Seed germination and seedling performance of *Pinus pinaster* Ait. populations from Coastal Central Portugal

Sofia Corticeiro[1*], Diana Rodrigues[1], Glória Pinto[2,], Carlos Fonseca[2,3], Paula Maia[2]

Abstract. Natural regeneration is crucial to maintaining local adaptative genetic pools of *Pinus pinaster* Ait. forests and their restoration following disturbance events. After a wildfire, weak-serotinous maritime pine populations may only depend on the viability of seeds exposed to fire to recover. Subsequently, summer drought during the early stages of the pine seedlings may seriously jeopardize the success of natural regeneration and the productivity of natural exploitable forests. The main objectives of this work were 1) to determine and compare the seed germination rates of weak-serotinous maritime pine populations along a geographic gradient within the Coastal Center of Portugal and 2) to investigate the ability of non-selected natural regenerated pine seedlings to survive under water limitation conditions. To accomplish those aims, seeds were collected from 8 populations distributed along a 100 km geographical gradient and left to germinate for 2 months. The seedlings were submitted to a water limitation experiment for 3 weeks.
Overall no relation was found between seed weight and germination rate, but different trends were observed amongst populations. No relation was found between seed traits and seedling growth. Under water limitation, results suggested differences in water use efficiency between populations, possibly justified by adaptation to specific site conditions.
Keywords: Wildfire, Climate change, Water limitation, Germination rates, Weak-serotinous

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Germinação de sementes e desempenho de populações de Pinus pinaster Ait. da Costa Central de Portugal

Sumário. A regeneração natural é crucial para manter os reservatórios genéticos das florestas de *Pinus pinaster* Ait. e a sua capacidade de adaptação local no seguimento de eventos de perturbação. Depois de um incêndio, as populações de pinheiro-bravo com baixo nível de serotinia dependem apenas da viabilidade das sementes expostas ao fogo para a sua recuperação. Posteriormente, a seca durante as fases iniciais das plântulas de pinheiro-bravo pode comprometer seriamente o sucesso da regeneração natural e a produtividade das florestas. Os principais objetivos deste trabalho foram 1) determinar e comparar as taxas de germinação de sementes de populações de pinheiro-bravo com baixa serotinia ao longo de um gradiente geográfico no Centro Litoral de Portugal e 2) investigar a capacidade das plântulas de pinheiro regeneradas naturalmente, não selecionadas, para sobreviverem em condições de limitação de água. Para atingir estes objetivos, foram recolhidas sementes de 8 populações distribuídas ao longo de um gradiente geográfico de 100 km e deixadas a germinar durante 2 meses. As plântulas foram submetidas a uma experiência de limitação de água durante 3 semanas.
No geral não foi encontrada nenhuma relação entre o peso das sementes e a taxa de germinação, apesar de terem sido observadas diferentes tendências entre as populações. Adicionalmente, não foi detetada qualquer relação entre as características das sementes e o crescimento das plântulas. Sob limitação de água, os resultados sugeriram diferenças na eficiência da utilização de água entre populações, possivelmente justificadas pela adaptação às condições específicas do local.
Palavras-chave: Fogos florestais, Alterações climáticas, Limitação de água, Germinação, Serotinia

Germination des semences et performance de semis des populations de *Pinus pinaster* Ait. De la Côte Central du Portugal

Résumé. La régénération naturelle est essentielle au maintien des pools génétiques adaptatifs locaux des forêts de *Pinus pinaster* Ait. et à leur restauration après des perturbations. Après un feu, les populations de pin maritime faiblement sérotines peuvent dépendre uniquement de la viabilité des graines exposées au feu pour se rétablir. Par la suite, la sécheresse estivale durant les premiers stades des semis de pin peut sérieusement compromettre le succès de la régénération naturelle et la productivité des forêts naturelles exploitables. Les principaux objectifs de ce travail étaient 1) de déterminer et de comparer les taux de germination des graines des populations de pin maritime peu sérotines le long d'un gradient géographique dans le centre côtier du Portugal et 2) d'étudier la capacité des semis de pin régénéré naturel non sélectionnés à survivre dans des conditions de limitation d'eau. Pour atteindre ces objectifs, des graines ont été collectées dans 8 populations réparties le long d'un gradient géographique de 100 km et laissées à germer pendant 2 mois. Les semis ont été soumis à une expérience de limitation de l'eau durant 3 semaines.
Dans l'ensemble, aucune relation n'a été trouvée entre le poids des graines et le taux de germination, mais différentes tendances ont été observées parmi les populations. Aucune

relation n'a été trouvée entre les caractéristiques des graines et la croissance des semis. Sous limitation d'eau, les résultats suggèrent des différences dans l'efficacité d'utilisation de l'eau entre les populations, probablement justifiées par l'adaptation aux conditions spécifiques du site.

Mots-clés: Feu de forêt, Changement climatique, Limitation de l'eau, Taux de germination, Faible-sérotineux

Introduction

Pinus pinaster Ait. is a medium size conifer, broadly distributed in Portugal, Spain, France, Italy, and North Africa (ALÍA and MARTIN, 2003; VIÑAS *et al.*, 2016) with high economic relevance at regional and, in most cases, at national scales. This species includes several ecotypes highly adapted to a wide range of soil and climatic conditions with substantial intraspecific phenotypic and genetic variations (TAPIAS *et al.*, 2001; CHARCO *et al.*, 2017). Such variability is probably related to the great diversity of ecological environments under which these forests have evolved, resulting in different survival, growth, and reproductive strategies, as well as adaptations to disturbances, such as fire (ESCUDERO *et al.*, 2002; PAUSAS *et al.*, 2008).

