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The effect of pollen performance on low seed fertility in a Greek population of *Juniperus excelsa*

Eleftherios K. Karapatzak[1], Georgios Varsamis[1], Irene Koutseri[2], Ioannis Takos[1], Theodora Merou[1]*

[1]Department of Forestry and Natural Environment Management, Eastern Macedonia and Thrace Institute of Technology, Kavala. Mikrochori-Drama, Greece
[2]Society for the Protection of Prespa National Park, Agios Germanos, Prespa, Greece
**Corresponding author: ekarapatzak@gmail.com*

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Abstract: The *Juniperus excelsa* (Cupressaceae) forest in northern Greece has been facing a documented severe decline of natural regeneration over the last few years. In order to dissect the physiological components of the low regeneration, pollen originated from different sites as well as from trees of different height was investigated in terms of viability and *in vitro* germination capacity. In addition, embryo development and viability of the first year immature seeds were further assessed. Pollen was found to present high viability (61.5–93.2%) in all sites and tree height classes. However, pollen germination was quite low (maximum germination percentage recorded was 30.2%). The low pollen germination capacity recorded in trees above 6 m in height was reflected in the subsequent low percentages of successful embryo development and viability found in the produced seeds. These results suggest that low seed fertility in *Juniperus excelsa* is associated with reduced pollen germination, suggesting a complex regeneration mechanism and is considered an additional step in shedding light on the physiological basis of the low natural regeneration of *Juniperus excelsa*.

Keywords: embryo development; embryo viability; pollen germination; regeneration; seed viability

Greek juniper (*Juniperus excelsa* M. Bieb.) occurs in the hills and mountains of the eastern Mediterranean basin, the Black Sea and a range of mountains arching around the south-west end of the Caspian Sea, while it becomes increasingly rare eastward (Strid, Tan 1997). It is usually met in regions with annual precipitation above 500 mm. The species is resistant to summer drought and heat, mainly occurring on stony, rocky calcareous slopes (Vrahnakis et al. 2011).

The Prespa Lake basin is one of the few areas in the Balkan Peninsula with a well-preserved forest of Greek juniper that forms the priority habitat type 9560 (Endemic forests with *Juniperus spp*.), an EU priority habitat type of limited distribution. Greek juniper woods (GJW) of the Prespa Lake are dominated by *J. excelsa* trees of wide canopy (Vrahnakis et al. 2011) constituting a relatively isolated population of significant size. After careful screening of the Prespa juniper forest, a significantly small number of individuals was found to be less than 1 meter in height, which represent the 0–30 years old age group (Vrahnakis et al. 2011). This suggests low regeneration over the last 20–30 years. Moreover, within the framework of LIFE12 NAT/GR/539 – JunEx research project, seed embryo viability was found to be extremely low. In particular 5.31% embryo viability with 82.92% of empty seeds was recorded in the first year immature seeds and 1.7% embryo viability with 90.03% of empty seeds

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was recorded in the second year mature seeds. On the other hand, Stampoulidis et al. (2013) found that the regeneration of *J. excelsa* in the Prespa National Park does take place either in facilitation or in open gaps under full light but the process of grazing through trampling and animal tread is the deterministic factor for successful regeneration. The low regeneration outcome, however, is also reported for other juniper species like European populations of *Juniperus communis,* which also showed a decline in natural regeneration (Garcia et al. 1999; Gruwez et al. 2013). In particular, Mediterranean populations of *J. communis* across mountain regions of southern Spain were dominated by mature individuals while seedlings and juveniles were absent, suggesting a gap in regeneration during the last 30–50 years. This low reproductive potential was attributed to many reasons such as poor fertilization and/or low seed viability and subsequent germination or high seedling mortality as a possible result of the current climate change (García 2001; Verheyen et al. 2009; Gruwez et al. 2013; Pinna et al. 2014; Gruwez et al. 2016). In addition, an increased amount of unfilled seeds, as a result of poor fertilization, was also reported enforcing the low regeneration capacity (Garcia et al. 2000). Poor fertilization that was connected with the processes of pollination and pollen tube growth was also suggested as a contributing factor to low seed viability in other gymnosperms like *Abies amabilis* (Owens, Molder 1977).

The lack of abundant natural regeneration in GJWs of the Prespa Lake is causing concerns about their future conservation, emphasizing the need for better understanding of the population reproduction dynamics of this highly resilient species, not only in the Prespa area, but also across the entire distribution range of this species. Successful seed production, in terms of seed quantity and embryo viability, is the prerequisite for natural regeneration. So it is considered vital to understand and record the mechanisms of pollination, pollen germination and tube growth that lead to seed formation.

The present study evaluates pollen performance along with seed setting and embryo viability of the first year immature seeds from *J. excelsa* individuals covering the entire range of its distribution across the Prespa basin. The outcomes of the study are expected to help elucidate the physiological basis of the observed low regeneration of *Juniperus excelsa* and provide important information on the strategic regeneration protocol design for this species.

