa* seeds cannot germinate in the first year without pre-germination treatments to release dormancy. The seeds of the study species exhibit combinational dormancy, both physical/mechanical and physiological dormancy, that prevent their germination as known for many *Rosa* species (Gao et al., 2022; Gudin et al., 1990; Haouala et al., 2013; Kazaz et al., 2010; Mowa and Maass, 2012; Pawłowski et al., 2020; Stoian-Dod et al., 2023; Younis et al., 2007). Moreover, if seeds overcome this combinational dormancy, a secondary dormancy can be induced when unfavorable conditions appear (Nadella et al., 2003; Pawłowski et al., 2020). Such secondary dormancy buffers against the adverse effects of temperature and humidity changes (Klupczyńska and Pawłowski, 2021), but it also poses a challenge for the natural regeneration of wild *Rosa* (Gao et al., 2021). However, during achene maturation, environmental factors, especially an increase in temperature, are likely to influence the embryo growth rate and contribute to an augmentation in

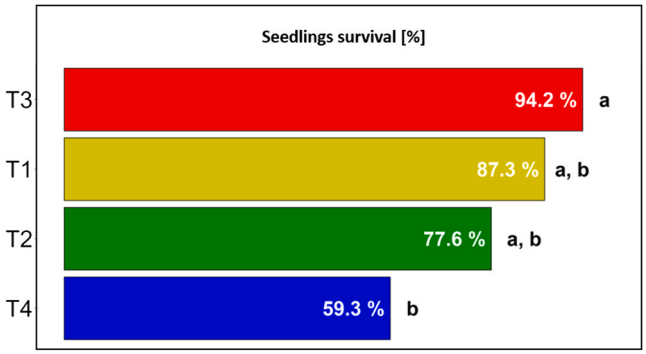

Fig. 5. Survival of seedlings that emerged from germinated seeds monitored in 1-liter (13 ×11 cm) pots filled with peatmoss. Posthoc letters correspond to generalized linear models testing for pairwise comparisons between the pre-germination treatments with respect to seedling survival (binomial). Treatments: warm-cold stratification (T1), warm-cold stratification +gibberellic acid (T2), warm-cold stratification +microbial fertilizer (T3), sulfuric acid 60 s+cold stratification (T4).

Table 6
Presents the survival percentages of transplanted *R. pulverulanta* seedlings resulted from each pre-germination treatment after germination tests at three different regimes.

	4 °C, 24 h dark				20/15 °C, 10 h light/14 h dark				25/20 °C, 12 h light/12 h dark			
	G	**E**	**S**	**S%**	**G**	**E**	**S**	**S%**	**G**	**E**	**S**	**S%**
T1	68	67	60	**89.6**	0	_	_	_	10	4	2	**50**
T2	67	65	50	**76.9**	0	_	_	_	2	2	2	**100**
T3	47	46	43	**93.5**	2	2	2	**100**	4	4	4	**100**
T4	28	27	16	**59.3**	0	_	_	_	0	_	_	_

Abbreviations: G: germinated seeds; E: emerged seeds S: survived seedlings; S%: survival percentage. Treatments: warm-cold stratification (T1), warm-cold stratification +gibberellic acid (T2), warm-cold stratification +microbial fertilizer (T3), sulfuric acid 60 s+cold stratification (T4).

endocarp thickness (Stoian-Dod et al., 2023). The latter is a critical in determining the degree of mechanical dormancy and, consequently, seed germination (Gudin et al., 1990).

The changes in temperature and precipitation patterns in Jordan over the past 44 years may have affected the degree of dormancy in *Rosa* seeds through maternal effects owing to the distinct climatic conditions experienced by the mother plants in their local environment (Huang et al., 2018; Stoian-Dod et al., 2023; VonAbrams and Hand, 1956). Such changes, either experienced by the mother plants during seed maturation or prevailed during germination periods, further complicate the already narrow germination requirements of *R. pulverulenta* (Al-Gharaibeh et al., 2017b; Baskin and Baskin, 2014). Together these factors may have contributed to declining population sizes and ultimately the local extinction of the remnant *R. pulverulenta* population in Jordan.

