The maternal environment determines the timing of germination in
Pinus pinaster

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Abstract

Optimizing the germination timing is crucial for the establishment of new generations. We hypothesized that environmental maternal effects may be relevant in the fine tuning of this trait in a long-lived Mediterranean model tree. We analyzed the influence of maternal genotype, maternal environment and their interaction on the germination success and germination phenology of 8725 *Pinus pinaster* seeds collected from genotypes clonally replicated in two contrasting environments. Besides maternal genetic effects, the maternal environment significantly affected both the percentage and the timing of germination. Seeds from the more favourable environment germinated 7.5 days earlier and showed higher germination rate (0.93 ± 0.01 vs 0.85 ± 0.03). Seed weight significantly influenced germination time, but seed weight differences between maternal environments were not enough to explain this form of transgenerational plasticity. The effect of the maternal environment varied depending on the genotype, indicating that genetic variation in the sensitivity to the maternal environment in this pine species does exist.

Highlights: > The maternal environment influences both the percentage and germination time > Favourable maternal environment favoured and speeded pinion germination > Differences were not explained by seed weight effects > Environmental maternal effects were genetically variable

Key-words: Adaptive traits; Carryover effects; Conifers; Forestry; Long-lived plants; Maritime pine; Maternal environmental effects; Seed weight; Seed size; Transgenerational plasticity

1. Introduction

Optimizing the timing of germination is vital for the establishment of an individual, especially in areas with strong seasonal variability (Baskin and Baskin, 1998; Castro, 2006). Among other limitations, germinating too early may expose the immature seedling to late frost injuries, whereas delaying germination may reduce the early competitive ability of the resulting offspring (Donohue et al., 2010). Plant populations have evolved to fine tune the timing of germination accordingly to these and other selective pressures. Environmental gradients within the distribution range of a plant species have commonly led, thus, to large among-population differentiation in this adaptive trait (see references in Baskin and Baskin, 1998; Donohue et al., 2010; Wagner and Simons, 2009).

Additive genetic variation within populations provides the fuel for adapting the timing of germination to the current environmental conditions. In addition to this, there is emerging evidence that environmental maternal effects are also relevant influencing this process (Donohue, 2009; Donohue et al., 2010; Tielborger and Petru, 2010). Environmental maternal effects are defined as the direct contribution of the maternal environment to the offspring phenotype, irrespective of the chromosomal contribution of the parents (Roach and Wulff, 1987). In angiosperms, there is increasing evidence that environmental maternal effects affects both germination percentage (Alboresi et al., 2005; Donohue, 2009; Figueroa et al., 2010; Tielborger and Petru, 2010) and germination phenology (Biere, 1991; Lacey, 1996). For instance, in resource limiting environments, seeds of semi-arid Mediterranean plant species developed in favourable years were found to have higher proportion of dormant seeds (Tielborger and Petru, 2010). Delaying the germination of a fraction of the seed yield may help to reduce competition between siblings in the current year, and to increase the available seed bank

in following unfavourable years (Tielborger and Petru, 2010). Environmental factors such as photoperiod and temperature during seed maturation have also been found to determine the environmental conditions needed to broke dormancy in *Arabidopsis thaliana*, and therefore significantly modulate germination timing (Boyko et al., 2010; Donohue et al., 2008). Moreover, it is well known that the environmental conditions where the mother plant grows may largely influence the size of the seeds, which in turn may influence germination timing and success (Castro et al., 2006). Identifying the environmental, genetic and epigenetic mechanisms involved in the transmission of maternal environmental effects through the seeds is important to understand their ecological function and adaptive value. This field is becoming a prolific research line in angiosperms (Boyko and Kovalchuk, 2011; Gehring et al., 2009; Mirouze and Paszkowski, 2011; Mosher and Melnyk, 2010) but available information for long-lived woody species such as gymnosperms is much more limited (Dolgosheina et al., 2008; Yakovlev et al., 2010)

