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Influence of tree age on seed germination response to environmental factors and inhibitory substances in *Pinus pinaster*

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Abstract. The present study analyses the reproductive behaviour of *Pinus pinaster* at different ages of the parent trees after subjecting the seeds to variations in environmental factors and inhibitory substances. An experiment was designed in which seeds from mature (43 years old) and young (13 years old) trees were subjected to different environmental factors: darkness, thermal shocks and the presence of inhibitory substances from the needles and the undergrowth humus of *P. pinaster*. A control treatment was also carried out for the seeds of each age. The results obtained indicate that the age of the parent trees has a significant effect on mean germination time after experimental treatments: shock temperatures, darkness, and leaf and humus exudates. Seeds from young populations have shorter mean germination times than those from adult populations. Similarly, mature *P. pinaster* populations show more diversity in the length of innate dormancy of their seeds than younger populations. However, age has no significant effect on total percentage germination. Significant reductions in germination were observed when seeds from mature and young trees were exposed to temperatures equal to or above 150°C for 5 min. Increased temperature also produces a delay in the onset of germination in both populations.

Additional keywords: germination; inhibitory components; photoperiod variations; seed age; thermal shock; viability.

Introduction

Maritime pine (*Pinus pinaster* Aiton) is an important species in the Mediterranean basin and is found throughout the western ranges, from France to Morocco and from Portugal to Tunisia. In Spain it covers 7.3% of forested land according to the Second National Forestry Inventory (Ministerio de Medio Ambiente y Junta de Castilla y Léon 1986–1996). Due to its high resin content this species is one of the most pyrophytic species among the Spanish conifers (Vélez 2000). As a result, fires frequently affect populations of this species. In addition, the number of different races of *P. pinaster* recognised in the Iberian Peninsula indicate that these populations have diverged significantly from their common origins. The Iberian populations belong to seven of these geographical races (Salvador *et al*. 2000). This indicates the extremely wide variety of ecological conditions under which they can develop – soils ranging from calcareous to sandy and climates from Mediterranean to Atlantic – resulting in specific adaptations in terms of growth patterns and survival (Alía *et al*. 1995). For this reason it is difficult to extrapolate the results from research in different areas.

Local populations (in NW Spain) have special adaptations to frequent disturbances such as fire. These adaptations include the capacity to bloom and bear fruit at an early stage, in addition to possessing a canopy seed bank and being capable of storing fertile seeds in the closed cones for up to 50 years (Tapias *et al*. 1998). The presence of these three characteristics is a function of both the population age and the zone of origin, in response to the local frequency of fires.

Pinus pinaster is an obligate seeder species (Martínez-Sánchez *et al*. 1995; Keeley and Zedler 1998) that depends on both its canopy and soil seed banks to regenerate. The advantage of germination is that it increases the genetic variability and stability of the populations (Baskin and Baskin 1998). Different environmental factors, such as the availability of light (Bonnet-Masimbert 1975; González 1993; Baskin and Baskin 1998) and temperature variations (Trabaud and Oustric 1989), affect seed germination. Many authors share the idea that seed viability and germination are influenced by the high temperatures reached during fire (Martínez-Sánchez *et al*. 1995; Reyes and Casal 1995; De las Heras *et al.* 1997; Saracino *et al*. 1997; Núñez and Calvo 2000). As occurs in

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other Mediterranean species, germination can also be limited by the presence of chemical substances that act as inhibitors (Gallet 1994; Pellissier 1994). Thus, leaf exudates from different species that are characteristic of the Mediterranean environment have an inhibitory effect on the germination of their own seeds (Chaves and Escudero 1997; Li and Romane 1997; Peñuelas and Llusiá 1998; Robles *et al.* 1999). López Mosquera and Guillén (1993) mention the allelopathic properties of *P. pinaster*, not only in the bark, which acts as an inhibitor of various species of herbs, but also in the needles. Fernández de Simón *et al.* (2001) have characterised the range of terpenes and acids present in the needles of *P. pinaster*, which are responsible for inhibiting seed germination in the undergrowth of mature forests. Substances such as phenolic compounds (Gallet and Pellissier 1997), which can act as germination inhibitors, are also produced in the acicular decomposition process. Some authors also point out that the germination of *P. pinaster* is affected by the year in which the seeds mature (Giannini *et al.* 1983; Prada 1992) and their size (Kandya and Ogino 1986; Escudero *et al.* 2000; Reyes and Casal 2001). Previous studies of the *P. pinaster* stands in the study area have shown that seed size is smaller in young pines (size: 8.98 ± 0.52 mm; weight: 67.88 ± 9.97 mg) than in adults (size: 6.36 ± 0.35 mm; weight: 32.04 ± 6.09 mg) (R. Alonso, L. Valbuena and L. Calvo, unpublished data). The age of the forest could therefore have an effect on the production of viable seeds and on their germination, as field observations have shown that a larger number of seedlings appear below the young stands than in the adult undergrowth (personal observation).