4.2. Dormancy release treatments and germination tests

The most common treatments to break combinational dormancy in *Rosa* seeds are stratification and scarification (Gao et al., 2022; Kazaz et al., 2010; Pawłowski et al., 2020; Stoian-Dod et al., 2023; Younis et al., 2007). Warm temperatures (25 °C), followed by several months of cold stratification (4 °C) was the most effective treatment to break dormancy in wild *Rose* seeds (Pawłowski et al., 2020; Stoian-Dod et al., 2023). The outcomes of our study validate and reinforce this observation, as the lowest seed dormancy was observed in seeds treated with warm-cold stratification (T1-T3). Cold temperatures after stratification appear to facilitate the completion of the dormancy-overcoming period, resulting in the highest germination percentage. This finding aligns with Alp et al. (2009), who observed a variation in the required cold period among various *Rosa* taxa. For natural regeneration, this cold period must persist for a defined duration in the field to reduce dormancy (Mattana et al., 2022; Nadella et al., 2003; Walck et al., 2011). Our findings suggest that the cold period should last longer than 24 weeks. However, the increasing daily minimum temperatures, inferred from a 44-year period of climatological data, suggest that seeds of *R. pulverulenta* may no longer undergo the necessary continuous cold stratification duration in their natural habitat during winter. Consequently, seeds remain dormant, leading to a lack of germination and recruitment in the field.

Chemical scarification using sulfuric acid (H_2SO_4), gibberellic acid, and microbial fertilizers are also methods employed to break dormancy in *Rosa* seeds, either independently or in combination with stratification (Kazaz et al., 2010; Mowa and Maass, 2012; Stoian-Dod et al., 2023). In our experiments, the application of gibberellic acid alone showed no influence in releasing dormancy. However, when used in combination with stratification, a significant release of dormancy was observed. Stratified seeds are more likely to rupture their hard pericarp enabling water and gas exchanges (Kazaz et al., 2010; Stoian-Dod et al., 2023). The use of chemical scarification (H_2SO_4) for short time (60 seconds) in combination with cold stratification also released dormancy. The corrosive effect of acid softens the seed pericarp, reducing physical restrictions for water absorption and gas diffusion (Lee et al., 2010; Younis et al.,

2007).

Together, these germination responses to different treatments indicate that seeds of our study species exhibit physiological and physical dormancy. All treatments, including warm-cold stratification (T1-T3), effectively released this combinational dormancy by rupturing endocarps and providing the required after-ripening conditions (Stoian-Dod et al., 2023), resulting in the highest germination rates. Conversely, the lower germination percentage in T4 shows that cold stratification alone is insufficient to release physiological dormancy, despite H_2SO_4 potentially softening the hard pericarp (Haouala et al., 2013). However, exposing the seeds to sulfuric acid (H_2SO_4) for a duration longer than 60 seconds significantly decreased seed viability and, consequently, their germination capacity. Prolonged exposure to this corrosive acid may have killed the embryos of some seeds as observed for other *Rosa* species (Younis et al., 2007). Although microbial fertilizers have been reported to significantly enhance seed germination in some *Rose* species (Belletti et al., 2003; Kazaz et al., 2010; Morpeth and Hall, 2000), our study did not find such effects. This discrepancy may be attributed to the duration of inoculation. Previous studies reported increased germination percentages when microbial inoculation was initiated at the beginning of stratification and lasted for at least 24 weeks. Therefore, further investigation may be needed to determine the effect of a longer duration of inoculation on *R. pulverulenta* seed germination.

Even with the application of specific dormancy-releasing treatments, germination percentages often remain below 50 % in roses (Gudin, 2017). However, in our results, more than 2/3 of the seeds from T1 and T2 germinated. Notably, this high germination probability was observed only at the germination regime of 4 °C with 24 hours of darkness, despite that temperatures range from 15 °C to 25 °C are considered optimal for seed germination in most *Rosa* species (Stoian-Dod et al., 2023). In the study of Alp et al. (2009), the overall germination of *R. pulverelanta* was 13.8 % at a temperature of 22 °C, which aligns with our results in T1 (25 °C). However, our results are concordant with Zhou et al. (2009b) who found that cold stratified *R. multibracteata* seeds germinated best at low temperatures (5 °C), irrespective of whether the temperatures remained constant or fluctuated. Exposing cold-stratified seeds to new conditions with temperatures exceeding 20 °C therefore appears to impede any release of dormancy as previously reported from *R. canina* seeds (Pawłowski et al., 2020).

