The Use of Sodium Hypochlorite and Plant Preservative Mixture Significantly Reduces Seed-Borne Pathogen Contamination When Establishing In Vitro Cultures of Wheat (Triticum aestivum L.) Seeds

Bošnjak Mihovilović, Anita; Kereša, Snježana; Lazarević, Boris; Topolovec Pintarić, Snježana; Martinko, Katarina; Marković, Zvjezdana; Turkalj, Katarina; Habuš Jerčić, Ivanka

Source / Izvornik: **Agriculture, 2024, 14**

Journal article, Published version
Rad u časopisu, Objavljena verzija rada (izdavačev PDF)

https://doi.org/10.3390/agriculture14040556

Permanent link / Trajna poveznica: https://urn.nsk.hr/urn:nbn:hr:204:875201

agriculture

MDPI

Article

The Use of Sodium Hypochlorite and Plant Preservative Mixture Significantly Reduces Seed-Borne Pathogen Contamination When Establishing In Vitro Cultures of Wheat (*Triticum aestivum* L.) Seeds

Anita Bošnjak Mihovilović [1,*], Snježana Kereša [1], Boris Lazarević [2,3], Snježana Topolovec Pintarić [4], Katarina Martinko [4], Zvjezdana Marković [5], Katarina Turkalj [1] and Ivanka Habuš Jerčić [1]

[1] Department of Plant Breeding, Genetics and Biometrics, University of Zagreb Faculty of Agriculture, Svetošimunska cesta 25, 10 000 Zagreb, Croatia; skeresa@agr.hr (S.K.); kate.turkalj@gmail.com (K.T.); ihabus@agr.hr (I.H.J.)
[2] Department of Plant Nutrition, University of Zagreb Faculty of Agriculture, Svetošimunska cesta 25, 10 000 Zagreb, Croatia; blazarevic@agr.hr
[3] Centre of Excellence for Biodiversity and Molecular Plant Breeding (CroP-BioDiv), Svetošimunska cesta 25, 10 000 Zagreb, Croatia
[4] Department of Plant Pathology, University of Zagreb Faculty of Agriculture, Svetošimunska cesta 25, 10 000 Zagreb, Croatia; stopolovec@agr.hr (S.T.P.); kmartinko@agr.hr (K.M.)
[5] Department of Viticulture and Enology, University of Zagreb Faculty of Agriculture, Svetošimunska cesta 25, 10 000 Zagreb, Croatia; zmarkovic@agr.hr
* Correspondence: amihovilovic@agr.hr; Tel.: +385-1-239-3850

Abstract: In vitro plants that are free of pathogens are crucial for biotechnological breeding methods. The present study investigates the effects of sterilization with sodium hypochlorite (NaClO) and the addition of Plant Preservative Mixture (PPM^{TM}) to the growth medium on pathogen elimination, germination, and seedling development of the winter wheat (*Triticum aestivum* L.) variety Mara. The sterilization treatments differed in the duration of seed sterilization in 4% NaClO and the PPM concentration added to the growth medium. Pathogenic fungi of the genera *Aspergillus*, *Cladosporium*, *Penicillium*, and *Bipolaris* were completely eliminated when the seeds were sterilized in NaClO and placed on growth media with the addition of PPM. Extending the duration of the sterilization treatment with NaClO to 50 min reduced *Fusarium* contamination, while the interaction between the 50 min sterilization treatment with NaClO and the addition of PPM to the growth medium reduced *Alternaria* contamination. Our results suggest that PPM could complement sterilization procedures with NaClO in the introduction of highly infected wheat seeds in vitro. Seed germination was not affected by sterilization with NaClO or by the addition of PPM. However, PPM at a concentration of 4 mL L^{-1} had a negative effect on seedling development.

Keywords: fungal pathogens; in vitro culture; PPM; sterilization; wheat seed; *Alternaria* sp.; *Aspergillus* sp.; *Cladosporium* sp.; *Penicillium* sp.; *Fusarium* sp.; *Bipolaris* sp.

Citation: Bošnjak Mihovilović, A.; Kereša, S.; Lazarević, B.; Topolovec Pintarić, S.; Martinko, K.; Marković, Z.; Turkalj, K.; Habuš Jerčić, I. The Use of Sodium Hypochlorite and Plant Preservative Mixture Significantly Reduces Seed-Borne Pathogen Contamination When Establishing In Vitro Cultures of Wheat (*Triticum aestivum* L.) Seeds. *Agriculture* **2024**, *14*, 556. https://doi.org/10.3390/agriculture14040556

Academic Editor: Bernard Hauser

Received: 10 February 2024
Revised: 25 March 2024
Accepted: 28 March 2024
Published: 31 March 2024

1. Introduction

Wheat (*Triticum aestivum* L.) is an important cereal crop with an estimated production of 803.1 million tons in 2022/23 [1]. Despite hopes that the world would emerge from the COVID-19 pandemic in 2021 and food security would begin to improve, global hunger continued to rise in 2021. It is estimated that between 702 and 828 million people worldwide (corresponding to 8.9 and 10.5 percent of the world population, respectively) faced hunger in 2021 [2]. Climate change, along with other factors, including the COVID-19 pandemic and the war between Russia and Ukraine, has contributed to the global rise in food prices in 2022 and continues to pose a threat to food security [3]. In order to ensure a sufficient amount of food for the growing world population in the shortest possible time, it is

necessary to accept both conventional and biotechnological plant breeding [4]. Tissue culture is an integral part of biotechnological breeding [5]. In wheat, immature embryos are the most commonly used explants to initiate plant tissue culture, but they have limited seasonal availability and are difficult to obtain in the off-season [4,6]. On the other hand, the use of mature embryos saves time and space [7] as they are convenient to collect and easy to store and are readily available as mature seeds [6]. In addition, zygotic embryos are one of the most suitable tissues for cryopreservation of germplasm from the point of view of genetic stability [8].