MATERIAL AND METHODS

Sites, tree selection process, pollen and seed collection

Pollen collection sites of different altitude and exposition were selected within the *Juniperus excelsa* forest of the Prespa National Park (Table 1). The selected sites represent nearly the entire distribution range of the studied population (Fig. 1). In order to further determine the microclimatic differences between the locations, the course of 19 bioclimatic parameters of temperature and precipitation over the latest years extracted by the DIVA GIS system (www.worldclim.org) were used. Details of the particular parameters are given in Table 2. A cluster analysis of the summarized data on the bioclimatic parameters is shown in Fig. 2, which outlines the overall climatic difference between the locations where pollen and seeds were collected for the study. The three groups of height that were selected in the study are abbreviated herein as A, B and C corresponding to the heights of 1–2 m, 2–6 m and ≥ 6 m, respectively (Table 1). The difference in height suggests a difference in age. A height up to 2 m most probably represents young trees (20–50 years old), 2–6 m could represent middle-aged trees (50–100 years old) and > 6 m most probably represents mature trees (more than 100 years old).

Table 1. Geographical and tree height class group details of the five pollen collection sites chosen within the Prespa National Park

Site code	Name	Coordinates (GPS)	Altitude (m)	Orientation	Tree height class group
L1	Vronteron	40.74157 21.02361	1145	180° (S)	B, C
L2	Oxia	40.73732 21.05116	888	160° (SSE)	C
L3	Agios Georgios	40.80893 21.06278	1114	235° (SW)	A, B, C
L4	Psarades	40.82407 21.02028	926	250° (WSW)	A, B, C
L5	Pyli	40.78629 21.05188	870	140° (SE)	C

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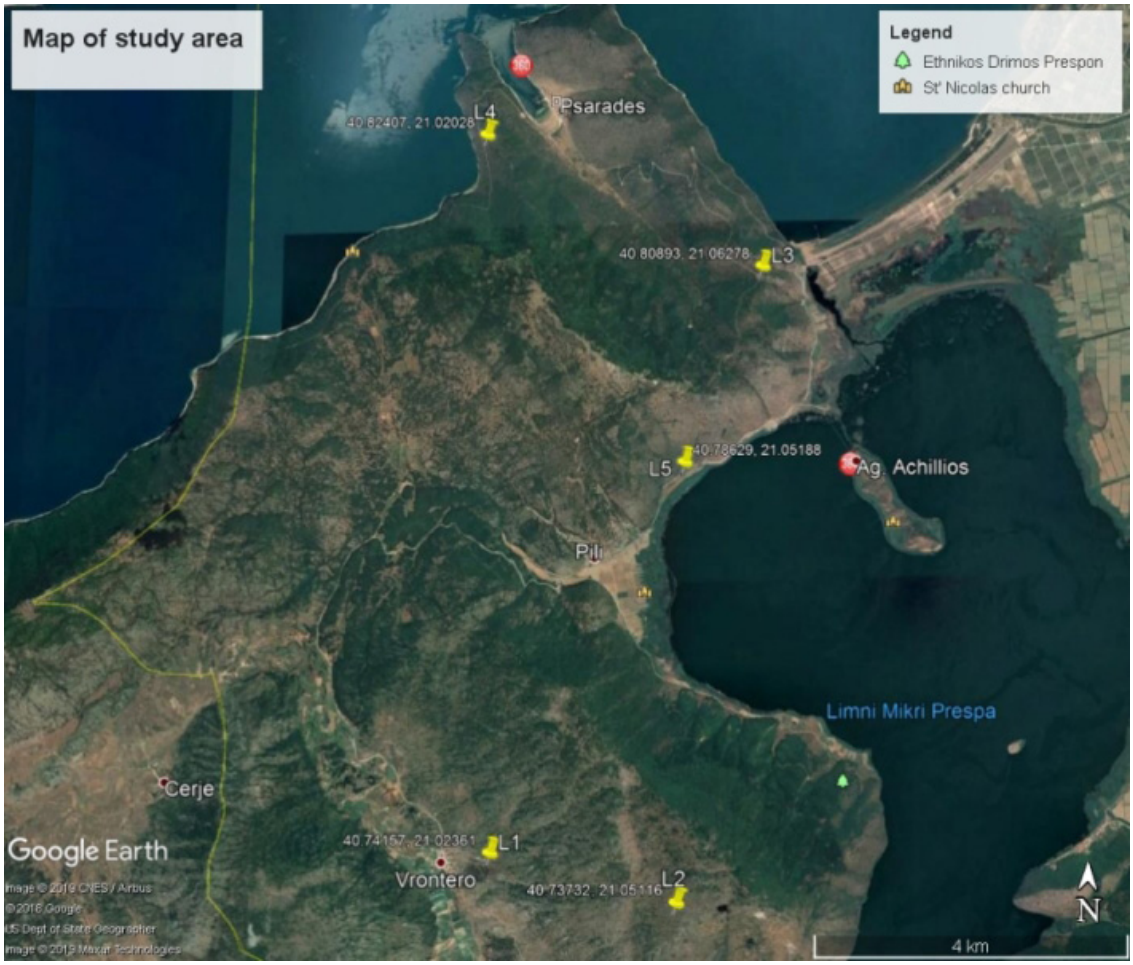

Fig. 1. A satellite image of the study area which includes pinpoints (in yellow) of the five sites where pollen and consequent seeds were collected (source: Google Earth software, Google Corp.)

