*Article*

# Impact of Fire Recurrence and Induced Water Stress on Seed Germination and Root Mitotic Cell Cycle of *Pinus pinaster* Aiton

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**Abstract:** Climate change will increase the frequency of drought, heat waves, and wildfires. We intended to analyse how fire recurrence and/or induced water stress can affect seed germination and root cell division in *Pinus pinaster* Aiton. Seeds from stands with no prior fire history and from post-fire regeneration (in areas burnt once, twice, and thrice) in northern Portugal were germinated in distilled water (control) and polyethylene glycol (PEG) to simulate water stress for four weeks, followed by a recovery period. Roots were analysed cytogenetically. The germination index of the *Pinus pinaster* seeds was not statistically influenced by the induction of osmotic stress, nor by the fire recurrence of the stands. The mean germination time (MGT) was 10–29 days and 1–36 days for the stress and recovery periods, respectively, and increased with PEG concentration. The 20% PEG treatment inhibited root growth after germination. The 10% PEG treatment induced a high frequency of cytogenetic anomalies, mostly in the sites which experienced fire exposure. While fire recurrence did not affect the germination rate, it seemed to reduce the water stress response, negatively impacting cell division and impair root growth.

**Keywords:** cytogenetics; drought; germination; mitosis; polyethylene glycol; maritime pine

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## 1. Introduction

Future climate scenarios for Mediterranean regions predict an increase in the frequency and severity of drought episodes, heat waves, and forest fires [1]. Dry years can have a negative impact on the different stages of forest development (seed production, germination, seeding, and seedling survival) [2,3]. Several researchers have widely reported the importance of water availability for *Pinus pinaster* Aiton seed germination (e.g., [4–7]). Furthermore, the delay or impairment of seed germination and seedlings' emergence caused by water stress are responsible for young pine seedlings' mortality [6,8–12]. This fact may be involved in the decreased natural regeneration ability of *Pinus* spp., namely, *Pinus pinaster* [6,13–15].

As with other abiotic stresses, water stress causes DNA damage and cell division anomalies that can compromise root and leaf growth and, consequently, plant development and yield [16–25]. The impact of water stress on seed germination and root cell division was previously analysed on *Pinus nigra* [18] by exposing the seeds to polyethylene glycol (PEG) under controlled conditions. PEG is an inert polymer with a high molecular weight [26] that

does not penetrate the cell and reduces the water potential in the extracellular medium, simulating water stress. The consequences and/or plant mechanisms of water stress response have also been studied in seedlings or in vitro cultured tissues exposed to PEG [18,27–33].

Adaptive management of *Pinus pinaster* is challenging in fire-prone areas, requiring knowledge of genetic variability, fire ecology, and stand dynamics. It has been reported that the fire regime has a relevant role in affecting the genetic diversity in the short term without generating genetic erosion of *Pinus pinaster* [34]. The difficulties in the natural regeneration of forests and the high risk of forest fires in the context of climate change in Southwestern Europe have been noticed and addressed in specific research programs (e.g., [35]). Fire can also negatively impact natural regeneration and the development stages of *Pinus pinaster*, as revised by Ribeiro et al. [3], mainly because high-severity fire can decrease or even impair seed germination [36–38]. *Pinus pinaster* populations vary widely regarding the ability to establish a canopy seed bank through serotinous cones, which remain closed until being exposed to fire and have evolved to ensure postfire regeneration [39,40]. Past exposure to fire is expected to change the proportion between serotinous and non-serotinous cones in pine populations, which can differ in their anatomy and morphology, potentially affecting the germination rate [39–42].

For these reasons, and given the expected increase in fire and drought episodes [1], it is highly relevant to analyse whether and how fire recurrence and water stress can affect the seed germination and root cell division of pine plants from the European Southwest region. We hypothesised that the recurrence of fire coupled or not with induced water stress could influence the germination ability of *Pinus pinaster* seeds and/or root development due to anomalies in cell division. Therefore, this work aimed to analyse the impact of fire recurrence alone and combined with induced water stress in (i) seed germination and (ii) root-tip cell division.

## 2. Materials and Methods

### 2.1. Study Area and Dendrometric Evaluation

The study area is the village of Seirós, in the municipality of 'Ribeira de Pena', 'Vale do Tâmega' region, in Northern Portugal (Figure 1). Three post-fire natural regeneration stands of *Pinus pinaster* with different fire recurrence (A1—once, B2—twice, D3—three times) and one unburned stand designated as control (C) were identified and selected for the study (Figure 1 and Table 1). The results presented throughout the article in the post-fire regenerated stands will be compared to those obtained from the unburned site.

