OPTIMISING *JUNIPERUS EXCELSA* (CUPRESSACEAE) GERMINATION FOR SUSTAINABLE ECOSYSTEM RESTORATION IN THE PRESPA AREA (WESTERN MACEDONIA, GREECE)

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In the Mediterranean Region, species of the genus *Juniperus* face conservation challenges due to human activities and climate change threats. *Juniperus* species are reported to present low regeneration rate, while their seeds often exhibit physical and physiological dormancy. Viability and germination of mature *Juniperus excelsa* seeds from population originating from the Prespa area (Florina, Greece), alongside with seedling survival were investigated to study the reproduction dynamic of species population known to represent a EU priority habitat type of limited distribution and with low regeneration rate. The seed cut test of an uncleaned seed lot revealed a high percentage of empty seeds (90.03%) and a low embryo viability (2%). Cleaned seeds were subjected to a total of 44 pretreatments, categorised into eight groups targeting physical and physiological dormancy, along with combinations of these techniques. Despite most treatments showing limited success in enhancing germination, three pretreatments exhibited promising results (germination between 22.22% and 39.40%). Subsequent germination tests involving these treatments in controlled chambers, as well as in field conditions, along with hydration-dehydration cycles, were conducted using newly collected seeds. Seed germination rates in controlled chambers remained low, suggesting a deep primary dormancy. Pretreated seeds, under controlled field conditions, passed two winter periods fully saturated, with 13 hydrating-dehydrating cycles in-between. Germination was maximised during the second year of the field experiment, reaching almost 100% after mechanical scarification for 6 sec., followed by immersion in 3% H_2O_2 for 2 h. However, seedling survival was low, reaching approximately 38.26%. The findings emphasise the challenges in germinating *Juniperus excelsa* seeds and highlight the importance of the optimised protocols for reforestation in order to conserve this habitat type.

Key words: climate resilience, Greek juniper restoration, seedling production, seedling survival, embryo viability, hydration-dehydration

Introduction

Juniperus species have a high ecological, cultural, and economic importance in the Mediterranean region. However, they are facing conservation threats due to habitat loss, over-logging, forest fires and other human activities (Ciesla, 2002; Carus, 2004; Giannakis et al., 2010). Moreover, their conservation is further challenged by projected climate change scenarios threats (Fatemi et al., 2018). This is recorded for several Mediterranean *Juniperus* species, such as *Juniperus thurifera* L., *J. communis* L., *J. excelsa* M.Bieb., and *J. foetidissima* Wild. (Vrahnakis et al., 2011; Broome et al., 2017).

Germinating seeds of *Juniperus* species is a crucial and challenging aspect for ecosystem restoration. Successful germination and subsequent growth of seedlings, which can then be reintroduced into their natural degraded habitats, contribute to their restoration. Many *Juniperus* species have seeds with a low viability (Wesche et al., 2005) or specific germination requirements (Broome, 2003). Understanding and addressing these requirements are critical to develop effective methods for breaking dormancy and successful germination.

In general, *Juniperus* species, including *Juniperus excelsa*, present a high proportion of empty seeds (Juan et al., 2006; Bonner, 2008; Gruwez et al., 2014; Pinna et al., 2014). This is probably due either to sub-optimal climatic conditions (Hedhly et al., 2009; Verheyen et al., 2009), which affect pollination, or due to inbreeding depression amongst the fragmented populations (García & Zamora, 2003; Castilleja-Sánchez et al., 2016). Moreover, seed viability of the genus *Juniperus* is generally low (García & Zamora, 2003) and it is intricately linked to ecological factors, and habitat suitability, and thus, it is minimised in the outer distribution limits or its geographical boundaries, beyond which the species is less likely to thrive due to environmental constraints (García et al., 2000).

Seeds of *Juniperus* species are under a strong combination of physical and physiological dormancy caused by both hard testa and embryo immaturity, which requires some time to develop (after-ripening) before germination (Baskin & Baskin,

2001; Bonner, 2008). The outer layer of the seed coat results in physical dormancy, while chemical components in the embryo cause physiological dormancy, both of which prevent seed germination (Tilki, 2007). Usually, seeds with such a complex type of dormancy require long periods of stratification in order to germinate (Baskin & Baskin, 2001; Feurtado & Kermode, 2011).