The transition of seeds from a dormant to a nondormant state involves a continuous series of alterations throughout the entire seed structure, depending on the degree of dormancy (Nadella et al., 2003; Soltani et al., 2019). The degree of dormancy is highly affected by environmental conditions the mother experienced, which varies among species, populations, and individuals (Bewley and Nonogaki, 2017; Haouala et al., 2013). Indeed, some seeds initiate germination during the beginning of stratification period, while others required longer time to germinate at the same temperature. Such variability in germination behavior within the individuals and populations represents an environmental and ecological adaptation to ensuring population survival under complex environmental conditions (Mitchell et al., 2017).

4.3. Terminal seeds viability and dormancy (post-treatments)

Seed viability declined for those exposed to dormancy release treatments and subsequent germination tests over a duration of 8–9 months. In contrast, viability over the same duration remained unaffected for non-treated seeds (control and GA3). The variation in viability loss could be attributed to the duration during which seeds were exposed to moisture and temperature. Both temperature and moisture content have been shown to negatively impact seed viability over time (Demir et al., 2009; Redden and Partington, 2019; Roberts and Abdalla, 1968). Even more strongly, seed viability declined in seeds treated with H_2SO_4 which is in accordance with previous studies (Fallah Imani et al., 2014; Mowa and Maass, 2012; Younis et al., 2007).

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4.4. Seedlings survival

Compared to chemical scarification, warm-cold stratification appears to be the most effective pre-germination treatment, particularly in promoting seedling survival. The corrosive effect of H_2SO_4 potentially exposes embryos, food reserves, and developing radicles to injuries (Dawar et al., 2024; Yeom et al., 2021), often resulting in the development of abnormal seedlings (Aliero, 2004; Amusa, 2011; Khalofah, 2022). The positive effect of microbial fertilizer inoculation after warm-cold stratification on seedling survival suggests a potential synergistic relationship that promotes seedling growth (Valle Expósito et al., 2022).

5. Conclusion

R. pulverulenta seeds exhibit combinational dormancy and their germination is affected by various environmental conditions. Unmet germination requirements in this context have likely restricted the population regeneration under natural conditions in Jordan. Climate change may further complicate dormancy releasing requirements by altering habitat conditions, thereby potentially negatively impacting regeneration of *R. pulverulenta* population. Dormancy-releasing treatments are essential for maximizing germination success in ex-situ conservation such as resurrection efforts. Understanding and categorizing dormancy types are critical for selecting effective treatments, prioritizing those optimizing germination and seedling establishment. These stages are vital for successful conservation efforts as they lay the groundwork for subsequent phases like reintroduction and reproduction in the wild.

Warm-cold stratification emerged as a key treatment for *R. pulverulenta*, highlighting its potential significance for other *Rosa* species and ecotypes. The optimal duration of this treatment may vary among different species, necessitating tailored approaches. Given that the resurrection of recently extinct populations or conservation in other *Rosa* species may face similar seed dormancy challenges, we advocate for further research into the effects of the maternal environment on dormancy types within this genus. These research efforts

could provide crucial insights into optimizing treatment combinations and durations, thereby enhancing the successful re-establishment of extinct or endangered *Rosa* populations. Moreover, this framework can be applied to other plant species facing similar seed dormancy and germination challenges, offering a valuable tool for global conservation practitioners working to prevent biodiversity loss.

CRediT authorship contribution statement

Mohammad M. Al-Gharaibeh: Conceptualization, Investigation, Methodology, Writing - Review & Editing, Visualization, Project administration, Funding acquisition. **Halim Adil Bakhit**: Investigation, Resources, Data Curation. **Shifaa Masadeh**: Investigation, Resources, Data Curation. **Dávid Nagy**: Formal analysis, Data Curation. **Christoph Rosche**: Formal analysis, Writing - Review & Editing, Visualization.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

Data will be made available on request.

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Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used [ChatGPT] in order to improve readability of a few sentences. After using this tool, we reviewed and edited the content as needed and we take(s) full responsibility for the content of the publication.

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