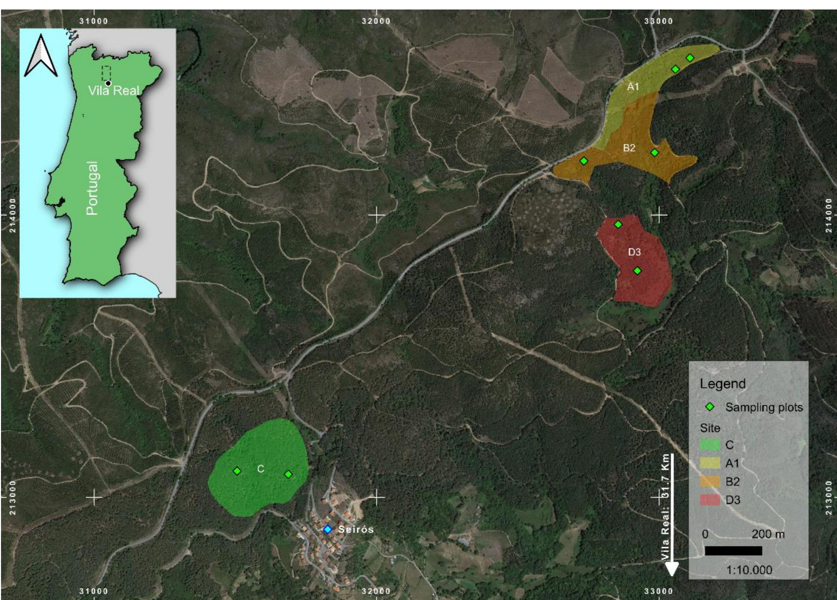


**Figure 1.** Geographic location of the study area.

**Table 1.** Total area of stands and years of occurrence of fires.

| Site | Area of the Stand (ha) | Fire Years |
|---|---|---|
| **C** | 8.6 | – |
| **A1** | 4.0 | 1987 |
| **B2** | 6.0 | 1987, 1998 |
| **D3** | 5.7 | 1989, 1990, 1998 |

Two temporary circular plots with individual area of 200 $m^2$ were installed at each site. The diameter at breast height (*d*) of all trees and the total height (*h*) of 10 trees distributed by diameter classes were measured. The trees with a *d* close to the central value of the diameter class were selected for height measurement. In addition, the total height of the dominant trees was also recorded. The *d* was measured with a caliper (1 mm precision) at 1.30 m above-ground. Total height was assessed with a Vertex hypsometer (10 cm resolution). The age of dominant trees was evaluated with a Pressler increment borer at 1.30 m above-ground. Five years were added to the total number of rings counted in the wood samples because the species takes an average of 5 years to reach the height level of 1.30 m. With the data collected in the field, the relative density of stands was assessed with the Reineke Stand Density Index (*SDI*) [43] considering an allometric coefficient (r) of 1.897 (Equation (1)), a value established by Luis and Fonseca [44] for pure *Pinus pinaster* stands in Portugal. The *SDI* value can be considered as the maximum expected number of trees ($N$, $ha^{-1}$) for a stand when its quadratic mean diameter ($dg$, cm) equals 25 cm. The *SDI* (%) was calculated using Equation (2).

$$SDI = N\left(\frac{dg}{25}\right)^{1.897} \quad (1)$$

$$SDI\ (\%) = \left(\frac{SDI}{1859}\right) \times 100 \quad (2)$$

The site index corresponding to the dominant height at a reference age of 35 years ($SI_{35}$, m) was evaluated with the Marques [45] site index curves. The estimates correspond to the medium quality class, representative of the study area. Table 2 presents the dendrometric characteristics of the trees and stands under study.

**Table 2.** Stand information: age (years), stand density ($N$, $ha^{-1}$), quadratic mean diameter ($dg$, cm), stand density index (*SDI*, %), site index ($SI_{35}$, m), mean tree diameter ($d$, cm) ± standard deviation (S.D.), and mean tree height ($h$, m) ± S.D.

| Stand | Age (Years) | $N$ ($ha^{-1}$) | $dg$ (cm) | *SDI* (%) | $SI_{35}$ (m) | $d$ ± S.D. (cm) | $h$ ± S.D. (m) |
|---|---|---|---|---|---|---|---|
| **C** | 58 | 550 | 24.2 | 28 | 16.8 | 24.0 ± 3.7 | 16.6 ± 2.0 |
| **A1** | 36 | 1000 | 22.4 | 44 | 17.3 | 21.3 ± 5.6 | 16.1 ± 2.7 |
| **B2** | 22 | 1075 | 16.1 | 25 | 17.9 | 15.1 ± 4.8 | 10.4 ± 2.6 |
| **D3** | 24 | 1800 | 13.4 | 30 | 16.6 | 13.0 ± 4.9 | 10.5 ± 3.5 |

Data presented in Table 2 are the averaged values of the two inventory plots installed per site. The diameters of the total number of trees in the two plots per population were used to calculate the mean diameter. Mean height was calculated in the same way.

### 2.2. Plant Material

In each stand, various pinecones were collected from the ground, given the difficulty of collecting them directly from the tree. An attempt was made to collect pinecones in the same

month in all four study sites to avoid the influence of annual variability in environmental conditions on seed characteristics [46]. When collecting the pinecones, it was ensured that they had no visible signs of insect attack or disease to guarantee that the sanitary condition of the seeds did not interfere with the result of the study. This collection took place in later February 2022. In the laboratory, at ambient temperature, as the pinecones lost moisture, some of the pinecones collected as fully closed opened spontaneously, indicating that an opening had already occurred in the field. Soares et al. [47] have already reported this phenomenon of opening and closing of the pinecone with humidity. These pinecones without or with a low number of seeds were replaced with others collected at the beginning of April 2022.

Twenty fully closed (never opened) pinecones per stand were selected for the study, with a mean size representative of the pinecones collected in the stand. The pinecones were placed in a dry air oven [48–51] at 100 °C to promote their opening. The opening of the pinecones was monitored frequently. Soon after their opening, they were removed from the oven to prevent the hot air from invalidating the seed embryo. On average, the pinecones took 1 h to open. Seed viability was assessed by manual compression, as referred by Santos et al. [52]. Cracked or soft seeds were eliminated. The seeds were stored for a few days at room temperature [49] until the germination and water stress tests started. For the present study, we used 100 seeds isolated from eight pinecones per stand, casually selected to ensure the existence of genetic variability.