However, germination can be highly improved through pretreatments that can release seed dormancy. Seed coat-imposed dormancy is a type of dormancy, in which the seed coat restricts water uptake and prevents the embryo from germinating. Several pretreatments are proposed to overcome this, such as mechanical or chemical scarification and hot water (Piotto et al., 2003). Sulfuric acid and hydrochloric acid are the most commonly used acids for chemical scarification (Hartmann et al., 2010), while hydrogen peroxide is also referred. Both sulfuric and hydrochloric acids can act as scarifiers by softening hard seed coats, promoting water absorption and enhancing germination (Baskin & Baskin, 2001). Hydrogen peroxide (H_2O_2) also plays a role in scarification, but its action is more complex. Although it can contribute to scarification of the seed coat tissues, its multifaceted role extends beyond scarification. When dissolved, it releases oxygen promoting cellular respiration and providing the energy required for germination. It can also act as a signaling molecule, potentially triggering the activity of enzymes involved in breaking down inhibitors present in the seed or initiating biochemical changes necessary for germination (Fontaine et al., 1994; Liu et al., 2010; Wojtyla et al., 2016; Gammoudi et al., 2021). Hot water treatment of seeds with hard or impermeable seed coats is also a method used in scarification to break seed dormancy and enhance germination (Hartmann et al., 2010).

On the other hand, overcoming physiological dormancy typically involves specific treatments to trigger the necessary physiological changes for germination such as cold-moist stratification or two periods of cold stratification separated by warm stratification or hormone application (Grzesik & Joustra, 1990; Broome, 2003; Daneshvar et al., 2016). Plant hormones can be used to promote or enhance seed germination. Gibberellic acid is often used to overcome dormancy in seeds with hard coats. It stimulates the synthesis of enzymes that break down stored nutrients in the seed, facilitating the mobilisation of reserves and promoting the growth of the embryo (Gupta & Chakrabarty, 2013).

Hydration-dehydration cycles play also a crucial role in breaking seed dormancy in many plant species (Hegarty, 1978). These cycles mimic natural environmental conditions, particularly the fluctuation in soil moisture levels that seeds experience in their native habitats. The process of hydration and dehydration involves the uptake and loss of water by seeds, triggering physiological changes that lead to the breaking of dormancy and the initiation of germination. Natural environmental conditions, such as seasonal changes, rain, and dry periods, often contribute to these hydration-dehydration cycles. The seeds may go through several cycles before dormancy is fully broken (Bai et al., 2015).

The Lake Prespa basin is one of the few areas in the Balkan Peninsula with well-preserved forests of *Juniperus excelsa* that form the *9562 «Grecian juniper woods (*Juniperetum excelsae*)» (GJWs), an EU-priority habitat type of limited distribution. Vrahnakis et al. (2011), in the frame of LIFE12 NAT/GR/539 project, documented the near absence of natural regeneration in the Lake Prespa *Juniperus excelsa* forests.

The current research targeted in clarifying the key factors influencing the seed biology of *Juniperus excelsa*. The outcomes of this investigation have significant implications for the ecological restoration efforts of its population within its natural habitat. The aim of the paper was to investigate the reproduction dynamics of a Greek *J. exselsa* population, known to represent a priority habitat type where natural regeneration is found to be rather low. Furthermore, the results of the study can be useful for national forest services to follow the protocols developed in the study and to produce seedlings for targeted plantings.

Material and Methods

Study area

The Greek juniper woods cover an area of 21.921 km^2 in Greece, situated at an altitude ranging from 900–1300 m a.s.l., on various exposures and inclines. The forest features a loose structure with an open crown canopy predominantly composed of *Juniperus excelsa* (Vrahnakis et al., 2011). Occasionally, these woods form poorly developed shrublands. The trees reach a maximum height of 20 m and a diameter at breast height (DBH) of 1.5–2.5 m in mature specimens. The primary branches spread out or ascend and become crooked in older trees, while the higher-order branches in younger trees are generally more ascending. The crown of young trees is broad-pyramidal, becoming broader as the trees mature.

Berry-shaped cones were collected from a natural population of *Juniperus excelsa* located in the area of Prespa, Florina, Greece (21.0191° N, 40.8254° E). These cones were harvested in early September from mature trees, approximately 10 m in height, from 50 trial plots (10 × 10 m, one tree was selected in each plot) scattered throughout the juniper forests to represent the total population. The trees were grown in the same altitudinal zone. The trial plots were spaced approximately 325 m apart to cover most of the study population. A total of 50 parental trees were selected as the seed source, and seeds were collected (Ivetić et al., 2016). Seed germination was monitored for two years, from 2016 to 2018.

Experimental procedures

The collected cones were slightly cut with a scalpel and placed for two days in tap water changed twice a day. Afterwards, the fleshy part was removed and seeds were collected. A batch of seeds from all trees (equal number of 100 seeds per tree from 50 trees in total) were mixed and formed a composite sample, which was shaken many times in a bucket in order to distribute the seeds evenly and randomly inside the sample. The initial seed moisture content (26.8%) was calculated according to ISTA (1999) specifications. A seed cut test was further performed according to ISTA (1999) in order to assess the internal characteristics of seeds quality. The test involved cutting seeds open and making observations to distinguish various categories based on their internal features. Three distinct seed categories were distinguished, namely empty, filled with resin, and seeds with fully developed embryo.

