



# The age of black pine (*Pinus nigra* Arn. ssp. *salzmannii* (Dunal) Franco) mother trees has no effect on seed germination and on offspring seedling performance

Reyes Alejano<sup>1</sup> · Marta Domínguez-Delmás<sup>2</sup> · Ignacio García-González<sup>2</sup> · Tomasz Wazny<sup>3,4</sup> · Javier Vázquez-Piqué<sup>1</sup> · Manuel Fernández-Martínez<sup>1</sup>

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## Abstract

- **Key message** We sampled *Pinus nigra* cones in 29 trees in an age range of 90 to 725 years. The mother tree age did not significantly influence the pinecone or pine seed size, seed germination capacity, or plant size or vigor displayed during the first year of growth in the nursery.
- **Context** *Pinus nigra* Arn. ssp. *salzmannii* is a long-lived Mediterranean species, with millenary trees residing in an old-growth forest in the Cazorla Mountain Range in SE Spain which is home to the oldest known trees in the Iberian Peninsula.
- **Aims** This study aimed to assess how the mother tree age in *Pinus nigra* influences seed viability, germination capacity, and the seedling survival and growth during the first year under nursery conditions.
- **Methods** Twenty-nine trees aged 90 to 725 years were selected and 60 cones were harvested per tree to study the cone characteristics (size and weight), seed viability, and germination capacity in relation to the mother tree age. Eighty germinated seeds per tree were transferred to the nursery and seedling survival and growth were measured after the first growing season.

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**Contribution of the co-authors** Marta Domínguez Delmás took part in designing the experiment, fieldwork, and tree ring analysis for age determination, and coordinating the research project.

Ignacio-García González participated in designing the experiment and fieldwork.

Tomasz Wazny took part in designing the experiment, fieldwork, and tree ring analysis for age determination.

Javier Vázquez Piqué participated in designing the experiment and fieldwork, running the data analysis, and writing the paper.

Manuel Fernández participated in designing the experiment, supervising lab and nursery work with seeds and seedlings, running the data analysis, and writing the paper.

Reyes Alejano had the first idea, participated in designing the experiment, and field sampling. He coordinated the works in the lab (cones and seeds assessment and germination tests) and nursery (plantation and follow up of seedlings). He participated in designing the statistical analysis and writing the paper.

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This article is part of the topical collection on *Mediterranean Pines*

✉ Reyes Alejano  
ralejan@uhu.es

Extended author information available on the last page of the article

- **Results** Significant between-tree differences were detected for cone characteristics (cone and seed weight, number of seeds per cone), as well as for germination capacity. Notably, however, the mother tree age did not significantly influence the aforementioned parameters.
- **Conclusion** Forest management and regeneration practices of *Pinus nigra* should take into account that trees of this species up to at least 725 years old produce seeds with a fairly high reproductive capacity.

**Keywords** *Pinus nigra* · Aging · Seed viability · Old-growth forests

## 1 Introduction

In face of the increasing human population pressure and resource demands and of a changing global climate, management of the remaining world's old-growth forests, either for conservation, for production, or for multiple use, is a global issue of critical importance (Beadle et al. 2009; Lindenmayer and McCarthy 2002). It is nowadays well understood that such forests play important roles in harboring biodiversity (Lindenmayer and Franklin 2002), and in terrestrial carbon storage and sometimes in sequestration (Carey et al. 2001). Nevertheless, there is little data on the ability of trees in old-growth forests to maintain their regeneration capacity (i.e., to produce viable seeds in enough quantity) as they may approach senescence. The regeneration phase is a key part of forest conservation and therefore of sustainable forest management (Gordo et al. 2012; Guariguata and Pinard 1998). Forest regeneration is a complex process driven by the interplay of numerous biotic and abiotic factors impacting on the life cycle stages of plants (Smith et al. 1997). The first pre-requisite for regeneration is suitable seed production, which depends on stand maturity, fertility, and masting (Lucas-Borja et al. 2012; Viglas et al. 2013). Further, natural regeneration depends not only on seed production, but also on seed germination and site conditions (Kitajima and Fenner 2000), with the seed and the seedling stages being recognized as the most important (Lucas-Borja et al., 2017; Mao et al. 2014).

The plant life cycle comprises several stages (juvenile, mature, and senescence). All of these stages are strictly coordinated by phytohormones and induced and modified by environmental factors. It is widely assumed that the senescence is generally characterized by a slowdown in metabolic activity, a decrease in apical and cambial growth, the production of smaller leaves and fewer flowers, and seeds with lower germination potential (Larcher 2003). However, as it is very difficult to know when a tree reaches senescence, this statement remains somewhat ambiguous (Stephenson et al. 2014). Although there is ongoing debate about the terminology used to describe the process, it is generally agreed that senescence can be due to aging (deterioration in performance not under the control of an endogenous biological clock) or to senescence (deterioration in performance depending directly on endogenous processes, like metabolite accumulation, that cause DNA damage) (Brutovská et al. 2013, Thomas 2013). Therefore, in order to avoid misunderstandings, in this article, the term senescence is used to define the last stage of plant life, but taken