### 2.3. Water Stress Induction by PEG and Seed Germination

In this work, a total of 24 experiments were performed regarding two repetitions of four stands × three PEG treatments. Per repetition, 10 seeds of each stand (burned A1, B2, D3, and unburned C) were exposed to 0%, 10%, or 20% PEG. The seeds were placed in Petri dishes containing filter paper moistened in distilled water (0% PEG, control treatment) or aqueous solutions of 10% and 20% ($w/v$) PEG, molecular weight 6000 (Biochemica, Applichem GmbH, Darmstadt, Germany), corresponding to the osmotic potentials of −0.4 MPa (or −4 bar) and −0.8 MPa (or −8 bar), respectively. The PEG–6000 solutions were prepared according to Topacoglu et al. [11]. The seeds germinated in the dark at 25 °C for four weeks. The PEG solutions were renewed daily to maintain the osmotic potential. After four weeks (28 days) of water stress induction, the seeds were kept in the Petri dishes and watered with distilled water (recovery period) for 67 days.

Germination was monitored daily during the water stress induction and recovery period. During the water stress induction and the recovery periods, seeds presenting a radicle of 1–2 mm length were considered germinated [53].

The percentage of germination was determined per stand (S), treatment (T), and S × T interaction during the water stress induction (28 days) and recovery (67 days) periods. Since the germination percentage has a binomial distribution, it was necessary to proceed with its transformation so that the transformed data are approximately normally distributed and intended to make the means and variances independent, with the resulting variances homogeneous. In this case, Steel and Torrie [54] and Sokal and Rohlf [55] recommend transforming the data by the angular arcsine function, which stretches out both tails of the distribution of percentages or proportions and compresses the middle. So, the percentage of germination was converted to angle (Equation (3)), resulting in the germination index (GI):

$$\mathrm{GI} = \frac{Arcsin\left(\frac{\sqrt{percentage}}{100}\right)}{\frac{\pi}{180}} \tag{3}$$

The mean germination time (MTG) was also calculated per stand (S), treatment (T), and S × T interaction during the water stress induction (28 days) and recovery (67 days) periods, using the Equation (4), where: n = number of seeds germinated on each day,

d = number of days since the imbibition of the seed (beginning of the germination test), and N = total number of seeds germinated at the end of the experiment [56].

$$MGT = \frac{\sum(n \times d)}{N} \quad (4)$$

### 2.4. Cytogenetic Analysis of the Root Meristematic Cells

During water stress induction and recovery periods, the primary root with a 1.5 cm length in each germinated seed was collected and immediately fixed in a solution of absolute ethanol and glacial acetic acid in the proportion of 3:1 ($v/v$). After fixation, roots were kept at −20 °C and then stained in 2% aceto-carmine for 48 h, at room temperature, to further prepare mitotic chromosome spreads using the squashing method [57]. One root per chromosomal spread was used. Three chromosomal spreads (n = 3) per stand × treatment interaction were performed whenever possible. Exceptions occurred in the cases of null germination or emission of one or two roots. At least 50 microscope observation fields were chosen randomly and analysed per chromosome spread. All interphase and dividing cells were scored, and the mitotic phases and eventual chromosomal or mitotic spindle anomalies per observation field were recorded. Based on these data, the mitotic index (MI) (Equation (5)) was calculated, where the scored cells constitute the sum of interphase and mitotic cells and the dividing cells with anomalies (DCA) (Equation (6)):

$$MI = \frac{\text{n of dividing cells}}{\text{n of scored cells}} \times 100 \quad (5)$$

$$DCA = \frac{\text{n of dividing cells with anomalies}}{\text{total n of dividing cells}} \times 100 \quad (6)$$

### 2.5. Statistical Analyses

The statistical analyses were performed with the IBM SPSS Statistics 25 software. Germination test and cytogenetic results are presented as mean values ± standard error (S.E.). In the germination test, the results are those obtained from two observations (i.e., data from two plots that work as replications) per stand and PEG treatment. The mean cytogenetic values resulted from scoring three chromosome spreads per stand × treatment interaction.

The Shapiro-Wilk test and the Kolmogorov-Smirnov test were used to test data normality. To study the effects of the stand (S), treatment (T), and their interaction (S × T), we performed two-way analyses of variance (ANOVA). The homogeneity of variances was tested using Levene's test and plotting predicted versus residual values. Tukey's test was used for the comparison of means between factors [58,59].

The $p$-value significance of all statistical analyses performed for the individual effects and their interaction were established for probabilities lower than 5% ($p$-value < 0.05).

## 3. Results

### 3.1. Germination Index and Mean Germination Time during Water Stress Induction and Recovery

After four weeks of water stress induction, no noticeable differences were evident in GI values among stands or treatments, with mean GI values ranging from 8.61 to 20.54 and from 9.78 to 15.22, respectively (Table 3). The same was verified for the GI values achieved in the recovery period (Table 3). Therefore, the mean differences in GI achieved in both stress and recovery periods per S, T and S × T, translated into $p$-values higher than 0.05 ($p$-values presented at the bottom of Table 3), and such absence of statistical significance did not allow us to claim the existence of a potential effect of fire recurrence or PEG treatment on the germination index.