In the present study we examine the hypothesis that population age of *P. pinaster* affects germination response to darkness, thermal shock and inhibitory substances from the leaves and undergrowth humus of a *P. pinaster* stand.

Materials and methods

Seed material

The biological materials used in this study were seeds of *Pinus pinaster* Aiton. The seeds were collected in two nearby *Pinus pinaster* stands in the Sierra del Teleno, SW León province (UTM 29TQG2984), at an approximate altitude of 1100 m. One stand had mature trees (43 years old) and the second stand had young trees (13 years old). The two stands were characterised by acid brown soils of sandy texture with a very stony C horizon. Both were characterised by a Mediterranean climate with 2–3 months' summer dryness and annual precipitation that varied between 650 and 900 mm (Rivas-Martínez 1987). These two stands were in close proximity to one another and had similar site indices, soils and exposure. They were selected in order to assume that the weather conditions, site indices and soils were equal.

Seeds were collected in October 2002, when the pine cones had matured as premature collection would have led to reduced germination rates (Catalán Bachiller 1991). Collection was carried out at the same time in both zones to avoid effects due to the annual variability of environmental conditions on seed characteristics (Molina *et al.* 1997). The pine cones were opened by placing them in a dry air oven at 45°C. This temperature was chosen in accordance with the bibliographic information available on the opening of *P. pinaster* serotine cones (Tapias and Gil 2000; Tapias *et al.* 2001; Reyes and Casal 2002*a*). The temperature used to open the cones (45°C) has been shown not to affect the germination response of *P. pinaster* seeds (Reyes and Casal 2002*b*, 2002*c*). The resin sealing the scale joints melts at this temperature, thus releasing the seeds. The seeds were stored in open paper bags, which permitted ventilation, and at laboratory room temperature in a dry place until they were used.

Germination tests

The *P. pinaster* leaves and litter used in the autotoxicity treatments were collected from *P. pinaster* populations located in the Sierra de Teleno.

In order to analyse the effect of darkness, thermal shocks and inhibitory components on germination, a method used widely by various authors (Trabaud and Casal 1989; Tárrega *et al.* 1992; Martínez-Sánchez *et al.* 1995; Escudero *et al.* 1999) was employed. A total of 100 seeds per treatment were placed on Petri dishes. There were four replicates of 25 seeds for each treatment.

The *P. pinaster* seeds from different-aged trees (young and mature) were subjected to the following experimental treatments:

- Complete darkness: the Petri dishes were completely covered by aluminium foil, so that no light could enter and were watered with demineralised water.
- Thermal shocks: the seeds were exposed to high temperatures for short periods of time to simulate the action exercised by fire under natural conditions. The *P. pinaster* seeds were subjected to the following combinations of temperature and exposure time using a dry air oven: 70°C, 90°C, 110°C, 150°C and 200°C for two exposure periods of 1 and 5 min.
- Effect of leaf exudates: the seeds were watered with an aqueous extract obtained by macerating *P. pinaster* leaves. The aqueous extract of *P. pinaster* leaf exudates was obtained by placing the leaves in a receptacle with cold distilled water and allowing them to macerate for 48 h at ambient temperature, using the method proposed by Sánchez (2001). The pH of these exudates was 6.5.
- Effect of humus exudates: the seeds were watered with an aqueous extract of humus obtained by macerating underground humus from *P. pinaster*. The aqueous extract of *P. pinaster* humus exudates was obtained by placing *P. pinaster* undergrowth humus in a receptacle with cold distilled water and allowing them to macerate for 48 h

at ambient temperature, using the method proposed by Sánchez (2001). The pH of these exudates was 5.