into account that it could be due to senescence, aging, or both simultaneously. Plant type differences (monocarpic vs. polycarpic; genet vs. ramet) must also be taken into account (Brutovská et al. 2013; Munné-Bosch 2008). In addition, within woody plant maturity and senescence stages, fruit production competes with vegetative growth of the supporting and photosynthetic tissues (Thomas 2013). The fertility and the reproductive capacity of perennial plants are believed to progressively increase up to a peak level, which is maintained for some years, and thereafter start decreasing or else completely disappear during the last stage of life (Larcher 2003; Müller et al. 2014). These parameters have been linked to genetic constitution, to climatic conditions, or otherwise to resource availability, yet their relationship with the mother tree age has hardly been studied (Ganatsas et al. 2008; Herrera 1991). Nonetheless, the aforementioned statements about an aged-linked decreases in reproductive capacity and fertility have been refuted by some authors, age having been considered a relative physiological parameter of plants or animals, depending on a given relative longevity (Kleksovski and Godfrey 1989). As some pine species' life spans are rather long, particularly mountain habitat dwelling species (e.g., 300 to 500 years for temperate *Pinus* sp., 1200 years for *P. cembra*, 2000 to 4000 years for *P. aristata* and more than 4000 years for *P. longaeva*), it is therefore not easy to establish whether senescence has been reached (Larcher 2003; Thomas 2013).

A great mass of data has been accumulated in relation to developmental processes when animals age, especially with regard to *Homo sapiens*. However, little beyond conjecture has been written on the effect of aging with respect to the longest lived organisms, that is to say trees (Lanner and Connor 2001). As a matter of fact, few studies exist on the relationship between seed viability and germination capacity and mother tree age for *P. nigra* (Tiscar Oliver 2002) or other forest species (Ganatsas et al. 2008; Lanner and Connor 2001; Mao et al. 2014). Lanner and Connor (2001) compared putative biomarkers of aging in Great Basin bristlecone pine (*Pinus longaeva* D. K. Bailey) ranging in age from 23 to 4713 years. They found no significant age-related differences in xylem and phloem characteristics, annual shoot growth increments, pollen viability, seed weight, seed germinability, seedling biomass accumulation, or frequency of putative mutations. On the contrary, Tiscar Oliver (2002) found that oldest trees produced a higher quantity of empty seeds, with lighter seeds and lower germination vigor, although old trees maintained a high germination rate and

differences with mature and young trees were not found to be significant. Therefore, the relationships between tree age and seed production and seed quality is unclear (Tiscar Oliver 2002; Müller et al. 2014; Munné-Bosch 2008; Thomas 2013).

*Pinus nigra* Arn. ssp. *salzmannii* (Dunal) Franco is a long-lived Mediterranean species, with millenary trees enduring in an old growth stand located in Puertollano-Cabañas, at the Cazorla Mountain Range, positioned in the South East of Spain (Creus 1998; Dominguez-Delmás et al. 2013). This stand constitutes the oldest known forest in the Iberian Peninsula. The inaccessibility of some areas, such as Puertollano-Cabañas, has constituted a natural self-defense for the ecosystems, later supported by protective management after the establishment of the Biosphere Reserve and Natural Park.

This study is aimed to assess how the mother tree age in *Pinus nigra* Arn. ssp. *salzmannii* influences seed viability and germination capacity, and the seedling survival and growth during the first year under controlled conditions in a nursery. We hypothesized that the oldest trees would have already entered senescence and, as a consequence, (i) the cone characteristics would show evidence of poorer reproductive performance (lower weight, fewer seeds per cone, lower seed-to-cone weight fraction, higher number of empty seeds); (ii) the germination capacity in oldest trees would be lower; and (iii) the survival, growth, and vigor of seedlings under controlled conditions from seeds of oldest trees would be lower.

## 2 Material and methods

### 2.1 Study area

The study area (Puertollano-Cabañas Peak) is located in the Poyo de Santo Domingo Forest (Jaén, Spain, 37° 49' N, 2° 57' W), home to the oldest pine trees of the Iberian Peninsula (Creus 1998). Altitude ranges from the 1700 m a.s.l. of Puertollano to the 2028 m a.s.l. of the Cabañas Peak (Fig. 1). The area is contained within the Cazorla, Segura, and Las Villas Biosphere Reserve and Natural Park, with high ecological value due to the high biodiversity and the presence of endemic plant species (Dominguez-Delmás et al. 2013).

The area's main woody species comprises *Pinus nigra* Arn. ssp. *salzmannii* representing up to 97% of the forest trees. The climate is Mediterranean with a dry summer and marked differences between the minima and maxima temperatures over the year. The average annual precipitation amounts to 1100 mm, ranging from 400 to 1900 mm. The average annual temperature is 11.7 °C, rising from a minimum mean temperature of 4 °C in January to a maximum mean temperature of 21 °C in August. The lithology consists mainly of limestone and dolomite.