**Table 3.** Mean (±standard error, S.E.) values of germination index and mean germination time (MGT) during the stress and recovery periods per stand (S), as well as PEG treatment (T).

| | | Germination Index (Stress) | Germination Index (Recovery) | MGT (Days) (Stress) | MGT (Days) (Recovery) |
|---|---|---|---|---|---|
| Stand (S) | C | 8.61 ± 5.77 *a* | 11.26 ± 8.15 *a* | 25.50 ± 3.50 a | 28.88 ± 6.88 a |
| | A1 | 9.22 ± 4.12 *a* | 13.21 ± 6.17 a | 21.67 ± 5.90 a | 12.00 ± 8.54 a |
| | B2 | 8.61 ± 5.77 *a* | 13.82 ± 4.54 a | 23.84 ± 1.17 a | 16.75 ± 8.01 a |
| | D3 | 20.54 ± 4.70 a | 7.76 ± 5.06 *a* | 25.53 ± 0.99 a | 11.75 ± 10.75 a |
| Treatment (T) | 0% PEG | 15. 22 ± 4.95 a | 12.09 ± 6.56 *a* | 20.53 ± 2.68 *a* | 21.08 ± 8.90 a |
| | 10% PEG | 9.78 ± 4.97 *a* | 12.21 ± 4.88 *a* | 25.22 ± 0.40 ab | 15.25 ± 7.65 *a* |
| | 20% PEG | 10.23 ± 3.98 *a* | 10.23 ± 3.98 *a* | 28.25 ± 0.75 *b* | 15.00 ± 7.58 a |
| ANOVA *p*-value | S | 0.336 | 0.863 | 0.030 | 0.706 |
| | T | 0.654 | 0.948 | 0.008 | 0.780 |
| | S × T | 0.445 | 0.127 | 0.061 | 0.701 |

Note: Different lowercase letters per column indicate statistically significant differences ($p < 0.05$). The italic letters indicate that the assumptions for the applicability of the Tukey test (normality of the data and/or equality of variance between sets of observations) were not fully met. C—unburned stand (control); A1, B2, and D3—stands burned once, twice, and thrice, respectively.

Concerning the mean germination time (MGT), the values achieved during the stress induction showed statistically significant differences ($p$-value < 0.05) among S ($p$-value = 0.030) and T ($p$-value = 0.008), being very close to the reference $p$-value of 0.05 in their interaction (S × T, $p$-value = 0.061) (Table 3). The MGT detected in the 20% PEG treatment (28.25 ± 0.75) was significantly higher ($p$-value < 0.05) than that of the control treatment (20.53 ± 2.68) (Table 3). In fact, the MGT values increased with the PEG concentration (Table 3), with statistically significant differences between the 0% and 20% PEG treatments (Table 3). In general, considering the S × T interactions, higher stress levels (higher %PEG) are reflected in higher MGT values, with the increase in the number of days varying with the number of times the sites experienced fire exposure.

In the recovery period, the MGT values did not show statistically significant differences ($p$-values > 0.05) among S, T, or S × T interactions (Table 3).

Overall, the statistical analyses performed for the germination data revealed that stand and PEG treatment influence the MGT variable during stress ($p$-value = 0.030 and $p$-value = 0.008, respectively) but not during the recovery, as shown in Table 3.

### *3.2. Root Mitotic Cell Cycle Analysis*

Due to the globally low GI and high MGT values, the cytogenetic analysis was performed with 1.5-cm length roots collected during both stress and recovery periods to ensure the cell cycle evaluation in most of the S × T interactions. In addition, most of the seeds germinating in the 20% PEG treatment showed root growth inhibition a few days after, hampering the cytogenetic analysis in most of the S × 20% PEG interactions.

Based on the cytogenetic data achieved, we determined the parameters of mitotic index (MI) and dividing cells with anomalies (DCA), both expressed in percentages (Table 4; Figure 2).

The mean mitotic index (MI) values showed statistically significant differences ($p$-value < 0.0001) among S, T, and their interaction (S × T) (Table 4).

The root-tip seeds from the stand that burned three times (D3) showed the highest mean MI (65.63 ± 16.42) and differed significantly ($p$-value < 0.0001) from the remaining stands, except for the one that burned twice (B2, 63.06 ± 9.72) (Table 4). Seeds from the unburned stand (C) presented the lowest MI value (Table 4). Regarding the treatment factor, the mean MI values decreased significantly with the increase in PEG concentration, being highest at 0%PEG (74.84 ± 7.34) and lowest at 20% PEG (25.00 ± 13.06) (Table 4). Concerning the MI values for S × T interactions, focusing on A1 and B2, 0 and 10%PEG, the MI values obtained with the 10%PEG treatment were higher than those obtained with

0%PEG (Figure 2). The 20% PEG treatment inhibited the root growth, and the cytogenetic analysis was performed only in the B2 × 20% PEG interaction, preventing comparative analysis for the three treatment levels.