High fire recurrence and severity are strong drivers for the decrease of pine forest areas (FERNÁNDEZ-GARCÍA *et al.*, 2019), a scenario that may aggravate as a consequence of the foreseen climate changes in the Mediterranean region (PAUSAS and KEELEY, 2009; VALBUENA *et al.*, 2019). A successful natural regeneration is crucial to maintain local adapted genetic pools and consequently to the re-establishment of forest related ecosystem services (CALVO *et al.*, 2016).

As an obligate seeder, maritime pine relies on the release and germination of canopy seed bank, mainly inside serotinous cones, to recolonize post-fire environments (PAUSAS *et al.*, 2004; PROENÇA *et al.*, 2010; MAIA *et al.*, 2012). Serotiny level is highly variable between individuals and populations (PAUSAS *et al.*, 2008), ranging from 2 to 82% of serotinous trees per population, in the Iberian Peninsula (TAPIAS *et al.*, 2001). High serotinous populations can retain viable seeds inside serotinous cones during decades, demonstrating higher probability to survive severe crown fires and to guarantee a successfully post-fire regeneration (resilience) (TAPIAS *et al.*, 2004). Weak serotinous populations developed alternative features to better cope with wildfire effects (resistance), like a thick bark or early flowering (TAPIAS *et al.*, 2001, 2004). Due to selective pressures, non-serotinous seeds may have increased seed mass and developed a thicker seed coat to improve embryo insulation (ESCUDERO *et al.*, 2000; ALÍA *et al.*, 2014). Previous studies demonstrated that weakly serotinous populations had heavier seeds with higher germination rates and produced taller seedlings when compared to highly serotinous populations (CALVO *et al.*, 2016). Weakly serotinous maritime pine populations often depend on the viability of seeds directly exposed to fire, to recover from the disturbance (MAIA *et al.*, 2012; RODRIGUES *et al.*, 2022). Local conditions, such as seed bed or post-fire soil surface conditions, play a determinant role in seedling emergence and establishment (REYES and CASAL, 2004; PAUSAS *et al.,* 2008; MADRIGAL *et al.,* 2010; MAIA *et al.,* 2012).

After recruitment, seedlings are under high selective pressure in natural conditions (SUÁREZ-VIDAL *et al.,* 2017), particularly in a burnt area, so adaptation to local conditions is an advantage to cope with abiotic and biotic stresses during the early stages, enhancing the probability of a successful establishment (ALÍA *et al.,* 2014). Summer drought during early development stages of seedlings is even more challenging in natural regeneration (DE LA MATA *et al.,* 2014; CHARCO *et al.,* 2017), especially to seedlings that germinated during spring. This is particularly true in Mediterranean regions, where the warmer and dryer periods coincide (FERNANDES *et al.,* 2017). Foreseen aggravation of summer drought (BARROS *et al.,* 2014) increases the threats posed to young seedlings (DE LA MATA *et al.,* 2014), with direct implications in the success of naturally established pine populations (DE LA MATA *et al.*, 2014; CHARCO *et al.,* 2017).

P. pinaster forest in the Central Coastal area represents a significant part of the maritime pine area in Portugal, highly relevant in terms of local provisioning, regulating and cultural ecosystem services (ALMEIDA *et al.,* 2002; FREIRE *et al.,* 2003; FABIÃO *et al.,* 2006; GUERRA, 2011; OLIVEIRA, 2014). Portuguese coastal maritime pine populations are generally classified as weakly serotinous (TAPIAS *et al.,* 2001), so natural regeneration after fire mostly occurs by germination of seeds that were able to maintain their viability after such disturbance. The dynamic between the number of viable seeds, the germination success and the initial seedling recruitment may be particularly challenging in preserving these populations under climate changes scenarios.

The present study presents a pioneer approach to characterize the potential of germination and establishment of non-selected seeds of 8 *P. pinaster* populations from the Coastal Center of Portugal. This work was delineated with the purpose of verifying two main aims 1) to determine and compare seed germination rate of weak-serotinous *P. pinaster* populations along a geographic gradient within the Coastal Center of Portugal and 2) to investigate the ability of non-selected natural regenerated pine seedlings to survive under water limitation conditions.

Methods

Study area and populations description

The study area comprises 40 years old *P. pinaster* stands selected amongst 8 populations, located along a geographic gradient of 100 km along Coastal Central Portugal (Figure 1): Mira (M), Tocha (T), Quiaios (Q), Osso da Baleia (OB), Praia de Pedrogão (PP), Praia da Vieira (PV), Leiria (L) and Marinha Grande (MG). This region belongs to the Mediterranean Csb climate zone, temperate climate with dry and mild summers, according to the Köppen classification (IPMA, 2020). Mean temperatures range from 13.3 to 15.9 º C (Table 1) and mean annual precipitation from 822 to 945 mm. (IPMA, 2020). These pine stands are established on dunes and sand dunes, coarse-textured soils (FABIÃO *et al.*, 2006; MARQUES, 2010; OLIVEIRA, 2014), from 15 to 108 m a.s.l.

