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Development of New Preventive Strategies for Pine Pitch Canker Caused by *Fusarium circinatum* in Irrigation Water and Evaluation in a Real Nursery Context

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Abstract: *Fusarium circinatum* is one of the many threats to forests and *Pinus* nurseries all over the world, being classified as a quarantine organism by several organizations and governing bodies, such as the European and Mediterranean Plant Protection Organization (EPPO) and the European Union (EU), with associated phytosanitary measures in place to prevent its spread through the various means of dispersal. One such means of dispersal is the water used for irrigation in nurseries, which can contain fungal propagules. Three different treatments, namely, Desogerme, Intra Hydrocare and sodium hypochlorite (NaClO), were tested for their efficacy in eliminating *F. circinatum* spores in water at several concentrations. Those that showed 100% disinfection rates were selected for further assays regarding seed germination and water quality impact. From these studies, Desogerme 1% and Intra Hydrocare 4% were then selected for large-scale seed germination and plant certification assays in nurseries, where they showed promising results in regard to the prevention of infections in nurseries, and in this way, contribute to the efforts of mitigating this disease.

Keywords: pine pitch canker prevention; nurseries; irrigation water; irrigation water quality assessment

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1. Introduction

Fusarium circinatum Nirenberg & O'Donnell is a well-known pathogen that is responsible for the pitch canker disease in pine trees and *Pseudotsuga menziesii* [1]. This disease is a very dynamic one, where the symptom expression and severity depend on the host and surrounding biotic and abiotic conditions [2]. Typical symptoms in adult trees include large resinous cankers, which can strangle the treetop and lead to its death. Other symptoms include chlorosis and death of needles and branches due to *Fusarium circinatum*'s ability to obstruct the plant vascular system, which can happen in both seedlings and in young branches of adult trees [2,3]. In seedlings, the symptoms encompass resin exudations, caulk lesions and chlorosis, as well as wilting and dieback [3]. Infected host seeds are asymptomatic, presenting three different types of infection: superficial, non-superficial-active and non-superficial-latent [4].

This disease was first reported in the state of North Carolina in the south-eastern region of the United States of America in 1946 [5] and has since spread to other countries [1,6]. Although its first formal reported presence in Europe dates to 2005 [7], reports of it being present in the Basque Country region of northern Spain go back to 1997 [8], with other European countries following suit, including France [9] and Italy [10]. In April 2008, it was first reported in the central region of Portugal in a nursery [11]. Currently, the pathogen is catalogued in the EPPO as a quarantine organism (A2 list), which is a classification that is also attributed by the EU in accordance with the Commission Implementing Regulation (EU) 2019/2072 of 28 November 2019 (Annex II, Part B), and phytosanitary measures are in place to prevent its spread.

While the spore dispersal of *F. circinatum* in forests occurs mostly during the autumn period, it was shown that it occurs all year round, with no clear temporal trends besides higher air inoculum being favoured by previous lower air temperatures and lower leaf wetness [12]. The dispersal of these spores can be caused by both abiotic (wind and rain, among others) and biotic (insects or other animal vectors and human activity) factors, with human activity being more prevalent in nurseries. One of the abiotic factors that can lead to infection by *F. circinatum* in nurseries is the use of irrigation water contaminated by fungal propagules. Most nurseries use naturally occurring water for irrigation, which at the surface level or in groundwater wells can easily be contaminated with spores or other propagules that can be transported via the air, insects or even rainwater [12]. One such example of irrigation water contaminating a nursery was that of the Karatara nursery in South Africa, where the nearby pond used for irrigating pine seedlings was found to contain *F. circinatum* spores [13].

With its presence in Portugal still being both an ecological and economical threat, an operational group (GO + PrevCRP) was formed as part of the Programa de Desenvolvimento Rural (PDR2020) program to develop new preventive strategies across several important factors in the spread of the disease within nurseries. These preventive strategies must be based on experimental results while working closely with nursery owners to assure the feasibility of them and their implementation in the nurseries. One such factor was the aforementioned irrigation water. As such, the main goals of the present study were to assess the efficacy of certain treatments in eliminating *F. circinatum* spores from contaminated water, to assure the water quality after treatment application, to assess the impact in watered plants with the treated water and to transpose these experimental results into the field by conducting large-scale assays in nurseries, providing both a larger sample size and a way to assess how feasible treatment application can be in a practical context.

2. Materials and Methods

2.1. Culture Media

During this study, two different culture media were prepared: PDAS (potato dextrose agar supplemented with 0.5 mg mL^{-1} of streptomycin sulphate salt) and SNA (Spezieller Nährstoffarmer agar), following the EPPO standard [14] for appropriate media for isolation and morphological identification of *F. circinatum*. Both media were prepared in accordance with the EPPO standard [14].