A critical stage in the introduction of mature wheat seed into tissue culture is to obtain cultures that are free from biological contamination. The vegetation period of wheat is almost 8 months. During this time, the seed is exposed to many pathogens. Various *Alternaria*, *Bipolaris*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Penicillium*, *Tilletia*, and *Ustilago* species are the most common seed-borne fungi of wheat worldwide and cause significant quality losses. The term "seed-borne" describes the condition of any microorganism carried with, on, or in the seed [9]. Detection and control of seed-borne diseases through quality control programmes that monitor seeds from harvest to purchase, marketing, and field sowing are critical to ensure high-quality, pathogen-free, and genetically pure seed [10]. Infected wheat seed can be a passive carrier of disease into new areas. Not only the growth of the seed industry and the global seed market but also the international exchange of plant germplasm for breeding programmes and research purposes have contributed to the risk of seed-borne pathogens spreading to new areas [9]. For vegetatively propagated plants, many countries regulate the import of vegetative germplasm, and the FAO-IPGRI technical guidelines recommend pathogen-tested in vitro plants for import between countries [11].

Because in vitro conditions provide an optimal growth environment for bacteria and fungi, unsuccessful sterilization hinders the progress of tissue culture studies [12]. During disinfection, all microorganisms, such as bacteria and fungi, that contaminate the explant exogenously and endogenously must be removed without damaging or destroying the plant tissue [13]. A range of chemicals, such as sodium hypochlorite, ethanol, hydrogen peroxide, bromine water, mercuric chloride, and silver nitrate, have been used for surface sterilization of plant material. However, sodium hypochlorite (NaClO) is the most commonly used disinfectant. It has a strong oxidizing property that makes it reactive with amino acids, nucleic acids, amines, and amides and is very effective against all types of bacteria, fungi, and viruses [12]. Due to phytotoxicity, a balance between the concentration and duration of exposure to sodium hypochlorite must be determined empirically for each type of explant [14]. Over-sterilization leads to a complete removal of microorganisms and also has a lethal effect on plant tissue. Therefore, it is important to determine the optimal conditions for a particular tissue or crop [15,16].

In addition to the surface sterilization of plant materials, Plant Preservative Mixture (PPM^{TM}, Plant Cell Technology, Washington, DC, USA) can be added to the tissue culture medium. It is a robust, broad-spectrum biocide formulated for use in plant tissue culture. In general, biocides have a broad spectrum of effects on bacteria, fungi, and algae and cause extreme physical or chemical damage to cells and toxicity [16]. PPM is specially formulated to protect plant tissue cultures from endogenous and exogenous contamination without damaging the explants. The active components of PPM penetrate microbial cells by inhibiting enzymes required for important metabolic processes, such as the citric acid cycle and the electron transport system, and inhibit the transfer of monosaccharides and amino acids into the cell [17]. PPM is autoclavable at 1.05 kg cm^{-2} (15 psi) and 121 °C for 20 min. As reported for several plant species, PPM, when added to media prior to sterilization, is effective in preventing and eliminating microbes [18]. However, no data are available for wheat.

Plant breeding programmes using biotechnological approaches require pathogen-free in vitro plants. However, contamination of plant cell and tissue cultures remains a problem that needs to be solved to ensure the successful introduction of plant material under in vitro conditions [19,20]. Due to the long growing season of winter wheat, an increased incidence

of seed-borne fungi is to be expected. The aim of this study was to detect fungal pathogens in winter wheat seed and to determine the effect of surface sterilization treatments (1) on the elimination of contamination by seed-borne pathogens in wheat seed cultivated in vitro and (2) on germination and seedling development.

2. Materials and Methods

2.1. Plant Material

The winter wheat variety Mara was provided by the University of Zagreb, Faculty of Agriculture. It was selected for this study because it was natural and had no protective fungicide coating.

2.2. Sterilization Treatments and In Vitro Culture Establishment

In this experiment, 12 different treatments for the sterilization of wheat seeds were tested (Table 1). The sterilization treatments differed in the surface sterilization procedures (N, C, S40, S50) and in the concentration of PPM added to the growth medium (0, 2, or 4 mL L^{-1}) (Table 1).

Table 1. Sterilization treatments used for sterilization of wheat (*Triticum aestivum* L.) seeds.

Sterilization Treatment Abbreviation	Surface Sterilization Procedure Abbreviation/ Surface Sterilization Procedure Description		Amount of PPM Added (mL L^{-1}) to the Growth Medium
N-PPM0 N-PPM2 N-PPM4	N	The dry control procedure: the dry seeds were placed on a sterile medium without washing or sterilization	0 2 4
C-PPM0 C-PPM2 C-PPM4	C	The wet control procedure: seeds were washed under tap water for 30 min, soaked in 70% ethanol for 30 s, treated with sterile dd H_2O + 0.05% Tween20 for 40 min, and then washed four times with sterile dd H_2O	0 2 4
S40-PPM0 S40-PPM2 S40-PPM4	S40	Seeds were washed under tap water for 30 min, soaked in 70% ethanol for 30 s, treated with 4% NaClO + 0.05% Tween20 for 40 min, and then washed four times with sterile dd H_2O	0 2 4
S50-PPM0 S50-PPM2 S50-PPM4	S50	The same as S40 but treated with 4% NaClO + 0.05% Tween20 for 50 min	0 2 4

The seed growth medium was prepared by dissolving 7 g of L^{-1} Plant agar (Duchefa Biochemie, Haarlem, Netherlands) in distilled demineralized water. Then, 20 mL of the growth medium was placed in glass tubes and autoclaved at 121 °C for 25 min at a pressure of 1.1 bar. For each treatment, 20 seeds were placed in tubes separately. The cultures were maintained in a growth room at 23 °C for a 16 h photoperiod with cool white fluorescent lamps. The light intensity was 40 µE $m^{-2}s^{-1}$.

2.3. Evaluation of Pathogen Elimination, Germination, and Seedling Development after Sterilization Treatments

The estimation of the number of contaminated seeds and the number of germinated seeds, the determination of the pathogen type, and the analysis of the root and shoot system of the seedlings developed in vitro were carried out 14 days after the start of the experiment. The pathogens were detected with a stereo binocular magnifier (Olympus SZ1145; Olympus, Tokyo, Japan).

The root system was scanned using WinRHIZO Pro software, version 2016a (Regent Instruments, Quebec, QC, Canada) and the total root length, total root surface area, average root volume, number of root tips, and average root diameter were determined. The total shoot length of germinated seeds was determined through image analysis using ImageJ software 1.8.0.