### 2.2 Tree selection, cone harvesting, and measurements

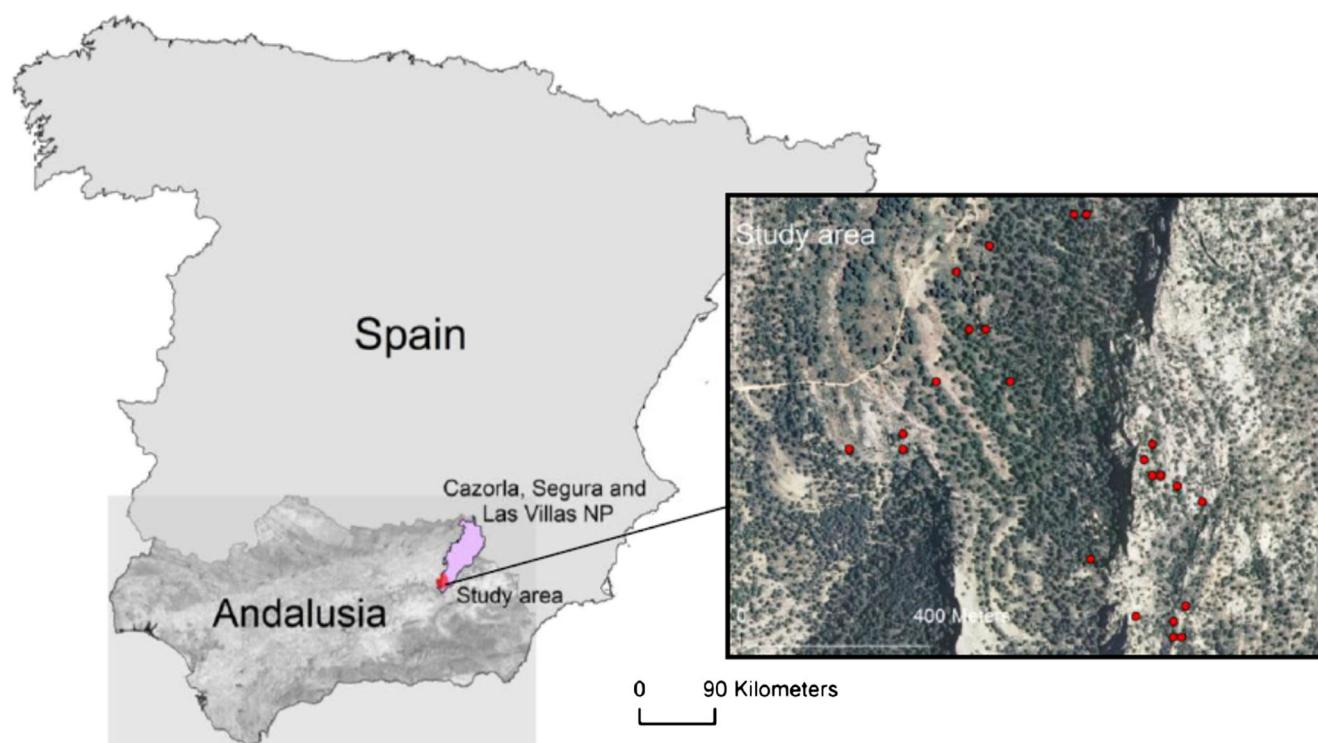
An initial sample of 80 trees was chosen in the Puertollano-Cabañas region in an area of 75 ha, in order to determine their respective ages by establishing tree ring chronologies according to standard dendrochronological procedures (Dominguez-Delmás et al. 2013). For the aims of this paper, 29 trees were randomly chosen from among these 80 trees. The 29 selected trees were 90 to 725 years old representing all age classes (Fig. 1, ESM Table 1).

In December of 2013, when cones were mature but seed dispersal had not started, a tree climber collected at least 60 cones from each of the 29 selected trees, while the rest of the cones were left on the trees. Cones were cold stored (3 °C) in the lab for 6 days and then placed in open trays at room temperature for measurement of the fresh weight of each cone. Sixty cones per tree were separated into two groups. One group of 30 cones was characterized for cone length ( $L_{cone}$ ) and maximum diameter ( $Wd_{cone}$ ) assessed with a caliper ( $\pm 0.01$  mm). Afterwards, these 30 cones were placed into individual paper bags and further stored at room temperature (18 to 25 °C) and a relative humidity of 30 to 50% for a further 2 months. This first group was later used for measuring cone characteristics (see the next paragraph). The other group was left in the trays and also stored for a period of 2 months at room temperature. This second group was used for obtaining seeds for seed viability and germination tests.

In February of 2014, all cones in both groups were placed into a convection oven at 30 °C for 40 h to accelerate their opening. Once the cones of the first group had opened, cone mass including seeds, cone mass without seeds, and the seed mass with and without wings were recorded ( $\pm 0.001$  g). The cone and seed moisture contents of a subsample were determined after oven-drying at 80 °C to a constant weight. Afterwards, dry mass per cone ( $DM_{1cone}$ ), number and dry mass of seeds per cone ( $N_{seeds}$  and  $DM_{seeds}$ , respectively), dry mass per seed ( $DM_{1seed}$ ), and the seed dry mass fraction ( $SDMF = DM_{seeds}/DM_{1cone}$ ) were calculated for each tree. For the cones of the second group, seeds were extracted and wings were removed manually; the pine seeds were stored in sealed plastic containers under cold conditions (3 °C), and then used for the seed viability and germination tests.