2.2. Spore Suspension Preparation

In order to test the effectiveness of the treatments, a suspension of *F. circinatum* spores was prepared. For this, a *F. circinatum* isolate supplied by the national reference laboratory and project partner INIAV (Instituto Nacional de Investigação Agrária e Veterinária) was grown in several SNA plates and incubated at room temperature (22 $\pm$ 6 °C) for 30 days to develop reproductive structures. After 30 days, and with the confirmation of the presence of conidia through microscopy, the cultures were washed with sterile MilliQ water and the resulting product of said wash was collected. Neubauer chamber counting was performed, and a solution with an approximate concentration of 1.5 $\times$ 10^6 spores mL^{-1} was prepared.

2.3. Treatment Selection

The tested treatments were Desogerme SP Vegetaux (Laboratoires ACI, Cabriès, France), sodium hypochlorite (Biochem Iberica, Montijo, Portugal) and Intra Hydrocare

(Intracare BV, Veghel, The Netherlands). This selection was based on the potential of these products to successfully eliminate *F. circinatum* spores in a water suspension. Desogerme SP Vegetaux is a commercially available surface disinfectant that contains quaternary ammonium compounds that were shown to be effective fungicides against other *Fusarium* species [15]. Sodium hypochlorite's history as an effective fungicide against both *Fusarium oxysporum* f. sp. *cubense* [15] and other phytopathogenic fungi [16], alongside its ease of access and use, made it an ideal candidate. Finally, Intra Hydrocare presents a special formulation of hydrogen peroxide stabilized by chelated silver nanoparticles that gives it a highly oxidative power due to the Fenton reaction that occurs between hydrogen peroxide and transition metal ions, such as silver, leading to the formation of hydroxyl radicals. This, compounded with its commercial availability, non-residual nature and the ability of hydrogen peroxide to eliminate *Fusarium circinatum* in contaminated irrigation water in a South African pine nursery with no phytotoxicity to pine seedlings [13], as well as in *Pinus* seeds [17], made it a worthy candidate as well.

2.4. Treatment Application

The previously prepared *F. circinatum* spore suspension was then subjected to the chosen treatments. For this, 5 mL of spore suspension was used for each treatment, and Desogerme SP Vegetaux, sodium hypochlorite and Intra Hydrocare were added to each 5 mL sample in the necessary quantities to achieve the final concentrations of 1% for Desogerme SP Vegetaux; 1%, 2% and 2.5% for sodium hypochlorite from a stock solution of 10% NaClO; and 1%, 2%, 3%, 4% and 5% for Intra Hydrocare.

The resulting suspensions were then plated onto PDAS plates and incubated at room temperature (22 ± 6 °C). Serial dilutions of the original spore suspension were prepared (1:1000 up to 1:1,000,000) and were also plated onto PDAS and incubated in the same conditions as a positive control. The same procedure was done with the water used to prepare the solutions as a negative control. The number of colony-forming units (CFUs) was determined by counting the *F. circinatum* colonies in the PDAS plates, with 5 repeats per treatment. Both the mean and standard error were determined, and statistical analysis was conducted as described below. The percentage of disinfection was determined by considering 0 CFUs counted as 100% and by using the following formula for when the mean number of CFUs was higher than 0:

$$\%\ of\ disinfection_{treatment} = 100 - \left(\frac{Treatment\ mean}{C^{+} mean} \times 100\right). \quad (1)$$

2.5. Statistical Analysis

The results of the treatment disinfection (number of CFUs) were analysed via the non-parametric Kruskal–Wallis test and Dunn's post hoc analysis with $p < 0.001$ using PAST software, version 4.03 [18].

2.6. Preliminary Germination Assay

The best-performing treatments from the previous step of disinfection were then selected for a preliminary germination assay to assess their impacts on the germination of *F. circinatum* host species seeds, with distilled water being used as a negative control. In test tubes containing agar as a solid media, seeds from three species of *Pinus* (*Pinus radiata*, *Pinus pinaster* and *Pinus pinea*) were sown, one seed per tube, using 10 seeds per treatment, for a total of 40 seeds per species. These seeds were then irrigated with the treatment solutions at regular intervals and their germination was monitored over 50 days.

2.7. Water Quality Assessment

Desogerme SP Vegetaux, sodium hypochlorite and Intra Hydrocare were added to tap water in the necessary quantities to achieve the final concentrations of 1% for Desogerme

SP Vegetaux, 1% sodium hypochlorite (from a stock solution of 10% NaClO) and 4% for Intra Hydrocare. Tap water, without treatment, was used as the control.

Treated and control waters were analyzed as follows: the pH and electrical conductivity (EC) were measured with a pH meter (model Orion 3 Star, Thermo Fisher Scientific, Waltham, MA, USA) and an EC meter (model Orion Star A212, Thermo Fisher Scientific, Waltham, MA, USA), respectively. Sodium, calcium and magnesium were quantified via inductively coupled plasma optical emission spectrometry (ICP-OES) using an ICP Spectrometer (iCAP 7000 Series ICP Spectrometer, Thermo Fisher Scientific, Waltham, MA, USA). Chloride was quantified via the Mohr method, using silver nitrate for the titration.