2.4. Data Analyses

After conducting the experiment, the types of pathogens were identified. Statistical analyses of the effects of the NaClO sterilization procedures and the addition of PPM to the growth medium were performed separately for each pathogen type detected. The influence of the sterilization procedure and the addition of PPM to the growth medium on germination efficiency as well as on the *Fusarium* and *Alternaria* contamination of sterilized seeds was analyzed using logistic regression. Germination efficiency and *Fusarium* and *Alternaria* contamination were coded as binary variables for each seed.

The influence of the sterilization procedure and the addition of PPM to the growth medium on the number of seeds contaminated with *Aspergillus niger*, *Cladosporium abietinum*, *Penicillium* sp., and *Bipolaris sorokiniana* was not subjected to statistical analysis, as the sterilization treatments with NaClO and PPM completely eliminated these pathogens. This resulted in unbalanced samples of observations and made the validity of the model fit questionable. Therefore, the results are recorded, and the percentage of seeds contaminated with *Aspergillus niger*, *Cladosporium abietinum*, *Penicillium* sp., and *Bipolaris sorokiniana* is reported.

The data on root and shoot development (total root length, total root surface area, average root volume, number of root tips, and average root diameter as well as total shoot length of germinated seeds) were subjected to an analysis of variance (ANOVA). Fixed effects are the surface sterilization procedure (N, C, S40, and S50) and the addition of PPM to the growth medium (PPM 0, PPM 2, and PPM 4). The Bonferroni post hoc test at $p \leq 0.05$ was used to examine the difference between the group means. All statistical analyses of the data were performed using the SAS/STAT® (2010) programme package [21].

3. Results

3.1. The Effect of Surface Sterilization with Sodium Hypochlorite and the Addition of Different Doses of PPM to the Growth Medium on the Elimination of Pathogens

The combination of NaClO surface sterilization procedures and the addition of PPM to the growth media tested in this study completely eliminated contamination with *Aspergillus niger*, *Cladosporium abietinum*, *Penicillium* sp., and *Bipolaris sorokiniana*. The pathogenic fungal species *A. niger*, *C. abietinum*, and *Penicillium* sp. only occurred in the sterilization treatment in which the seeds were treated with sterilized ddH_2O and no PPM was added to the growth medium (wet control: C-PPM0 surface sterilization procedure; Figure 1). The pathogenic fungus *Bipolaris sorokiniana* occurred in 4.5% to 4.7% of the sterilization treatments N0, C2, and S40-0 (Figure 1).

The presence of the pathogenic fungal species *A. alternata* and *Fusarium* spp. was observed in all sterilization treatments tested in this experiment; however, contamination with these fungal species was significantly affected by the sterilization treatments (Table 2). The surface sterilization procedures with 4% sodium hypochlorite for 50 min (S50) significantly reduced the contamination with *A. alternata* compared to the surface sterilization procedures S40, N, and C (Table 2). In addition, the interaction between the surface sterilization procedure and the addition of PPM to the growth medium had a significant effect on *A. alternata* contamination (Figure 2a, Supplementary Table S1). In Figure 2b, we have plotted separate lines for each sterilization procedure over a continuous range of PPM to highlight the PPM effect in each sterilization procedure. The addition of PPM 2 mL L^{-1} or 4 mL L^{-1} and surface sterilization with 4% sodium hypochlorite for 50 min (surface sterilization procedure S50) significantly reduced contamination with *A. alternata*. An extended duration of the sterilization procedure with 4% sodium hypochlorite (surface sterilization procedure S50) significantly reduced the contamination with *Fusarium* sp. compared to S40 and the wet control surface sterilization procedure (C) with sterile water (Table 2).

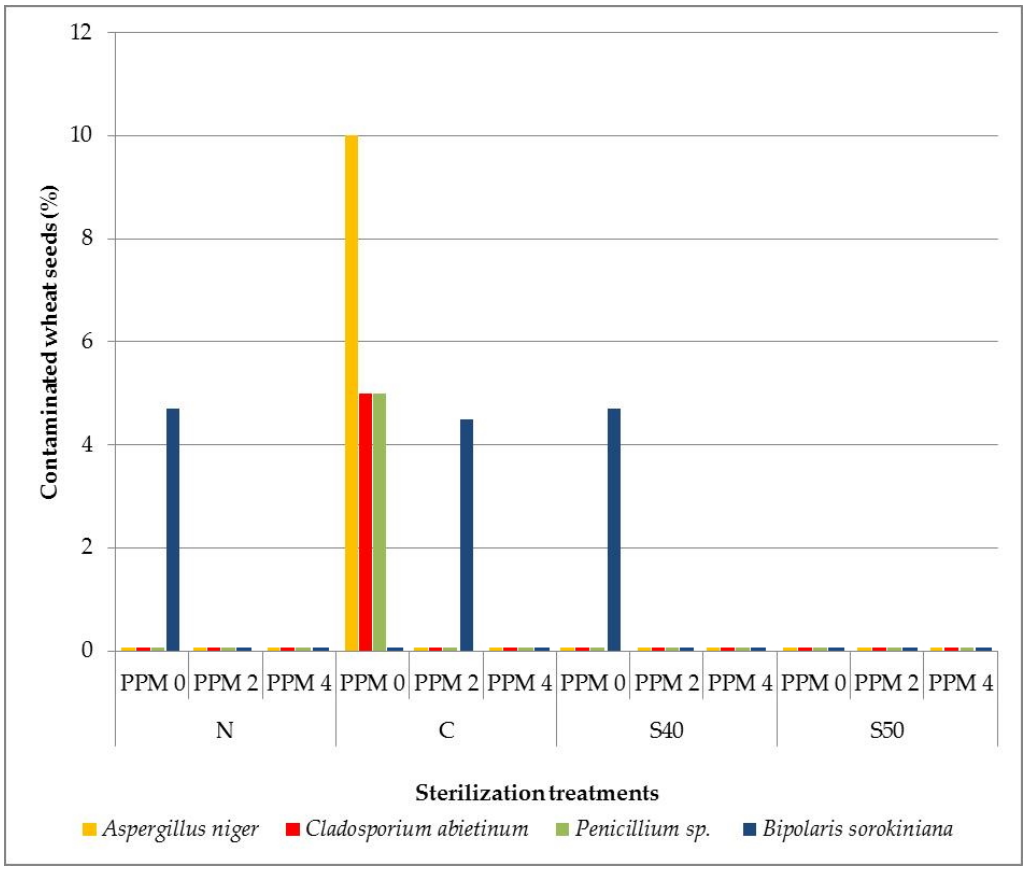

Figure 1. Wheat (*Triticum aestivum*) seeds (%) contaminated with the pathogenic fungi *Aspergillus niger*, *Cladosporium abietinum*, *Penicillium* sp., and *Bipolaris* sorokiniana after sterilization treatments.