**Table 4.** Mean (±S.E.) percentage values of mitotic index (MI) and dividing cells with anomalies (DCA) determined per stand (S), PEG treatment (T), and for their interaction (S × T).

| | | MI (%) (Mean ± S.E.) | %DCA (Mean ± S.E.) |
|---|---|---|---|
| Stand (S) | C | 28.15 ± 14.14 a | 7.82 ± 4.89 a |
| | A1 | 59.70 ± 15.28 b | 22.05 ± 13.13 a |
| | B2 | 63.06 ± 9.72 b,c | 30.03 ± 5.88 a |
| | D3 | 65.63 ± 16.42 c | 14.63 ± 10.73 a |
| Treatment (T) | 0% PEG | 74.84 ± 7.34 c | 24.63 ± 8.09 b |
| | 10% PEG | 62.57 ± 12.28 b | 27.90 ± 9.99 b |
| | 20% PEG | 25.00 ± 13.06 a | 4.04 ± 3.62 a |
| ANOVA *p*-value | S | <0.0001 | 0.2222 |
| | T | <0.0001 | 0.0444 |
| | S × T | <0.0001 | 0.0826 |

Note: Different lowercase letters per column indicate statistically significant differences ($p < 0.05$). C—unburned stand (control); A1, B2, and D3—stands burned once, twice, and thrice, respectively.

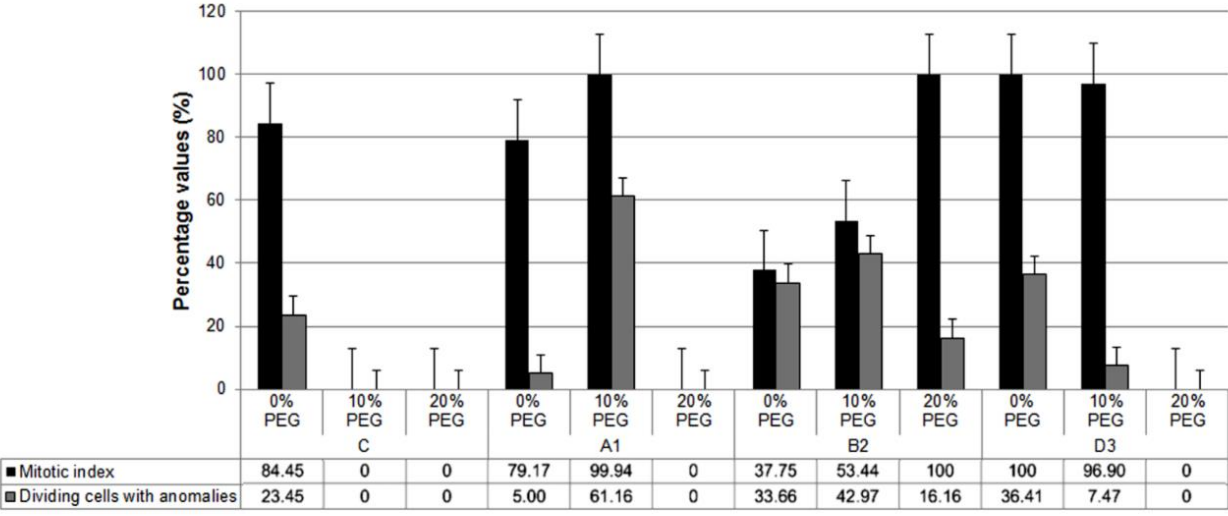


**Figure 2.** Mean values of mitotic index (MI) and dividing cells with anomalies (DCA) determined per stand × PEG treatment interaction. Results are expressed in percentage ± standard error (S.E.). Notes: C—unburned stand (control); A1, B2, and D3—stands burned once, twice, and three times, respectively.

As for the mean values of DCA, no statistically significant differences among stands or S × T interactions (*p*-value > 0.05) were found (Table 4). However, the mean DCA values differed significantly (*p*-value < 0.05) among PEG treatments (Table 4). The lowest mean DCA value was found in the 20% PEG treatment (4.04 ± 3.62), being statistically different from the mean values presented by the 0%PEG (24.63 ± 8.09) and 10%PEG (27.90 ± 9.99) treatments (Table 4).

The meristematic root cells presented normal and irregular dividing cells, mainly in prophase (Figure 3; Table S1). Nonetheless, the mean number of normal and irregular prophase cells did not show significant differences (*p*-value > 0.05) among S, T, or S × T (Table S1).