Figure 1 - *Pinus pinaster* selected populations Mira (M), Tocha (T), Quiaios (Q), Osso da Baleia (OB), Praia de Pedrogão (PP), Praia da Vieira (PV), Leiria (L) and Marinha Grande (MG) distributed along the North-to-South geographic gradient within the study area

Table 1 - Mean characterization of the selected populations Mira (M), Tocha (T), Quiaios (Q), Osso da Baleia (OB), Praia de Pedrogão (PP), Praia da Vieira (PV), Leiria (L) and Marinha Grande (MG) distributed along the North-to-South geographic gradient within the study area

***P. pinaster* Population**	**Stand age (years)**	**Soil**	**Eleva-tion (m)**	**Mean annual precipi-tation (mm)**	**Mean annual tempera-ture (°C)**	**Presence of serotinous cones (closed and +3years)**		
						Trees (%)	**Cones *per* tree**	**Mean cone age (years)**
M	40	Dunes and sand dunes (coarse-textured)	15	845	13.3	6.7	2.1	4.2
T			21	945	15.2	10	1.5	3.5
Q			108	928	15.2	7.5	1.5	3.5
OB			15	900	15.4	0	0	-
PP			29	822	15.5	0	0	-
PV			19	822	15.5	0	0	-
L			63	790	15.9	0	0	-
MG			75	810	15.3	0	0	-

The studied maritime pine populations were mature plantations, even aged and approximately 40 years old, with no evidence of recent management operations or fires. From each population, 30 trees bearing male and female cones (year 0), 1-year old cones (mostly green, closed) and 2-year-old cones (mostly brown, some open) were sampled.

Serotiny was evaluated by considering the proportion of sampled trees that presented closed cones, still attached after maturation (cohorts ≥3 years old) and the age of the cones. At each site, 30 trees were sampled for serotiny. Cone age was estimated by counting the number of internodes (annual growth) from the tip of the tree in relation to the location of the cone. Overall, the trees did not present serotinous cones on the canopy, and the observed fallen cones were open, corroborating the low serotiny levels of these populations (TAPIAS *et al.*, 2004).

Cone sampling and processing

Cone sampling was done in the 8 maritime pine populations described previously. A minimum of 3 mature two-year old cones were collected per tree, on a minimum of 30 trees per population. Cones were dried during 2 to 3 days in a forced air circulation oven at 45° C, to melt the resin seal closing the scales and releasing the seeds without damage. Immediately after cone opening, seeds were manually extracted. 450 seeds were randomly selected from each site from a sub sample of 30 trees and 3 cones per population. This resulted in a sub-set of 15 seeds per tree, 5 seeds per cone. Seeds were randomly mixed within the same site, divided by sets of 30 seeds, 12 sets per site (each set corresponded to a replicate). Each replicate was weighted, and the average weight was calculated for each replicate (CALVO *et al*., 2016).

Seed germination trials

Seeds were soaked in deionized water for 16 hours (overnight). Floating seeds were discarded. Soaked seeds were placed into Petri dishes with a moist cotton disk bed. When necessary, cotton disks were re-moistened with deionized water. Seeds were left to germinate in a laboratory at room temperature (24/ 18 °C day/ night temperatures), under a 14h-10h photoperiod. Seeds were left to germinate for 2 months and were inspected on a daily basis. Once the radicle was > 1mm, germinated seeds were removed from the Petri dishes.

Seedling nursing and water limitation

The first 10 germinated seeds from each site were transplanted into individual 300 ml plastic pots, filled with a volumetric proportion of a 2:1 peat:perlite mixture and placed into a controlled air humidity and temperature growth chamber. The growth chamber was programmed with 16/8h light/dark photoperiod, 25/20 °C day/night temperature, 65%/60% day/night relative humidity, and 500 µmol m^{-2} s^{-1} photon flux density (AMARAL *et al*., 2019). Seedlings were watered to field capacity once a week and were left to grow for a period of 3 months for acclimatization. Once a month, the seedlings were supplied with a standard NPK nutritional solution (12:4:6). Stem height and shoot basal diameter of seedlings were measured once a week during the acclimatization period.

Three months after acclimatization, stem height and shoot basal diameter of seedlings was measured once more to obtain the initial biometry of plants regarding the water limiting experiment. At the beginning of the water limiting experiment, seedlings were watered to field capacity and placed again in the controlled air humidity and temperature growth chamber. Field capacity can be defined as the amount of water retained in the pot growing medium after soil saturation and drainage (TIMMER and ARMSTRONG, 1989). The growth chamber was maintained with 16/8h light/dark photoperiod, 25/20°C Day/night temperature, 65%/60% day/night relative humidity, and 500 μmol m^{-2} s^{-1} photon flux density (AMARAL *et al.*, 2019). Experiment conditions were created by allowing the soil from seedlings pots to dry until seedlings remained greenish but with visually withered shoots, prior to any loose of leaves. Soil pots with seedling were weighed once a week. The experiment lasted for 3 weeks. At the end of that period, seedlings were removed from the growth chamber and their final biometry and weight were assessed. As the experiment duration was of only 3 weeks, weight increment of the seedlings was considered negligible. Soil content per pot was oven-dried for 48 h at 60º C and weighted once more to obtain soil dry weight. Soil water content (SWC) was determined by the gravimetric method (ROYO *et al.*, 2001), according to the expression: %SWCw=[TW-(T+SFW+MDW)]/MDW. TW refers to total weight, T refers to weight of the pot, SFW refers to seedling fresh weight and MDW refers to soil dry weight.