Table 2. Effect of surface sterilization procedure and PPM addition to seed growth medium on the probability of contamination of wheat (*Triticum aestivum*) seeds with *A. alternata* and *Fusarium* spp., as revealed by logistic regression analysis.

	A. alternata **Contamination**		***Fusarium*** **spp. Contamination**	
	Wald Chi-Square	**Pr > Chi-Square**	**Wald Chi-Square**	**Pr > Chi-Square**
sterilization procedure	34.68	<0.0001 ***	11.90	0.0077 ***
PPM	2.06	0.36	-	-
sterilization procedure × PPM	15.04	0.019 *	-	-
sterilization procedure C vs. N	0.13	0.71	1.85	0.17
sterilization procedure C vs. S40	0.62	0.43	0.05	0.83
sterilization procedure C vs. S50	11.34	0.0008 ***	8.77	0.003 **
sterilization procedure S50 vs. S40	8.08	0.0045 ***	9.96	0.0016 **
sterilization procedure S50 vs. N	14.04	0.0002 ***	3.36	0.07
sterilization procedure S40 vs. N	1.43	0.23	2.53	0.11

*, **, and *** indicate significance at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively; there is no information on the dependence of the occurrence of *Fusarium* spp. on the PPM added to the culture medium and the interaction of PPM and the sterilization procedure because the most informative model (based on the AIC criterion) is the one that only considers the sterilization procedure.

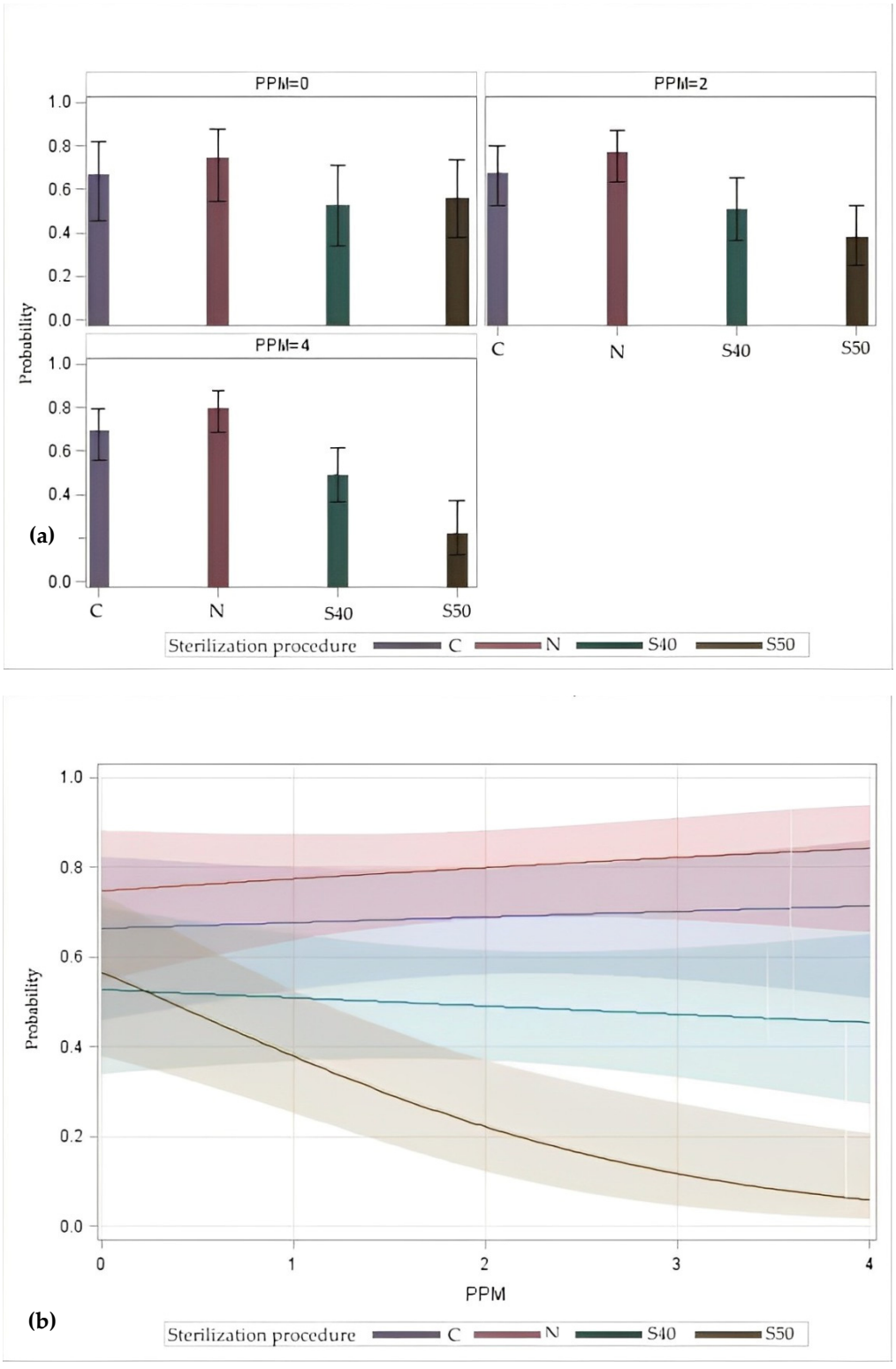

Figure 2. Predicted probabilities of *Alternaria alternata* contamination showing: (**a**) differences between surface sterilization procedures at different PPM concentrations; (**b**) each surface sterilization procedure over a continuous range of PPM shown in colored lines. Different color bands represent 95% confidence limits for each sterilization procedure.