In conifers, the maternal environment is known to influence the offspring frost-hardiness (Johnsen et al., 1996; Lindgren and Wei, 1994), but there are only few examples of significant environmental maternal effects on germination phenology (Stoehr et al., 1998). Curiously, however, certain particularities of the reproduction in gymnosperms could favour their sensitivity to environmental maternal effects. In conifers, both the seed coat and the endosperm had maternal origin whereas in angiosperms both parents genetically contribute to form the endosperm (Linkies et al., 2010). Additionally, the period during which the seed remain connected to the mother is, in some genera such as *Pinus*, remarkably large, lasting up to two years in Maritime pine (*Pinus pinaster* Ait.) and even more in other species. Although the underlying mechanisms involved in the transmission of environmental maternal effects are largely

unknown, they are likely activated during this period (Donohue et al., 2008; Lacey et al., 1997; Roach and Wulff, 1987).

The main objective of this paper was to evaluate the influence of the maternal environment on the germination timing of *Pinus pinaster*. This pine species is an emblematic Mediterranean forest tree that occupies more than 4 million ha in southwest Europe and north Africa, and it is becoming a model species for genetic and ecological studies in the Mediterranean region (González-Martínez et al., 2004). We followed the germination timing of pre-weighted seeds collected from several genotypes clonally replicated in two contrasting environments, one with favourable conditions for the development of this pine species (in terms of growth and reproduction rate), and the other one with less favourable conditions (see Table 1). Our experimental design allowed to separate and quantify the influence of the maternal genotype, the maternal environment and their interaction, providing a quantification of the relative contribution of genetic and epigenetic effects on both the germination success and the germination timing, two relevant life-history traits that represent the main change within a plant cycle, the switch from adult to offspring.

2. Material and methods

2.1. Site location and plant material

Seed material was collected from two clonal seed orchards with exactly the same genetic material and experimental design but contrasting site qualities. Both seed orchards belong to the Galician Tree Breeding Program (Consellería do Medio Rural, Xunta de Galicia) and include 116 unrelated *P. pinaster* genotypes. These genotypes were selected in natural stands or mature plantations within the coastal area of Galicia (NW Spain, provenance ‘1a. Noroeste Litoral’ sensu Alía et al. (1996)). Within each

stand, individual trees were mass selected for their superior growth, stem form and branching habit by comparing its phenotype with that of the surrounding trees (see Zas et al., 2004 for details). Scions were collected in each selected tree using climbing tools and grafted on two-year old seedlings (height ~80 cm, basal diameter ~1.5 cm) by replacing the terminal bud. Each genotype was clonally replicated by grafting in 10 individual trees (ramets) within each site. The experimental layout in each site was a randomized block design with 10 blocks and one ramet of each genotype per block. The favourable seed orchard (Sergude, 42.82° N, 8.45° W) is located in a high quality site for *P. pinaster* development, with mild temperatures, adequate moisture all over the year, and well drained and deep soil (Table 1). The other seed orchard (Monfero, 43.52° N, 7.93°W) is located in the top of a hillside on a site especially harmful for *P. pinaster*. Pine growth and reproduction in this site are strongly limited by low winter and spring temperatures, extreme wind exposure, and water-logging and thin soils (Table 1). In average, individual pine growth (measured as the annual increment in basal area at breast height) was more than eight times higher in Sergude than in Monfero (Table 1). The reproduction effort (both at a common age and at a common tree size) was also much higher in Sergude. Based on the extreme differences in growth and reproduction rates we will refer to Sergude as the *favourable* maternal environment, and to Monfero as the *stressful* maternal environment.

2.2. Cone and seed sampling, processing and sowing

Ten genotypes, randomly selected among the 116 genotypes included in the seed orchards, were used in this study. In January 2007, four to six ramets of each of these genotypes were selected for sampling within each seed orchard. In each ramet, three to four cones were randomly selected within the tree at the stage of flowers (March 2007),

adequately labelled and allowed to be open-pollinated during spring 2007. In January 2009, after the regular maturation time for this species (two seasons), all the cones were carefully collected using ladders and climbing tools. Total sample size was 377 cones. Diameter at breast height was measured in all mother trees.