Four replicates per treatment were prepared. Data were subject to one-way analysis of variance (ANOVA). Whenever significant differences were found ($p \leq 0.05$), a post hoc Fisher's least significant difference (LSD) test was used to further elucidate differences between treatments at a significance level of $\alpha = 0.05$. The statistical analysis was performed using the Statistix software package (version 9.0; Analytical Software, Tallahassee, FL, USA).

2.8. Nursery Germination Assays

Nursery assays were conducted in 4 different partnered nurseries in similar controlled conditions in order to accommodate the scale of said assays. Two different *Pinus* species were used: *P. pinaster* and *P. pinea*. Whilst it was one of the species studied in the preliminary germination assays, *P. radiata* was not used due to the unavailability of seeds during the experimental period. The treatments tested were Desogerme 1% and Intra Hydrocare 4% in accordance with the results obtained in the water quality assays (see the Results and Discussion section).

The assays were put in place in each of the 4 nurseries as shown in Table 1, where one seed was sowed per container cell, and seeds/seedlings were regularly irrigated with treated irrigation water. Here, the water used to prepare the treatments was used untreated as the control. Seed germination was assessed one and two months post-sowing.

Table 1. Nursery germination assays: treatments and species tested per nursery.

Nursery	Species	Treatment	No. of Containers per Treatment	No. of Individual Cells per Container	Total No. of Cells per Treatment
A	*Pinus pinaster*	Intra Hydrocare 4% Control	24	60	1440
B	*Pinus pinea*	Desogerme 1% Control	25	54	1350
C	*Pinus pinaster*	Desogerme 1% Control	34	40	1360
D	*Pinus pinea*	Intra Hydrocare 4% Control	30	54	1620

Considering that, in accordance with the applicable Portuguese legislation (Law-Decree nº 205/2003, from 12 September, amended and republished by Law-Decree n.º 13/2019, from 21st January), there are defined general standards applied to the production and commercialization of forest plants, the same methodology of plant certification used by ICNF, I.P., was conducted to evaluate the development of the plants resulting from this assay to assess whether they fulfilled the prerequisites for plant certification. To this end, 7 to 8 months post-sowing, the plants from each assay were observed and the average height, average collar diameter, and the absence or presence of deficiencies were assessed. Statistical analysis of the data obtained in each nursery for both the germination and plant certification results was undertaken via the two-sample *t*-test using PAST software, version 4.03 [18].

3. Results and Discussion

3.1. Effect of Treatments on Fusarium circinatum Spores

The number of CFUs was determined by counting the *F. circinatum* colonies in five PDAS plates per treatment, and the obtained results are displayed in Table 2.

Table 2. Mean of the *Fusarium circinatum* CFUs per treatment and corresponding percentage of disinfection. Data (n = 5) associated with different letters represent significantly different results in accordance with Dunn's post hoc test, $p < 0.001$. The percentage of disinfection was considered non-applicable (n.a.) for the negative control group.

Treatment	Mean (n = 5)	Standard Error	% of Disinfection
C^+	8.6×10^5 a	1.9×10^4	0
C^-	0 e	0	n.a.
NaClO 1%	0 e	0	100
NaClO 2%	0 e	0	100
NaClO 2,5%	0 e	0	100
Desogerme 1%	0 e	0	100
Intra Hydrocare 1%	3280 b	3.16	99.62
Intra Hydrocare 2%	401 c	5.06	99.53
Intra Hydrocare 3%	5.4 d	3.82	99.99
Intra Hydrocare 4%	0 e	0	100
Intra Hydrocare 5%	20 d	10.96	99.98

As we can observe, the treatments had a significant effect when compared with the control, with several being able to achieve a 100% disinfection of the *F. circinatum* spores in suspension, namely, sodium hypochlorite at all tested concentrations, Desogerme at 1% concentration and Intra Hydrocare at 4% concentration (Table 2). The statistical analysis showed that these treatments were significantly similar to one another and the negative control, and significantly different from the positive control and the treatments that showed a CFU mean higher than 0 ($p < 0.001$). It also demonstrated that all other tested treatments were significantly different from both the positive and negative controls, meaning that they showed a marked effect on *F. circinatum* spores in suspension, but were not able to achieve 100% disinfection.