3.2. The Effect of Surface Sterilization with Sodium Hypochlorite and the Addition of Different Doses of PPM to the Growth Medium on Germination and Seedling Development

Seed germination was not significantly affected by any of the factors investigated in this study (Supplementary Table S2).

Increased PPM concentrations in the growth medium had a negative effect on root growth (Table 3). The addition of 4 mL L^{-1} of PPM to the growth medium resulted in a significant reduction in root length, surface area, and volume and an increase in average root diameter. This negative effect was less pronounced at lower PPM concentrations (2 mL L^{-1}).

Table 3. Effects of PPM addition to the growth medium and surface sterilization procedures on root and shoot development of winter wheat (*Triticum aestivum*).

Sterilization Treatment	TRL (cm)	RSA (cm^2)	RV (cm^3)	RTN	ARD (mm)	S(C)L (cm)
sterilization procedure (*p* value)	0.041 *	0.076	0.089	0.023 *	0.481	0.634
PPM (*p* value)	<0.0001 ***	<0.0001 ***	<0.0001 ***	<0.0001 ***	<0.0001 ***	<0.0001 ***
sterilization procedure × PPM (*p* value)	0.107	0.204	0.369	0.034 *	0.144	0.0012 **
N	31.318 a	4.78 a	0.061 a	34.72 a	0.51 a	10.36 a
C	29.568 a	4.72 a	0.063 a	26.88 ab	0.49 a	8.99 a
S40	28.184 a	4.12 a	0.049 a	25.03 ab	0.45 a	9.95 a
S50	34.36 a	4.86 a	0.058 a	22.92 b	0.47 a	10. 58 a
PPM 0	47.31 a	6.68 a	0.076 a	33.35 a	0.37 c	16.31 a
PPM 2	38.78 b	5.68 a	0.068 a	38.72 a	0.45 b	9.54 b
PPM 4	8.878 c	1.89 b	0.034 b	14.19 b	0.62 a	5.10 c

*, **, and *** indicate significance at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. Values followed by the same letter within a column do not differ significantly according to the Bonferroni test at $p < 0.05$; TRL, total root length; RSA, root surface area; RV, root volume; RTN, number of root tips; ARD, average root diameter; S(C)L, shoot, i.e., coleoptile length.

The number of root tips was influenced by the surface sterilization procedure, the PPM, and their interaction (Table 3). The highest PPM concentration (4 mL L^{-1}, PPM 4) had a negative effect on the number of root tips compared to 2 mL L^{-1} of PPM (PPM 2) and the treatments in which no PPM was used (PPM 0). The procedure of surface sterilization with 4% sodium hypochlorite for 50 min (S50) significantly reduced the number of root tips compared to procedure N, in which the dry seeds were placed on a sterile medium without washing and sterilization (Table 3). However, the addition of PPM (PPM 2 or PPM 4) to the surface sterilization procedures with 4% sodium hypochlorite for 40 or 50 min (S40 or S50, respectively) did not significantly change the number of root tips compared to S40 and S50 surface sterilization procedures without the addition of PPM (S40-PPM0, S50-PPM0) (Figure 3).

On the other hand, shoot growth was negatively affected by the highest dose of PPM (4 mL L^{-1}) added to the growth medium when surface sterilization procedures C, S40, or S50 were applied (Figure 4).

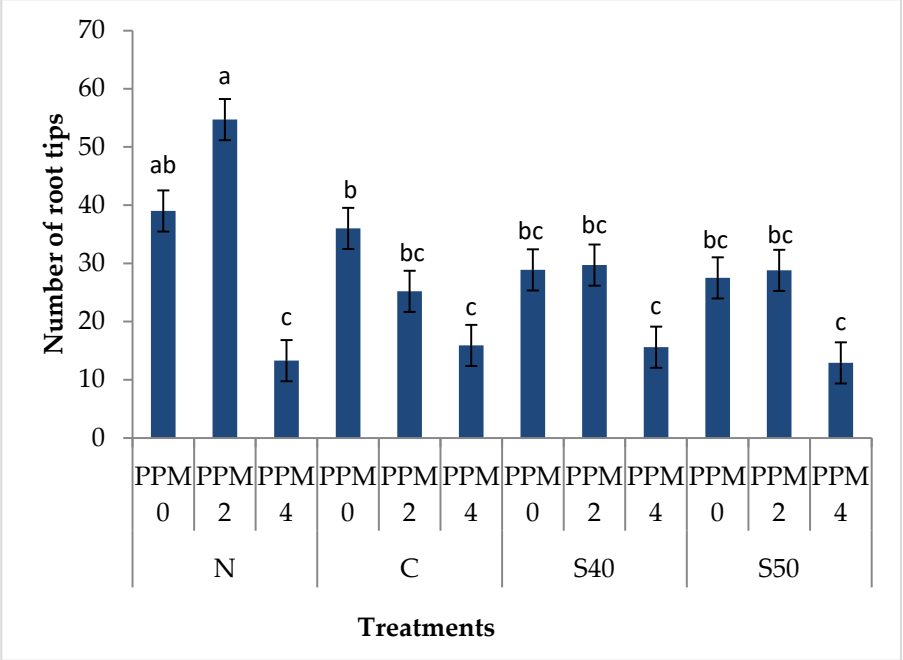

Figure 3. Effects of the addition of PPM to the growth medium and the surface sterilization procedure on the number of root tips (root architecture) of winter wheat (*Triticum aestivum*). Values followed by the same letter do not differ significantly according to the Bonferroni test at $p < 0.05$. Different letters indicate significant differences between sterilization treatments in the number of root tips.