Pollen was collected early in the spring of 2016 when male cones started to dehisce naturally on the trees. A central point was selected in each location and pollen was collected from 8–10 trees within a radius of 100 m from that point at different directions. Pollen was mixed into a composite sample from each location and height group in accordance with established pollen sampling methods (Shivanna, Rangaswa-

Table 2. Details of the bioclimatic parameters of temperature and precipitation used in order to microclimatically separate the five locations used in the study

No.	Parameter	No.	Parameter
1	Mean annual temperature	11	Mean temp of coldest quarter
2	Mean monthly temperature range	12	Sum of annual precipitation
3	Isothermality	13	Precipitation of wettest month
4	Temperature seasonality	14	Precipitation of driest month
5	Max temp of warmest month	15	Precipitation seasonality
6	Min temp of coldest month	16	Precipitation of wettest quarter
7	Annual temperature range	17	Precipitation of driest quarter
8	Mean temp of warmest month	18	Precipitation of warmest quarter
9	Mean temp of driest quarter	19	Precipitation of coldest quarter
10	Mean temp of warmest quarter		

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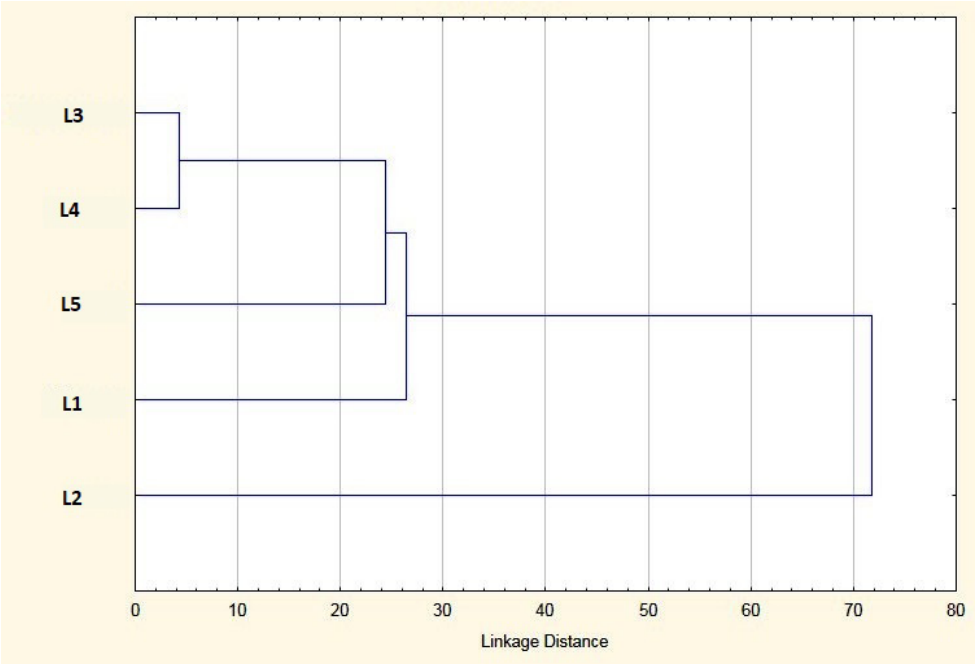

Fig. 2. Cluster analysis showing the microclimatic difference between the five locations of the study based on the Euclidian distances (k-Means Cluster analysis) of the summarized data from 19 bioclimatic parameters for each location from 1971 until today using DIVA-GIS. The particular bioclimatic parameters used are given in Table 2

my 1992). Using this method, pollen sample homogeneity can be increased whereas response variability is minimized. However, in three out of the five sites, no pollen was collected from height class A trees due to either lack of male cones or their immaturity. The same problem was encountered in height class B trees in two out of the five sites, leaving trees of height class C the only group whose pollen was collected from all studied sites. Table 1 provides an overall view of the combinations of site and height groups within which pollen was collected. Pollen was collected from trees not growing under shaded conditions and from twigs of the same age (by counting the number of internodes from the base of the stem) at the middle and upper nodes in height class A and middle and lower nodes in height classes B and C. In addition, all trees chosen for the study were visually examined for infections (no infected branches and <10% leaf loss in the entire tree) and signs of pests (Table 3). Immediately after collection, pollen samples were placed into a thermal cabinet and, on the same day, they were transferred to the laboratory where they were stored at 2°C before any assessment.

Immature seeds (seeds of green colour produced in the first year after pollination) were collected about 10 months later (late 2016) from female trees in close proximity to the 'marked' male trees from which pollen had been collected in all five sites. Seeds were collected only in the height class C trees, since it was the only height group that pro-

Table 3. Chosen prerequisites for the selection of male pollen donor trees as well as nearby female trees from which seeds were collected for each of the three height classes across the five sites of the study within the Prespa National Park

Height class	Shade conditions	Selected twigs sprouted	Diseased	Pest infestation	Whole tree health status
A	no shade	from the 15^{th}–30^{th} internode	no visible signs of infection	no visible signs of infestation	< 10% leaf loss
B	no shade	from the 1^{st}–10^{th} internode	no visible signs of infection	no visible signs of infestation	< 10% leaf loss
C	no shade	from the 1^{st}–10^{th} internode	no visible signs of infection	no visible signs of infestation	< 10% leaf loss

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vided pollen for collection in all sites. At least 500 seeds from 10–15 trees were collected from each location, stored at 2°C and evaluated consecutively.