Cones were air dried for a few weeks and then oven-dried (7d at 35 °C) to induce opening. After this time, cones were weighed with a precision scale (0.001 g) and all the seeds were removed and stored in PET vials at 4 °C. For each individual cone, empty seeds were separated by decantation in cold water for some seconds, where empty seeds remained floating at the surface. Empty and filled seeds were immediately oven-dried again at 35 °C for seven days, and then the number and total weight of seeds per cone were recorded. Twenty four filled seeds per cone were randomly selected, individually weighed (0.0001 g), identified by their position in 96 wells microplates and stored at 4 °C. These identified seeds were individually sown on sterilized vermiculite (0.5 – 2.0 mm coarse), using 96 wells sowing trays with 9 cm tall wells. Trays were moisturized and disposed at random inside a glass greenhouse in October 2009 at temperatures fluctuating in the range of $22 \pm 4^{\circ}\text{C}$. Germination was individually assessed every second or third day during three months. Total sample size was 8990 identified seeds of known individual seed weight.

2.3. Statistical analyses

The effects of design factors on the percentage of filled seeds and the germination percentage per cone were analyzed with a generalized linear mixed model using the PROC-GLIMMIX procedure of the SAS System, assuming a binomial residual distribution. A hierarchical model with two levels of nested experimental units (ramets and cones) was considered (Littell et al., 2006). This model includes the fixed effect of

the maternal environment (the two twin seed orchards with contrasting site qualities), the random effect of the maternal genotype (the 10 genotypes clonally replicated in each seed orchard), and the random interaction between both factors, which represents the genetic variation in the transmission to the progeny of environmental maternal effects. The model also include the effect of blocks nested within each seed orchard, which accounted for the environmental variation within each seed orchard, and the random effect of ramets, accounting for micro-environmental variation at scales lower than the block size and other phenotypic effects of the ramets such as the effect of the root genotype in which the genotypes were grafted, ontogeny, biotic interactions, etc. Measures from different cones within the same ramet were considered as a within-subject factor (Littell et al., 2006). As cones were open-pollinated, any environmental and genetic paternal effects would be included into the residual term. Diameter at breast height of each tree was also included as a fixed covariate in this model in order to account for possible allometric effects, but it was removed because it was not significant and did not improve the resolution of the model.

The effects of design factors on germination time were analyzed using a linear mixed model with the PROC-MIXED procedure of the SAS System fitting a hierarchical model similar to those used to solve a split-split design with three levels of nested experimental units: ramets, cones and seeds (Littell et al., 2006). Both, values within a given cone, and values from different cones within a ramet were dependent measures within the same subject (cone or ramet, respectively). Besides the factors included in the former model and described above, the model also included the random effect of cones, the covariation of the germination time with the individual seed weight, and the interaction of the seed weight with the maternal environment, which accounted for the possible variation between environments in the seed weight covariation. Both

fixed covariates were highly significant and clearly improved the model. Diameter of the mother tree and cone weight were included as covariates in a former version of this model, but then removed as they did not improve the likelihood of the model. Both the ramet and cone effects were considered within-subject random effects, thus conforming together with the residual error term, the three experimental units of this hierarchical model (ramet, cone and seed).

The statistical significance of the variance components for each random factor in both statistical models was assessed using likelihood or pseudo-likelihood ratio tests (Littell *et al.* 2006), where the differences in two times the log-likelihood of the models including and excluding that random factor are distributed as one tailed χ^2 , with one degree of freedom.

Cumulative germination over time was modelled by fitting exponential and Gompertz models using nonlinear regression procedures in SAS. The time needed to reach the inflexion point (i.e. the time needed for germination of the 50% of the sowed seeds) was used to quantitatively compare the timing of germination between both environments.