A viability test was also performed using 1% (w/v) of 2,3,5-Triphenyl-tetrazolium chloride (hereinafter – TTC). Characterisation of embryos as viable (hereinafter – stained) and non-viable (hereinafter – unstained) was performed by taking into account ISTA (1999) specifications (Fig. 1).

The rest of the initial seed lot (individually per tree) was subjected to a cleaning procedure through air blowing followed by imbibition in tap water for the empty seeds to remove. Then, an equal number of 150 cleaned seeds per tree were chosen and the new composite sample was re-shaken inside the bucket again many times for creating a uniform seed distribution.

A total of 44 pretreatments (including a control), were employed to overcome seed dormancy, categorised into eight distinct groups. The purpose of these pretreatment groups was to elucidate the type of seed dormancy and explore methods to enhance germination rates. All the seeds used in germination experiments have been surface sterilised for 2 min., with 1% (v/v) sodium hypochlorite and thoroughly washed.

These pretreatments included mechanical and chemical scarification, to overcome physical dormancy, with diverse acids and durations, and varying stratification periods, as well as stratification, hormone applications and hydrating-dehydrating cycles to address physiological dormancy. Additionally, combinations of these techniques were employed to target the combination of both dormancy types. In addition to the aforementioned pretreatments, the study was aimed to explore the impact of high temperature and smoke as cues for germination related to forest fire. These factors were examined both individually and in various combinations (Table 1).

Following this, a standard germination test was conducted in a germination chamber (SANYO Inc., Japan), with alternating temperatures of +25°C/+15°C for 8 h in light and 16 h in darkness. The outcomes of the pretreatments were largely disappointing, with less than 5% germination observed over an 8-month period. However, three pretreatments showed promise, exhibiting a 20–30% germination percentage: i) Mechanical scarification for 6 sec., followed by a 2 h. immersion in 3% hydrogen peroxide (H_2O_2) (abbreviated as MSH); ii) Immersion in 10% hydrogen peroxide for 20 min., followed by immersion in water for 48 h. (abbreviated as HW); iii) Immersion in 10% hydrogen peroxide for 20 min., followed by immersion in 400 ppm gibberellic acid (GA_3) (abbreviated as HGA) for 48 h.

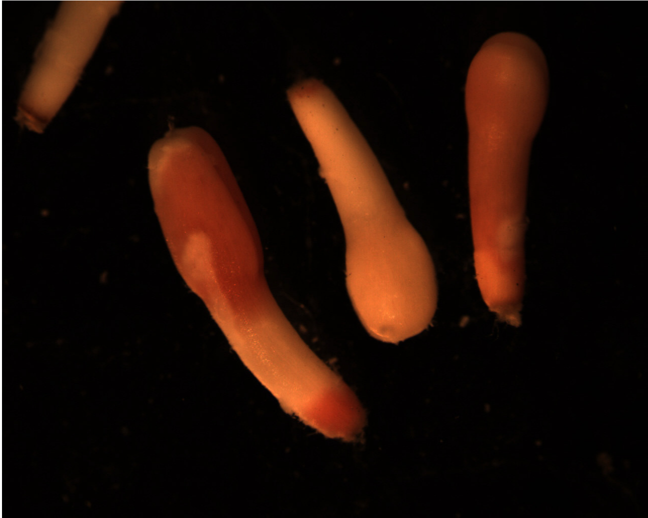

Fig. 1. *Juniperus excelsa* embryos after staining with 1% (w/v) 2,3,5-Triphenyl-tetrazolium chloride.

Table 1. Groups of treatments in *Juniperus excelsa* seeds before they were placed for germination

№	Group of pretreatments	Description
1	Mechanical scarification	Seed mechanical scarification for 4 sec. or 6 sec. using mechanical scarifier.
2	Chemical scarification	Immersion of seeds in various chemical solvents separately (not in combination) as follows: Sulfuric acid (H_2SO_4) (98%) for 30 min. and 45 min. respectively; Hydrochloric acid (HCl) (37%) for 20 min., 40 min., and 60 min., respectively; Hydrogen peroxide (H_2O_2) in two distinct concentrations 3% or 5% (v/v) for 2 h.
3	Stratification	Seeds were subjected to either warm (two months duration) or cold (three months duration) stratification as well as to combined cold-warm and cold-warm-cold stratification for the same intervals as above.
4	Hormone treatments	Imbibition for 48 h. in 400 ppm of gibberellic acid (GA_3) under dark.
5	Combination of each of the treatments №1, №2, and №3 with treatment №4	Combination of scarification treatment and imbibition in GA_3 treatment as above.
6	Hydrating-dehydrating cycles	Seeds immersion in tap water for four days, then left to dry for six days. The seed were subjected to a total number of four cycles of hydrating-dehydrating at room temperature and then placed in the germination chamber.
7	Combination of each of the treatments №1, №2, and №3 with treatment №6	Combination of scarification or stratification treatment with hydrating-dehydrating cycle treatment as above.
8	Simulated forest fire	Exposure to 200°C temperature, 2.5 h in a box full of smoke, 48 h. in smoke water and combination of the aforementioned.