Pollen viability and germination measurements

The acetocarmine staining solution (1% in 45% glacial acetic acid) was used for the viability assessment. This stain has been extensively used in pollen viability studies with a high level of accuracy (Porch, Jahn 2001; Karapatzak et al. 2012; Gaaliche et al. 2013). Several hydration times were tested, namely 1, 2, 4, 6, 8, 10 and 12 hrs of hydration. The thick exine followed by the thinner intine of pollen grains was found to be successfully ruptured when pollen was hydrated for at least 4 hrs, which was the chosen hydration time used in the study (Southworth 1988; Danti et al. 2011). Pollen was mixed with the stain (added after hydration) and was assessed for viability after a period of approx. 1 hour at ambient room temperature. The numbers of stained against non-stained pollen grains were recorded for each slide/culture as a mean value from 10 random microscopic fields and the percentage of viable pollen grains was calculated.

Pollen germination capacity was assessed *in vitro* using the sitting drop culture method' (Shivanna, Rangaswamy 1992). The Brewbaker and Kwack (1963) pollen germination medium was used with an extra of 2% sucrose. The modified medium consisted of 120 g·l^{-1} sucrose, 100 mg·l^{-1} boric acid, 300 mg·l^{-1} calcium nitrate, 200 mg·l^{-1} magnesium sulphate and 100 mg·l^{-1} potassium nitrate. The cultures were hydrated for a period of 4 hours before the medium was added and subsequently incubated for a period of 12 days at a temperature of 24 ± 2°C, in agreement with Nepi et al. (2005). Percentage of germination (pollen tube growth) was evaluated immediately after incubation, using the same measurement method as for viability. When the developing pollen tube was equal or longer to the diameter of the grain, it was considered germinated (Nepi et al. 2005; Williams 2009). A high-powered microscope (Nikon Optiphot microscope, Japan) was used throughout at a 100× magnification level.

Seed cut and embryo viability test

Seeds were dissected and embryo development was macroscopically observed under a high-powered stereoscope (Leica MZ6, Germany). Four classes of seeds were scored: empty seeds (no visible signs of embryo development), seeds filled with a resinous like substance, seeds filled with an undifferentiated mass of starchy cells and seeds containing a fully developed embryo. The production of a fully developed embryo was interpreted as the successful growth of the pollen tube within the ovule and successful fertilization of the female part (Williams 2009). Subsequently, the fully developed embryos were extracted and tested for viability applying the tetrazolium chloride test (TTC) at a 1% concentration, prepared according to ISTA (1999) specifications.

Experimental design and statistical analysis

Pollen viability and pollen germination assessment

For each combination of site and height class group a composite pollen sample was collected as described above. From each composite pollen sample 10 viability tests and 10 germination cultures were randomly performed which represented the replicates in a completely randomized design. Height class and site were the experimental factors with pollen viability and germination being the dependent variables. An average viability and germination percentage was deduced for each combination which was arcsine transformed. The transformation was conducted in order to meet the assumptions for running an analysis of variance (ANOVA) because large mean differences between treatment groups were observed and all the subsequent analyses were run on the transformed form of the percentage data.

Separate analyses of variance at a significance level of 0.05 were conducted as appropriate in order to assess the effects of the studied factors on the variables measured and dissect the individual mean differences since the height class factor did not have all three levels across all sites. In particular, an ANOVA was conducted between the three height classes for the two sites that had all three levels of height (L3 and L4) and a separate ANOVA was conducted between height classes B and C for site L1. Similarly, an ANOVA was conducted between the three sites for height class B and a separate ANOVA was conducted between all five sites

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Table 4. Average pollen viability (%) and pollen germination (%) for 10 combinations of site and height class from which pollen was obtained

Site code	Height class A	Height class B	Height class C	ANOVA (*P*) between height classes
Pollen viability				
L3	89.1 (1.109 ± 0.029) ^{abc}	86.3 (1.051 ± 0.032) ^{bc}	92.6 (1.191 ± 0.024) ^{ab}	0.066
L4	89.7 (1.116 ± 0.015) ^{abc}	89.6 (1.120 ± 0.029) ^{abc}	89.1 (1.105 ± 0.022) ^{abc}	
L1		86.8 (1.058 ± 0.028) ^{bc}	93.2 (1.209 ± 0.025) ^{a}	0.001
L2			61.5 (0.664 ± 0.022) ^{d}	
L5			84.7 (1.022 ± 0.035) ^{c}	
ANOVA (*P*) between sites		0.220	0.000	
Pollen germination				
L3	00.0 (0.000 ± 0.000) ^{A}	00.0 (0.000 ± 0.000) ^{A}	22.90 (0.232 ± 0.014) ^{B}	0.000
L4	3.28 (0.032 ± 0.006) ^{A}	2.06 (0.020 ± 0.003) ^{A}	10.80 (0.108 ± 0.010) ^{C}	
L1		20.1 (0.202 ± 0.008) ^{B}	30.18 (0.306 ± 0.015) ^{D}	0.000
L2			0.70 (0.007 ± 0.002) ^{A}	
L5			10.37(0.103 ± 0.007) ^{C}	
ANOVA (*P*) between sites		0.000	0.000	