- Control: the seeds were placed on Petri dishes in the germination chamber with a 15 h light/9 h dark photoperiod. The seeds were watered with demineralised water.

Immediately after treatment the seeds were sown in 8.5-cm diameter Petri dishes on four layers of filter paper saturated with demineralised water. The dishes were placed in a controlled environment cabinet at a temperature of 20°C ± 1°C with 15 h light/9 h dark photoperiods. A temperature of 20°C was used, comparable with other germination studies where temperatures have varied between 20°C and 23°C (Trabaud and Oustric 1989). The seeds were examined every week. A seed was considered to have germinated when the radicle could be seen with the naked eye (Côme 1970). The experiment was continued for 70 days, using the method proposed by Martínez-Sánchez *et al*. (1995), according to which the experiment ends after ~15 days without germination.

Before carrying out these treatments, viability assays were also carried out using the Tetrazolium test (Besnier Romero 1989) on a total of four replicates of 25 seeds for each replicate, which was a total of 100 seeds for the young population and another 100 for the adult population.

Statistical analysis

The total percentage viability was calculated for each population using the data obtained in the Tetrazolium test. The total germination percentage and the mean germination time were also calculated for each of the experimental treatments.

The average germination time was estimated using the following expression:

$$t_m = \frac{N_1T_1 + N_2T_2 + \cdots + N_nT_n}{N_1 + N_2 + \cdots + N_n},$$

where N_1 is the number of seeds that germinated during the time T_1, N_2 the number of seeds that germinated between time T_1 and time T_2, and so on (Côme 1970).

The effects of heat shock temperature and duration on the number of seeds that germinated from different population ages were analysed by a factorial analysis of variance, including temperature, duration and tree age as factors and germination as the dependent variable. Similarly, the effects of dark, leaf exudates and humus exudates on the number of seeds germinating from the different-aged population were analysed by factorial analysis of variance considering treatment and tree age as factors and germination as the dependent variable. Finally, a two-way analysis of variance (treatment and age) was carried out to check for the existence of significant differences in the mean germination time between treatments and in terms of tree age. The Tukey test was performed to determine the significance of the differences. All statistical analyses were carried out using the statistical package Stastistica '98 Edition. Before analysis, sampling normality was checked using the David test (David *et al*. 1954) and the homogeneity of variances was assessed using the Cochran test (Cochran 1941). For statistical analyses, data expressed as germination percentage were arcsine transformed before analysis and the average germination time data were transformed using log (mean germination time).

Results

The *P. pinaster* seeds showed 98% viability for the adult population and 97% for the young population. This means that all of the seeds collected in the field were able to germinate and that possible delays in germination may be due to the existence of factors extrinsic to the seed itself. There were no appreciable effects on viability due to age.

Global germination percentages

The global percentage germination of the *P. pinaster* seeds was relatively high in both the mature (Fig. 1*a*,*b*) and young (Fig. 1*c*,*d*) populations, exceeding 80% germination in most treatments. Germination after thermal shock (Fig. 1*a*,*c*) presented significant differences with regard to temperature ($F = 74.04$; $P < 0.05$) and exposure time ($F = 261.64$; $P < 0.05$). Thus, at temperatures equal to or above 150°C and exposure times of 5 min, germination was almost zero. There were no significant differences in comparison with the control when the temperatures or exposure times were low. Tree age did not significantly affect the percentage germination after thermal shock ($F = 0.82$; $P > 0.05$).

When the percentage germination after the total darkness treatments, humus exudates and leaf exudates were compared (Fig. 1*b*,*d*), no differences were observed in terms of treatment ($F = 5.514$; $P > 0.05$) or age ($F = 1.946$; $P > 0.05$). However, watering with humus exudates significantly decreased the percentage germination in comparison with the control. This decrease was more marked in the adult population ($F = 4.12$; $P < 0.05$) than in the young population. No significant difference in the percentage germination was observed between the control and total darkness. In both cases high germination percentages (90%) were observed.

On comparing all of the experimental thermal treatments, significant differences ($F = 50.93$; $P < 0.05$) were only detected among the thermal shock treatments at 150°C for 5 min and 200°C for 5 min compared with the rest of treatments.