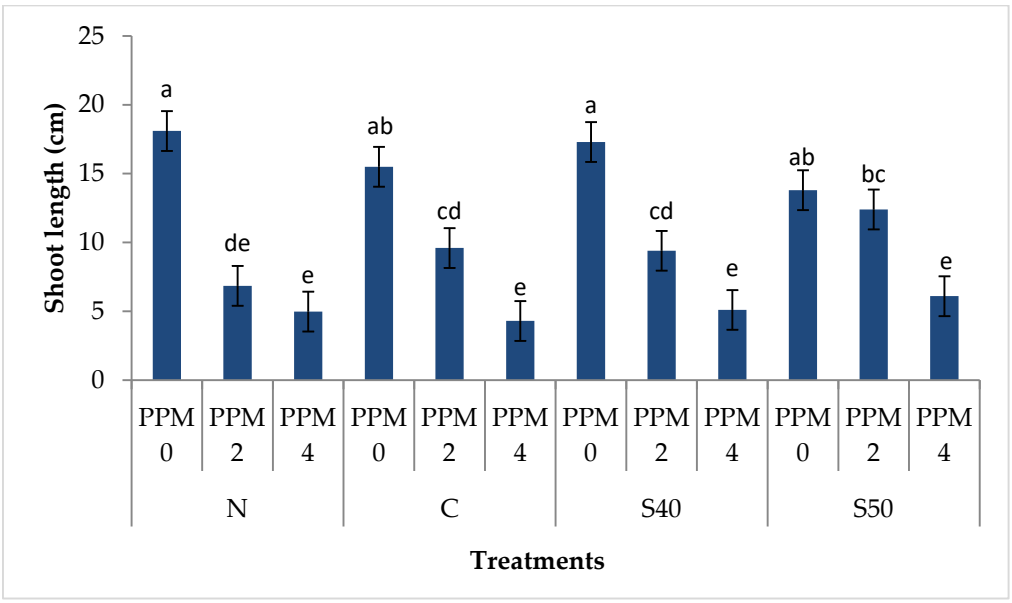

Figure 4. Effects of the addition of PPM to the growth medium and surface sterilization procedure on shoot length of winter wheat (*Triticum aestivum*). Values followed by the same letter do not differ significantly according to the Bonferroni test at $p < 0.05$. Different letters indicate significant differences between sterilization treatments in shoot length.

4. Discussion

This study investigated the effects of sterilization procedures and the addition of different concentrations of PPM to the growth medium on the elimination of some fungal pathogens, seed germination, and seedling development when wheat seeds were introduced into in vitro culture. Fourteen days after the start of the experiment, the seeds were

examined with a binocular stereo magnifier, and different fungal species, *Alternaria alternata* (Fr.) Keissl, *Aspergillus niger* Tiegh, *Cladosporium abietinum* (Pers.) Link, *Fusarium* spp., *Penicillium* sp., and *Bipolaris sorokiniana* (Sacc.) Shoemaker, were identified.

Fusarium, *Alternaria*, *Cladosporium*, and *Bipolaris* are common fungi in fields. The damage caused by these fungi occurs before harvest and does not increase during storage. On the other hand, *Aspergillus* and *Penicillium* are storage fungi. They are widely distributed and almost always present. In addition, *Aspergillus*, *Fusarium*, *Alternaria*, and *Penicillium* are considered to be the most important mycotoxin-producing fungi [9]. The accumulation of mycotoxins presents a major risk to food safety and the health of humans, animals, and natural ecosystems. In this experiment, the pathogenic fungal species *A. niger*, *C. abietinum*, and *Penicillium* sp., as well as contamination with *B. sorokiniana*, were completely eliminated by the sterilization treatments, which included NaClO surface sterilization procedures and the addition of PPM to the growth media.

Pathogenic fungi have been associated with seeds for more than 130 million years and have evolved mechanisms for seed-borne disease transmission [22]. There are several ways in which fungi infect and colonize seeds of host plants, and they can be hosted in different parts of the seed. Microbial colonization is generally restricted to the outer layers of cereal grains: the husk, between the husk and pericarp, and within the pericarp tissue [23]. There are fungal species (*Alternaria*, *Bipolaris*, and *Fusarium* spp.) that have been reported to be able to penetrate into the endosperm and cause internal infections [24]. Due to their oxidizing effect, chlorine and hypochlorite treatments are some of the most commonly used methods to control microbial spoilage of cereal grains. However, it has been reported that disinfection with sodium hypochlorite removes only 10–15% of *Alternaria* and *Bipolaris* contamination, indicating that the grains are contaminated below the pericarp [25].

In this study, contamination with *B. sorokiniana* occurred in the sterilization treatments N-PPM0, C-PPM2, and in the S40-PPM0 (Figure 1). However, in the sterilization treatments with NaClO surface sterilization procedures and the addition of PPM to the growth media, the pathogenic fungal species *B. sorokiniana* was completely eliminated. These results suggest that the addition of PPM can complement seed sterilization procedures to reduce *B. sorokiniana* contamination.

Alternaria sp. is one of the most important wheat pathogens and causes Alternaria leaf blight [26,27]. The dormant mycelium of *Alternaria alternata* is found in the seed coat and pericarp in asymptomatic and weakly symptomatic seeds. Moderately to severely infected seeds carry the fungal mycelium in all parts: in the seed coat or pericarp, in the endosperm, and in the embryo [28,29]. The synergistic effect of PPM in the growth medium (2 mL L^{-1} and 4 mL L^{-1}) and surface sterilization with 4% sodium hypochlorite for 50 min (Figure 2a,b) significantly increased the efficiency of elimination of the fungus *Alternaria* sp.

To eliminate pathogens of the genus *Fusarium*, the seeds had to be exposed to 4% sodium hypochlorite for 50 min (S50, Table 2). While *Alternaria* sp. spores are present closer to the surface of the seed, *Fusarium* sp. spores are often located deeper in the seed, so it is possible that only a longer sterilization time of 50 min allows the agent to penetrate the pathogen. Indeed, the degree of invasion of *Fusarium culmorum* into the wheat kernel tissues depends on the time of infection. If infection takes place in the early stages of kernel development, colonization can reach deeper tissues, including the thick cuticle of the endosperm and the aleurone layer, where mycelial cushions may form. If infection occurs shortly before maturity, only the pericarp is affected [29]. When natural, dry seeds were placed on the growth medium without any sterilization (surface sterilization procedure N), less contamination with *Fusarium* sp. was observed than in the surface sterilization procedures with sterile water (surface sterilization procedure C) and surface sterilization with sodium hypochlorite for 40 min (surface sterilization procedure S40). It can be argued here that the influence of water in surface sterilization procedures C and S40 activates enzymes to initiate the germination process, which stimulates the growth of fungi of the genus *Fusarium*. Another possible explanation is that the C and S40 surface sterilization procedures reduced the infection of seeds by the pathogen *Alternaria* sp., which reduced the

competition for space and nutrients between these two pathogens and allowed *Fusarium* sp. to develop mycelium on the surface of the seeds (Supplementary Figure S1).