3. Results

The maternal environment significantly affected both the percentage of germination (Table 2) and the emergence time (Table 3). Specifically, seeds from trees of the high quality site germinated more successfully (0.927 ± 0.013 and 0.854 ± 0.029 in the favourable and unfavourable maternal environments respectively) and around seven days earlier than those from the unfavourable maternal environment (Figure 1). The percentage of filled seeds did not significantly differ between the two maternal

environments (Table 2, 0.927 ± 0.01 and 0.922 ± 0.01 in the favourable and unfavourable maternal environments, respectively).

Germination time was genetically variable (Table 2), with average emergence time varying among maternal genotypes from 23.6 to 39.0 days since sown. The significant genotype by environment interaction also revealed that pine families varied in their ability to express maternal environments effects in their germination time (Table 2). Comparison of variance components for the G and G×E effects indicates that the variation among genotypes in this form of transgenerational plasticity was even higher than the genetic variation in the average germination time (Table 2). Differences between environments in the timing of germination ranged from 0.6 days for pinions of genotype G1 to 14.5 days for pinions of genotype G9 (Figure 2b). Germination time of seeds from the favourable maternal environment was shorter for all the studied genotypes (Figure 2b).

Percentage of filled seeds also significantly varied among maternal genotypes, but no genetic variation and just marginally significant G×E interaction were observed for the germination percentage (Table 2). While seeds from the favourable environment germinated earlier in all cases, the effect of the maternal environment in the percentage of germination showed contrasting trends, depending on the family (Figure 2a).

Germination time significantly covaried with seed weight (Table 2), with heavier seeds germinating earlier. The significant SW×E interaction revealed that the dependence of the timing of germination on the seed weight was not the same for the two maternal environments (Table 2). The slopes of the covariation between seed weight and germination time significantly differed between the two environments. The covariation for seeds from the unfavourable environment (slope in Monfero = -0.187 ± 0.021) was steeper than that for seeds from the favourable environment (slope in

Sergude = -0.026 ± 0.017). This result indicates that the effect of seed weight on germination time was more pronounced in the unfavourable environment.

4. Discussion

Our results showed that the maternal environment had a strong influence on both the germination viability and the timing of germination of *P. pinaster* pinions. Seeds from the stressful maternal environment germinated later, and the proportion of seeds that remained ungerminated at the end of the experiment was higher for this maternal environment. There is growing evidence that maternal effects are involved in the expression of key characters controlling the adaptation of populations to environmental conditions (Bossdorf et al., 2009; Donohue, 2009; Donohue et al., 2008; Donohue et al., 2010; Richards et al., 2010; Tarutani et al., 2010). Previous studies have also identified environmental maternal effects as a significant factor explaining the variation in germination timing and dormancy in different plant species (Biere, 1991; Donohue et al., 2005; Stoehr et al., 1998; Teixeira et al., 2009; Tielborger and Petru, 2010). Particularly, *Picea glauca* seeds from mother trees grown at colder conditions have been shown to germinate earlier and in greater proportion, and the resulting seedlings were more frost hardy (Stoehr et al., 1998). These last results contrast with the results reported here, in which seeds from mother trees grown in the colder environment germinated later and in lower proportion. This discrepancy may arise because the environmental factors that more critically limit seedling performance differ between the two studies. For example, under boreal climates, the plants may benefit from starting its development soon, as they would have more chance to exploit the short growing season (Bevington, 1986). Furthermore, differences between the maternal environments in the Stoehr et al. (1998) study also include differences in maternal photoperiod, which has

276 been reported to modulate offspring germination time (Donohue et al., 2005). Seeds
277 from the northern location were developed under shorter photoperiods, and thus could
278 tend to germinate earlier than seeds from the southern location when planted in a
279 common environment. In our case, latitude differences between the two maternal
280 environments were minimal and the photoperiod was almost the same in both sites.