Average germination time

The mean germination time was significantly greater ($F = 260.606$; $P < 0.05$) in seeds from the adult population than from the young population (Table 1). Seeds from the mature *P. pinaster* population had a mean germination time of between 3 and 4 weeks in most treatments (Table 1). Seeds from the young *P. pinaster* population had lower mean germination time (between 2 and 3 weeks). There were also significant differences ($F = 7.155$; $P < 0.05$) between treatments.

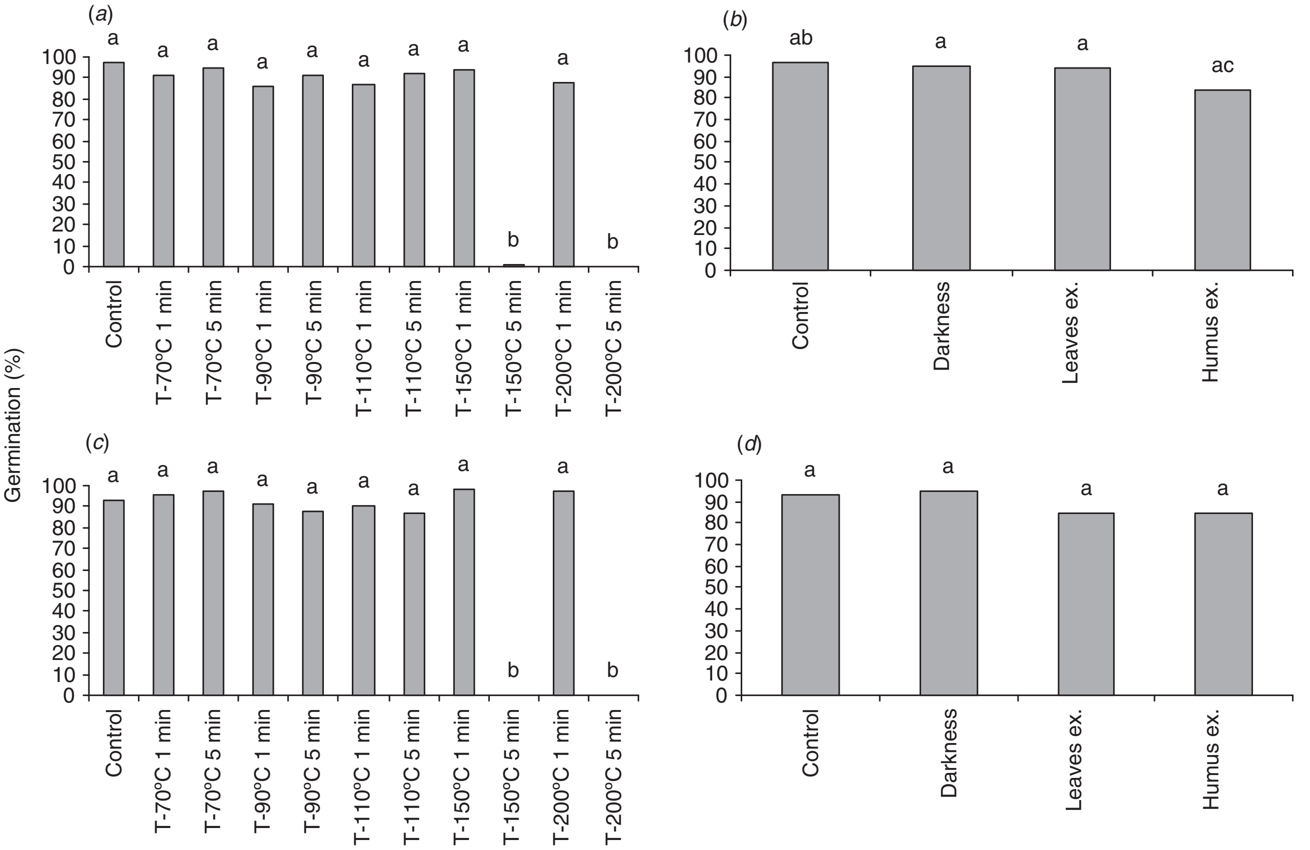

Fig. 1. Percentage germination of *Pinus pinaster* seeds in the control situation and after different treatments: darkness, inhibitory effects of leaf exudates (leaf ex.), inhibitory effects of humus exudates (humus ex.) and thermal shock treatments at 70, 90, 110, 150 and 200°C (exposure times 1 and 5 min) in the (*a*,*b*) mature and (*c*,*d*) young populations. Different letters indicate significant differences between treatments at 95% ($P < 0.05$).

