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Use of Phosphite Preparations to Protect Ash Seedlings *Fraxinus excelsior* L. against *Phytophthora* spp. and *Hymenoscyphus fraxineus* Pathogens

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Abstract: In this study, greenhouse tests were conducted on 240 *Fraxinus excelsior* seedlings to investigate the simultaneous damage caused by thea pathogenic fungus and oomycetes. The experiment was performed under controlled conditions in the greenhouse of the Institute of Forest Research in Sękocin Stary (Poland). Three species of oomycetes were used for the experiment: *Phytophthora plurivora*, *Phytophthora* taxon *hungarica*, *Phytophthora megasperma*, and the fungus *Hymenoscyphus fraxineus*. Inoculations using the fungus were carried out on shoots and in plant pots in which the soil was mixed with the three *Phytophthora* species mentioned above, both simultaneously and separately, which made it possible to recognize the cumulative effect of the related plant infection. The aim of the study was to investigate the effect of phosphite-containing preparations on the health of common ash under conditions of threat to the roots by *Phytophthora* spp. and damage to the aerial parts of the plant by the fungus, as well as the possible occurrence of synergistic effects. Two types of protective preparations (Actifos and Phos60 of the nitrogen and potassium forms, respectively) were used. It was found that the inoculation of ash seedlings with the fungus *H. fraxineus* resulted in plant mortality, while the mixture of *Phytophthora* did not cause significant damage. It was confirmed that when pathogens coexist, a phenomenon occurs that leads to an acceleration in the development of disease symptoms and, thus, to plant mortality. In vitro tests confirmed the usefulness of phosphite preparations for the protection of ash seedlings.

Keywords: phosphonates; dieback; IPM; inoculations; root damage; nursery; disease

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1. Introduction

This article deals with a current problem in Europe, namely, the phenomenon of ash dieback [1,2], the first symptoms of which were observed in 1992 in northeast Poland [3,4]; this concerned the death of seedlings in tree nurseries, young trees in forest plantations, and old trees in tree stands. The leaves were infected by the ascospores of the fungus, and, as a result, the entire shoots withered (Figure 1). When the tissue necrosis covered the entire circumference of the trunk, the entire plant died. Ash dieback spread across the whole country and was soon no longer a purely localized phenomenon. The death of trees is observed in all age classes, but is particularly intense in young stands due to artificial regeneration. The disease is very widespread in the habitats typical for ash cultivation.

Several years of research have not yet found a solution to this problem. However, the main cause of ash dieback has been identified. The first scientific report in this regard pointed to the fungus *Chalara fraxinea* [5]. This was a fundamental step that initiated a series of further activities to investigate the mechanism of ash dieback. After numerous studies conducted in the following years, the Latin name of the causal agent of ash dieback was adopted as the official name in May 2014: *Hymenoscyphus fraxineus* (T. Kowalski), according to Baral, Queloz, and Hosoya [6,7].

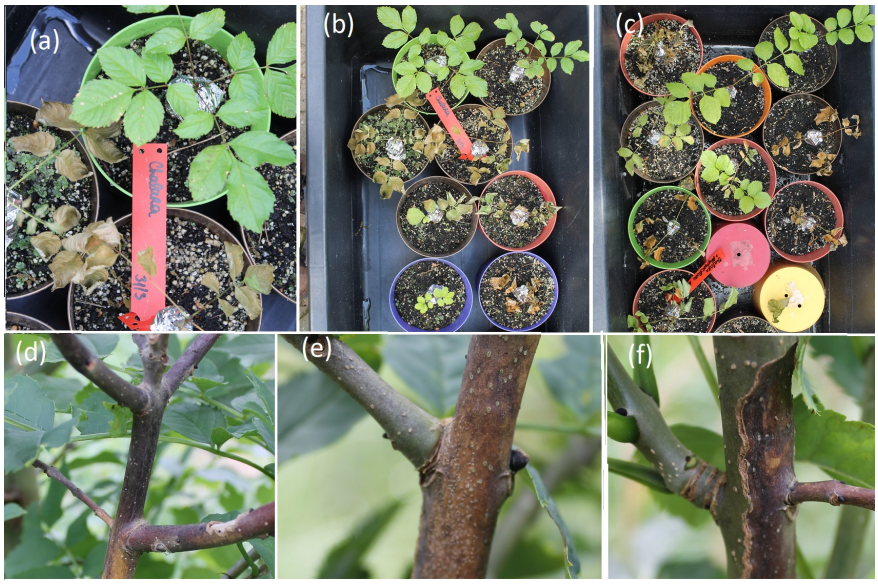

Figure 1. Ash seedlings inoculated with the pathogen *H. fraxineus* alone (**a**,**b**) and with the interaction of *Phytophthora* spp. (**c**). Symptoms of the disease: infection and dieback of the main shoots (**d**,**e**) and development of the disease from the side shoot to the main stem (**f**).