281 Our results seem to agree with the idea that the delay in the germination of the
282 seeds collected in the colder environment can help the offspring to ‘escape’ from late
283 frost damage. This would be in the same line that several studies in boreal conifers that
284 have reported that environmental maternal effects are mechanisms for cold adaptation
285 (Andersson, 1994; Johnsen et al., 2005; Lindgren and Wei, 1994; Webber et al., 2005).
286 Johnsen et al. (2005) observed that mother trees of *Picea abies* subjected to photoperiod
287 and temperature conditions of higher latitudes during embryo development and seed
288 maturation lead to offspring phenotypes with biological rhythms adapted to cold.
289 However, it should be noted here that the environmental differences between the two
290 maternal environments of our study are not limited to the temperature regime, but also
291 include large differences in other characteristics such as edaphic properties or wind
292 exposure that have strong influences in primary productivity and tree growth (Table 1).
293 All these environmental factors are also known to be potential drives of
294 transgenerational plastic responses (e.g. Latzel et al., 2010). Besides, adaptation to late
295 spring frosts is probably not the main driver of the transmission of maternal effects
296 under Mediterranean conditions, although it cannot be discarded because of the
297 biogeographic history of this species during glacial periods.

298 Although our results suggested strong environmental maternal effects on
299 germination time and viability, different male parent donors in the two environments
300 may have also contributed to genetically differentiate the seeds in the two maternal

environments. Several reasons led us to think, however, that environmental maternal effects prevail upon male genetic effects. Firstly, the most plausible source of parental differences may arise from contamination by foreign pollen, which is very unlikely, as both environments are controlled seed orchards, i.e. plantations designed for seed production that are established further away from other *P. pinaster* stands. Pollen introgression is, thus, assumed to be low. Secondly, both seed orchards include exactly the same genetic material and have the same spatial designs, so spatial differences in the male gametic contribution within each plantation are likely to be low. Thirdly, although variation in reproductive phenology and allocation could contribute in part to imbalance the parental contributions within each orchard (Zas et al., 2003) the reproductive effort of *P. pinaster*, although plastic, has been shown to present low genotype $\times$ environment interaction (Santos-del-Blanco et al., 2010), so the average pollen contribution in both seed orchards is expected to be similar. Finally, and more importantly, the seed tissues that most influence germination (the coat and the endosperm) are completely maternal in gymnosperms (Linkies et al., 2010). Specifically, all hormones and provisioning transcripts regulating germination are directly transmitted from the mother (Nakabayashi et al., 2005). Indeed, it is widely assumed that the female genetic contribution has much more influence on germination than the male contribution (Baskin and Baskin, 1998; Donohue, 2009; Roach and Wulff, 1987). Despite all these convincing explanations about the relevance of environmental maternal effects on the observed differences in germination between the two maternal environments, further experiments with control-pollinated seeds are necessary to confirm the results presented here.

The contrasted climatic conditions between the two maternal environments may have also favoured differences in cone and pinion maturation. The observed differences

in germination time of the seeds from the two environments could thus be explained, at least in part, by differences in seed maturity between environments. However full embryo maturation in *Pinus* is typically complete in the second autumn after fertilization (Keeley and Zedler, 1996). Particularly, *P. pinaster* embryos in Portugal, not far from our seed orchards, has been reported to be full matured at the end of the second summer (Miguel et al., 2004). Because we harvested the cones in January, long after the complete maturation of the seeds, differences in embryo maturation between the two maternal environments in our study can be discarded.

Another confounding factor that could have differentially affected the germination of the seeds from the two maternal environments is the possible differential effect of the rootstocks in the two seed orchards. The genotype of the rootstock is known to potentially influence the performance of the grafted scion, affecting, among others, its growth and reproduction, disease resistance, drought tolerance, or fruit quality (reviewed for conifers by Jayawickrama et al., 1991). Particularly, because rootstock can modulate the genetic expression patterns of the scions (Jensen et al., 2010) it could potentially affect the epigenetic transmission of maternal effects through the seeds. However, the effect of the rootstock is commonly only relevant when comparing very different rootstock genotypes, even of different species (Jayawickrama et al., 1991). In our case, we used as rootstocks relatively homogeneous commercial material from the same provenance. Rootstock effects are, thus, expected to be low. Furthermore, the same rootstock material was used in the two seed orchards so we can assume that rootstock effects are unlikely contributing to differentiate the seeds from the two maternal environments.