Table 1. Average germination time (days) and standard error (s.e.) of *Pinus pinaster* seeds of different ages (young and mature) for each treatment

Treatment	Mature *Pinus pinaster*		Young *Pinus pinaster*	
	Average	s.e.	Average	s.e.
Control	18.62	0.15	16.71	1.89
Darkness	25.97	2.70	19.25	4.32
Leaf exudates	25.43	2.58	17.06	2.27
Humus exudates	29.30	2.31	17.74	1.17
T-70°C 1 min	23.65	2.32	17.17	0.78
T-70°C 5 min	27.28	3.54	17.19	0.70
T-90°C 1 min	29.48	2.65	19.56	1.37
T-90°C 5 min	26.79	2.28	19.28	1.28
T-110°C 1 min	26.32	2.30	19.07	1.66
T-110°C 5 min	29.92	4.43	21.16	2.13
T-150°C 1 min	26.29	2.63	20.23	1.76
T-150°C 5 min	28.00	0.00	–	–
T-200°C 1 min	31.28	1.67	20.45	2.25
T-200°C 5 min	–	–	–	–

The mean germination time in both populations (mature and young) was increased after all of the experimental treatments in comparison with the control. In the young population, temperatures above 110°C significantly increased the mean germination times ($F = 2.34$; $P < 0.05$) in comparison with the rest of the experimental treatments. In the adult population, temperatures above 150°C significantly increased the mean germination time ($F = 6.16$; $P < 0.05$).

Temporal distribution of germination

The temporal distribution of germination was fairly concentrated during the first week for control, darkness and exudates in mature and young populations (Figs 2, 3). The thermal treatments delayed the onset of germination in both populations in comparison to control, darkness and washing with exudates.

In the mature *P. pinaster* population, the treatments placed the peak of maximum germination at 17 days. These peaks were sharper after thermal shocks, except where temperature and exposure time resulted in no germination. In the young *P. pinaster* population, the treatments placed the peak of maximum germination between days 11 and 21. These peaks were more pronounced than in the same treatments in the mature population.

It can be seen from the results that the mature *P. pinaster* population had a wider range of dormancy intensities, as