### 2.3 Seed viability and germination

When there were enough seeds, 300 seeds per tree were randomly selected from the containers of seeds to be used for the germination tests. Before starting the tests, the seeds were disinfected by immersion in a 30 mL L<sup>-1</sup> solution of H<sub>2</sub>O<sub>2</sub> for 10 min followed by mixing seeds with wetted perlite and spraying with a fungicide (1.2 g L<sup>-1</sup> Captan). The mixture was placed into plastic containers under cold conditions (3 °C) in the



**Fig. 1** Map of the *Pinus nigra* study area in “Poyo in Santo Domingo” forest, municipality of Cazorla (Jaén, Spain), within the Cazorla, Segura, and Las Villas Natural Park. The red circles displayed in the aerial photograph indicate the location of the 29 sampled trees, ranging from 90 to 725 years old

dark for a month. In April 2014, 200 pine seeds per tree were randomly selected and 50 pine seeds were distributed across four 8-cm-diameter Petri dishes. These Petri dishes had been prepared beforehand by placing five sheets of absorbent paper at the bottom which had been wetted with distilled water and sprayed with fungicide. Dishes were kept at room temperature (18 to 25 °C) exposed to a 14-h light/10-h dark photoperiod. Twice a week, for 4 weeks, germinated seed number was recorded. The germination energy (GE, defined as the percentage of the seeds germinated within the first week of analysis) and germination percentage at the end of the test (GP), as well as germination values according to Czabator and Djavanshir and Pourbeik ( $GV_{CZ}$  and  $GV_{DP}$ , respectively) were assessed (FAO 1985).

In 17 trees (ranging from 158 to 725 years old), an additional 30 seeds were used to perform a tetrazolium test to assay seed viability (FAO 1985). In the remaining  $29 - 17 = 12$  trees, the cone yield in the year of collection was very small and we were unable to obtain enough seeds to perform the viability test. Before the test, the number of full and empty seeds ( $F_{seeds}$  and  $E_{seeds}$ , respectively) were counted and the percentage of full seeds was calculated ( $PF_{seeds} = 100 \times F_{seeds}/(F_{seeds} + E_{seeds})$ ). Empty seeds were recognized by their low weights and because they could be crushed applying a slight pressure with the fingertips. Briefly, seeds were first soaked in distilled water for 24 h at room temperature, and later cut in half with a scalpel. Each half seed, classified according to the tree from which it came, was immersed in a tetrazolium solution ( $5 \text{ g L}^{-1}$  of 2,3,5 triphenyl-2H-tetrazoliumchloride). Containers were stored under dark conditions for 80 min at

20 °C. Thereafter, viable seeds (VS, i.e., those in which embryo and nutritive tissue stained red) from each tree were counted and the following variables were calculated: percentage of viable seed fraction with respect to  $F_{seeds}$  ( $VS_{fs} (\%) = 100 \times VS/F_{seeds}$ ) and the percentage of viable seed fraction with regard to the total number of seeds ( $VS_{ts} (\%) = VS/(F_{seeds} + E_{seeds})$ ).

#### 2.4 Nursery growth of seedlings and plant quality

The first 80 seeds that had germinated per tree (0.5 to 1.5 cm radicle length) were transferred to the nursery and planted in 250-cm<sup>3</sup> forestry containers (45 containers per tray, one seed per container) filled with a mixture of peat (75% by volume) and perlite (25% by volume) plus 1 kg m<sup>-3</sup> of added Nitrofoska® blue 12-12-17 + 2 MgO (BASF). Seedlings were sprayed with fungicide every week, and were kept well-watered according to their needs with tap water and rainfall under a shade mesh that reduced sunlight by 30%. At the end of January 2015, survival was assessed by counting the live seedlings. At the same time, the length of the epicotyl stem (EL) of all seedlings was measured.

In addition, the shoot and root dry weights (SDW and RDW, respectively, after oven-drying at 80 °C), the total dry weight of the plant (TDW = SDW + RDW), and the shoot-to-root ratio (SRDW = SDW/RDW) were determined for a sample of six seedlings per tree, evaluating a total of 8 randomly selected trees, aged 90 to 725 years drawn out of a 100-year-old range class starting at the < 100-year age class and

extending all the way up to the 700–800-year-old class. Furthermore, for another six seedlings from each of these eight mother trees, a root growth capacity test was performed (Fernández et al. 2007). Briefly, the test was carried out over 6 weeks (from early February to mid-March), and upon extracting seedlings out of their containers, making every possible effort to keep the root balls intact, the pre-existing white roots (usually fewer than 2 and shorter than 1 cm) grown out from each ball were detached. Next, plants were transferred to 2.5-L containers filled with moistened perlite before relocation in a greenhouse with favorable temperature (25/18 °C, day/night) and moisture (50/80% relative humidity) conditions. Seedlings were watered daily and fertilized once a week, and after a 6-week growth period in the greenhouse plantlets were removed from their containers. We then measured the total length of the new roots that had emerged from the root ball as a measure of root regeneration capacity (RGC).