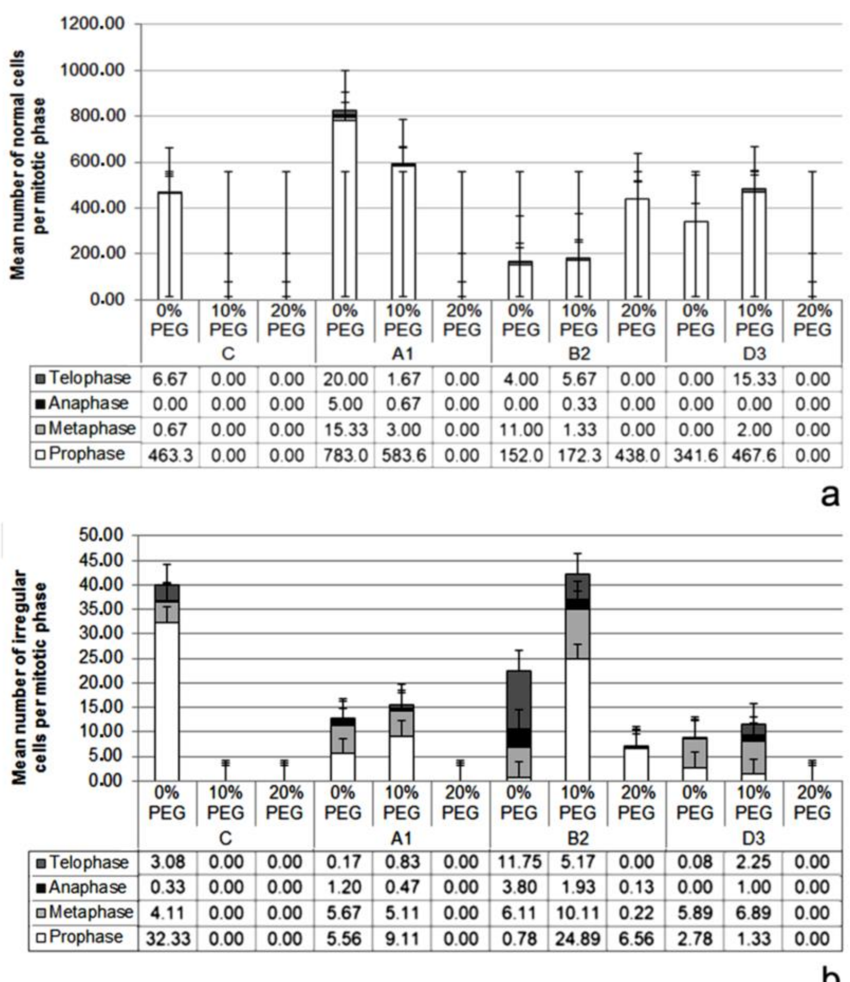


**Figure 3.** Mean (±S.E.) number of normal (**a**) and irregular (**b**) dividing cells per mitotic phase.

For the mean number of normal metaphase cells, the effects of stand (S), treatment (T), and S × T interaction were statistically significant ($p$-value < 0.05) (Table S1). For this mitotic phase, the mean number of normal cells was higher for 0%PEG than for 10%PEG treatment. The mean normal cell values of seeds collected from the once-burned stand (A1) were distinct from the others (C, B2, D3) ($p$-value = 0.0282) (Table S1). The mean number of normal and irregular anaphase cells showed statistically significant differences ($p$-value < 0.05) among S, T and/or S × T interactions (Table S1). Regarding the telophase cells, only the mean values of the irregular ones showed statistically significant differences ($p$-value < 0.05) among S, T, or S × T (Table S1).

The S factor significantly influenced the mean number of irregular anaphase and telophase cells, high in B2 (Table S1). The mean number of irregular metaphase, anaphase, and telophase cells differed significantly among PEG treatments (Table S1). In the 10% PEG treatments, a higher number of irregular telophase cells were identified in stands that burned twice (B2) and three times (D3) (Table S1). Concerning the S × T interactions, the highest mean values of irregular telophase cells were determined for B2 × 0% PEG, B2 × 10% PEG, C × 0% PEG, and D3 × 10% (Figure 3).

Independently of the stand or PEG treatment, the irregular dividing cells showed three to five different types of anomalies per mitotic phase (Figure 4; Table S2). The anomalies presented in Figure 4 are representative of those observed in all stands and/or PEG treatments.