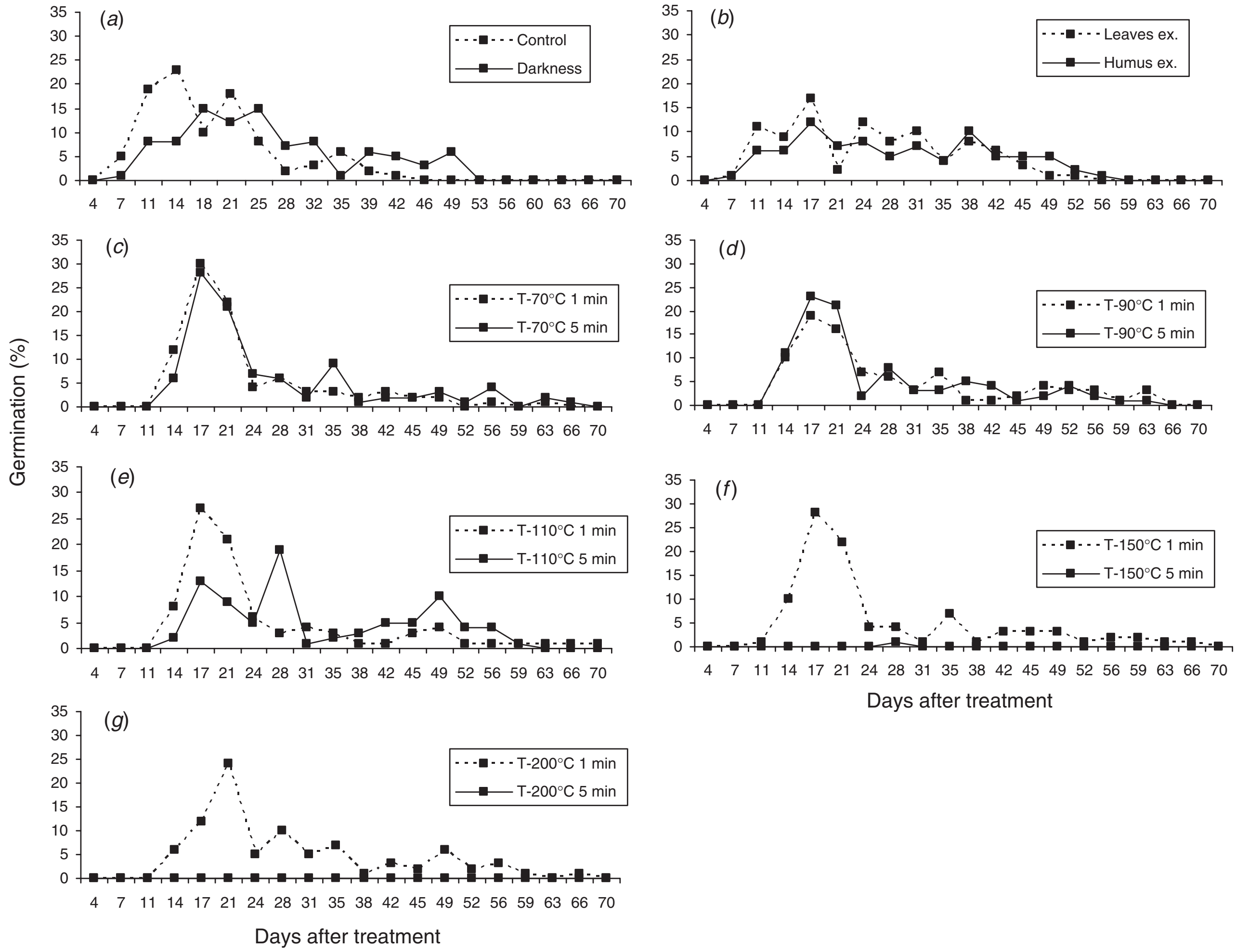

Fig. 2. Distribution of germination times of mature *Pinus pinaster* in each treatment: (*a*) darkness, (*b*) inhibitory effects of leaf exudates (leaf ex.) and humus exudates (humus ex.), and thermal shock treatments at (*c*) 70, (*d*) 90, (*e*) 110, (*f*) 150 and (*g*) 200°C (exposure times 1 and 5 min).

seed germination occurred over a prolonged period. After the thermal shock treatments germinations were observed from day 11 until approximately day 66 (Fig. 2). In the control, total darkness and washing with exudates treatments, germination occurred from day 7 until approximately day 56 (Fig. 2). However, the germination periods of the young seed population following thermal treatment were concentrated between days 11 and 38, while after the other treatments the germination period was between days 7 and 38. That is to say, the young population of seeds showed a smaller range of dormancy intensities. Therefore, population age affects seed germination over time, creating diversity in the length of innate dormancy.

Discussion

Pinus pinaster is an obligate seeder (Keeley and Zedler 1998) and so depends on its seeds to regenerate after any type of disturbance. The main reproductive strategy consists of storing a large number of seeds in serotine cones with high percentage viability (almost 100%) over long periods of time (Tapias *et al.* 1998; Sánchez-Fernández 1999; Tapias *et al.* 2001).

Kuhnholtz-Lordat (1938) considered the genus *Pinus* as pyrophytic because plants are able to regenerate rapidly after a fire due to the stimulation of germination in the soil seed bank. However, in the present study some very high germination percentages, similar to those for the control, were observed for *P. pinaster* for all of the thermal treatments, regardless of the population age. The seeds did not germinate more readily with increasing temperature or exposure time. It can be concluded from this that seed germination, from this present population, was not significantly stimulated by thermal shock, which corroborates the opinion of many authors that this species is not pyrophytic (Trabaud 1987; Martínez-Sánchez *et al.* 1995; Reyes and Casal 2002*a*; Torres Carretero 2002).