Sodium hypochlorite can have a negative effect on the germination rate and the survival rate of seedlings. However, in our experiment, the sterilization procedure with sodium hypochlorite had no significant effect on the germination rate or seedling growth (Supplementary Table S2). The addition of PPM to the growth medium also had no effect on the germination rate. On the other hand, the development of roots and shoots was significantly influenced by the amount of PPM added to the growth medium. PPM at a dose of 2 mL L^{-1} significantly increased the efficiency of sterilization and did not reduce the total root area, the volume of the root system, or the number of root tips. Therefore, the use of 2 mL L^{-1} of PPM can be recommended for the control of pathogenic fungi of the genus *Alternaria* when wheat seeds are introduced under sterile conditions.

Increasing the PPM dose from 2 to 4 mL L^{-1} significantly reduced shoot length, total root length, root surface area, and root volume, as well as the number of root tips, while the average root diameter increased (Table 3). The reduced root growth, especially the reduced root length accompanied by an increase in the average root diameter, observed in seeds grown in crops with elevated PPM concentrations suggests a toxic effect of PPM on roots. A similar response, namely an inhibition of root growth and an increase in root diameter, was observed in wheat under aluminum (Al) toxicity [30]. In addition, increased PPM concentration reduced shoot length (Table 3, Figure 4). The observed phenotype could be explained as a response of the plant to the stress caused by PPM and as a possible effect of increased ethylene concentrations. Increased ethylene concentrations can cause a reduction in root elongation and a strong swelling of the radial tissue under stress conditions [31,32].

5. Conclusions

This study provides information on the pathogens to be expected when using wheat seed to obtain mature embryos for the establishment of an in vitro culture. Contamination with *Aspergillus niger*, *Cladosporium abietinum*, *Penicillium* sp., and *Bipolaris sorokiniana* was completely eliminated by the combination of NaClO surface sterilization procedures and the addition of PPM to the growth media tested in this study. Sterilization in 4% NaClO for 50 min had the best effect on reducing *Alternaria* and *Fusarium* contamination, and for *Alternaria*, the addition of PPM at a concentration of 2 or 4 mL L^{-1} was also found to have a significant effect on reducing contamination. However, because the dose of 4 mL L^{-1} of PPM significantly increases the negative impact on the development of roots and shoots of seedlings, a 50 min surface sterilization procedure with sodium hypochlorite with the addition of 2 mL L^{-1} of PPM to the growth medium can be recommended when establishing in vitro cultures of wheat seeds. The sterilization of the seeds with sodium hypochlorite and the addition of PPM to the medium had no negative effects on the germination of the seeds.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agriculture14040556/s1, Supplementary Figure S1: Wheat (*Triticum aestivum*) seeds of the variety Mara (%) contaminated with the pathogenic fungi of the genus *Alternaria* and *Fusarium* after sterilization procedures. Supplementary Table S1: Odds Ratio Estimates and Wald Confidence Intervals for *Alternaria* contamination of wheat (*Triticum aestivum*) seeds of the variety Mara. Supplementary Table S2: Effect of surface sterilization procedure and PPM addition to the seed growth medium on the probability of germination of wheat (*Triticum aestivum*) seeds, as revealed by logistic regression analysis.

Author Contributions: A.B.M., I.H.J. and S.K., conceptualization; A.B.M., I.H.J. and B.L., methodology and data analysis; S.T.P., K.M., B.L., K.T., A.B.M. and I.H.J., experiments and measurements; I.H.J. and A.B.M., writing—original draft preparation; S.K., B.L., Z.M. and S.T.P., review and editing. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the University of Zagreb's financial support for scientific research.

Institutional Review Board Statement: Not applicable.