Numbers in brackets are the arcsine transformed mean values ± SEMs (n = 10) (P < 0.05). Mean values that share the same letter are not significantly different (P < 0.05). Mean comparisons were made using Tukey's (HSD) test (P < 0.05) for pollen viability and Dunnett's T3 test for pollen germination (P < 0.05). E.g. In the height class C column in Pollen Viability 92.6 is not significantly different from 89.1 or from 93.2, but it is significantly different from 61.8, which is in itself different from all other values as well as from 84.7, which is also different from all other values of that column apart from 89.1. Similarly, for the first two rows (L3 and L4 sites) all values are not significantly different from each other, which is also reflected in the relevant *P*-value of 0.066.

for height class C (Table 4). For pollen viability, comparisons of means were made at a significance level of 0.05 using Tukey's HSD as a post-hoc test in ANOVA which assumes equal variances. For pollen germination, on the other hand, Dunnett's T3 test was used for treatment mean comparison at a significance level of 0.05 since the transformed pollen germination data ware not found homogenous according to Levene's test and the sample sizes were relatively small (n = 10). The statistical software used for pollen viability and germination was SPSS v19 (IBM Corp.). Graphs were produced using the Microsoft Excel (2007) graph facility.

In addition, pollen germination data for height class C from which pollen was assessed across all sites were tested against the binomial distribution fit (Eq. 1):

$$\text{Expected probability (r successes out of n trials)} = \left[\frac{n!}{r!(n-r)!}\right] p^r (1-p)^{n-r} \quad (1)$$

where:

p – probability of success with a single trial or the overall proportion of success: r/n (Mead et al. 2003).

r – no of expected successes out of a total no of trials (eg. 0, 1, 2, 3, 4 or 5),

n – total no of trials (eg. 10), see note (column 2, Table 5).

The observed pollen germination frequencies were compared with the calculated expected frequencies using a X^2 test. The values of X^2 were calculated as (Observed frequency – Expected frequency)^2/Expected frequency or (Eq. 2):

$$(O - E)^2/E \quad (2)$$

where:

O – observed germination frequencies from the data,

E – expected probability calculated from Eq. 1.

which were then compared with the statistical value of the X^2 distribution at a significance level of 0.05 and having degrees of freedom the number of

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frequency classes minus 2 (Mead et al. 2003). This has been carried out in order to identify whether pollen grains were germinating independently of each other or whether there was a significant pollen population effect within the culture as the total number of pollen grains in each culture varied. If the binomial distribution fits the observed frequencies of pollen germination (observed and expected frequencies not significantly different according to the X^2 test), then each pollen grain has equal probability for germinating and thus acts independently from its surrounding grains not affecting the environment of the other grains to make it either more or less likely that any other grain will germinate (MEAD et al. 2003).

Seed cut test, embryo viability

The seed cut test was conducted on seeds from height class C (> 6 m) since this was the only height class when pollen was assessed throughout all sites. As such, 'site' was the only treatment factor. A composite seed batch from each of the 5 sites was collected and 4 replicate working samples of 50 immature seeds each (200 seeds/site) were dissected for the seed cut test in a completely randomized design (n = 50). An average percentage estimate was deduced for each of the four seed classes mentioned in the previous section from each replicate sample. Percentage data were arcsine transformed in order to meet the assumptions for running an analysis of variance (ANOVA) and all the subsequent analyses were run on the transformed form of the percentage data.

Finally, analysis of variance was conducted on the effects of the 'site' on embryo development and seed internal morphology. Comparisons of means were made at a significance level of 0.05 using the least significant differences (LSD) as a post-hoc test in ANOVA which assumes equal variances. Full embryo development frequency data were correlated with pollen germination data (from height class C group) using a power type function ($y = ax^b$).

Embryo viability was assessed on extracted embryos from the above seed samples. An average percentage estimate of viable embryos out of the total number of seeds was deduced for each site. Percentage data were arcsine transformed in order to meet the assumptions for conducting an analysis of variance (ANOVA) and all the subsequent analyses were run on the transformed form of the percentage data.

Analysis of variance was conducted in the effects of the 'site' on the measured proportions of embryo viability. Comparisons of means were made at a significance level of 0.05 using the least significant differences (LSD) as a post-hoc test in ANOVA which assumes equal variances. The statistical software used for seed cut test and embryo viability was SPSS (Version 19, IBM). Graphs were produced using the Microsoft Excel (Version 2007, Microsoft) graph facility.

RESULTS

Pollen viability and germination

High rates of pollen viability were observed throughout sites ranging from 61.5% to 93.2%. The lowest viability (61.5%, $P < 0.05$) was observed in pollen collected from height class C (most probably mature trees) in L2 (Table 4). However, the observed rates of pollen germination were quite low, ranging from 0% to 3.3% in pollen collected from height class A (young trees), from 0% to 20.1% in height class B and from 0.7% to 30.2% in height class C across all sites (Table 4).

L2 location presented the lowest pollen germination capacity within the height class C group (0.7%, $P < 0.05$ after 12 days in culture), even though being adequately viable (62.5%). As far as L3 location is concerned, only pollen from height class C trees showed a capacity to germinate up to 23%, while pollen from height classes A and B did not germinate at all, even though being viable (Table 4). In addition, pollen from L1 and L4 demonstrated a pattern where pollen from height class C (older) trees showed better germination capacity than pollen from the other height classes with samples from L4 giving 3.3% and 2% germination in height classes A and B, respectively, and 10.8% in height class C, a significantly higher value ($P < 0.05$) (Table 4). Similarly, height class B from L1 presented approx. 20% germination and height class C 30.2% ($P < 0.05$). The statistical analysis showed a significant effect of both site and height class on the pollen germination capacity ($P < 0.05$).