Another factor that can affect the germination of this species is the presence of terpenes and acids in both the needles and bark of mature populations. *P. pinaster* is characterised by the presence of allelopathic substances in both

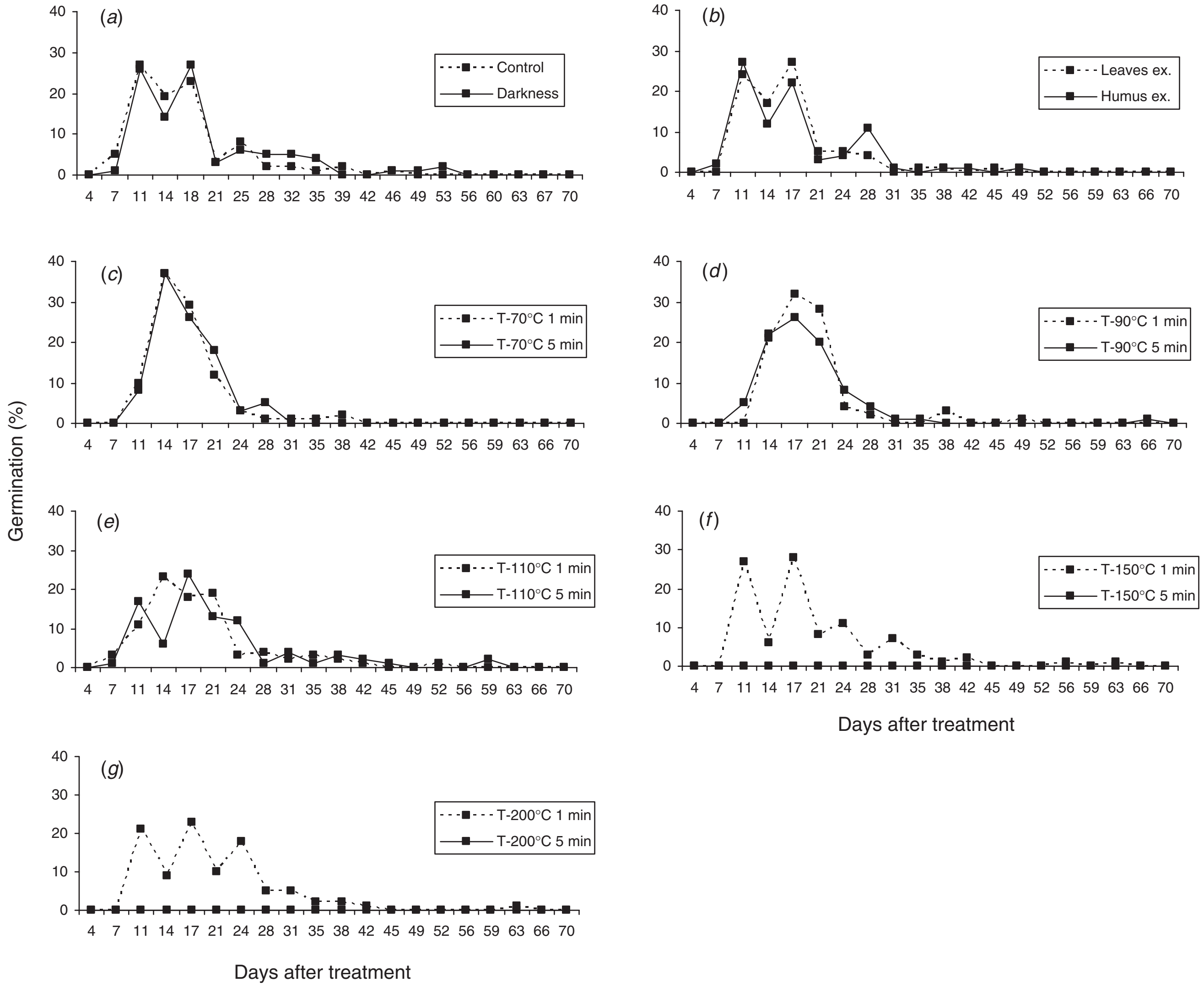

Fig. 3. Distribution of germination times of young *Pinus pinaster* in each treatment: (*a*) darkness, (*b*) inhibitory effects of leaf exudates (leaf ex.) and humus exudates (humus ex.), and thermal shock treatments at (*c*) 70, (*d*) 90, (*e*) 110, (*f*) 150 and (*g*) 200°C (exposure times 1 and 5 min).