## 2.5 Data analysis

### 2.5.1 Influence of mother tree age on cone and seed traits and germination capacity

As a first step, we checked the significance of the between-tree variability in cone and seed traits and germination capacity by building a random effect model considering tree as a random effect. The model was:

$$y_{ij} = t_j + e_{ij}$$

where  $y_{ij}$  referred to cone and seed trait or germination capacity of cone  $i$  from tree  $j$ ,  $t_j$  is the tree random effect with  $t_j \sim N(0, \sigma^2_t)$ , and  $e_{ij}$  is the residual error with  $e_{ij} \sim N(0, \sigma^2_e)$ . In order to satisfy the assumptions of normality,  $N_{\text{seeds}}$  and SDMF values were transformed to  $\sqrt{N_{\text{seeds}} + 0.5}$  and  $\text{arcsin}\sqrt{\text{SDMF}}$ . We predicted for each variable the covariance parameter at tree level and checked the covariance parameter equal-to-zero hypothesis with a  $\chi^2$  test. We also calculated the percentage of variance explained by the tree effect. This first step is relevant because, if the tree effect is not significant, it is not appropriate to check whether there are covariates at the tree level (tree age) that explain a significant part of such variability.

In a second step, for variables with a significant tree effect, we introduced the age of the tree as a covariate in a linear and quadratic form and assessed the significance of the coefficients with an  $F$  test. We introduced the quadratic term to check for non-linear relationships of tree age with cone and seed traits or germination capacity.

For variables with only one value per tree ( $\text{PF}_{\text{seeds}}$ ,  $\text{VS}_{\text{fs}}$ ,  $\text{VS}_{\text{ts}}$ ), a regression analysis was performed with age and  $\text{age}^2$  as independent variables. These variables were arcsin transformed.

### 2.5.2 Influence of mother tree age on seedling evolution and plant quality

As in Section 2.5.1, we analyzed first the between-tree variability with a random tree effect model. The variables analyzed were EL, SDW, RDW, TDW, SRDW, and RGC. For variables with significant between-tree variability (after a  $\chi^2$  test), we introduced the age of the tree as a covariate in a linear and quadratic form. For the survival analysis, we had only one value for each of the 29 trees so we performed a regression analysis with age and  $\text{age}^2$  as independent variables, and having previously arcsin transformed the survival percentage.

## 3 Results

### 3.1 Cone and seed properties

The cone, seed size, and weight parameters showed significant differences between trees with 52 to 63% of the variance explained by the tree (Table 1). The mean values (SD, range) of cone size were  $L_{\text{cone}} = 53.7$  mm (0.27, 28 to 81 mm), and  $Wd_{\text{cone}} = 27.3$  mm (0.1, 18 to 36 mm). The moisture content of the pinecone seeds at collection time was 14.9% (1.6, 5 to 40%), with no significant differences between trees ( $p = 0.889$ ).

Tree age did not significantly influence cone or seed size, or the number of seeds per cone (Table 1). The perceived trends to lower  $\text{DM}_{\text{1seed}}$  and SDMF values with increasing tree age were non-significant and had weak correlations (Fig. 2).

Seed viability analysis evidenced that on average, (a) over three quarters of all seeds were full,  $\text{PF}_{\text{seeds}} = 78.5\%$  (3.3), range 50 to 100%; (b) a very high percentage of these full seeds were viable,  $\text{VS}_{\text{fs}} = 96.1\%$  (0.8); (c) three quarters of all seeds were viable,  $\text{VS}_{\text{ts}} = 75.2\%$  (3.0); and (d) none of these variables, neither  $\text{PF}_{\text{seeds}}$ , nor  $\text{VS}_{\text{ts}}$ , nor  $\text{VS}_{\text{fs}}$ , were related to the age of the mother tree (Table 1).

Seed germination-related parameters (GE, GP,  $\text{GV}_{\text{Cz}}$ ,  $\text{GV}_{\text{DP}}$ ) were highly correlated among themselves ( $0.81 < r < 0.98$ ,  $p < 0.001$ ,  $N = 17$ ). The correlation between  $\text{GV}_{\text{Cz}}$  and  $\text{GV}_{\text{DP}}$  was so prominent ( $r = 0.98$ ,  $p < 0.001$ ,  $N = 17$ ) that the second attribute has been omitted from Fig. 3. In the case of these four parameters, significant differences were detected between trees ( $p < 0.001$ , Table 1), the tree effect accounting for 58 to 84% of the total variability, but again the age of the mother tree was not significant (Table 1). We observed the following mean values (SD, range):  $\text{GE} = 67.1\%$  (1.9, 22 to 94);  $\text{GP} = 76.3\%$  (1.7, 41.5 to 100);  $\text{GV}_{\text{Cz}} = 68.0$  (3.9, 10 to 148); and  $\text{GV}_{\text{DP}} = 66.5$  (3.3, 14.5 to 123). In the same way, the dry mass of a single seed ( $\text{DM}_{\text{1seed}}$ ) was positively correlated with GP and  $\text{GV}_{\text{Cz}}$  ( $0.53 < r < 0.57$ ,  $p < 0.003$ ,  $N = 29$ ), and SDMF with GP ( $r = 0.47$ ,  $p = 0.011$ ,  $N = 29$ ).