A second germination test was conducted aiming to explore the potential for enhancing seed germination. This involved implementing the three aforementioned treatments followed by exposure to fluctuating temperature field conditions and undergoing repeated cycles of seed dehydration and rehydration. The selection of these particular combinations of dormancy-breaking treatments was predicated on the hypothesis that employing the aforementioned methods, along with subjecting seeds to hydration-dehydration cycles, would effectively address both physical and physiological dormancy. This approach aimed at creating conditions favourable for germination to occur.

A new completely randomised block experimental design was applied that begun the following October and lasted 18 months in total. A new seed lot was collected with the same procedure as the previous year. Seeds were extracted from berry-shaped cones and subjected to a cleaning procedure. In each of the three selected pretreatments, four repetitions of 100 seeds were used. Control treatment was also included. Pretreated and control seeds were fully imbibed in tap water for four days to reach a maximum saturation and placed under field conditions. Seed moisture content was monitored once a week according to ISTA (1999) specifications. The pretreatments HGA, MSH, and HW underwent a re-evaluation in controlled chambers. This re-testing was aimed to compare germination outcomes independent of the experimental design involving hydration-dehydration cycles and the field conditions. Untreated seeds were additionally exposed to hydration-dehydration cycles (control).

During the first winter period (November – February) moisture was maintained above 90% by covering seeds with moist woolen sacks in order to simulate winter moist chilling. In order to prevent any possibility for mold to appear, the woolen sacks were changed every day. In addition, the germination substrate was sprayed once every ten days with a Captan solution of low concentration (0.5% w/v). From March to mid-October, as temperatures began to rise and air moisture decreased, the treated seeds underwent a series of 13 alternating cycles. Each cycle involved hydration for four days followed by dehydration, during which the seeds were left to dry for 14 days, reaching a moisture content of approximately 10%. These cycles were conducted under field conditions inside insect rearing cages (24 × 24 cm with a 1350-μm mesh) and were sheltered to protect from rain. However, in mid-April, mid-May and mid-June, germination tests were conducted to assess the release of dormancy. Throughout the subsequent winter period from mid-October to March, the same procedure was replicated. Monitoring of the germination process occurred on a weekly basis. Germination was finally detected in June. The germinated seeds were counted, recorded by pretreatment and put in trays with sand. In December, when no germination was observed, all the non-germinated seeds were manually cut and separated as belonging to one of the following categories: empty, filled with resin, and seeds with fully developed embryo. When an embryo was found, it was excised and subjected to a viability staining test to evaluate treatment effectiveness. Seed viability was calculated using the following formula:

$$TI = IG + SE,$$

where TI – total viability, IG – initial germination (%), SE – stained embryos (%).

Seed germination percentage was corrected according to an embryo viability staining test as follows:

$$CG = \frac{NGS}{NGS + SE} \times 100\%,$$

where CG – corrected germination (%), NGS – number of germinated seeds, SE – stained embryos.

Statistical analysis

Seed cut test results were expressed on percent basis of the total seed sample used. Germination was calculated on a percent basis as mentioned above. Viability results were also calculated on a percent basis of the embryos stained by the TTC test. Since normality of data could not be achieved any differences among treatments were assessed with Kruskal-Wallis analysis of variance using STATISTICA v. 10 software (StatSoft GmbH, Germany). Additionally, due to non-normal distribution of data, except for the results of the initial seed cut test, their dispersion around the mean was estimated by calculating Interquartile Range. For the results regarding the initial seed cut test the coefficient of variation was used as a measure of data distribution around the mean values of seed categories.

Results

The seed cut test revealed a remarkably high percentage of empty seeds, with 90.03% and 90.01% recorded for both collection years (Table 2). Additionally, nearly 5% of seeds lacked embryos but were instead filled with resin. Consequently, only a minimal fraction of seeds contained embryos (4.72% and 5.10%, respectively). Subsequent embryo viability testing indicated that only 2% of the embryos were viable, as evidenced by TTC.

The seed germination rates in the chambers, following the pretreatments HGA, MSH, and HW, were relatively low in both collection years, ranging from approximately 22% to around 40%, whereas control exhibited minimal germination, with recorded germination below 4% (see Table 3). The results of the TTC tests of the non-germinated seeds for chamber experiments, at the end of the experiment in terms of mean values and variability, are shown in Table 3 and Table 4.

As far as the field experiment is concerned, a low seed germination (15% in MSH and HGA and 9% in HW) was recorded in March, i.e. eight months after the beginning of the experiment, while no germination was recorded in the control (Fig. 2). No germination was recorded in the following autumn in any treatment (Fig. 2). Seed germination was finally recorded after a total of 18 months and when corrected according to seed cut test reached almost 100% (Table 5).