Seedlings were partitioned into roots and shoots and weighted for seedling fresh weight (SWF) determination (CHAMBEL *et al.*, 2007). Then they were oven-dried during 48 h at 60 °C to determine seedling dry weight (SDW). Dry weights of shoots and roots were determined, and seedling total dry weight was calculated. Plant water (PWC) content was computed from values of fresh and dry weight (%PWCw=(SFW-SDW)/SDW). SFW refers to seedling fresh weight and SDW refers to seedling dry weight.

Statistical Analysis

Differences in seed germination rate among the eight *P. pinaster* populations (Mira (M), Tocha (t), Quiaios (Q), Osso da Baleia (OB), Praia de Pedrogão (PP), Praia da Vieira (PV), Leiria (L) e Marinha Grande (MG)) were tested through a One-way ANOVA, followed by a post hoc Tukey test. All the other seed and seedling related variables were analyzed by population through a Kruskal-Wallis test, followed by the post hoc pairwise Mann-Whitney U-test, as they did not meet the One-way ANOVA assumptions. Correlations and differences between the biometric variables, measured before and after water depletion experiment, were investigated by grouping values from all populations. Pearson correlations were applied to determine the relation between plant and soil water contents at the end of the water limitation trial. Differences in seedling height before and after the water limitation experiment were evaluated with a t-test, while differences in basal shoot diameter were analyzed with a Kruskall-Wallis test, followed by a pairwise Wilcoxon with the Bonferroni correction on the p value. Data analysis was performed on R software, version 4.1.1. using the packages stats (4.1.1), ggpubr (0.4.0), and dplyr (1.0.8).

Results

The proportion of floating seeds, which were discarded, did not vary significantly between populations, corresponding on average to 20 % of the initial number of seeds. Seed weight was significantly different among populations ($\chi 2 = 81.616$, $p < 0.05$), varying between 0.042 and 0.066 g. The lightest seeds were found in PV and the heaviest in T (Figure 2). However, no geographical pattern correlated to seed weight was identified.

The first seeds germinated 11 days after the beginning of the experiment from M, Q, MG, OB, PV and PP populations. Despite having run the germination experiment for 2 months, a considerable time according to most studies, no plateau was achieved for the cumulative germination in any of the populations. The weekly germination pattern was very similar between replicates of different populations, with generally higher germination rates during the third and seventh weeks (Figure 3).

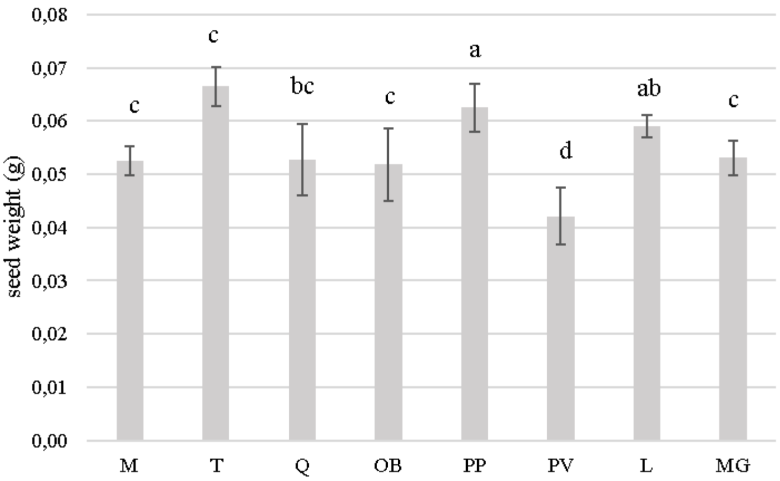

Figure 2 - *Pinus pinaster* mean seed weight of Mira (M), Tocha (T), Quiaios (Q), Osso da Baleia (OB), Praia de Pedrogão (PP), Praia da Vieira (PV), Leiria (L) and Marinha Grande (MG) populations, along the North-to-South geographic gradient within the study area. Error bars represent the standard deviation. Different letters indicate significant differences ($p<0.05$) in germination rate as detected by Tukey's HSD test

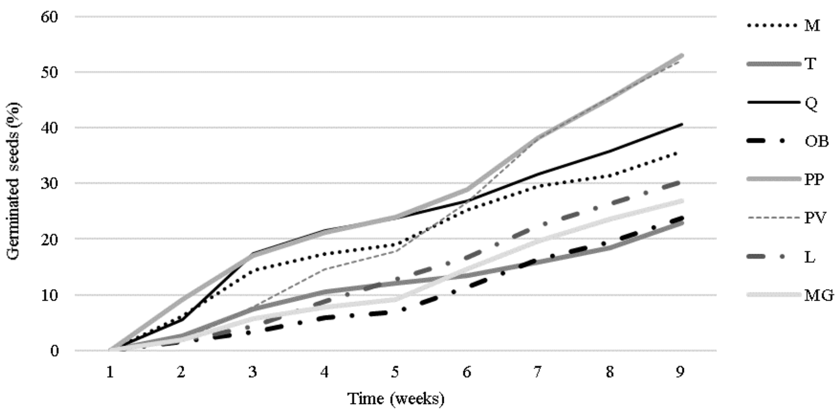

Figure 3 - *Pinus pinaster* cumulative germination, over time, of Mira (M), Tocha (T), Quiaios (Q), Osso da Baleia (OB), Praia de Pedrogão (PP), Praia da Vieira (PV), Leiria (L) and Marinha Grande (MG) populations, along the North-to-South geographic gradient within the study area

The final germination percentage was significantly different amongst sites ($F2 = 12.2$, $p < 0.05$), being higher in PV and PP, with mean germination percentages of 52 % and 54 % respectively. The lowest germination value was 23 % in T, although with no significant differences from M, OB, L and MG (Figure 4).