Sodium hypochlorite showed for *F. circinatum* the same fungicidal activity as it had previously been reported both in other Fusarium species, such as *Fusarium oxysporum* f. sp. *cubense* [15], and in other phytopathogenic fungi, namely, *Botrytis cinerea* [16], achieving a disinfection percentage of 100%, even at the lowest concentration tested (1%). The same effect was also verified for Desogerme, which was also previously reported as an effective fungicide *against Fusarium oxysporum* f. sp. *cubense* [15] at the only concentration tested (1%). Regarding Intra Hydrocare, it showed disinfection rates above 99% for all concentrations tested, and all treatments were revealed to be significantly different from the positive control in terms of the CFU numbers ($p < 0.001$). Moreover, a 100% disinfection was also achieved with a concentration of 4% and was significantly similar to the negative control ($p < 0.001$). As such, Intra Hydrocare's special formulation of hydrogen peroxide and chelated silver particles was shown to be successful in the elimination of *F. ciricnatum* spores.

Thus, we could observe that several treatments achieved promising results in lab conditions, and these were selected for further assays to test the feasibility of their application in a real nursery context.

3.2. Preliminary Germination Assays

Since all sodium hypochlorite (NaClO) treatments were 100% effective in terms of disinfection, the lower concentration of 1% was selected for further experiments, with the intent of lowering the potential harmfulness for plants, as well as lowering the eventual cost of its application in nurseries. As such, the treatments selected for this assay were

NaClO 1%, Desogerme 1% and Intra Hydrocare 4%. The number of germinated seeds was recorded throughout 50 days, and the results obtained were as follows (Figure 1).

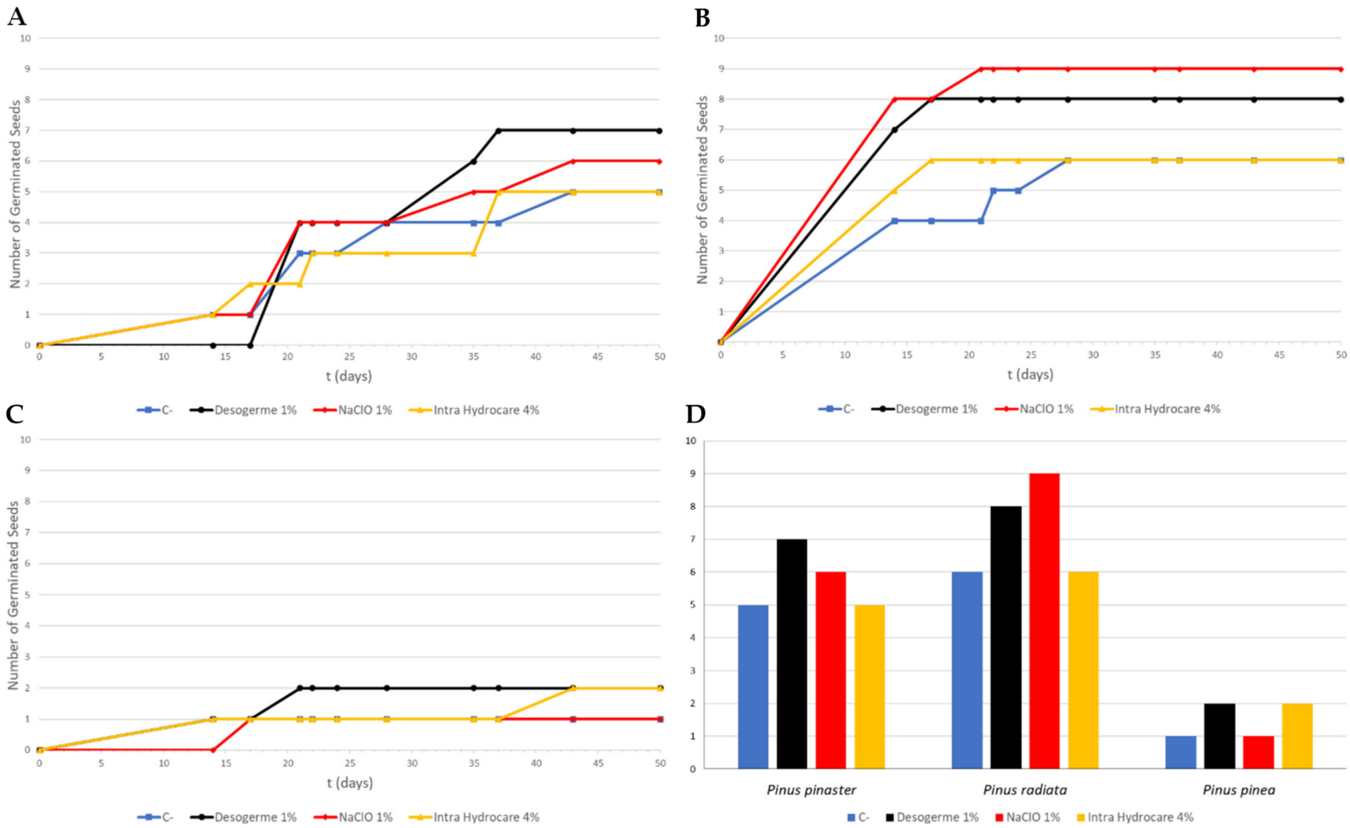

Figure 1. Number of germinated seeds of (**A**) *Pinus pinaster*, (**B**) *Pinus radiata* and (**C**) *Pinus pinea* throughout 50 days per treatment. (**D**) Total number of germinated seeds after 50 days per treatment and species.