Moreover, the observed pollen germination frequencies for height class C from which pollen was assessed across all sites do not fit the binomial distribution that was tested across all samples ($P < 0.05$, Table 5), which provided significant evidence that pollen grains within each culture did not share an equal probability for germination. Conse-

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Table 5. Binomial distribution tests for pollen germination frequencies observed for height class C trees across the five sites of the study

Height class	Germinating grain frequency classes	Frequency		$(O - E)^2/ E$
		observed	expected	
C	0	1	1	1
	1	2	0.3281	8.52
	2	1	0.2048	3.08
	3	1	0.1323	5.69
	4	0	0	0
	5 or more	0	0	0
	Total	5	1.6652	$X^2 = 18.29 > 9.49$ ($P < 0.05$)

Five classes of germinating pollen grains were set for testing the binomial distribution fit. Zero to 5 or more grains germinating out of a total of 10 (the upper 5 neighbouring classes were combined in order to avoid very small expected frequencies, because there were no data above 50% germination and in order to avoid very large factorial terms (5! = 120). The frequencies are taken from all the sites across height class C (Table 4, pollen germination) since it was the only height class where pollen was assessed across all sites (this is why the observed frequencies add up to 5). The percentage of germination observed for each measured date was taken and divided by 10; for example a germination rate of 30.18 % = 3.018 grains germinating out of 10, or ≈ 3 grains germinating out of 10. The degrees of freedom for any binomial distribution dataset are the number of classes –2; thus here they are 4. The observed X^2 value was compared with the *P*-value of the Chi-squared distribution at the 99.5% confidence level for 4 d.f., which is 9.49.

quently, each grain did not germinate independently from its surrounding grains, which means that there was a significant population effect in pollen cultures.

Seed cut test and embryo viability

As far as the seed cut test is concerned, low proportions of seeds with complete embryo development were observed throughout all sites with L2 presenting the lowest percentage (4%) (Fig. 3). L1 and L4 sites showed the highest rates of embryo development (20% and 17.6%, respectively) (Fig. 3). The overall low complete embryo development that was observed corresponds to a certain extent to the low pollen germination rates found across all sites concerning height class C (mature) trees (R^2 = 64.2%, $y = 0.107x^{1.8712}$, $P < 0.05$). On the other hand, proportions of empty seeds ranged from 25.5% in L4 to 72% in L2 (Fig. 4). In addition, measured percentages of seeds with no embryo but with an undifferentiated mass of starchy cells varied among sites. The lowest value was recorded in L1 (5%), and the highest one in L4 (53.5%) (Fig. 4). A significant number of seeds did not have any embryo at all or even an undifferentiated mass of starchy cells and were full of resinous substance instead, ranging from 1% in L4 to 10.5% in L3 and 19.6% in L1 (Fig. 4). Nevertheless, the cumulative frequencies of empty seeds, seeds filled with resin and seeds with incomplete embryo development were high throughout all sites ranging from 80% in L4 to 96% in L2 of the evaluated seeds (Fig. 3). The analysis of variance of seed data showed a significant effect of the location on seed internal morphology and embryo development ($P < 0.05$). Similarly, the overall proportions of viable embryos (extracted from seeds with full embryo development) according to the TTC test were very low ranging from 0.5% to 4.5% across all sites (Fig. 3) without, however, being significantly affected by the location ($P < 0.05$).

DISCUSSION

Pollen viability and germination

In the present study pollen showed low germination capacity following 12 days in culture across all sites. Although the staining test showed that pollen was viable, the potential of the grain to produce a functioning pollen tube that will eventually fertilize the female part leading to embryo development (Williams 2009) remained unclear as only a small fraction of the collected pollen germinated. Even though the population of pollen grains within each culture was sufficiently large to promote ger-