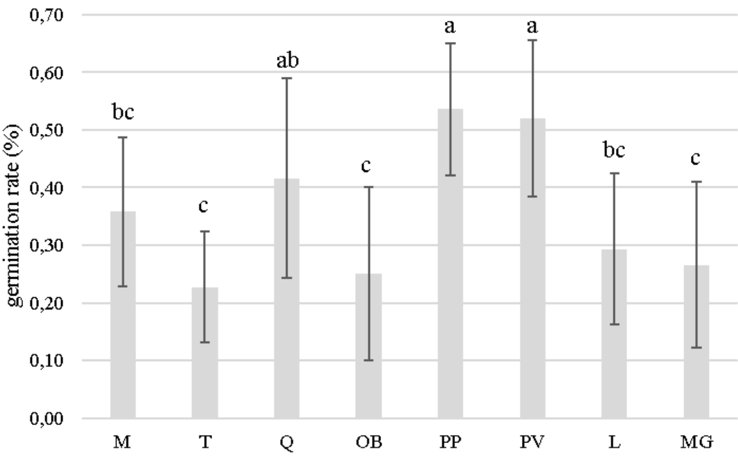

Figure 4 - *Pinus pinaster* mean germination percentage of Mira (M), Tocha (T), Quiaios (Q), Osso da Baleia (OB), Praia de Pedrogão (PP), Praia da Vieira (PV), Leiria (L) and Marinha Grande (MG) populations, along the North-to-South geographic gradient within the study area. Error bars represent the standard deviation. Different letters indicate significant differences ($p<0.05$) in germination rate as detected by Tukey's HSD test

Overall no relation was found between seed weight and germination rate, regarding the joint analysis of data from all the populations (Figure 5). At the population level, different trends were observed. A positive relation between seed weight and germination rate was found in the PP population. This population had in average heavy seeds (62.5 mg) and an average high germination rate (54 %). Two other different trends were observed in T and PV populations. The T population had in average the heaviest seeds (66.5 mg), but the average lowest seed germination rate (23 %). The population from PV presented in average the lightest seeds (42.1 mg) but with high germination success (average of 52 %).

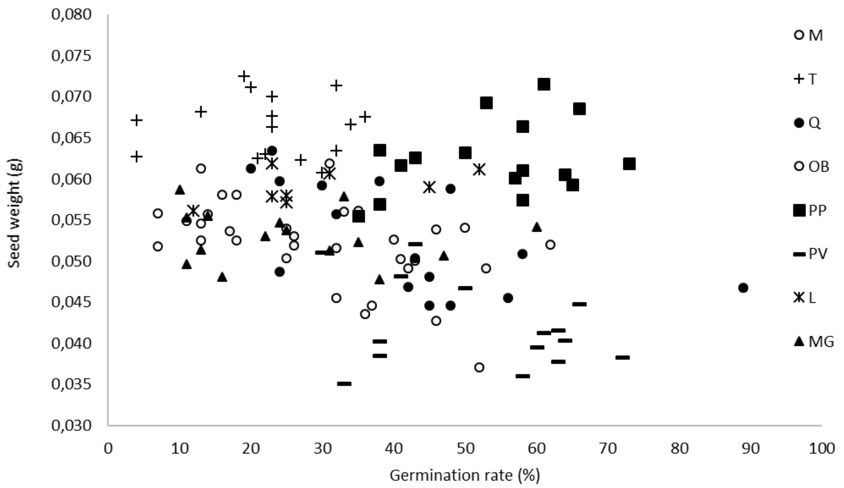

Figure 5 - Relation between seed weight (g) of Mira (M), Tocha (T), Quiaios (Q), Osso da Baleia (OB), Praia de Pedrogão (PP), Praia da Vieira (PV), Leiria (L) and Marinha Grande (MG) populations of *Pinus pinaster* and the respective germination rate (%)

Despite some evident differences in seed weight and germination rates between populations, seedling mortality during the first three months was negligible to all populations. Additionally, no significant differences were found between the average height of seedlings amongst populations ($\chi 2 = 10.931$, $p > 0.05$), under standard watering regime. Therefore, no correlation was found between seed weight and seedling's height, or between seed weight and seedling's basal shoot diameter, for the individual or the overall populations set under non-stress conditions.

During the water limitation experiment, soil water content generally decreased in all populations, from an average value of 199% at the beginning of the experiment, to significantly different ($\chi 2= 14.56$, $p<0.05$) values amongst populations, ranging from 17% in OB to 11% of water in soil in M. Seedlings water content (Table 2) varied between populations ($p < 0.05$), being the higher values found in Q and OB, 72% and 69% respectively. The lower values were those of PV and M populations, 37% and 38 %, respectively.