Molecular studies showed that the pathogen was introduced from East Asia, where it occurs on the Manchurian ash *Fraxinus mandshurica* Rupr. [8]. The fungus *H. fraxineus* has been detected in forest nurseries, suggesting that the disease spreads with infected planting material [9,10]. For many years, the ash tree *F. excelsior* was considered a disease-resistant species, even against the pathogenic *Phytophthora* spp. [11]. In 2011, studies in older dying ash stands in Poland and Denmark shed new light on the mortality process [12]. They confirmed the presence of pathogenic oomycetes in the root rhizosphere of ash trees. This experience and the discovery of seedlings in nurseries infected with new organisms of the genus *Phytophthora* [13] were an incentive to investigate their role in the process of tree mortality. Therefore, it was necessary to conduct a comprehensive study to investigate the relationships (possible synergies) between the infections caused by already identified pathogenic organisms; on the shoots, this was the fungus *H. fraxineus*, and on the roots, it was the algal-like species of the genus *Phytophthora* (pathogenicity tests under controlled conditions). Tests for new possibilities in terms of plant protection were carried out by inducing resistance with the help of phosphite preparations.

In 1929, the first accurate method for analyzing the phosphite content of plants was described, and in the early 1930s, Mengdehl (1933) began research into the effects of phosphite on plant metabolism [14,15]. Phosphites (also known as phosphonates) are the salts of phosphoric acid H_3PO_3 (orthophosphoric acid III), which differ in structure from phosphoric acid H_3PO_4 (orthophosphoric acid V) in that way they have one less oxygen atom [16]. This difference means that phosphonates have greater mobility in soil and plant tissue than phosphates and are more easily transported into plants via leaves, stems, and roots. As a result, they are also more easily transported through the xylem and phloem [17]. To date, the vast majority of studies conducted on the use of phosphites are aimed at protecting plants from the negative effects of pathogenic *Phytophthora* pathogens.

Phosphites act both directly on pathogenic oomycetes and indirectly by stimulating the host plant's defense [16]. When combined, both mechanisms lead to limiting the development of a pathogen, e.g., of the genus *Phytophthora*, and reducing its sporulation [18]. These mechanisms probably also work for other pathogens, such as the fungus *H. fraxineus*, which has been identified as one of the main causes of ash dieback.

The aim of this study was to investigate the effect of phosphite preparations on the health of ash trees threatened by *Phytophthora* roots and the damage to above-ground plant parts caused by the fungus *H. fraxineus*; the researchers also investigated the possible occurrence of synergistic infections of ash (*F. excelsior*) shoots by the fungus *H. fraxineus* and roots by pathogens of the genus *Phytophthora*. A further aim of the study was to determine the effects of mixed infections with *H. fraxineus* and *Phytophthora* spp. on the health of ash trees and to determine the role of *Phytophthora* spp. in damaging the roots of ash trees.

2. Material and Methods

2.1. Preparation of Ash Seedlings

The experiment was carried out under controlled conditions in the greenhouse of the Forest Research Institute (IBL), where two-year-old ash seedlings were grown on ash breeding plates of *F. excelsior* L. from seeds from the Forest Gene Bank in Kostrzyca (Figure 2). Before sowing, the seeds were subjected to the prescribed 32-week stratification (heat/cold). For the first 16 weeks, the seeds were kept in a stratification medium at about 19 °C and, for the next 16 weeks, at 3 °C. After leaving stratification, the seeds were sown in 1.5-L pots, to which vermiculite was added to improve soil aeration and moisture properties. The soil used to plant the seedlings in pots was purchased as garden soil with a pH of 5.5–6.5. It contained a mixture of different types of soil, such as sand, clay, peat, and other organic ingredients. It was not additionally enriched with mineral fertilizers or other additives, such as compost or organic fertilizers. To ensure sterility, the soil substrate was autoclaved before being placed in the pots. Sterilization was carried out in an autoclave at 120 °C for 20 min. The pots used for the experiment were previously disinfected with sodium hypochlorite and placed in 60-L containers, with 10 pots in one container (Figure 2) so that they could be regularly flooded with water.

A total of 240 ash seedlings were used in the experiment, which were inoculated with pathogens and/or sprayed with phosphite preparations in 12 different treatments (Table 1). During the summer, the temperature in the greenhouse box was maintained at 27 °C. Ceiling-mounted, automatically operated curtains were used to help maintain the desired temperature. During the winter, a temperature of around 18 °C was maintained. Automatic sprinklers activated for approximately 1 min were used to cool the air temperature. The seedlings were watered as required. The humidity in the greenhouse was maintained at 70%, which allowed the plants to develop properly. During the growing of the ash trees, the natural day photoperiod was used with no additional lighting.