its bark and its needles (López Mosquera and Guillén 1993; Fernández de Simón *et al*. 2001). These substances act as germination and growth inhibitors to some herb species found in the same habitat (López Mosquera and Guillén 1993). However, germination of its own seeds is not affected. Another source of seed germination inhibition is the presence of phenolic compounds that appear on humus decomposition (Gallet and Pellissier 1997). The substances present in the humus affect the *P. pinaster* seeds studied. This effect has been demonstrated for other coniferous species, such as *Picea abies* (Pellissier 1994).

Therefore, total percentage germination of the *P. pinaster* populations studied is not negatively affected by either darkness (as can occur in other species [González 1993]) or by the inhibitory effects of allelopathic substances from leaves of *P. pinaster*. They are, however, negatively affected by high temperature and exposure time, as has been demonstrated in other coniferous species by Thanos and Doussi (2000).

Some authors found that the percentage of germination for *P. pinaster* is affected by seed size (Kandya and Ogino 1986; Escudero *et al*. 2000; Reyes and Casal 2001). In the present study, comparing the effect of age, and therefore seed size, on a young population (smaller size) with an adult population (larger size) (R. Alvarez, L. Valbuena and L. Calvo, personal observation), no significant effects were observed on global percentage germination. However, the age of the parent trees had a significant effect on mean germination time. The mature population had a greater mean germination time than young trees from the same zone. This may be due to the fact that seeds from the mature population have greater physical dormancy; that is, the seeds are harder or more impermeable so they delay the first stage of germination (imbibing water) (Bradbeer 1988). Another possibility is that these seeds have greater mechanical dormancy: the seeds have a harder endosperm or head, thus delaying germination as these tissues mechanically resist embryo growth (Bradbeer 1988).

In general, after a fire, these seeds take a few days to start to germinate, allowing environmental conditions to become more suitable due to a reduction in competition and a possible decline in pressure from predators (Reyes and Casal 2002*b*). This confers an advantage to the mature population, which is recurrently subjected to fires. However, this population produces seeds with a wider range of dormancy intensities than those of young *P. pinaster* populations, so that germination of its seeds occurs over a wider range of days. This is an advantage to the plants trying to regenerate in seasons with irregular meteorological conditions, as is the case in the Mediterranean climate (Molina *et al*. 1997).

In addition to the age of the parent trees, all the experimental treatments in this study increased the mean germination time. The thermal treatments also affect the seeds by delaying the onset of germination in both the adult and the young populations. The seeds can respond to these thermal shocks with delayed dormancy. Prada (1992) related delayed dormancy to temperature and recorded that high temperatures at the end of summer induce higher percentage dormancy in *P. pinaster*, although these differences were only observed for trees less than 12 years old. Molina *et al*. (1997) also recorded this relationship between dormancy and temperature, and found that batches of mature *P. pinaster* seeds in the northern aspect of the crown (where lower temperatures are reached) reach maximum germination in a shorter time and that their accumulated germination is greater after 25 days. The fact that high temperatures delayed the onset of germination suggests an effective strategy for overcoming recurrent disturbances like forest fires (Molina *et al*. 1997).

Conclusion

In these *P. pinaster* populations there were no significant differences in the percentage of germination behaviour between a young and a mature population. Global percentage germination was not significantly affected by the experimental treatments of darkness, washing with leaf exudates or thermal shocks below 150°C for 5 min and 200°C for 5 min. Differences were only observed when the seeds were subjected to 150°C or more for at least 5 min and when they were watered with humus exudates. The main differences between the treatments was the mean germination time, which increased in the face of more unfavourable conditions, such as darkness, high temperatures or humus exudates.

In these *P. pinaster* populations, age had a significant influence on the mean germination time and on the length of the innate dormancy of seeds. The mature population had a longer mean germination time and a wider range of innate dormancy intensities. These characteristics would provide a great advantage in unfavourable environmental conditions, as the seeds would ensure that environmental conditions were suitable by taking a few days to germinate and dormancy variability also ensures regeneration in seasons with irregular meteorological conditions.

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