Table 2 - Height, basal shoot diameter, soil water content and plant water content (%), at the beginning (week 0), during (weeks 1 and 2) and at the end (week 3) of the water limitation experiment, of Pinus pinaster seedlings from Mira (M), Tocha (T), Quiaios (Q), Osso da Baleia (OB), Praia de Pedrogão (PP), Praia da Vieira (PV), Leiria (L) and Marinha Grande (MG) populations localized along the North-to-South geographic gradient within the study area. values represent the mean ± sd. Different letters indicate significant differences between populations to the specific variable ($p<0.05$) as detected by Wilcoxon test with the Bonferroni correction

Parameter	Week	M	T	Q	OB	PP	PV	L	MG
Height (cm)	0	9.86±1.31 ^{a}	9.92±1.36 ^{a}	9.68±1.85 ^{a}	10.20±1.21 ^{a}	10.38±0.84 ^{a}	9.88±1.03 ^{a}	9.74±0.64 ^{a}	10.86±0.91 ^{a}
	1	9.96±1.05 ^{a}	9.54±1.59 ^{a}	9.72±2.12 ^{a}	10.04±1.15 ^{a}	10.04±1.06 ^{a}	9.40±0.93 ^{a}	9.84±0.81 ^{a}	10.80±0.91 ^{a}
	2	9.86±1.14 ^{a}	9.64±1.41 ^{a}	9.60±2.04 ^{a}	10.14±0.82 ^{a}	10.14±1.05 ^{a}	9.76±0.79 ^{a}	10.14±0.51 ^{a}	10.84±0.90 ^{a}
	3	9.68±1.06 ^{a}	9.60±1.61 ^{a}	9.52±1.90 ^{a}	10.14±0.90 ^{a}	9.96±0.91 ^{a}	9.38±0.93 ^{a}	9.90±0.69 ^{a}	10.60±0.80 ^{a}
Basal shoot diameter (cm)	1	2.62±0.26 ^{ab}	2.90±0.31 ^{ab}	2.38±0.36 ^{a}	2.78±0.34 ^{ab}	2.68±0.16 ^{ab}	2.74±0.13 ^{ab}	3.06±0.92 ^{ab}	3.16±0.11 ^{b}
	2	2.18±0.26 ^{b}	2.62±0.31 ^{ab}	2.14±0.24 ^{b}	2.52±0.08 ^{ab}	2.56±0.17 ^{ab}	2.46±0.27 ^{ab}	2.78±0.74 ^{ab}	2.84±0.05. ^{b}
	3	2.04±0.23 ^{a}	2.18±0.28 ^{a}	2.08±0.26 ^{a}	2.22±0.26 ^{a}	2.12±0.18 ^{a}	2.16±0.15 ^{a}	2.46±0.64 ^{a}	2.46±0.25 ^{a}
Soil water (%)	0	211±34 ^{a}	203±40 ^{a}	234±40 ^{a}	163±12 ^{a}	219±12 ^{a}	167±19 ^{a}	186±32 ^{a}	171±27 ^{a}
	3	9.6±2.3 ^{bc}	11.7±2.9 ^{abc}	9.5±1.6 ^{c}	14.2±0.8 ^{a}	11.6±1.6 ^{bc}	10.7±1.1 ^{abc}	10.4±2.6 ^{abc}	12.4±1.9 ^{ab}
Plant water (%)	3	38.2±18.3^{ab}	72.2±32.8^{ab}	47.3±13.8^{ab}	69.2±17.5^{a}	48.9±12.0^{ab}	36.9±8.8^{b}	52.5±12.4^{ab}	48.8±16.1^{ab}

Seedlings and soil water content, at the end of the water stress experiment, showed to be positively correlated (R= 0.67, $p < 0.05$) indicating that seedlings that maintained higher water contents also used less water from the soil (Figure 6). Results demonstrated high variability within some populations, such as T and L, and different tendencies regarding the overall correlation, amongst populations. Despite the high variability of water content in seedlings from T, the values appeared always above the correlation line, while seedlings from M and PV had the opposite tendency, with water contents below the correlation line. Seedlings from OB had the higher water content percentage, together with higher levels of water in the soil.

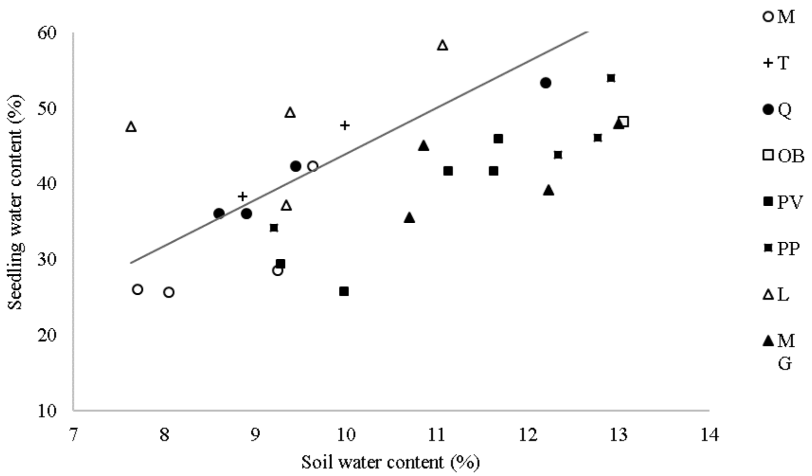

Figure 6 - Correlation between seedling water content (%) Mira (M), Tocha (T), Quiaios (Q), Osso da Baleia (OB), Praia de Pedrogão (PP), Praia da Vieira (PV), Leiria (L) and Marinha Grande (MG) populations of *Pinus pinaster* and the respective soil water content (%) determined after the water limitation experiment. Correlation value of 0.67 ($p<0.05$)