The number of germinated seeds throughout the 50 days (Figure 1) showed that, as far as we could determine in this small-scale study, no negative impact was observed for any treatment when compared with the control. This was observed for all species of *Pinus* tested. In *Pinus pinaster*, the control showed 5/10 seeds germinated, with Intra Hydrocare 4% matching that same germination rate and NaClO 1% and Desogerme 1% showing higher rates of germination, namely, 6/10 and 7/10 germinated seeds, respectively. In *Pinus radiata*, the control showed 6/10 germinated seeds, with Intra Hydrocare 4% once again matching that germination rate, and both NaClO 1% and Desogerme 1% showing higher germination rates, in this case, 9/10 and 8/10, respectively. Finally, in *Pinus pinea*, the germination rate was very low across all treatments and the control. However, once again, no treatment showed a lower germination rate than that of the control group.

While these results on their own were not very significant due to the small scale of the assay, they were, however, a good hint of the potential application of these treatments in a real context. Having this in mind, water quality assessment assays (see the Materials and Methods section) were performed for NaClO 1%, Desogerme 1% and Intra Hydrocare 4% to determine the effects of these treatments in terms of possible plant toxicity effects based on water chemical parameters.

3.3. Water Quality Assessment

Table 3 presents the effect of the different treatments on the quality of the water for irrigation.

Table 3. Mean values of the pH; electrical conductivity (EC); concentrations of chloride (Cl^-), sodium (Na), calcium (Ca) and magnesium (Mg); and sodium adsorption ratio (SAR) of the irrigation water, which was untreated (control) or treated with NaClO 1%, Desogerme 1% or Intra Hydrocare 4%. In each column, values followed by the same letter did not significantly differ by LSD test ($\alpha = 0.05$).

Treatment	pH	EC (mS cm^{-1})	Cl^- (mg L^{-1})	Na (mg L^{-1})	Ca (mg L^{-1})	Mg (mg L^{-1})	SAR
Control	7.87 b	0.28 b	32.8 b	50.6 b	15.3	3.11 b	3.1 b
NaClO 1%	11.88 a	33.5 a	11650 a	2211 a	15.9	4.66 a	125 a
Desogerme 1%	7.24 c	0.59 b	178.5 b	11.62 b	13.7	3.21 b	0.74 b
Intra Hydrocare 4%	6.63 d	0.27 b	33.5 b	16.98 b	11.5	2.25 c	1.20 b

Statistical analysis of the data shows that the treatments significantly affected ($p < 0.001$) the water pH. Water pH is an important parameter to be considered since it influences the relative solubility of certain nutrients and can impact the solubility of certain chemicals or pesticides used by growers [19], with the recommended pH range for nursery irrigation water ranging from 5.5 to 6.5 [20]. The irrigation water used in this experiment (control) had a pH of 7.87, slightly higher than the recommended range, and disinfection with NaClO 1% caused a very sharp increase in pH to 11.88, which is a very high value for irrigation water. High pH values of the irrigation water can cause an increase in the growing media pH, which causes the insolubilization of several nutrients, reducing their absorption and leading to a deficiency in these nutrients in the plants [21]. In contrast, treatment with Desogerme 1% and Intra Hydrocare 4% reduced the water pH to 7.24 and 6.63, respectively, which are values closer to the recommended pH range.

Compared with the control (untreated water), treatment with NaClO 1% led to a very sharp and significant ($p < 0.001$) increase in the electrical conductivity (EC) of the water, which increased from 0.28 mS cm^{-1} (control) to 33.5 mS cm^{-1} (NaClO 1%). The optimal EC range for nursery irrigation water is 0 to 0.50 mS cm^{-1}, and an EC higher than 1.50 mS cm^{-1} is considered unacceptable [20]. A high EC is a consequence of a high salt concentration in the water, meaning that a salinity problem exists if salt accumulates in the crop root. Yield reductions occur when salts accumulate in the root zone to such an extent that the crop is no longer able to extract sufficient water from the salty soil solution, resulting in water stress for a significant period [22]. Symptoms vary with the growth stage, which is more noticeable if the salts affect the plant during the early stages of growth, as happens in forest nurseries. In the other treatments, the EC values remained within the acceptable value range: Desogerme 1% led to a slight EC increase (to 0.59 mS/cm); Intra Hydrocare 4% did not affect the EC of the water.