Data Availability Statement: Data are contained within the article or Supplementary Materials.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. FAO. Food Outlook—Biannual Report on Global Food Markets. Food Outlook. November 2023. Rome. Available online: https://www.fao.org/documents/card/en/c/cc8589en (accessed on 30 January 2024).
2. FAO; IFAD; UNICEF; WFP; WHO. The State of Food Security and Nutrition in the World 2022. Repurposing Food and Agricultural Policies to Make Healthy Diets More Affordable. Rome, FAO. Available online: https://www.fao.org/documents/card/en/c/cc0639en (accessed on 30 January 2024).
3. von Grebmer, K.; Bernstein, J.; Wiemers, M.; Reiner, L.; Bachmeier, M.; Hanano, A.; Chéilleachair, R.N.; Foley, C.; Sheehan, T.; Gitter, S.; et al. Global Hunger Index: The Power of Youth in Shaping Food Systems. 2023. Available online: https://www.globalhungerindex.org/pdf/en/2023.pdf (accessed on 8 January 2024).
4. Kumar, R.; Mamrutha, H.M.; Kaur, A.; Venkatesh, K.; Grewal, A.; Kumar, R.; Tiwari, V. Development of an efficient and reproducible regeneration system in wheat (*Triticum aestivum* L.). *Physiol. Mol. Biol. Plants* **2017**, *23*, 945–954. [CrossRef] [PubMed]
5. Parmar, S.S.; Sainger, M.; Chaudhary, D.; Jaiwal, P.K. Plant regeneration from mature embryo of commercial Indian bread wheat (*Triticum aestivum* L.) cultivars. *Physiol. Mol. Biol. Plants* **2012**, *18*, 177–183. [CrossRef] [PubMed]
6. Yin, G.X.; Wang, Y.L.; She, M.Y.; Du, L.P.; Xu, H.J.; Ye, X.G. Establishment of a highly efficient regeneration system for the mature embryo culture of wheat. *Agric. Sci. China* **2011**, *10*, 9–17. [CrossRef]
7. Zale, J.M.; Borchardt-Wier, H.; Kidwell, K.K.; Steber, C.M. Callus induction and plant regeneration from mature embryos of a diverse set of wheat genotypes. *Plant Cell Tissue Organ Cult.* **2004**, *76*, 277–281. [CrossRef]
8. Dale, P.J.; Webb, K.J. Germplasm Storage and Micropropagation. In *Advances in Agricultural Biotechnology*; Bright, S.W.J., Jones, M.G.K., Eds.; Springer: Dordrecht, The Netherlands, 1985; Volume 15, pp. 79–96.
9. Martín, I.; Gálvez, L.; Guasch, L.; Palmero, D. Fungal Pathogens and Seed Storage in the Dry State. *Plants* **2022**, *11*, 3167. [CrossRef] [PubMed]
10. Bashyal, B.M.; Rawat, K.; Sharma, S.; Gogoi, R.; Aggarwal, R. Major Seed-Borne Diseases in Important Cereals: Symptomatology, Aetiology and Economic Importance. In *Seed-Borne Diseases of Agricultural Crops: Detection, Diagnosis & Management*, 1st ed.; Kumar, R., Gupta, A., Eds.; Springer: Singapore, 2020; pp. 371–426.
11. Kumar, P.L.; Cuervo, M.; Kreuze, J.F.; Muller, G.; Kulkarni, G.; Kumari, S.G.; Massart, S.; Mezzalama, M.; Alakonya, A.; Muchugi, A.; et al. Phytosanitary Interventions for Safe Global Germplasm Exchange and the Prevention of Transboundary Pest Spread: The Role of CGIAR Germplasm Health Units. *Plants* **2021**, *10*, 328. [CrossRef] [PubMed]
12. Yildiz, M. The Prerequisite of the Success in Plant Tissue Culture: High Frequency Shoot Regeneration. In *Recent Advances in Plant In Vitro Culture*; Leva, A., Rinaldi, R., Eds.; InTech: Rijeka, Croatia, 2012. [CrossRef]
13. Teixeira da Silva, J.A.; Winarto, B.; Dobránszki, J.; Zeng, S. Disinfection procedures for in vitro propagation of Anthurium. *Folia Hortic.* **2015**, *27*, 3–14. [CrossRef]
14. Oyebanji, O.B.; Nweke, O.; Odebunmi, O.; Galadima, N.B.; Idris, M.S.; Nnodi, U.N.; Afolabi, A.S.; Ogbadu, G.H. Simple, effective and economical explant-surface sterilization protocol for cowpea, rice and sorghum seeds. *Afr. J. Biotechnol.* **2009**, *8*, 5395–5399.
15. Chawla, H.S. *Introduction to Plant Biotechnology*, 3rd ed.; Science Publishers: Enfield, NH, USA, 2009; pp. 28–32.
16. Niedz, P.R. Using Isothiazolone Biocides to Control Microbial and Fungal Contaminants in Plant Tissue Cultures. *HortScienece* **1998**, *8*, 598–601. [CrossRef]
17. Kraj, W.; Dolincki, A. The influence of PPM upon the sterility of the in vitro cultures in european birch (*Fagus sylvatica* L.). *Acta Soc. Bot. Pol.* **2003**, *72*, 303–307. [CrossRef]
18. Plant Cell Technologies. 2017. Available online: http://www.plantcelltechnology.com/ppm-references/ (accessed on 30 October 2023).
19. Omamor, I.B.; Asemota, A.O.; Eke, C.R.; Eziashi, E.I. Fungal contaminants of the oil palm tissue culture in Nigerian institute for oil palm research (NIFOR). *Afr. J. Agr. Res.* **2007**, *2*, 534–537.
20. Orlikowska, T.; Nowak, K.; Reed, B. Bacteria in the plant tissue culture environment. *Plant Cell Tissue Organ Cult.* **2016**, *128*, 487–508. [CrossRef]
21. *SAS/STAT*; SAS Institute: Cary, NC, USA, 2010.
22. Buller, A.H.R. *Researches on Fungi*, 1st ed.; Universit Toronto Press: Toronto, ON, Canada, 1950; pp. 415–428.
23. Nierop, S.V. The impact of microorganisms on barley and malt quality—A review. *J. Am. Soc. Brew. Chem.* **2006**, *64*, 69–78. [CrossRef]
24. Los, A.; Ziuzina, D.; Bourke, P. Current and Future Technologies for Microbiological Decontamination of Cereal Grains. *J. FoodSci.* **2018**, *83*, 1484–1493. [CrossRef] [PubMed]
25. Andersen, B.; Thrane, U. Food-borne fungi in fruit and cereals and their production of mycotoxins. *Adv. Exp. Med. Biol.* **2006**, *571*, 137–152. [PubMed]
26. Schiro, G.; Verch, G.; Grimm, V.; Müller, M. Alternaria and Fusarium Fungi: Differences in Distribution and Spore Deposition in a Topographically Heterogeneous Wheat Field. *J. Fungi* **2018**, *4*, 63. [CrossRef] [PubMed]

27. Jevtić, R.; Župunski, V.; Lalošević, M.; Tančić Živanov, S. Colonization of winter wheat grain with Fusarium and Alternaria species and influence on pest control management. *J. Gen. Plant Pathol.* **2019**, *85*, 273–281. [CrossRef]
28. Agrawal, K.; Sharma, J.; Tribhuwan, S.; Dalbir, S. Histopathology of Alternaria tenuis infected black-pointed kernels of wheat. *Botanic Bull. Acad. Sinica* **1987**, *28*, 123–130.
29. Singh, D.; Mathur, S.B. Location of Fungal Hyphae in Seeds. In *Histopathology of Seed-Borne Infections*, 1st ed.; Singh, D., Mathur, S.B., Eds.; CRC Press: Boca Raton, FL, USA, 2004; pp. 118–186.
30. Lazarević, B.; Sturrock, C.J.; Poljak, M.; Mooney, S.J. Quantification of Aluminum-Induced Changes in Wheat Root Architecture by X-ray Microcomputed Tomography. *Commun. Soil. Sci. Plant Anal.* **2016**, *47*, 263–274. [CrossRef]
31. Guzmán, P.; Ecker, J.R. Exploiting the triple response of Arabidopsis to identify ethylene-related mutants. *Plant Cell* **1990**, *2*, 513–523. [PubMed]
32. Sharp, R.E.; LeNoble, M.E. ABA, Ethylene and the Control of Shoot and Root Growth under Water Stress. *J. Exp. Bot.* **2002**, *53*, 33–37. [CrossRef]