**Table 1** Between-tree variability and effect of tree age on cone and seed traits and germination parameters.  $DM_{1cone}$ , dry mass per cone.  $N_{seeds}$ , number of seeds per cone.  $DM_{1seed}$ , dry mass per seed.  $SDMF = DM_{seeds}/DM_{1cone}$ , seed-to-cone dry mass fraction. GE, germination energy. GP, germination percentage.  $GV_{CZ}$ , germination values according to Czabator.  $GV_{DP}$ , germination value according to Djavanshir and Pourbeik. PF<sub>seeds</sub>, percentage of full seeds. VS<sub>fs</sub>, viable seed fraction as a percentage of full seeds. VS<sub>ts</sub>, viable seed fraction as a percentage of total seeds

Variables	Covariance parameter estimates			Variance explained by tree effect (%)	$p(\sigma_t^2 = 0)$	Tree age effect	
	Tree ( $\sigma_t^2$ )	Residual ( $\sigma_e^2$ )	Total			Age ( $p > F$ )	Age <sup>2</sup> ( $p > F$ )
$DM_{1cone}$	7.28	4.32	11.60	63	< 0.0001	0.07	0.13
$N_{seeds}$	2.30	1.37	3.67	63	< 0.0001	0.23	0.13
$DM_{seeds}$	0.0532	0.0391	0.0923	58	< 0.0001	0.22	0.15
$DM_{1seed}$	13.10	12.02	25.12	52	< 0.0001	0.73	0.51
$SDMF$	0.0032	0.0021	0.0053	61	< 0.0001	0.57	—
GE	224.4	164.5	388.9	58	< 0.0001	0.20	0.31
GP	221.4	95.8	317.2	70	< 0.0001	0.37	0.57
$GV_{CZ}$	1428.3	273.9	1702.2	84	< 0.0001	0.18	0.43
$GV_{DP}$	999.9	220.9	1220.8	82	< 0.0001	0.21	0.43
PF <sub>seeds</sub>		0.0317				0.57	0.42
VS <sub>fs</sub>		0.0187				0.77	0.56
VS <sub>ts</sub>		0.0144				0.75	0.79

### 3.2 Nursery growth of seedlings and plant quality

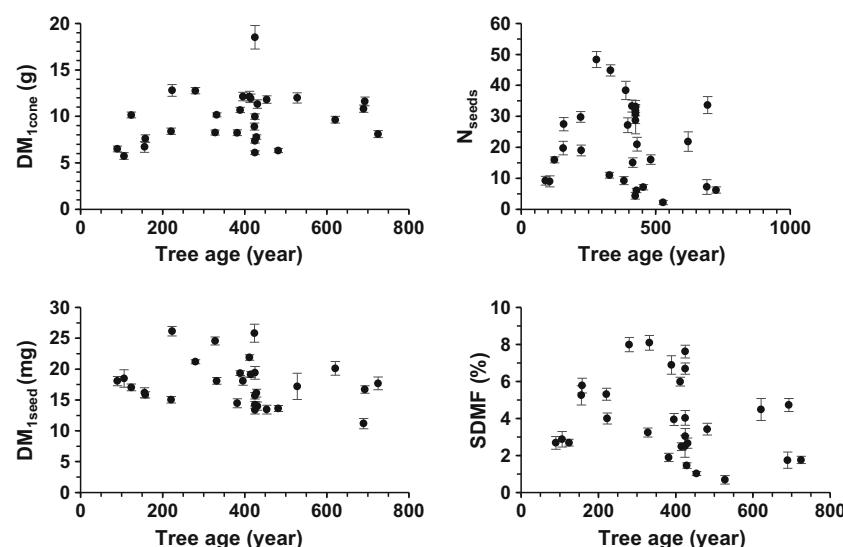
At the end of the study period, neither plant size (EL, SDW, RDW, and TDW) nor shoot-to-root ratio (SRDW) nor the new root regeneration capacity (RGC) were significantly associated with mother tree age (Table 2). The mean total plant dry weight (TDW) was 281 (8) mg, this varying significantly between trees ( $p = 0.02$ , Table 2). The aforementioned differences were due to the above-ground plant portion as between-tree differences in RDW were not significant ( $p = 0.27$ ). Likewise, neither the mean EL (7.81 (0.3) mm) nor the SRDW (0.79 (0.03)) differed significantly between trees (Table 2). As for the de novo root regeneration, while RGC (4.80 (0.82) cm) of the newly regenerated roots differed significantly between trees ( $p = 0.04$ ), it was also not significantly related to the mother tree age.