Considering the concentration of the specific ions evaluated, the NaClO 1% treatment led to a very marked and significant increase ($p < 0.001$) in the concentration of chlorides and sodium in the water, which was in agreement with the increase in EC found in the same treatment. Chloride (11,650 mg L^{-1}) and sodium (2211 mg L^{-1}) concentrations in this treatment were much higher than the maximum values (70 and 50 mg L^{-1}, respectively) recommended for irrigation water [20]. Besides affecting the water EC, chloride and sodium can be directly toxic to the plant [19]. Regarding the other treatments (Desogerme 1% and Intra Hydrocare 4%), the concentrations of chlorides and sodium in the water were not significantly different from the control. The treatments had no effect ($p > 0.05$) on the calcium concentration in the water and the effect on the magnesium concentration was not relevant.

The sodium concentration in irrigation water needs to be evaluated in terms of the amounts of calcium and magnesium because of the close interactions between the three nutrients. To quantify this relationship, the sodium adsorption ratio (SAR) was calculated [16]. In accordance with the effect on the sodium concentration, water treated with NaClO 1% showed a very high SAR value (125), which was significantly higher than the SAR values of the other treatments. Generally, the higher the SAR value, the greater the potential for harmful effects. High-SAR water applied using an overhead irrigation system in a climate with low humidity (i.e., evaporates quickly) can cause leaf injury [19]. An excessive sodium proportion (high SAR) in irrigation water promotes soil dispersion and structural breakdown, often resulting in a severe water infiltration problem due to the lack of sufficient calcium to counter the dispersing effects of the sodium. Other related problems, such as soil crusting, poor seedling emergence, lack of aeration, plant and root diseases, and weed control problems caused by the low rate of infiltration may further complicate crop management [22].

The results obtained showed that treatment with Desogerme 1% and Intra Hydrocare 4% did not negatively affect the water quality for irrigation. On the other hand, due to the very high values of pH, EC, chlorides and sodium concentrations, and SAR, the water treated with NaClO 1% was not suitable for watering plants in nurseries.

3.4. Nursery Germination Assays

After the water quality assays determined that the NaClO 1% treatment made the treated water unusable for irrigation, large-scale germination assays were conducted in the partnered nurseries for the Intra Hydrocare 4% and Desogerme 1% treatments. It was possible to verify that none of the two tested treatments negatively affected the germination of *P. pinaster* and *P. pinea* seeds. Moreover, it was possible to obtain germination rates similar, or even superior in some cases, to the control (Table 4). Statistical analysis of the data showed that for most nurseries, both treatment and control germination rates were significantly similar to one another after two months, with the exception being nursery C, where Desogerme 1% showed a significantly higher germination rate than that of the control group. The overall reduced germination rate registered in nursery B was likely due to a late sow. A better germination rate would have been obtained had the sowing taken place earlier, i.e., during March.

Table 4. Germination rate (%) after watering with the Desogerme 1% and Intra Hydracare 4% treatments in nurseries 1 and 2 months post-sowing. In each column, data followed by * were significantly different according to a *t*-test ($p < 0.05$).

	Germination Rate Post-Sowing (%)							
Species	***Pinus pinaster***				***Pinus pinea***			
Nursery	**A**		**C**		**B**		**D**	
Treatment	**1 month**	**2 months**	**1 month**	**2 months**	**1 month**	**2 months**	**1 month**	**2 months**
Desogerme 1%	-	-	68	79 *	31	39	-	-
Intra Hydrocare 4%	73 *	90	-	-	-	-	0 *	51
Control	85 *	89	62	66 *	32	39	9 *	46

The plant certification process evaluated *P. pinaster* and *P. pinea* plants that were subjected to each of the two treatments in three of the nurseries (A, B and C), and the results were those presented in Table 5. Nursery D showed generalized plant death for both Intra- Hydrocare-treated and control plants, and as such, no plant certification evaluation took place.

The obtained results showed that the percentages of certified *Pinus pinaster* plants were very similar between the treated and non-treated plants, even if slightly lower for the former. Some variability could be seen in the results between some of the nurseries, which could have been due to several factors, namely, late sowing and extreme heat during the summer that preceded the plant certification.

Nursery A presented a percentage of certification above 90% for both the treated and control *P. pinaster* plants (90.38% and 93.70%, respectively) and a low rate of failures. While the mean height was significantly different between the treatment and control, both groups still showed an average height that was higher than the one required for certification in this species [23]. In nursery B, the *P. pinea* plants did not display the necessary values of height and diameter for certification, and as such, no plant certification data can be shown.

However, these results were the same for both the treated and control plants, with statistical analysis showing the mean height and diameter values to be significantly similar between the treatment and control, and thus, could not be attributed to irrigation with Desogerme 1%; instead, this result was likely caused by the aforementioned factors that conditioned the plant growth, namely, the late sowing and high temperatures experienced. Finally, nursery C showed similar plant certification rates for both the treated and non-treated plants (78.26% and 79.41%, respectively) (Table 5). Statistical analysis of the data from nursery C showed that the differences in the mean height and diameter of the plants were not significant.