According to Galloway and Etterson (2007), maternal effects are adaptive if i) the maternal environment influences the offspring phenotype, ii) the effect of the

maternal environment is genetically based, and iii) the transgenerational effects enhance the fitness of the progeny under environments similar to the maternal environment. Our results support with enough empirical evidence the first of these arguments, i.e. the maternal environment does significantly influence the germination of *P. pinaster* seeds. Additionally, the significant and large G×E interaction supports that within population genetic variation in the offspring sensitivity to the maternal environment does exist, and thus, these plastic responses can evolve enhancing the adaptation of populations to environmental variation. Results reported here do not allow, however, to determine whether the observed maternal effects increase offspring fitness. Further reciprocal transplant experiments are needed to demonstrate the adaptive value of the observed maternal effects (e.g. Galloway and Etterson, 2007).

Seed weight is a character of great evolutionary importance (Moles et al., 2005; Ramirez-Valiente et al., 2009) and it is known to be strongly influenced by the maternal environment (Linkies et al., 2010). Many studies have found that the significant effect of the maternal environment on germination time was, in fact, driven by differences on seed weight (reviewed in Castro et al., 2006). In our case, the germination time significantly covariates with seed weight, with bigger seeds tending to germinate earlier. This trend, which was more apparent for the stressful environment, has been previously observed, and interpreted in terms of the increased chance of embryo development within seeds with higher reserves (Castro et al., 2006). Nevertheless, although seed weight appeared to affect germination, differences in the timing of germination between seeds obtained in the two maternal environments were not only due to differences in the seed weight between the two sites. The effect of the maternal environment remained highly significant even when the statistical model properly accounted for the seed weight covariation. Our results suggest, thus, that other

mechanisms not related to seed size must be involved in the transmission of the observed maternal effects regarding germination traits. Nowadays, there is increasing evidence that epigenetic mechanisms, including changes in the frequency of homologous recombination, changes in DNA methylation patterns, micro and small RNAs, transposons and histone modifications, are involved in the transgenerational plastic responses to stress conditions (Boyko and Kovalchuk, 2011; Mirouze and Paszkowski, 2011). Additionally, direct environmental effects on the accumulation of metabolites, proteins and/or mRNAs in the seeds can also play a significant role in the transmission of maternal effects. Our experimental approach does not allow, however, to discriminate whether this heritable or non-heritable transgenerational effects (sensu Boyko and Kovalchuk, 2011) are prevailing in our case.

In conclusion, this work reports significant environmental maternal effects on the germination time and the viability of seeds of a temperate conifer species. Although different male genetic contributions may be also affecting germination, our results suggest that environmental maternal effects are significant drivers of these highly important adaptive traits in *P. pinaster*. Additionally, although germination timing was highly influenced by the seed weight, seed weight differences between the two maternal environments were not enough to explain the observed differences in germination timing. Our results indicate, thus, that other epigenetic mechanisms rather than the maternal quantitative investment in seed size may be involved in the transmission of this form of transgenerational plasticity. Genetic variation in the sensitivity to the maternal environmental variation for both the timing of germination and the viability of seeds constitute the basis for the evolution of these maternal effects as adaptive traits in this Mediterranean pine species.

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Table 1. Climatic, edaphic and dasometric characteristics of the two maternal environments of contrasting site quality, the high (Sergude), and the low quality clonal seed orchards (Monfero).