Table 2. Various categories of *Juniperus excelsa* seeds in percent basis of the two collection years (%) from the initial seed cut test (format: mean value ± standard error)

Seed category	1st collection year		2nd collection year	
	Mean value	Coefficient of variation	Mean value	Coefficient of variation
Empty	90.03 ± 1.80	4.00	90.01 ± 0.88	1.96
Seeds containing embryo (seeds containing viable embryo, TTC test)	4.72 ± 0.57 (1.72 ± 0.19)	24.30 (23.11)	5.10 ± 0.07 (1.75 ± 0.10)	3.01 (11.71)
Seeds with resin	5.25 ± 0.38	14.63	4.89 ± 0.62	25.40
Total	100.00	–	100.00	–

Table 3. Germination and cut-test results of non-germinated *Juniperus excelsa* seeds at the end of the experiment inside chambers (cleaned seed lot) (mean values ± standard error)

Treatment	Initial germination, %*	Stained embryos, %	Unstained embryos, %	Resinous seeds, %	Total, %	Total viability, %**	Corrected germination, %
1st collection year							
	(1)	(2)	(3)	(4)	(5)	(1)+(2)	(1)/(1)+(2)
HGA	4.07 ± 0.44[ab]	14.65 ± 1.38[ab]	32.84 ± 1.90[a]	48.45 ± 1.89[a]	100.00	18.72 ± 0.98	22.27 ± 3.43
MSH	6.97 ± 0.24[a]	13.72 ± 0.45[ab]	30.92 ± 1.68[a]	48.38 ± 1.58[a]	100.00	20.70 ± 0.43	33.73 ± 1.26
HW	7.39 ± 0.70[a]	11.36 ± 0.86[a]	30.83 ± 2.19[a]	50.42 ± 2.35[a]	100.00	18.75 ± 0.26	39.52 ± 4.11
Control	0.74 ± 0.46[b]	17.37 ± 0.27[b]	32.02 ± 1.91[a]	49.88 ± 1.63[a]	100.00	18.11 ± 0.54	3.89 ± 2.42
2nd collection year							
Treatment	Initial germination, %*	Stained embryos, %	Unstained embryos, %	Resinous seeds, %	Total, %	Total viability, %**	Corrected germination, %
	(1)	(2)	(3)	(4)	(5)	(1)+(2)	(1)/(1)+(2)
HGA	5.19 ± 0.86[ab]	13.30 ± 1.15[ab]	31.52 ± 1.19[a]	49.99 ± 1.45[a]	100.00	18.49 ± 0.36	28.29 ± 4.98
MSH	6.10 ± 0.43[ab]	13.85 ± 0.48[ab]	30.04 ± 1.32[a]	50.01 ± 1.15[a]	100.00	19.95 ± 0.40	30.55 ± 2.01
HW	6.95 ± 0.83[b]	11.40 ± 0.83[b]	33.52 ± 1.27[a]	48.12 ± 1.36[a]	100.00	18.36 ± 0.92	37.81 ± 3.85
Control	0.49 ± 0.51[a]	17.37 ± 0.32[a]	35.01 ± 1.73[a]	47.13 ± 1.56[a]	100.00	17.86 ± 0.33	2.63 ± 2.91

Note: * – values were calculated in total of seeds with embryo (stained or unstained) as well as those with resinous filling; ** – values were calculated by summing up the percentages of germinated seeds plus non-germinated ones but with viable embryo. Designations of treatments: HGA (immersion in 10% of hydrogen peroxide for 20 min., followed by immersion in 400 ppm of gibberellic acid (GA_3), MSH (mechanical scarification for 6 sec., followed by 2 h. immersion in 3% of hydrogen peroxide (H_2O_2), HW (immersion in 10% of hydrogen peroxide for 20 min., followed by immersion in water for 48 h.); values within columns followed by the same letter do not significantly differ at 0.05 p-level of significance for each parameter separately.

Table 4. Interquartile range for means of germination, viability and resinous *Juniperus excelsa* seeds at the end of the experiment inside chambers (cleaned seed lot)

Treatment	Initial germination, %	Stained embryos, %	Unstained embryos, %	Resinous seeds, %	Total viability, %	Corrected germination, %
1st collection year						
	(1)	(2)	(3)	(4)	(1)+(2)	(1)/(1)+(2)
HGA	1.38	4.52	5.11	5.97	3.14	11.19
MSH	0.86	1.39	4.82	5.10	1.39	3.68
HW	1.71	2.52	6.61	7.41	0.80	10.95
Control	1.47	0.90	5.64	4.24	1.39	7.77
2nd collection year						
Treatment	Initial germination, %	Stained embryos, %	Unstained embryos, %	Resinous seeds, %	Total viability, %	Corrected germination, %
	(1)	(2)	(3)	(4)	(1)+(2)	(1)/(1)+(2)
HGA	2.70	3.23	3.58	4.71	1.12	15.26
MSH	0.87	1.53	4.41	3.75	1.22	5.25
HW	2.60	2.45	3.13	3.77	2.73	8.40
Control	0.98	1.07	5.98	5.25	0.90	5.26

Note: Designations of treatments: HGA (immersion in 10% of hydrogen peroxide for 20 min., followed by immersion in 400 ppm of gibberellic acid (GA_3), MSH (mechanical scarification for 6 sec., followed by 2 h. immersion in 3% of hydrogen peroxide (H_2O_2), HW (immersion in 10% of hydrogen peroxide for 20 min., followed by immersion in water for 48 h.).