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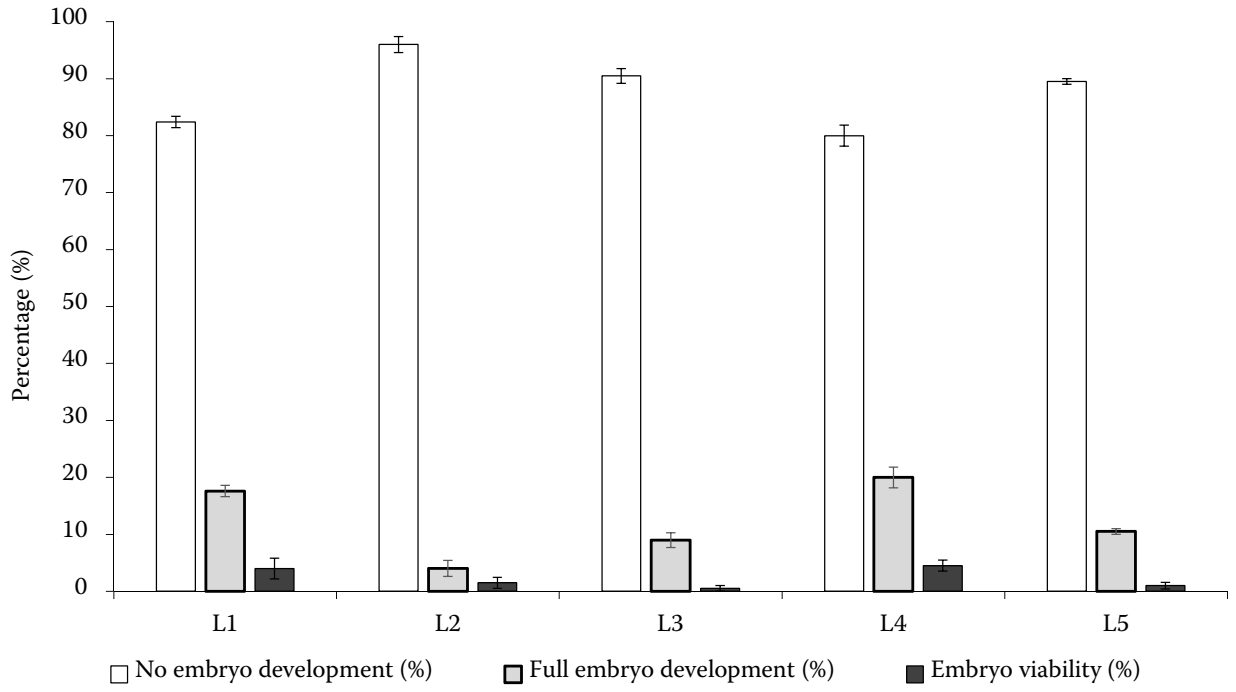

Fig. 3. Average percentages of seeds with a fully developed embryo (grey bars) from the five sites of the study along with the average percentages of seeds without embryo development (cumulative white bars, representing the three seed classes: empty seeds, seeds with an undifferentiated mass of starchy cells and seeds filled with resin) and percentage of embryo viability (black bars) out of the total number of seeds per sample. Details of the L1–L5 sites are given in Table 1. SEM bars are shown in the graph ($P < 0.05$).

mination based on the significant pollen population effect observed (Table 5) (Karapatzak et al. 2012) and thus eliminating misinterpretation of the results due to low pollen density (Boavida, McCormick 2007; Chen et al. 2000). It is documented that pollen density within *in vitro* culture affects germination and it seems as if there was a positive linear relationship between pollen density and germination, known as the pollen population effect (Pasonen, Käpylä 1998).

The low pollen germination rates observed were correlated with the subsequent low embryo formation rates. As such, pollen germination capacity contributes to the low reproduction dynamics of the species. However, it has been reported in Pinaceae that in the first year after pollen release only a fraction of pollen grains forms pollen tubes while the rest remain viable and enclosed in the ovule for one or even two years. A pollen tube seems to be dormant and revives in response to some unknown cues when the formation of the female gametophyte has been completed (Williams 2009). As such, a pollen dormancy mechanism may be in place. Consequently, if pollen possesses a kind of dormancy, then, although the staining test will evaluate the grain as viable, no germination would be recorded. Considering the above, if a pollen dormancy mechanism exists, it should be either physical or physiological. In the present study it was observed that the exine layer of almost all pollen grains and the intine of most grains were ruptured shortly after hydration and water was absorbed, even though it did not result in germination. So, the exine and intine structures do not most probably serve as a physical barrier enforcing dormancy, which indicates that if there is any dormancy mechanism, it might have a physiological character, which, in turn, suggests the need for further work on the pollen ultrastructure.

In addition, an examination of the cellular structure of pollen grains produced under different microclimatic conditions might further clarify the impact of environment considering the significant effect of location (microclimate) observed on pollen germination capacity, especially under the ongoing climate change. Environmental conditions affect the fertilization process in several stages as the pollen tube growth can be postponed up to a