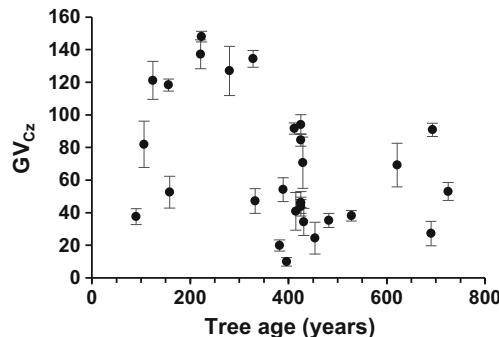
**Fig. 2** Cone and seed parameters of the 29 *Pinus nigra* sampled trees as a function of tree age (symbols are mean  $\pm$  SE).  $DM_{1cone}$ , dry mass of one cone.  $N_{seeds}$ , number of seeds into a cone.  $DM_{1seed}$ , dry mass per seed.  $SDMF = DM_{seeds}/DM_{1cone}$ , seeds to cone dry mass fraction

### 4 Discussion

For the sampled trees and corresponding age range, the average cone size (9.8 g, length of 54 mm, diameter of 27 mm), pine seed weight (18 mg), number of pine seeds per cone (21), and the percentage of empty pine seeds (21.5%) were all within the average range for the species (Derridj et al. 2011; Ruiz de la Torre 2006; Tíscar Oliver 2002). Although not an objective of this study, it should be highlighted that some trees in the sample did not have sufficient production of pine cones to allow the harvest of more than 60 cones. This is common for *P. nigra* as being a masting species whose cone and pine seed production varies from one year to another (Tíscar and Linares 2011; Tíscar and Lucas 2010).

The between-tree differences in the parameters related to pine cone size ( $DM_{1cone}$ ) and seed size as well as number ( $DM_{1seed}$ ,  $N_{seeds}$ ,  $SDMF$ ) highlight the between-tree variability





**Fig. 3** Germination value ( $GV_{Cz}$ ) of *Pinus nigra* seeds as a function of tree age. Symbols are mean  $\pm$  SE

which can be expected to be more greatly influenced by aspects related to genetics, physiology, or the prevailing environment of the collection year (Ayari and Khouja 2014; Tíscar Oliver 2007; Viglas et al. 2013) than the age of the mother tree. The lack of significance of tree age in the analysis of covariance is indicative of there being no evidence that these pine trees had passed beyond the stage of maturity and entered into the senescence stage, despite some trees advanced ages (Brutovská et al. 2013; Lanner 2002; Lanner and Connor 2001; Mencuccini et al. 2014). As in the case of *P. nigra*, some authors have established significant decreases in seed dry mass ( $DM_{1\text{seed}}$ ) coupled with increases in the number of empty seeds ( $E_{\text{seeds}}$ ) with plant age (Ganatsas et al. 2008; Müller et al., 2014; Tíscar and Linares 2011; Tíscar Oliver 2002). Nonetheless, these same authors claimed that either the differences found were very small in absolute terms, despite being significant, and were only detected for very elderly trees at lower altitudes, and not at the altitudes of this study, 1755–1953 m a.s.l. (Tíscar and Linares 2011; Tíscar Oliver 2002), or had no effect on seed viability or seed germination (Ganatsas et al. 2008; Müller et al. 2014). This suggests that there may be some interaction between the species' reproductive performance and its environment (see also Xu et al. 2016), but this does not apply to our study since all the mother trees were from the same site, with similar climate, elevation, and coordinates notwithstanding variation attributable to microsite differences. As no trees over 725 years

were sampled in this study, we are unable to extrapolate our findings to trees of more advanced ages.

With respect to the seed viability and germination capacity, the values obtained ( $VS_{fs} = 96\%$ ,  $GP = 76\%$ ,  $GV_{Cz} = 68$ ) also lay within the usual range for the species (Escudero et al. 1997; Lucas-Borja et al. 2011; Temel et al. 2011; Tíscar Oliver 2002). Analogous to the pinecone and pine seed sizes, the seed germination-related parameters evidenced significant between-tree differences yet were not related to the mother tree age. That is, there was no evidence of a decline in seed germination or seed viability in the oldest trees studied, as has been shown in other species (Ganatsas et al. 2008; Müller et al. 2014; Viglas et al. 2013). The positive correlation between the individual seed weight ( $DM_{1\text{seed}}$ ) and the germination capacity parameters (GE, GP, GV), despite presenting a moderate correlation coefficient ( $0.49 < r < 0.57$ ), have indicated a trend towards heavier seeds implying a more advanced state of development. The significant relationship between seed weight and germination parameters was not surprising, since larger seed size is generally, although not always, associated with more rapid germination (Xu et al. 2016). The latter association may be due to a greater amount of biochemical reserves, for example, proteins, involved in the germination process (Wahid and Bounoua 2013). However, our experiment was carried out under controlled conditions but, even though under natural conditions fast germination and emergence are essential for successful establishment, other seedling morphophysiological characteristics (e.g., growth rate, phenology, frost and drought resistance), as well as local conditions such as herbivores, weeds, and light, must be taken into account (Parker et al. 2006). In addition, the positive correlations of SDMF with  $N_{\text{seeds}}$  and GP indicate that pinecones whose weight portion contributed by the internal pine seeds was greater also tended to contain more seeds with a higher germination potential. This could be a consequence of the accumulation and activity of phytohormones, mainly auxins, during embryo and seed development (Holloway et al. 2016) that contribute to the pinecone growth when developing seeds are inside (Sauer et al. 2013).