Table 1. Factors analyzed in the greenhouse experiment and the experimental variants.

Preparations		**Pathogens**		
A (Actifos) P (Phos60) C (Control) —		H (*Hymenoscyphus fraxineus*) M (*Mixture of Phytophthora*—3 species) HM (*H. fraxineus* + *Phytophthora* spp.) N (Control)		
	Treatment	**Symbol**	**Prep.**	**Pathogen**
1	Control	C-N	C	N
2	Actifos	A-N	A	N
3	Phos60	P-N	P	N
4	*H. fraxineus*	C-H	C	H
5	*Phytophthora* spp.	C-M	C	M
6	*H. fraxineus* + *Phytophthora* spp.	C-HM	C	HM
7	Actifos + *H. fraxineus*	A-H	A	H
8	Actifos + *Phytophthora* spp.	A-M	A	M
9	Actifos + *H. fraxineus* + *Phytophthora* spp.	A-HM	A	HM
10	Phos60 + *H. fraxineus*	P-H	P	H
11	Phos60 + *Phytophthora* spp.	P-M	P	M
12	Phos60 + *H. fraxineus* + *Phytophthora* spp.	P-HM	P	HM

Figure 2. Common ash trees (2 years old) prepared for inoculation; spraying with preparations containing phosphites.

2.2. Preparation and Inoculation of Ash Trees with the Fungus H. fraxineus

In November 2013, shoots of ash trees (*F. excelsior*) (in the Chojnów forest district) that showed symptoms of dieback due to infection with the fungus *H. fraxineus* (anamorphic stage = *C. fraxinea*) were harvested. The necrotic shoots were cut into fragments several centimeters long and then successively disinfected by immersion in 96% ethanol (1 min), in 4% sodium hypochlorite (5 min), and again in 96% ethanol (0.5 min) [19]. After drying on sterile filter paper and cutting off the top layer of bark, the 5 × 2 × 2 mm wood fragments were placed on 2% maltose agar medium (MEA) in Petri dishes. Six fragments of diseased tissue were placed in each dish, totaling 42 pieces. They were then incubated at 20 °C, and every few days, the growing colonies were detached and then morphologically examined to determine if they were the typical *Chalara* stage phialides with spores. In order to confirm that these were pure cultures of *H. fraxineus*, selected cultures of the fungus were molecularly tested using a molecular biology method. The presence of *H. fraxineus* was confirmed by polymerase chain reaction (PCR). The primer set Hym_F 5′-GCGAATGAATATATATATGGGCTTACA-3′ and Hf_R 5′-GCATAGCGTGGGGCTCTCTGG

3′ were used for the reaction [20]. In this way, material was obtained that was later used in the experiment to inoculate ash trees growing in pots in the greenhouse.

The next step was to obtain healthy ash shoots, which served as carriers for the inoculum of the pathogen *H. fraxineus*. The shoots (with a diameter of about 1 cm) were obtained from the Chojnów Forest Administration in the Kraśnicza Wola forest complex (close to Warsaw, Poland). After removing the bark and phloem, 120 pieces of wood measuring 1 × 0.2 × 0.2 cm were prepared, which were then sterilized in an autoclave under pressure at 120 °C. After cooling, they were placed on the surface of 2-week-old pure cultures of the fungus *H. fraxineus*. After a 4-week incubation period in the dark in a thermostat at 24 °C, the well-grown mycelium-containing pieces of wood were used as inoculum. The inoculation of ash trees with the mycelium fungus *H. fraxineus* was carried out by making scalpel incisions of 1 × 0.2 × 0.2 cm in the ash tree stems, up to the wood, at a height of about 5–10 cm above the ground, into which pieces of wood overgrown with the mycelium fungus *H. fraxineus* were inserted using sterile instruments. In order to prevent the injured tissues and fungi from drying out, the sites where the inoculations were placed were protected with parafilm and then wrapped in cotton wool moistened with distilled sterile water and wrapped in aluminum foil.

2.3. Preparation and Inoculation of Soil with Phytophthora Organisms

Pure cultures of *P. plurivora*, *P. megasperma*, and *P.* taxon *hungarica*, which are kept in the collection of the IBL, were used for the experiment. These species were isolated from the rhizosphere of an ash stand in the Wolica Reserve (section 374-c) in the Chojnów Forest District. Pure cultures of the pathogens were cultivated on PDA medium at 24 °C and were then transferred to a medium containing vermiculite and oats in Elenmayer flasks. After 6 weeks of growth, the prepared inoculum, consisting of three *Phytophthora* species, was placed in the soil near the root systems. In each hole (two holes per pot), 25 g of the inoculum was added. Inoculation with the *Phytophthora* species mixture was carried out on 31 March 2014. The ash trees inoculated with *Phytophthora* species (a description of the treatments can be found in Table 1) were watered regularly and flooded monthly for 72 h to stimulate zoospores production, which are a natural source of infection for the fine roots.