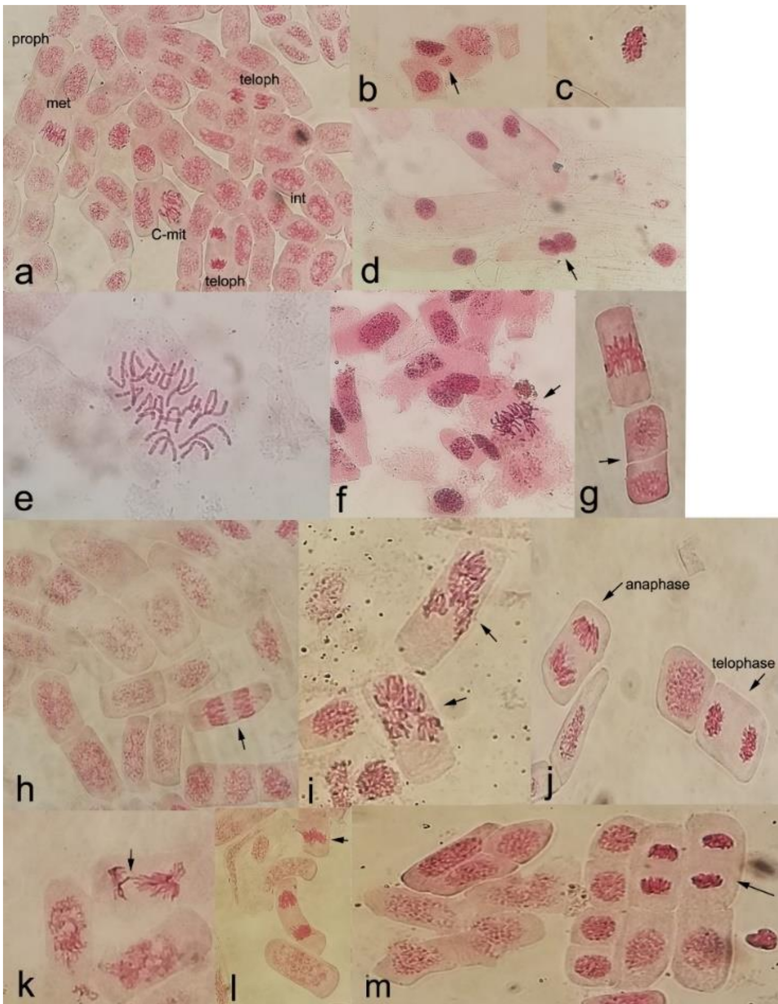


**Figure 4.** Root meristematic cells of *Pinus pinaster* Aiton (2n = 2x = 24) stained with 2% aceto-carmine showing: (**a**) cells in interphase (int), prophase (proph), and metaphase (met), as well as irregular metaphase (C-mitosis) and telophase (teloph) cells; (**b**) one irregular prophase with one micronucleus (arrow); (**c**) a sticky prophase; (**d**) a binucleate prophase (arrow); (**e**) C-mitosis; (**f**) metaphase with disturbance in chromosomal orientation, and laggard chromosomes (arrow); (**g**) one metaphase (upper cell) and one irregular telophase with disturbed orientation (arrow); (**h**) anaphase cell with laggard chromosomes (arrow); (**i**) multipolar anaphases; (**j**) one anaphase with chromosomal disturbance and one normal telophase; (**k**) anaphase with one chromatin bridge (arrow); (**l**) unipolar anaphase (arrow) and one sticky telophase; (**m**) two sticky telophase cells (arrow).

Among the different types of anomalies, the stickiness of chromatin was detected in all mitotic phases (Figure 4), and it was significantly influenced ($p$-value < 0.05) by the stand and PEG treatment, except in prophase (Table S2). In general, for the remaining types of anomalies and mitotic phases, the mean number of irregular dividing cells was higher in the 10% PEG or 20% PEG treatments (Table S2). The lower mean number of irregular cells detected in the S $\times$ 20% PEG interactions was due to the inhibition of root development in this treatment, except for the B2 $\times$ 20% PEG interaction.

Table 5 presents a summary of the variables that were significantly influenced by the stand and PEG treatment factors.

The summarized statistical data revealed that the MGT was significantly influenced by the PEG treatment only during the water stress induction (Table 5). Additionally, different cytogenetic variables were affected by the stand and/or the PEG treatment factors (Table 5).

**Table 5.** Summary of the variables that were significantly influenced by the stand and/or PEG treatment factors.

| Variables Influenced by the Stand Factor (Unburned or Burned One, Two or Three Times) | Variables Influenced by the PEG Treatment Factor (0% PEG, 10% PEG, and 20% PEG) |
|---|---|
| – | MGT (water stress period) |
| MI (%) | MI (%) |
| Mean number of normal metaphase and anaphase cells | Mean number of normal metaphase cells |
| – | DCA (%) |
| Mean number of irregular metaphase, anaphase, and telophase cells | Mean number of irregular metaphase, anaphase, and telophase cells |
| Stickiness of chromatin (metaphase, anaphase, telophase) | Stickiness of chromatin (metaphase, telophase) |
| Laggard chromosomes (anaphase, telophase) | Laggard chromosomes (anaphase, telophase) |
| Binucleate prophase cell | – |
| – | C-mitosis (metaphase) |
| Chromatin bridges in anaphase | Chromatin bridges in anaphase |
| Disturbed chromosomal orientation in anaphase and telophase | Disturbed chromosomal orientation in anaphase and telophase |

Note: MGT—mean germination time, MI—mitotic index, DCA—dividing cells with anomalies.

## 4. Discussion

### 4.1. Impact of Fire Recurrence and/or Induced Water Stress on Seed Germination

The statistical analysis of the germination indexes determined for both water stress induction and recovery periods did not reveal an impact of fire recurrence or PEG treatment on the seed germination ability. The seed germination indexes were low for all stands (S), PEG treatments (T), S $\times$ T, and interactions. Besides, we did not find a pattern of increase or decrease in the germination index between the stress and recovery periods or related to the fire recurrence. Previous studies on different pine species [26,53,60,61] reported that the induction of water stress decreased the seed germination capacity. The low germination capacity under osmotic potentials of $-4$ bar (10% PEG) and $-8$ bar (20% PEG) was previously referred for *Pinus pinaster* [61] and *Pinus ponderosa* [62] seed morphological traits (e.g., size) or reproductive strategies, particularly after fire exposure [63,64].

Although the tree age has been mentioned as a factor influencing germination success [41], all the studied stands can be considered able to produce viable seeds, including the stand D3 (24 years), since the production of viable seeds in maritime pine generally starts at 15 to 20 years age in Portugal, or even lower ages [3]. Additionally, the studied stands share similarities regarding edaphoclimatic and site quality, factors that could influence germination capacity [65]. Hence, the different germination indexes observed during this work may be due to different ratios of serotinous and non-serotinous pinecones per stand, which we did not assess.

Contrary to the germination index, the treatment significantly influenced the mean germination time (MGT). The increase in PEG concentration delayed seed germination. The significant impact of PEG treatment in the MTG, under equal concentrations to those used in this research, was previously observed in *Pinus nigra* [18].

Seeds from the stand that burned three times (D3) took longer to germinate under water stress induction. The MGT during stress increased with PEG concentration, evidencing that water stress delays seed germination. This pattern was not observed in the recovery period. On recovery, seeds from site (C) showed higher MGT values. The low seed germination index found in the interaction stand C $\times$ 0% PEG may arise from abiotic stresses spontaneously occurring in the field.

### 4.2. Impact of Fire Recurrence and/or Induced Water Stress on Root Cell Division

Given the low germination index, the long mean germination time, and the inhibition of root growth, the seeds were maintained beyond the period of water stress induction (28 days). The seeds of the different experiments were kept in the Petri dishes, watered, and monitored daily for an additional period of 67 days (recovery period) in an attempt to collect enough roots to ensure the cytogenetic analysis in most of the S $\times$ T interactions.