Seedlings height was not significantly influenced by the water limitation experiment. Seedlings basal shoot diameter was, overall, significantly affected during the water limitation period ($\chi 2=780$; $p < 0.05$), being the onset of growth cessation during the second week to all populations (Table 2, Figure 7). Albeit not significantly different from the majority of the remaining populations, the seedlings from Q showed a lesser decrease in shoot diameter (-12.1%), while the seedlings from T showed the higher reduction in basal shoot diameter (-23%) ($p < 0.05$) (Figure 5). Regarding the variation diameter, seedlings had significant reductions during the period of water limitation, varying from -12.1% in Q population to -24.5% in T population. Mean biomass allocation at the end of the study was highly balanced between roots and shoots (shoots ≈ 49%; roots ≈ 51%), with no overall significant differences amongst the 8 populations.

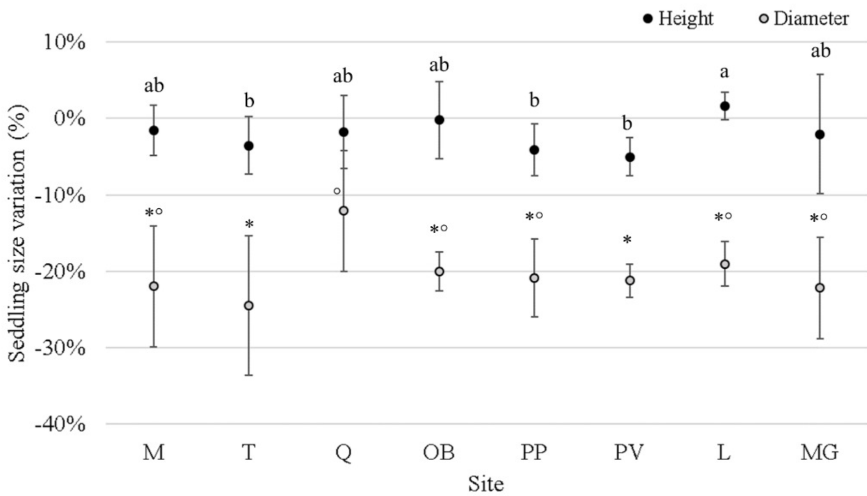

Figure 7 - Variations (delta) in hei.ght (black) and in basal shoot diameter (grey) of *Pinus pinaster* seedlings during the first and third weeks of the water stress experiment. Results are presented by site, according to a North to South geographic gradient within the Central Coastal of Portugal. Dots represent the mean ± sd of height and diameter of seedlings by population

Discussion

This work aimed to characterize the germination potential of 8 *P. pinaster* populations, classified as non-serotinous according to TAPIAS *et al.* (2004), distributed along 100 km within the Central Coastal region of Portugal.

Seed weight within the present study varied in the same range as reported by other authors in *P. pinaster* populations (WAHID and BOUNOUA, 2013). However, seed weight cannot be seen as an indicator of the degree of serotiny in Portuguese *P. pinaster* Coastal populations, as our results showed higher variability in seed weight than the reported to low serotinous populations in Spain (CALVO *et al.*, 2016). In particular, the seed weight range in the present study encompasses the entire variability found by CALVO *et al.* (2016) in Iberian maritime pine populations with low to high serotiny degrees.

Populations with heavier seeds are widely reported to have more developed embryos, higher germination rates, lower germination times and higher seedling lengths, than populations with lighter seeds (ESCUDERO *et al.*, 2000; REYES and CASAL, 2001; ALVAREZ *et al.*, 2007; HERNÁNDEZ-SERRANO *et al.*, 2013; CALVO *et al.*, 2016; VALBUENA *et al.*, 2019). The present study found some different trends to those previously reported (ESCUDERO *et al.*, 2000; REYES and CASAL, 2001; ALVAREZ *et al.*, 2007; HERNÁNDEZ-SERRANO *et al.*, 2013; CALVO *et al.*, 2016; VALBUENA *et al.*, 2019) that were population dependent. Accordingly, the heaviest seeds found in T had one of the lowest germination rates, while seeds from PV were the lightest and were amongst the ones that showed higher germination rates. On the other hand, seeds from PP presented a high germination rate, similar to the neighbour PV population, but with much heavier seeds, contrasting with the overall trend found in this study.

The seed germination rates observed in the present work, with a maximum of 54% obtained after 2 months, were much lower than those reported in several other studies, where more than 90% of seeds germinated in the first month (ALVAREZ *et al.*, 2007; CALVO *et al.*, 2016), the standard time for the duration of similar experiments. CORREIA *et al.* (2014), reported a final germination of 29% after 21 days in seeds from Leiria, that increased to 68% after a prechilling dormancy-breaking treatment.

The reported delayed germination of the seeds within Portuguese Coastal populations, in relation to other populations, could imply local adaptations to environmental stressors. The environmental conditions where mother tree grows has a direct influence in the size of the seeds, as well as in their germination timing

(CASTRO, 2006). Factors such as light and temperature during seed maturation determine the environmental conditions that some species need to brake dormancy (CENDÁN *et al.,* 2013; CORREIA *et al.,* 2014). The formation and thickness of seed coat and the viability of the endosperm are highly determined by the maternal environment (LINKIES *et al.,* 2010). The extreme drought during 2017 in Portugal (IPMA, 2017), coinciding with the time of cone formation, may have influenced seed production and maturation (CENDÁN *et al.,* 2013), resulting in seeds with thicker coats and therefore modulating the germination phenology found in our study.