2.4. Spraying Ash Trees with Phosphite Preparations

On 29 March 2014, the ash trees were sprayed with two phosphite preparations: an ammonium form (Actifos from Agropak, Poland) with a total nitrogen (N) content of 10.2% (NH4), and water-soluble micronutrients: boron (B): 0.02%, copper (Cu): 0.008%, iron (Fe): 0.06%, manganese (Mn): 0.04%, molybdenum (Mo): 0.004%, and zinc (Zn): 0.02%, and the potassium form (Phos60® EU from Kazgod, Poland), which, in addition to nitrogen, contains 10.0% phosphorus, 43.0% (P_2O_5), and potassium 5.0% (K_2O). These preparations were applied once to the foliage at a concentration of 0.6 %, according to the manufacturer's recommendations. The preparations used in the study are commercially available as so-called resistance fertilizers. The formulation was applied using a hand-held pressure sprayer with nozzles providing a fine droplet spray of 300–500 micrometers. Spraying the leaves with a phosphite preparation was chosen because it has been shown in the literature [21] to be more effective than watering the plants. The reason for this is that the soil bacteria utilize the phosphorus source to develop their populations, and only a small amount of the preparation would reach the seedlings. Thanks to the foliar application of the preparation, we interfere less with the natural environment, as the preparation is absorbed by the plant tissues on which it is supposed to act as an immune trigger.

2.5. Health Evaluation of Ash Trees in a Greenhouse Experiment

In the last part of the experiment, 3 months after inoculation with *H. fraxineus* and a mixture of *Phytophthora* and after the application of phosphite preparations, a visual assessment of the health status of the ash trees was carried out, taking into account the condition of the shoots and leaves. For the purposes of this experiment, our own method

was developed to assess the health of the tested seedlings. During the assessment, each ash seedling was categoriszed into one of three groups: healthy, damage of up to 30% of the shoots, diseased, damage of between 30 and 50%, dying and dead, damage of over 50%.

2.6. Measurement of Biometric Characteristics of Ash Trees

After the experiment, the selected biometric characteristics of the ash seedlings were measured (Table 2). The roots of the ash trees were analyzed using WinRhizo® software Regent Instruments Inc. (Québec City, QC, Canada) (https://regent.qc.ca/assets/winrhizo_software.html (accessed on 12 January 2024)) and an EPSON Perfection V700 Photo Water Scanner (Nagano, Japan). With this method, the root systems could be categorized according to their dimensionality (size) so that it was possible to measure them in individual categories (fine 0–2 mm and maternal 2–5 mm).

The specialized WinRhizo software made it possible to determine a number of root parameters, starting with root length, area and volume while at the same time dividing them into fine roots (up to 2 mm thick) and mother roots (from 2–5 mm thick) (Figure 3).

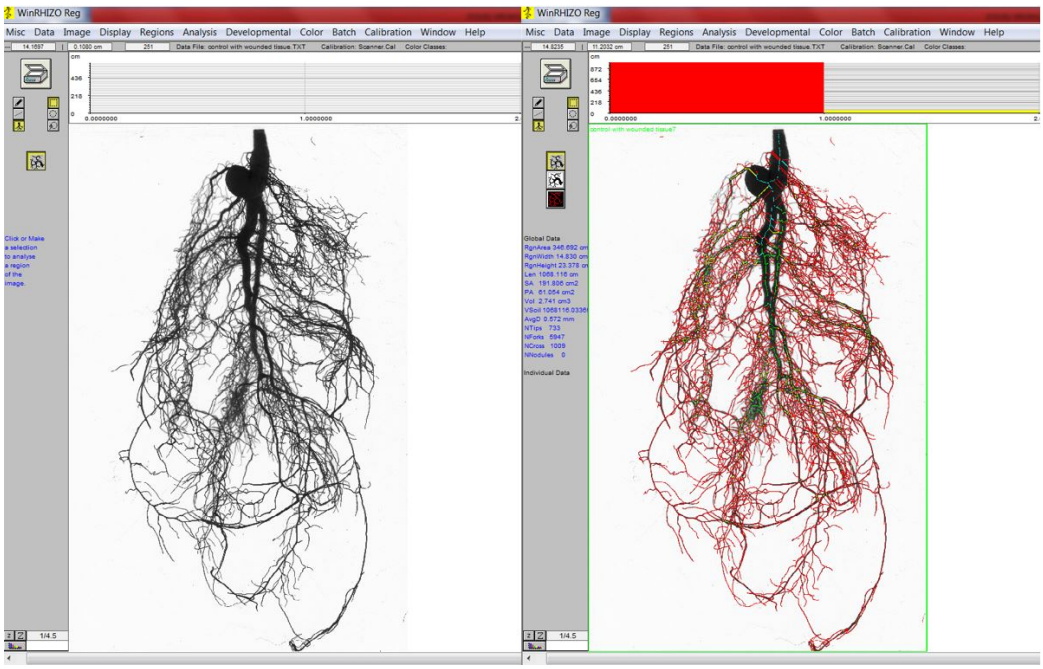

Figure 3. Scans of root systems in WinRhizo divided into fine roots up to 2 mm (red) and mother roots from 2–5 mm (yellow).