	Monfero	Sergude
Altitude (m)	615	258
Mean annual temperature (°C)	10.6	13.2
Maximum temperature (°C)	35.3	38.7
Minimum temperature (°C)	-8.0	-4.7
Mean temperature of the warmest month (°C)	15.5	19.2
Mean temperature of the coldest month (°C)	5.9	7.8
Number of frost-free months ^a	3	5
Annual precipitation (l m ⁻²)	1435	1445
Daily average wind speed (m s ⁻¹)	5.2	3.2
Number of windy days per year ^b	166	35
Soil pH (H ₂ O 1:2.5)	4.5	5.1
Soil depth (cm)	45.1 ± 3.2	> 120 cm
Tree age at sampling	20	27
Mean tree diameter at breast height (cm)	6.1 ± 0.3	20.9 ± 0.6
Annual individual tree growth in basal area (cm ² yr ⁻¹)	1.65 ± 0.15	13.6 ± 0.8
Number of cones per tree at age 9	3.8 ± 0.2	76.5 ± 11.0
Reproductive allocation (cones dm ⁻²) ^c	15.8 ± 2.8	47.8 ± 4.1

^a *sensu* Emberger et al. (1963) i.e. the period during which the average minimum temperature is over 7° C

^b average wind speed > 5 m s⁻¹

^c Number of cones per unit of basal area at breast height (modified from Climent et al., 2008)

Table 2. Results of the generalized mixed models for the analysis of the proportion of filled seeds and the germination rate of filled seeds. Degrees of freedom (DF) and F -ratios are shown for fixed effects; variance components (VarComp) and associated χ^2 values showing their statistical significance are shown for random factors. Both seed orchards have exactly the same genetic material, clonally replicated by grafting, and follow the same spatial design. Significance ($P < 0.05$) P values are typed in bold.

Effect	Filled seeds %			Germination %		
	DF or VarComp	F or χ^2	P value	DF or VarComp	F or χ^2	P value
Fixed factors						
Maternal environment [E]	1, 9	0.04	0.8542	1, 9	5.48	0.0439
Block(E) ^a	15, 68	1.09	0.3842	15, 68	0.86	0.6722
Random factors						
Mother genotype [G]	0.01 ± 0.11	2.82	0.0465	0.02 ± 0.16	0.27	0.3017
G × E	0.17 ± 0.15	2.12	0.0727	0.24 ± 0.22	1.97	0.0802
Ramet [G × B(E)]	0.64 ± 0.13	1860.9	<0.0001	0.86 ± 0.18	452.0	<0.0001

^a Block was nested within seed orchards

Table 3. Results of the general mixed models for the analysis of the emergence time. Degrees of freedom (DF) and *F*-ratios are shown for fixed effects; variance components (VarComp) and associated χ^2 values showing their statistical significance are shown for random factors. Both seed orchards have exactly the same genetic material, clonally replicated by grafting, and follow the same spatial design. Significance ($P < 0.05$) *P* values are typed in bold.

Effect	Emergence time		
	DF or VarComp	<i>F</i> or χ^2	<i>P</i> value
Fixed factors			
Maternal environment [E]	1, 67	378.18	<0.0001
Block(E) ^a	15, 67	1.38	0.1832
Seed weight [SW]	1, 7201	64.32	<0.0001
SW × E	1, 7201	37.55	<0.0001
Random factors			
Mother genotype [G]	5.8 ± 5.7	18.6	<0.0001
G × E	7.7 ± 4.8	10.9	0.0005
Ramet [G × B(E)]	10.9 ± 2.3	123.4	<0.0001
Cone(E × G × B) ^a	3.4 ± 0.6	69.0	<0.0001
Residual	71.6 ± 1.2		

^a Block was nested within seed orchards, and cones were nested within ramets [G × B(E)]

Figure 1. Germination progression of seeds collected in ten genotypes clonally replicated in two contrasting environments, one especially favourable for pine growth (black dots) and the other suffering multiple abiotic stresses (open circles). Dashed lines denote the time needed for germination of the 50% of the sowed seeds. A Gompertz model was fitted for each series. Sample size was 4628 and 4097 for the favourable and unfavourable environments, respectively.

Figure 2. Germination percentage and time to emergence of seeds of *P. pinaster* collected from ten maternal genotypes clonally replicated in two contrasting environments, one especially favourable for pine growth (black bars) and the other suffering multiple abiotic stresses (white bars). Means $\pm$ s.e. are shown.