Table 5. Germination and cut-test results of non-germinated *Juniperus excelsa* seeds at the end of the outdoor experiment (cleaned seed lot)

Treatment	Initial germination, %*	Stained embryos, %	Unstained embryos, %	Resinous seeds, %	Total, %	Total viability, %**	Corrected germination, %
HGA	20.01 ± 0.91^{a}	1.71 ± 0.46^{ab}	31.16 ± 0.54^{ab}	47.11 ± 0.87^{ab}	100.00	21.72 ± 1.21^{ab}	92.75 ± 1.71^{a}
MSH	10.46 ± 0.52^{ab}	0.00^{a}	32.35 ± 1.28^{ab}	57.19 ± 0.78^{a}	100.00	10.46 ± 0.52^{a}	100.00^{a}
HW	19.56 ± 1.08^{a}	15.58 ± 1.36^{ab}	34.96 ± 0.41^{a}	29.90 ± 1.25^{b}	100.00	35.13 ± 2.23^{b}	55.21 ± 1.61^{b}
Control	4.56 ± 1.47^{b}	27.42 ± 0.93^{b}	23.44 ± 0.58^{b}	44.58 ± 0.63^{ab}	100.00	31.99 ± 0.95^{b}	16.28 ± 4.22^{c}

Note: * – values were calculated in total of seeds with embryo (stained or unstained) as well as those with resinous filling; ** – values were calculated by summing up the percentages of germinated seeds plus non-germinated ones but with viable embryo. Designations of treatments: HGA (immersion in 10% of hydrogen peroxide for 20 min., followed by immersion in 400 ppm of gibberellic acid (GA_3), MSH (mechanical scarification for 6 sec., followed by 2 h. immersion in 3% of hydrogen peroxide (H_2O_2), HW (immersion in 10% of hydrogen peroxide for 20 min., followed by immersion in water for 48 h.); values within columns followed by the same letter do not significantly differ at 0.05 p-level of significance for each parameter separately.

In general, a profound number of stained (viable) embryos was recorded in the non-germinated treated seeds, kept in the chamber, while the relevant percentage in the non-germinated treated seeds under field conditions was very low (Table 3, Table 5). Regarding the variability of germination and viability data of the outdoor experiment the control treatment had generally a larger variability than the other treatments (Table 6).

A notable number of seedlings dried in 1–5 months' time after germination. The reasons for that were various and not always directly explicable as the seedlings were fully protected. In the first stage of cotyledon unfolding, it was observed that many seedlings (up to 20%) did not physically discard the seed coat. These seedlings did not grow and dried very quickly. Another considerable number of seedlings dried either before expanding the first needles or soon after the appearance of the first needles. Also, a little later, it was observed that a number of seedlings did not take height and formed a rather «spherical» crown and they gradually dried (Fig. 3). The recorded percentage of seedling establishment was approximately 38.26%, taking into account the percentage of germinated seeds that unfold cotyledons.

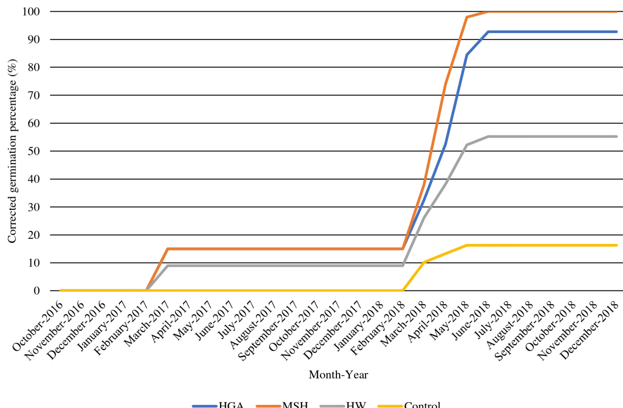

Fig. 2. Germination course of *Juniperus excelsa* seeds in the outdoor experiment. Designations: MSH – mechanical scarification for 6 sec., followed by a 2 h. immersion in 3% hydrogen peroxide (H_2O_2), HW – immersion in 10% hydrogen peroxide for 20 min., followed by immersion in water for 48 h., HGA – immersion in 10% hydrogen peroxide for 20 min., followed by immersion in 400 ppm gibberellic acid (GA_3) for 48 h.