The root systems of ash trees were analyzed to assess the impact of pathogenic oomycetes on the damage caused. In addition, the length of the ash shoots, their thickness in the root necks, and the dry biomass of the roots were measured. A total of six characteristics were used for the statistical analyses (Table 2).

Table 2. Ash seedling characteristics used for the statistical analyses.

Name	Symbol	Units
Fine roots surface	FRS	[cm^2]
Fine roots volume	FRV	[cm^3]
Total root volume	TRV	[cm^3]
Seedling length	SL	[cm]
Root collar thickness	RCT	[mm]
Root dry weight	RDW	[g]

2.7. Pathogen Re-Isolation and Identification

In accordance with Koch's postulate, 90 days after the inoculation of the ash trees with the fungus, the pathogen was re-isolated from the infected tissues to confirm that it was the same species used for inoculation. For this purpose, two ash trees were randomly selected from each variant to re-isolate the pathogens causing the disease symptoms. In the case of the greenhouse, ash seedlings were inoculated in nine treatments of the experiment: three treatments with *H. fraxineus*, three treatments with a *Phytophthora* mixture, and three treatments with *H. fraxineus* and a combination of *Phytophthora* species. A total of 18 ash trees were sampled and transferred to 24 Petri dishes (Table 3).

In the case of the experimental treatments containing specific pathogens, samples were taken from each ash seedling (shoots to confirm *H. fraxineus* and roots to confirm *Phytophthora* presence). Fine roots were collected from 12 ash trees (six from treatments containing only *Phytophthora* and six from treatments with two types of pathogens). Additionally, tissues were collected from the above-ground parts (shoots) of 12 ash trees.

Table 3. The number of prepared Petri dishes for the re-isolation of pathogens.

	Hymenoscyphus fraxineus	**Two Pathogens**	**Mix of *Phytophthora***
	C-H (2) A-H (2) P-H (2)		C-M (2) A-M (2) P-M (2)
		(2) C-HM (2) (2) A-HM (2) (2) P-HM (2)	
Total	6	12	6

For the *H. fraxineus* treatments, fragments of about 4 × 2 × 2 mm were taken from the area of the previous inoculation and transferred to a 2% MEA medium (malt extract agar) (Merck, Darmstadt, Germany). For treatments with a *Phytophthora* mixture, short sections of approximately 3–4 mm long fine roots were taken and then transferred to the selective PARPNH agar medium (V8 agar with the addition of Pimaricin, Ampicillin, Rifampicin, Pentachloronitrobenzene PCNB, Nystatin, and Hymexazol) [22].

The cultivated pure pathogen cultures were first identified on the basis of morphological characteristics and then DNA analyses were carried out to determine the species of the pathogen. The *Phytophthora* pathogens tested were restricted to genus identification using the universal ATP9-PhyG2 AAAGCCATCAATAAACARAATAAGC probe and primer PhyG-ATP9 AATAAATCATAACCTTCTTTACAAACAGAATTTAATG [23]. The samples submitted for analysis were in the form of hyphal fragments and were stored at 4 °C until molecular analysis. The polymerase chain reaction (PCR) was performed using the Phire® Plant Direct PCR Kit (Thermo Scientific®, FINNZYMES, Waltham, MA, USA).

2.8. Adopted Experimental Treatments

The ash trees prepared for the experiment (240 specimens) were randomly divided into 12 groups (treatments) of 20 ash trees each (Table 1). With the material thus prepared, a two-factor experiment was carried out using the preparations (A: Actifos, P: Phos60, and C: control) and a fungal inoculation (H: *H. fraxineus*, M: *Phytophthora* mix spp., HM: both pathogens, N and N: no pathogens) (Table 1).

2.9. Statistical Analysis of Measured Data

The empirical data collected were first analyzed in the form of descriptive statistics (minimum, maximum, mean, standard deviation, median, and the coefficient of the variation of the characteristic). Subsequently, the Shapiro-Wilk test was used to check the conformity with the normal distribution for the continuous quantitative variables, and the Levene test was used to check the homogeneity of variances.