In the pioneer species *P. pinaster,* seed size is reported to be a good indicator of seedlings ability to survive and to grow mainly during early-stages (ALVAREZ *et al.,* 2007; SUÁREZ-VIDAL *et al.,* 2017), contributing to species recruitment and establishment (CENDÁN *et al.,* 2013). Larger seeds are reported to produce more robust seedlings, with lower mortality rates than smaller seeds (FERNANDES *et al.,* 2017). Despite the differences in seed weight between populations of the present study, seedlings mortality under nursing conditions, during the first three months was negligible, and no differences were found between seedlings length amongst populations. The mean seedlings height of 10 cm is in accordance to results of other works (ALVAREZ *et al.,* 2007). No relation was found between seed weight and seedling height, again contrasting with other findings where a positive correlation between seed size and seedling length was observed (FERNANDES *et al.,* 2017; SUÁREZ-VIDAL *et al.,* 2017). These contrasting relations between seed weight, germination and early seedling length seem to support a distinct ecological dynamic of the Portuguese maritime pine Coastal populations, compared to other Iberian populations.

In line with the findings of SUÁREZ-VIDAL *et al.* (2017), the present study found no evident effect of seed weight on the performance of early seedlings on the course of the water limitation experiment. Water limitation induced a general reduction of basal shoot diameter of plants, as also found by CHAMBEL *et al.* (2007), except for the remarkable case of Leiria seedlings, that increased their length during the water limitation experiment, despite the reduction in stem diameter. Other studies found a continuous growth of *P. pinaster* seedlings in terms of height and diameter under water limitation (FEINARD-DURANCEAU *et al.,* 2018), confirming the high phenotypic plasticity of maritime pine populations also in terms of water use efficiency due to their transpiration patterns (CHAMBEL, *et al.,* 2007; DE LA MATA *et al.*, 2014; KATTGE *et al.,* 2020).

After water limitation, plant water content varied between 27% and 40%, although biomass allocation between roots and shoots was not significantly different amongst populations. Plant water content was highly variable between seedlings of the various sites, positively correlated to soil water content, even though nursing conditions and seedling manipulation remained homogenous. The water content of seedlings from T, although highly variable, was higher considering the soil water content estimated by the correlation model. Seedlings from M and PV had an opposite tendency, with lower plant water contents considering similar levels of water in the soil. Seedlings from OB had the higher water content, together with higher levels of water in the soil. Such differences between populations, may suggest contrasting degrees of efficiency in water use by seedlings from different populations. Higher plant water content should have resulted from physiological adjustments related to water use efficiency rather than different patterns of biomass allocation (GASPAR *et al.,* 2013). In line with other authors (FEINARD-DURANCEAU *et al.,* 2018), our results suggest variations in the physiological status of seedlings from different sites, irrespectively of biometric traits (FEINARD-DURANCEAU *et al.,* 2018), that may turn into ecological advantages to the success of *P. pinaster in situ* recruitment and establishment. It seems clear that Portuguese Coastal *P. pinaster* populations present a wide range of seed and seedling phenotypic traits, with complex interrelations. This high variability can be due to the high spatial environmental heterogeneity of the forest areas, not only in terms of local soil and climate conditions, but arguably dominated by management and disturbance history, even in such a narrow geographic scale as addressed by the present study (*e.g.*, MAIA *et al.,* 2014). Historical management (thinning and tree harvest selection, rotation period) and cultural forest uses (weed control, cone harvesting, pine seeding with allochthonous material) may have an important impact in the present phenotypical variability. There are evidences that role of genetics does not prevail over environmental conditions (DE MIGUEL *et al.,* 2012; ALBERTO *et al.,* 2013) and in particular, those of the maternal environment can carry transgenerational plasticity manifested as a high variability in germination phenology and seedling phenotype (CENDÁN *et al.,* 2013).

Even though the same general management guidelines apply to the entire study area, given the jurisdiction limits of the regional offices of Portuguese forest management authorities (ICNF), local variability in cultural uses and small-scale management variation is very noticeable in Portuguese forest areas (VALENTE *et al.,* 2015). Such local variability can drive specific phenotypic alterations within *P. pinaster* populations, from seed size and germination delay to seedlings with higher water use efficiency, derived from local adaptations to environmental stressors (DE SIMÓN *et al*., 2017; KATTGE *et al*., 2020).

Conclusion

In general, the results evidenced a high inter-population variability amongst all the studied variables, probably due to successive generations of non-unselected maritime pine individuals obtained by natural regeneration of seeds exposed to local stresses, wildfires, and other disturbances, but apparently with no implications for the viability of the maritime pine forest.

Even though, it was possible to demonstrate the existence of different germination rates among populations. Overall no relation between seed weight and the germination rate was found, likely due to the lack of a homogenous relation pattern between the studied populations. Divergent relationships between the seed weight and germination potential were observed, particularly striking between the neighbouring populations of PV and PP. Despite differences in seed weight values and germination rates, seedling mortality in nursing conditions was negligible in all the studied populations. By submitting 3 months-old seedlings to water limitation, results suggested differences in water use efficiency between populations, apparently independent of seed size or germination potential. Particularly noteworthy is the case of the OB population, where plant water content was the highest among all the populations, yet not reflected by any of the other variables analysed in this study.

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