Table 6. Interquartile range for means of germination and viability of *Juniperus excelsa* seeds in the outdoor experiment (cleaned seed lot)

Treatment	Initial germination, %	Stained embryos, %	Unstained embryos, %	Resinous seeds, %	Total viability, %	Corrected germination, %
HGA	2.99	1.35	1.30	2.90	3.64	5.55
MSH	1.25	0.00	4.40	2.60	1.25	0.00
HW	3.10	4.15	1.10	4.00	6.25	4.10
Control	4.90	2.95	1.60	1.85	2.05	14.36

Note: Designations of treatments: HGA (immersion in 10% of hydrogen peroxide for 20 min., followed by immersion in 400 ppm of gibberellic acid (GA_3), MSH (mechanical scarification for 6 sec., followed by 2 h. immersion in 3% of hydrogen peroxide (H_2O_2), HW (immersion in 10% of hydrogen peroxide for 20 min., followed by immersion in water for 48 h.).

Fig. 3. A newly emerged seedling of *Juniperus excelsa* with spherical crown that did not evolved into normal seedling.

Discussion

Juniperus species generally exhibit low regeneration rates and a high ratio of empty seeds (García et al., 2002; Wesche et al., 2005). In consistence with these findings, our research revealed a significant percentage of seed lacking embryos or gametophytic residues during the seed cut test, indicating a low fertilisation success. The literature data suggest that the main causes of this issue may be self-incompatibility, incomplete pollen development, or a high homozygosity ratio (Williams, 2009; Gruwez et al., 2013). For instance, Castilleja-Sánchez et al. (2016) documented similar challenges in endangered species, *Pinus rzedowskii* Madrigal & M.Caball., in Mexico, where high inbreeding levels were linked to low germination and seedling establishment issues, such as undeveloped primary needles and deformed stems.

In the Prespa area, there is strong evidence of inbreeding depression. Karapatzak et al. (2019) observed poor fertilisation linked to pollen viability and germination issues in Prespa's *Juniperus excelsa*. Additionally, our preliminary data indicate that embryo viability declines over time; immature seeds (green, 1-year-old) showed nearly double the embryo viability compared to mature seeds. The reason for this decline remains unclear. Future research on the duration and microphenology of the reproductive cycle (i.e. from the initiation of macrostrobil primordia to the complete morphological differentiation of the embryo) in *Juniperus excelsa* within the study area might help identify the stages of embryonic development where degeneration occurs. Supplementary genetic research is also essential for obtaining more robust results.

Apart from the high percentage of empty seeds, a profound number of seeds were filled with resin, drastically increasing the proportion of non-viable seeds. Resinous seeds are associated with resin canals, common in the Cupressaceae family, which are used for damage repair (Lin et al., 2002). Increased resin production might also be a response to insect infestation, as plants often activate defense mechanisms, such as resin production, to deter further insect feeding and protect against infection (Bracalini et al., 2013). In coniferous trees, insect infestations can lead to an increased resin production, protecting the seeds and cones from damage and potentially resulting in a higher ratio of resinous seeds. However, the high ratio of resinous seeds observed in this study requires further investigation.

The sum of empty and resinous seeds accounted for almost 95% of the total seeds, supporting *in situ* findings by Vrahnakis et al. (2011), reported an almost total absence of regeneration in the Prespa area. Seed germination under controlled conditions was generally low despite various pretreatments applied during the first collection year, such as mechanical or chemical testa rupture, hormone imbibition, and various combinations of these. This suggests the presence of deep primary dormancy in the seeds (Lee et al., 1995; Tilki, 2007; Khalofah, 2022). However, three treatments involving hydrogen peroxide (H_2O_2) combined with either mechanical scarification or gibberellic acid (GA_3) or water imbibition showed more effective germination results. Hydrogen peroxide can have both an abrasive action in medium or high concentrations and can stimulate germination in low con-

centrations (Rosner et al., 2003; Statwick, 2016). The increased germination observed with HGA and HW treatments indicates that seeds with hard testa are likely being ruptured by H_2O_2, suggesting physical dormancy (Baskin & Baskin, 2001). Similarly, mechanical scarification increased the germination percentage, further indicating physical dormancy.

Germination of HGA pretreated seeds was notably higher than those pretreated with HW, both under controlled conditions and in the field. The application of exogenous GA_3 appears to stimulate germination more effectively, suggesting the presence of physiological dormancy in the seeds (Baskin & Baskin, 2001). Mechanical scarification followed by low-concentration (3%) H_2O_2 imbibition also showed an increased germination, indicating a combination of physical and physiological dormancy.