The variables met the above assumptions, so a two-factor parametric analysis was performed using an ANOVA linear model (with interaction).

$$Y \sim preparation + pathogen + preparation : paghogen,$$

where Y consists of all the measured characteristics presented in Table 2. After checking, when the p-value of the interaction term in the resulting model ($preparation : pathogen$) was below the threshold of 0.05 we applied the simplified model with the main effects only

$$Y \sim preparation + pathogen$$

The Tukey's HSD post-hoc test (for equal sample sizes and a balanced system) was then applied to compare the means between the main effects of the treatments. The same significance level, $p = 0.05$, was used for all tests.

The statistical analyses were performed using the Statistica (version 10) program [Statsoft, Inc., 2010, Tulsa, OK, USA] and MS Excel 2010 spreadsheet.

3. Results

3.1. Assessment of the Health of Ash Trees at the End of the Experiment

The highest number of dying and dead trees (19, 95%) was recorded in the variant C-H, in which the ash trees were inoculated with the fungus *H. fraxineus*, as well as in the treatments C-HM, A-HM, P-H, P-HM, in which 18 ash trees died (90%).

In the A-H variant, a 15% (i.e., 3) improvement in the survival of ash trees was observed as a result of spraying with Actifos compared to the C-H variant (Figure 4).

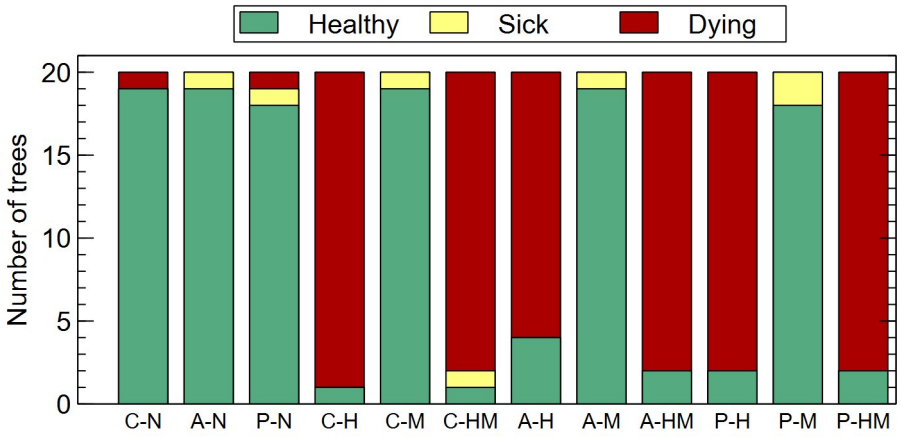

Figure 4. Assessment of the health of ash trees 90 days after inoculation with pathogens and spraying preparations containing phosphites. Healthy, up to 30% of damage, Diseased, 30%-50% of damage, Dying, above 50% of damage. The meaning of symbols in the x-axis is described in Table 1, the first character represents the application of: the C, Control, A, Actifos, P, Phos60 preparations, and the characters after the dash represent the application of N, Control (none), H, *H. fraxienus*, M, *Phythopthora* spp., HM, *H. fraxineus* + *Phythopthora* spp. pathogens.

The highest proportion of healthy ash trees (19 specimens, i.e., 95%) was found in the variant in which Actifos A-N was used as fertilizer; in the variant C-M, in which three inoculum *Phytophthora* species were introduced into the soil and in the variant A-M, in which Actifos was applied together with the *Phytophthora* spp. inoculum. A high survival rate of seedlings was also achieved with the P-N and P-M treatments (18 units, i.e., 90%) (Figure 4).

3.2. Results of Measuring Biometric Characteristics of Ash Trees

As part of the experiment, a total of 11 characteristics were measured and assessed for each of the 240 ash trees (Table 2).

The measured or estimated traits reflected the response of the trees to the experimental treatments. In order to limit the scope of this study, only the results for the traits for which the differences were statistically significant and the observations that are important for formulating conclusions are presented below. The controls are highlighted by a green frame. A detailed description of the treatments can be found in Table 1.

The application of phosphites alone did not lead to any statistically significant changes in the fine root surface (Figure 5). The simultaneous presence of pathogens significantly reduced the surface area of the fine roots. The addition of phosphites to pathogen treatments led to the protection of the fine root area, which was comparable to that of the control seedlings (Figure 5).