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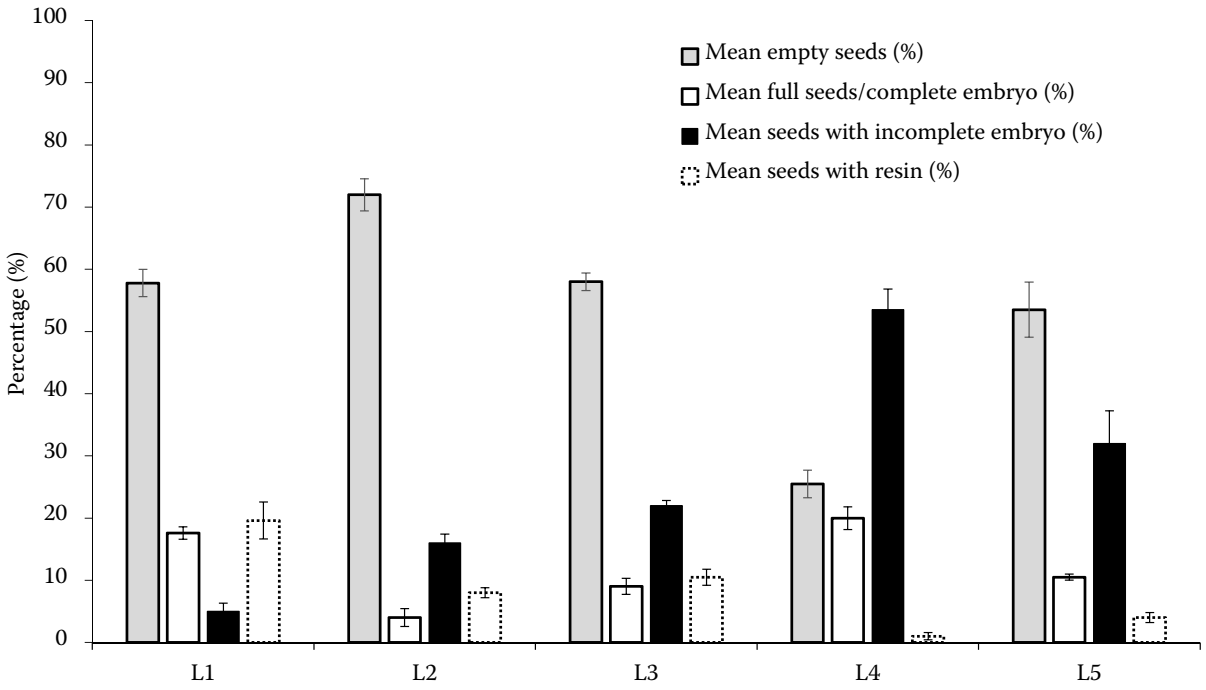

Fig. 4. Average percentages of empty seeds (grey bars), seeds with a complete embryo (white bars), seeds with undifferentiated mass of starchy cells (black bars) and seeds filled with resin (dashed bars). SEM bars are shown in the graph ($P < 0.05$). Details of the L1–L5 sites are given in Table 1. LSD = 14 for empty seeds, LSD = 6.5 for seeds with a complete embryo, LSD = 16 for seeds with undifferentiated mass of starchy cells and LSD = 7 for seeds filled with resin ($P < 0.05$)

year in Junipers (Gruwez et al. 2013). Pardi et al. (1996) noticed that in certain *Pinus* species environmental conditions from year to year can affect pollen performance and histology outlining the importance of the external environment on pollen tube formation. A rise in atmospheric temperature and nitrogen deposition, due to climate change, during pollen tube growth and female gametophyte development have been suggested to adversely affect seed viability in *J. communis* (Gruwez et al. 2014). In addition, the temperature rise has been suggested to affect the processes of pollen tube growth and fertilization in UK populations of *J. communis* (Ward 2010).

Female gametophyte abortion, on the other hand, was suggested as a contributing factor to the low seed set of *Picea sitchensis* (Owens, Molder 1980). Similarly, in *Pinus sylvestris* high rates of seed abortion were attributed to a maternal genotypic effect (Kärkkäinen et al. 1999). Nonetheless, evaluation of the receptiveness of the female part *in vivo* is suggested as a further means of assessing the reproduction dynamics of *J. excelsa*. It was shown in the past using *in vitro* fertilization assessments (IVF) in conifers that the pollen tube can grow into nonviable or even degenerated female ovules, suggesting a lack of the pollen tube ability to recognize viable or same species female gametophytes (Dumont-BéBoux et al. 1998; Fernando et al. 1997; Fernando et al. 1998). Thus, self-incompatibility is most probably being controlled by the female part.

Seed development

Apart from the contribution of pollen performance to the reproduction dynamics of *J. excelsa* that has been studied herein, poor fertilization and consequent poor seed development could also be the outcomes of genetic factors. Narrow genetic variability and high inbreeding rates can severely affect embryo viability and population reproduction (Williams et al. 2003, Williams 2009). However, it has been found that *J. excelsa* populations across the eastern Mediterranean basin (Prespa provenance was not included) had a high level of genetic diversity, at both species and population

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levels (Douaihy et al. 2011). Similar results have also been obtained in previous studies on *J. phoenicea* across the wider Mediterranean basin (Meloni et al. 2006). Vanden Broeck et al. (2011) concluded that the observed low seed viability of *J. communis* across northwestern Europe was not due to a genetic diversity bottleneck. Nonetheless, it is considered essential to investigate the genetic variability of the current provenances as inbreeding may better explain both poor fertilization and low embryo viability. Moreover, self-pollination causing embryonic death through the embryo lethal system (Williams 2009) may explain the high percentage of empty seeds recorded in the current research.

For a long-lived species like *Juniperus excelsa* its reproduction strategies may reflect a slow regeneration process. Nevertheless, the results of low fertilization potential and low embryo viability documented for the species in the Prespa National Park cannot be disregarded. From the results of the present study on *J. excelsa* it can be concluded that a complex regeneration mechanism is being employed. Apart from the observed low pollen germination capacity and the consequent low embryo formation rates, the regeneration potential of *J. excelsa* may also be affected by pollen dormancy, climatic variables or genetic reasons acting either independently or synergistically. If one would also include natural or anthropogenic disastrous events as potential threats to the habitat, the lack of natural regeneration may result in an inability of *Juniperus excelsa* forests to re-establish after such events. Thus, it is urgent to investigate the deeper causes of poor fertilization and low regeneration potential and propose measures for their elimination to preserve a vigorous and stable juniper population across the Mediterranean region and the rest of Europe.

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