The number of roots grown during the recovery period was low. Among the germinated seeds, the root growth was inhibited a few days after the emission of the radicle, hampering the cytogenetic analysis of various interactions, mainly of those involving the 20% PEG treatment.

Overall, the average MI increased significantly ($p$-value < 0.05) with the frequency of fire occurrence among the analysed stands (Table 4). The mean values of MI were high through the various S $\times$ T interactions. Since both normal and irregular dividing cells are included in the calculation, we focused on the mean values DCA to analyse the plant responses to fire recurrence and induced water stress. The average DCA values did not show statistically significant differences ($p$-value > 0.05) among stands, and the lowest DCA was found in the 20% PEG treatment, which differed significantly ($p$-value < 0.05) from the remaining treatments (Table 4). The results revealed that fire recurrence did not impact the frequency of DCA, but such a variable can be influenced by water stress. The cell cycle and chromosomal anomalies can occur spontaneously due to induced or naturally occurring abiotic stress. Besides, the plant has repair mechanisms to correct these anomalies to some extent. Additionally, the post-fire regenerated stands present a higher abundance of serotinous cones than the unburned ones to protect the seeds [66]. The canopy seed bank of *Pinus pinaster* is high enough to enable a massive natural regeneration after the fire, as determined by serotiny level and stand age [39]. Therefore, we considered the DCA values achieved more relevant during the stress period, where the highest mean DCA values were found in the A1 $\times$ 10% PEG and B2 $\times$ 10% PEG interactions, which presented cell cycle and chromosomal anomalies in all mitotic phases.

Most scored normal, and irregular mitotic cells were in prophase, suggesting cell cycle arresting. Similar results were found in *Pinus nigra* [18] and other plant species under drought or other abiotic stresses [16,17,19–21,67]. The cell cycle arresting in prophase delays the mitosis progression, justifying the long time required to root growth that, in most cases, allowed rooting and hampered the cytogenetic analysis. The highest numbers of irregular telophase cells were found in the B2 $\times$ 0% PEG and B2 $\times$ 10% PEG interactions, indicating that despite cellular disturbances, the roots from seeds of stand B2 were able to reach the last phase of mitosis.

The anomalies found in all mitotic phases occurred commonly in various plant species under different abiotic stresses [16–21,67]. As reported by these authors, chromatin stickiness is one of the most frequent anomalies in response to stress, and given its irreversibility, it can lead to cell death. Our results revealed that the appearance of this anomaly in the various stages of mitosis, except prophase, may be influenced by the recurrence of fire and PEG treatment. The abiotic stress induces the oxidative damage of DNA that it is the origin of chromosomal anomalies, as verified for *Pinus nigra* under PEG-induced water stress [18]. This latter pine species presented higher percentage values of germination than *Pinus pinaster* under the same conditions.

Nevertheless, the developed seedlings of *Pinus nigra* presented DNA damage weeks after the water stress induction on the seeds, demonstrating its long-term impact. Such a result can explain the reduced germination indexes in the present study during the recovery period. Besides, only two seedlings belonging to seeds from stand A1 developed in the recovery period.

Drought tolerance depends on the interplay of physiological, biochemical, anatomic-morphological, cellular, and molecular mechanisms, whose effectiveness may vary within species [68]. The present results, compared to those achieved previously in *Pinus sylvestris* [68] and *Pinus nigra* [18], confirmed the differentiated tolerance to water stress among pine species and within species, as seen for the infraspecific taxa of *Pinus nigra* [18].

The importance of the root system in plant survival and water stress response is well documented. The capacity of a seedling to absorb water is affected by the size and distribution of its root system, the root–soil contact, and the hydraulic conductivity of the root [69]. Lack of root development can result in increased seedling water stress ([69] and references therein) and therefore determine seedling survival.

## 5. Conclusions

In this work, the induction of osmotic stress nor the fire recurrence of the stands influenced the germination index of the *Pinus pinaster* seeds significantly. However, the induction of water stress delayed the mean germination time. There was statistical evidence suggesting that during stress, seeds exposed to 20% PEG took longer to germinate (about seven more days) than seeds not exposed to PEG treatment. Induction of water stress with 20% PEG inhibited root growth in most germinated seeds. DCA percentage was not affected by the fire occurrence. In contrast, the water stress induced by 10% PEG increased the %DCA, which may affect root growth.

The findings presented here suggested trade-offs in the impact of recurrent fire and drought on *Pinus pinaster* seed germination and root development of seedlings. The results of this study allow the anticipation of scenarios of the dynamics of *Pinus pinaster* natural regeneration in an unfavourable scenario of climate change, influencing management practices for this species from an adaptive management perspective. Namely, in case of constraints by stress factors, such as drought or low germination rates in situ, natural regeneration can be complemented by sowing or planting using as source genetic material more resistant to water stress and/or populations better adapted to fire to ensure better performance both in germination and in the subsequent development of seedlings.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f14010078/s1, Table S1: Mean (±S.E.) number of normal and irregular cells in prophase, metaphase, anaphase, and telophase determined per stand (S), PEG treatment (T) and for their interaction (S × T). Different lowercase letters per column indicate statistically significant differences ($p$-value < 0.05) among S or T based on the Tukey's test; Table S2: Mean (±S.E.) number of irregular dividing cells in different mitotic phases showing various types of anomalies determined per stand (S), PEG treatment (T) and for their interaction (S × T). Different lowercase letters per column indicate statistically significant differences ($p$-value < 0.05) among S or T based on the Tukey's test.

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