Table 5. Number of certified plants per nursery, species and treatment. Per nursery, mean height and mean diameter data followed by * were significantly different according to a t-test ($p < 0.001$).

	Plant Certification					
Species	*Pinus pinaster*				*Pinus pinea*	
Nursery	A		C		B	
Treatment	**Intra Hydrocare 4%**	**Control**	**Desogerme 1%**	**Control**	**Desogerme 1%**	**Control**
Alveoli (total)	1440	1440	1360	1360	1350	1350
No. plants/ lot	1330	1253	1343	1360	474	494
No. certified plants	1202	1174	1051	1080	-	-
% of certified plants	90.38	93.7	78.26	79.41	-	-
Mean height (cm)	11.07 *	12.75 *	6.83	6.71	6.32	6.58
Mean diameter (mm)	2.00	2.00	1.99	1.96	1.95	1.99

These results showed that while there was a decrease in the percentage of certified plants for both treatments, it was negligible in the case of the Desogerme-1%-treated plants. This decrease was more pronounced in the Intra-Hydrocare-4%-treated plants. However, both the treated and non-treated plants showed percentages of certification above 90%. Additionally, while there was a decrease in the percentage of certified plants for the Desogerme 1% treatment, the treated plants that were certified showed identical average heights and diameters to the control plants.

Lastly, it is important to note that due to the irrigation systems already implemented in the nurseries where these tests took place, the logistics of implementing these treatments were the biggest difficulty encountered in this study. Nevertheless, solutions that bypassed the problem were found and, while requiring more labour, the possible gains of preventing a disease as severe as pine pitch canker in a *Pinus* spp. nursery far outweigh the costs associated with these new irrigation procedures.

4. Conclusions

With this study, a new approach to *Fusarium circinatum* prevention was developed in close collaboration with nurseries that are prone to be affected by the spread of this disease. As such, this partnership allowed for a very complete overarching evaluation of the efficacy of the tested treatments, from the experimental results to their final application in a proper nursery context.

The data showed that, of the selected treatments, Desogerme 1%, Intra Hydrocare 4% and NaClO 1% were able to completely eradicate the *F. circinatum* spores in suspension, and thus, were selected for further evaluations of their applicability. Preliminary germination assays indicated that none of these treatments negatively impacted *Pinus* spp. seed germination and further water quality impact studies were conducted. From these, the obtained results showed that water treatment with NaClO 1% made it unsuitable for irrigation due to the very high values of pH, EC, chlorides and sodium concentrations, and SAR. However, the quality of the water treated with Desogerme 1% and Intra Hydrocare 4% was not significantly affected, and thus, could be used for irrigation.

Finally, large-scale germination and plant certification assays were conducted in four partnered nurseries for both treatments. With those assays, it was possible to conclude that the percentage of germinated *Pinus pinaster* and *Pinus pinea* seeds subjected to treatments was always equal or superior to that of the seeds irrigated with non-treated water 2 months post-sowing. Plant certification evaluation took place 7–8 months post-sowing, and it was possible to verify that while there were some differences between the treated and non-treated plants, they were negligible. As such, should nurseries be able to implement these measures in their *Pinus* spp. plant production, they could prevent plant infection by including these treatments in their irrigation practices. This, associated with other preventive practices, such as container disinfection, can lead to a reduction in *F. circinatum* spread in nurseries and, consequently, in forests.