Plant growth in the nursery, mortality, and root regeneration capacity during their first growing season were not correlated

**Table 2** Between-tree variability and effect of tree age on nursery parameters. EL, length of the epicotyl stem. SDW, shoot dry weight. RDW, root dry weight. TDW, total dry weight. SRDW, shoot-to-root ratio. RGC, total length of new roots

Variables	Covariance parameter estimates			Variance explained by tree effect (%)	$p (\sigma^2_t = 0)$	Tree age effect	
	Tree ( $\sigma^2_v$ )	Residual ( $\sigma^2_e$ )	Total			Age ( $p > F$ )	$Age^2$ ( $p > F$ )
EL	0.48	3.62	4.10	12	0.25		
SDW	176.62	460.11	636.73	28	0.02	0.71	0.6
RDW	157.16	1206.85	1364.01	12	0.27		
TDW	770.89	2176.96	2947.85	26	0.02	0.99	0.77
SRDW	0	0.03809	0.03809	0	1		
RGC	7.62	25.20	32.82	23	0.045	0.12	0.16
Survival		0.019				0.26	0.3

with the mother tree age, indicating that vigor differences were not influenced by age, at least during the first year of growth under controlled conditions. The lack of a between-tree difference in SRDW ratio points towards a similar tree biomass distribution strategy which is consequently more linked to the microsite environment (Parker et al. 2006), and less to the mother tree age or the individual. Nonetheless, the between-tree differences in SDW and root regeneration did indicate between-tree differences in vigor, likely linked to the trees physiological states reflected in the diverse reserve accumulations and growth rates (Fernández et al. 2007; Fernández et al. 2008), as neither was correlated with seed size. The root regeneration test is considered a good indicator for estimating the general plant physiological state and the plant quality index (Andivia et al. 2011; Davis and Jacobs 2005). Under favorable controlled conditions, seedling growth and biomass allocation can be more or less independent of seed size, but under unfavorable conditions (common under natural conditions) large seeds could have an advantage if they contain more biochemical reserves and produce larger seedlings (Parker et al. 2006; Xu et al. 2016). Tíscar Oliver (2002) as well as Tíscar and Lucas (2010) obtained a positive relationship between seed weight and plant biomass, possibly as they used weights of individual seeds or else of small pine seed groups in their analysis, either of which provide greater accuracy than tree averages as used in our study. In addition, seed weight differences between pinecones of the same tree can bring about additional variation, further masking the tree influence (Tíscar and Lucas 2010), and this may have occurred in our study. The plant sizes obtained at the end of the study lay within the normal range for the species (Tíscar Oliver 2002), except perhaps a little low for the epicotyl stem length, yet showed high values of biomass accumulation, especially with respect to the root system.

As a final consideration, in this study, we did not analyze the total cone production per tree or the masting behavior of the species in the area, or the synchronization of cone and seed production between trees. To do so, we would need to follow the tree cone production for several years. Such monitoring would provide interesting information about the synchronization of masting. If there is lack of synchronization between trees in seed production, this could be confounding the effect of mother tree age on reproductive ability.

## 5 Conclusions

For the *Pinus nigra* tree sample in an age range of 90 to 725 years, the mother tree age did not significantly influence the pinecone or pine seed size, seed germination capacity or plant size or vigor displayed during the first year of growth in the nursery. Therefore, a natural regeneration system's first requirements, namely, the production of viable seeds of sufficient quality, as well as the seed's ability to germinate and to produce potentially vigorous plants, were not affected by the

mother tree age, all of which should be taken into account with respect to the management and the administration of this natural *Pinus nigra* old-growth forest system.

Significant differences between trees were detected, evidencing either variability in genetic composition or a determined physiological condition present in the collection year or else of the microenvironment of the individual trees. The size of cones and seeds, and the seed viability and germination capacity, as well as the characteristics of the plants raised in the nursery all fell within the normal range for this species, despite having sampled trees of an advanced age. That is, no drop in reproductive vigor was detected and nor were symptoms of aging identified in the trees sampled. This constitutes valuable information for *Pinus nigra* old-growth forest management practices.

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**Data availability** The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflicts of interest.

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## Affiliations

Reyes Alejano<sup>1</sup> · Marta Domínguez-Delmás<sup>2</sup> · Ignacio García-González<sup>2</sup> · Tomasz Wazny<sup>3,4</sup> · Javier Vázquez-Pique<sup>1</sup> · Manuel Fernández-Martínez<sup>1</sup>

Marta Domínguez-Delmás  
mardodel@gmail.com

Ignacio García-González  
nacho.garcia.glez@gmail.com

Tomasz Wazny  
twazny@email.arizona.edu

Javier Vázquez-Piqué  
jpique@uhu.es

Manuel Fernández-Martínez  
manuel.fernandez@dcaf.uhu.es

<sup>1</sup> Agroforestry Sciences Department, University of Huelva, Campus El Carmen, 21071 Huelva, Spain

<sup>2</sup> Department of Botany, University of Santiago de Compostela, Rua Benigno Ledo s/n, Escuela Politécnica Superior, Campus de Lugo, 27002 Lugo, Spain

<sup>3</sup> Nicolaus Copernicus University, Institute for the Study, Conservation and Restoration of Cultural Heritage, ul. Sienkiewicza 30/32, 87-100 Toruń, Poland

<sup>4</sup> Tree Ring Lab, University of Arizona, 1215 Lowell St., Tucson, AZ, USA