Despite the deep primary dormancy of *Juniperus excelsa* seeds, a small percentage (approximately 15–30%) appeared to have shallower dormancy and germinated relatively easily, possibly due to a bet-hedging strategy (van Klinken et al., 2008). This strategy helps populations facing strong environmental pressures by distributing germination across years, ensuring population persistence even under suboptimal conditions.

Regarding the experimental conditions, the treatments did not significantly increase the total germination beyond the mentioned percentages. Field seed stratification for one winter period was insufficient to maximise germination, while a second winter stratification period significantly increased final germination percentages. This confirms the strong dormancy status of *Juniperus excelsa* seeds, though it is unclear whether the increased germination was due to the second stratification period alone or its combination with hydration-dehydration cycles. Nonetheless, it appears that a combination of two winter stratification periods with intermediate summer hydration-dehydration cycles effectively breaks seed dormancy.

The production of a high ratio of empty seeds combined with low ratios of seeds with viable embryos, as well as the low survival rates of produced seedlings, strongly suggest the need for further management actions to preserve this priority habitat. An extensive reforestation approach, utilising the seed dormancy break protocol described here, could be beneficial. This approach would not only enhance germination success but also improve the overall regeneration potential of *Juniperus excelsa* in the Prespa area. Additionally, understanding the ecological and genetic factors contributing to seed viability and dormancy will be crucial for developing effective conservation strategies. Therefore, continued research and adaptive management practices are essential to ensure the long-term survival and regeneration of this species and its habitat.

Conclusions

The study on *Juniperus excelsa* from the Prespa area (Florina, Greece) highlights significant challenges in the species' regeneration, with particularly low natural seed germination and survival rates, largely due to low embryo viability. A high percentage of seeds were found to be empty, and only 2% of embryos were viable, underscoring the low reproductive success of *Juniperus excelsa*. Despite applying a wide range of pre-treatments to overcome physical and physiological dormancy, only three treatments showed moderate improvement in germination rates. Further tests involving hydration-dehydration cycles and field experiments revealed that germination improved significantly (up to nearly 100%) after mechanical scarification and hydrogen peroxide treatment. While the optimised pretreatment protocols significantly improved germination, the low seedling survival emphasises the need for more effective strategies in reforestation and conservation efforts. This research provides valuable insights for preserving the limited-distribution habitat of *Juniperus excelsa*, but further studies are necessary to enhance seedling survival and ensure sustainable regeneration of the species.

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ОПТИМИЗАЦИЯ ПРОРАЩИВАНИЯ *JUNIPERUS EXCELSA* (CUPRESSACEAE) ДЛЯ УСТОЙЧИВОГО ВОССТАНОВЛЕНИЯ ЭКОСИСТЕМ В РЕГИОНЕ ПРЕСПА (ЗАПАДНАЯ МАКЕДОНИЯ, ГРЕЦИЯ)

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В Средиземноморском регионе виды рода *Juniperus* сталкиваются с проблемами их сохранения из-за деятельности человека и угроз изменения климата. Известно, что виды рода *Juniperus* демонстрируют низкую скорость регенерации, в то время как их семена часто проявляют физический и физиологический покой. Жизненность и всхожесть зрелых семян *Juniperus excelsa* из популяции в регионе Преспа (Флорина, Греция), а также выживаемость сеянцев были изучены с целью изучения динамики размножения популяции этого вида, который формирует приоритетный в ЕС тип местообитания с ограниченным распространением и низкой скоростью регенерации. При исследовании среза неочищенной партии семян была выявлена высокая доля пустых семян (90.03%) и низкая жизненность зародышей (2%). Очищенные семена были подвергнуты в общей сложности 44 предварительным обработкам, которые были разделены на восемь групп, нацеленных на физический и физиологический покой, а также комбинации этих методов. Несмотря на то, что большинство обработок показали ограниченный успех в повышении всхожести, три предварительные обработки показали многообещающие результаты (всхожесть от 22.22% до 39.40%). Последующий анализ прорастаний, включающий эти обработки в контролируемых камерах, а также в полевых условиях вместе с циклами гидратации-дегидратации были проведены с использованием недавно собранных семян. Скорость прорастания семян в контролируемых камерах оставалась низкой, что предполагает глубокий первичный покой. Предварительно обработанные семена в контролируемых полевых условиях прошли два зимних периода полностью насыщенными, с 13 циклами увлажнения-дегидратации между ними. Всхожесть была максимальной во второй год полевого эксперимента, достигнув почти 100% после механической скарификации в течение 6 с. с последующим погружением в 3% раствор H_2O_2 на 2 ч. Однако выживаемость саженцев была низкой и достигала в среднем 38.26%. Полученные результаты показывают трудности проращивания семян *Juniperus excelsa* и подчеркивают важность оптимизированных протоколов лесовосстановления для сохранения этого типа экосистем.

Ключевые слова: восстановление греческого можжевельника, выживаемость саженцев, гидратация-дегидратация, жизнеспособность зародыша, производство саженцев, устойчивость к климату