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References

1. Drenkhan, R.; Ganley, B.; Martín-García, J.; Vahalík, P.; Adamson, K.; Adamčíková, K.; Ahumada, R.; Blank, L.; Bragança, H.; Capretti, P.; et al. Global Geographic Distribution and Host Range of *Fusarium circinatum*, the Causal Agent of Pine Pitch Canker. *Forests* **2020**, *11*, 724. [CrossRef]
2. Wingfield, M.J.; Hammerbacher, A.; Ganley, R.J.; Steenkamp, E.T.; Gordon, T.R.; Wingfield, B.D.; Coutinho, T.A. Pitch canker caused by *Fusarium circinatum*—A growing threat to pine plantations and forests worldwide. *Australas. Plant Pathol.* **2008**, *37*, 319–334. [CrossRef]
3. Solel, Z.; Bruck, R.I. Relation between wilt rate and obstruction of water flow in stems of two families of loblolly pine affected by pitch canker. *Eur. J. For. Pathol.* **1990**, *20*, 317–320. [CrossRef]
4. Agustí-Brisach, C.; Pérez-Sierra, A.; Armengol, J.; García-Jiménez, J.; Berbegal, M. Efficacy of hot water treatment to reduce the incidence of *Fusarium circinatum* on *Pinus radiata* seeds. *Forestry* **2012**, *85*, 629–635. [CrossRef]
5. Hepting, G.H.; Roth, E.R. Pitch canker, a new disease of some southern pines. *J. For.* **1946**, *44*, 724–744.
6. Berbegal, M.; Pérez-Sierra, A.; Armengol, J.; Grünwald, N.J. Evidence for Multiple Introductions and Clonality in Spanish Populations of *Fusarium circinatum*. *Phytopathology* **2013**, *103*, 851–861. [CrossRef] [PubMed]
7. Landeras, E.; Garcia, P.; Fernández, Y.; Braña, M.; Fernández-Alonso, O.; Méndez-Lodos, S.; Pérez-Sierra, A.; Leon, M.; Abad-Campos, P.; Berbegal, M.; et al. Outbreak of Pitch Canker Caused by *Fusarium circinatum* on *Pinus* spp. in Northern Spain. *Plant Dis.* **2005**, *89*, 1015. [CrossRef]
8. Dwinell, D. Global Distribution of the Pitch Canker Fungus. In *Current and Potential Impacts of Pitch Canker in Radiata Pine, IMPACT Monterey Workshop, Monterey, CA, USA*; Citeseer: Princeton, NJ, USA, 1999; Volume 30, pp. 54–57.
9. EPPO Global Database. *First Report of Gibberella circinata in France*; EPPO: Paris, France, 2006; p. 104.
10. Carlucci, A.; Colatruglio, L.; Frisullo, S. First report of Pitch Canker caused by *Fusarium circinatum* on *Pinus halepensis* and *P. pinea* in Apulia (southern Italy). *Plant Dis.* **2007**, *91*, 1683. [CrossRef] [PubMed]
11. Bragança, H.; Diogo, E.; Moniz, F.; Amparo, P. First report of Pitch canker on Pines caused by *Fusarium circinatum* in Portugal. *Plant Dis.* **2009**, *93*, 1079. [CrossRef]
12. Dvořák, M.; Janoš, P.; Botella, L.; Rotková, G.; Zas, R. Spore Dispersal Patterns of *Fusarium circinatum* on an Infested Monterey Pine Forest in North-Western Spain. *Forests* **2017**, *8*, 432. [CrossRef]

13. Van Wyk, S.J.; Boutigny, A.L.; Coutinho, T.A.; Viljoen, A. Sanitation of a South African forestry nursery contaminated with *Fusarium circinatum* using hydrogen peroxide at specific oxidation reduction potentials. *Plant Dis.* **2012**, *96*, 875–880. [CrossRef] [PubMed]
14. EPPO. European and Mediterranean Plant Protection Organization. *EPPO Bull.* **2019**, *49*, 228–247.
15. Nel, B.; Steinberg, C.; Labuschagne, N.; Viljoen, A. Evaluation of fungicides and sterilants for potential application in the management of *Fusarium* wilt of banana. *Crop Prot.* **2007**, *26*, 697–705. [CrossRef]
16. Macnish, A.J.; Morris, K.; Theije, A.; Mensink, M.; Boerrigter, H.; Reid, M.; Jiang, C.; Woltering, E. Sodium hypochlorite: A promising agent for reducing *Botrytis cinerea* infection on rose flowers. *Postharvest Biol. Technol.* **2010**, *58*, 262–267. [CrossRef]
17. Dwinell, L.D.; Fraedrich, S.W. Contamination of Pine Seeds by the Pitch Canker Fungus. In *Forest and Conservation Nursery Associations*; General Technical Reports SRS-25; USDA, Forest Service, Southern Research Station: Asheville, NC, USA, 1999.
18. Hammer, Ø.; Harper, D.A.T.; Ryan, P.D. PAST: Paleontological statistics software package for education and data analysis. *Palaeontol. Electron.* **2001**, *4*, 9.
19. Robbins, J.A. *Irrigation Water for Greenhouses and Nurseries*; University of Arkansas: Little Rock, AR, USA; United States Department of Agriculture, and County Governments Cooperating: Washington, DC, USA, 2010.
20. Landis, T.D.; Wilkinson, K.M. Water Quality and Irrigation. In *Tropical Nursery Manual: A Guide to Starting and Operating a Nursery for Native and Traditional Plants*; Agriculture Handbook 732; Department of Agriculture, Forest Service: Washington, DC, USA, 2014; p. 376.
21. Voogt, W.; Bar-Yosef, B. Water and Nutrient Management and Crops Response to Nutrient Solution Recycling in Soilless Growing Systems in Greenhouses. In *Soilless Culture*; Elsevier: Amsterdam, The Netherlands, 2019; pp. 425–507. [CrossRef]
22. Ayers, R.S.; Westcot, D.W. *Water Quality for Agriculture*; Food and Agriculture Organization of the United Nations: Rome, Italy, 1985; Volume 29, p. 174.
23. Instituto da Conservação da Natureza e Florestas. Available online: https://www.icnf.pt/florestas/plantasesementes/mfr (accessed on 23 January 2023).