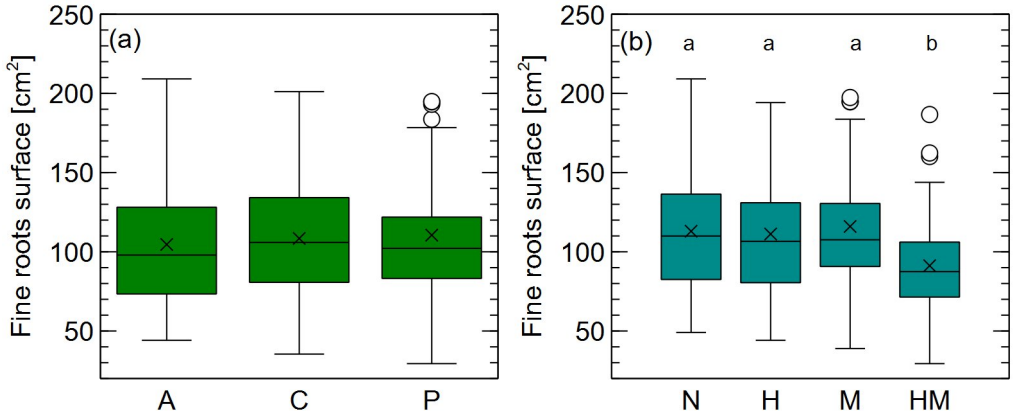

Figure 5. Fine root surface depending on the application of (**a**) phosphites and (**b**) pathogens. Different lowercase characters at the top of the figure (**b**) indicate the statistical significance of the difference between the treatments, according to the post-hoc Tukey test. No statistically significant difference at the $p < 0.05$ level was found for the main effect of phosphites application, see subfigure (**a**).

The application of phosphites alone did not lead to any statistically significant changes in fine root volume (Figure 6). Pathogens occurring together (HM) significantly reduced the volume of fine roots. The addition of phosphite to the pathogen treatments led to an equalization of fine root volume to that of control seedlings (Figure 6).

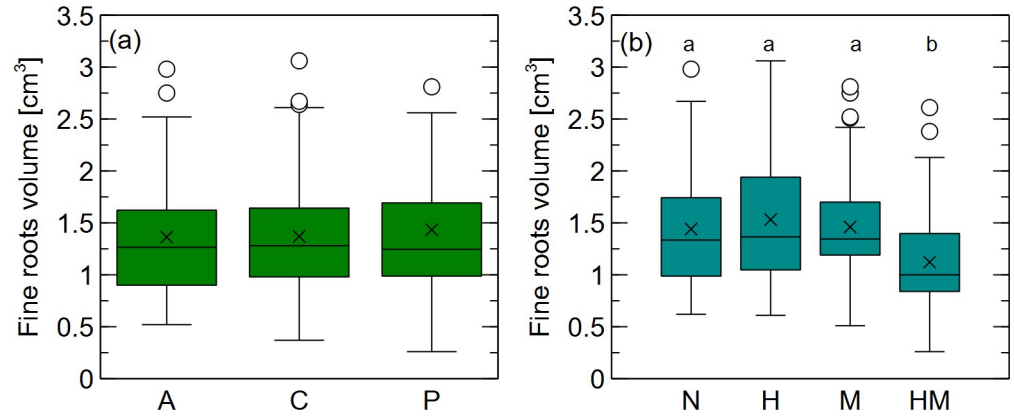

Figure 6. The volume of fine root depending on the application of (**a**) phosphites and (**b**) pathogens. The different lowercase characters at the top of the figure (**b**) indicate the statistical significance of the difference between the treatments, according to the post-hoc Tukey test. No statistically significant difference at the $p < 0.05$ level was found for the main effect of phosphites application, see subfigure (**a**).

The application of phosphites alone did not lead to any statistically significant changes in the total volume of fine and mother roots (Figure 7). Pathogens occurring together (HM) significantly reduced the root volume compared to treatments using a single inoculation of

fungus (H) or oomycetes (M). The application of phosphites to treatments with pathogens led to an equalization of the total root volume to the level of the control seedlings (Figure 7).

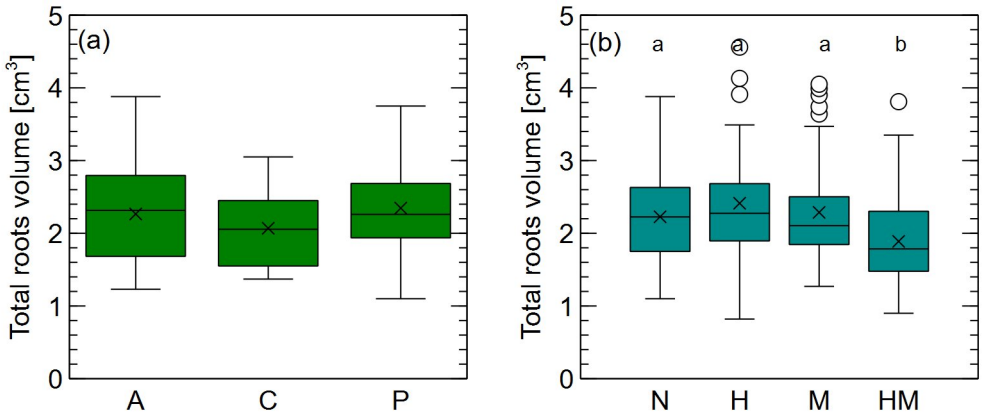

Figure 7. Total root volume depending on the application of (**a**) phosphites and (**b**) pathogens. Different lowercase characters at the top of the figure (**b**) indicate the statistical significance of the difference between the treatments, according to the post-hoc Tukey test. No statistically significant difference at the $p < 0.05$ level was found for the main effect of phosphites application, see subfigure (**a**).

The application of phosphites alone did not lead to any statistically significant changes in the length of the seedlings (Figure 8). A significant reduction in the length of the seedlings was observed for treatment with pathogens. Adding phosphite to the pathogen treatments resulted in the same stem length as the control seedlings (Figure 8).

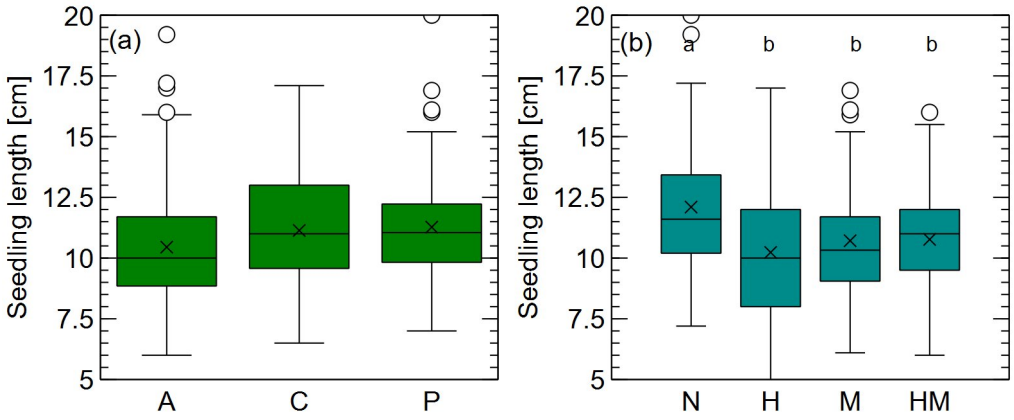

Figure 8. Seedling length depending on the application of (**a**) phosphites and (**b**) pathogens. Different lowercase characters at the top of the figure (**b**) indicate the statistical significance of the difference between the treatments, according to the post-hoc Tukey test. No statistically significant difference at the $p < 0.05$ level was found for the main effect of phosphites application, see subfigure (**a**).

The application of phosphites alone did not lead to any statistically significant changes in root collar thickness (Figure 9). A significant reduction in root collar thickness was observed in all treatments with pathogens. In this analysis, the interaction effect was found to be statistically significant at $p < 0.05$, but not at $p < 0.01$. In addition, in this case, we decided to use the statistical model without interaction.

The application of phosphites alone did not lead to any statistically significant changes in root dry mass (Figure 10). In a treatment using a fungal pathogen (H) and in combination with oomycetes (HM), a significant reduction in the thickness of the root necks was observed compared to the variant inoculated with oomycetes (M) (Figure 10).

In this analysis, the interaction effect was found to be statistically significant at $p < 0.05$, but not at $p < 0.01$ level. In addition, in this case, we decided to use the statistical model without interaction.

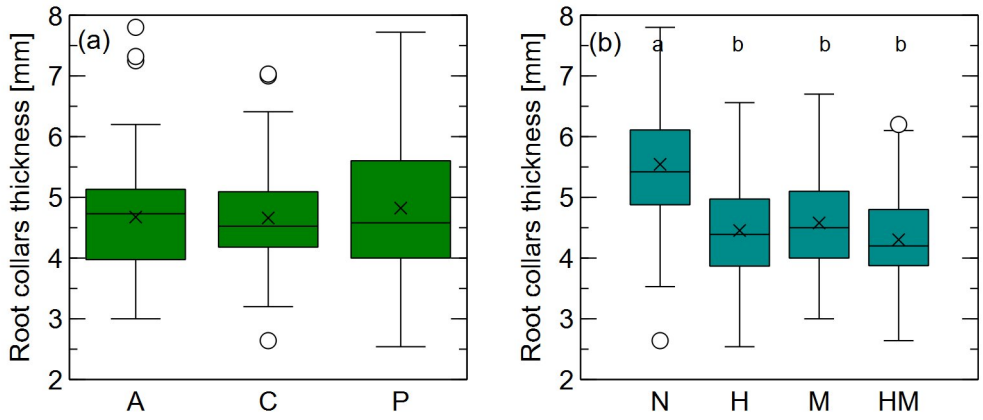

Figure 9. Root dry matter depending on the application of (**a**) phosphites and (**b**) pathogens. Different lowercase characters at the top of the figure (**b**) indicate the statistical significance of the difference between the treatments, according to the post-hoc Tukey test. No statistically significant difference at the $p < 0.05$ level was found for the main effect of phosphites application, see subfigure (**a**).

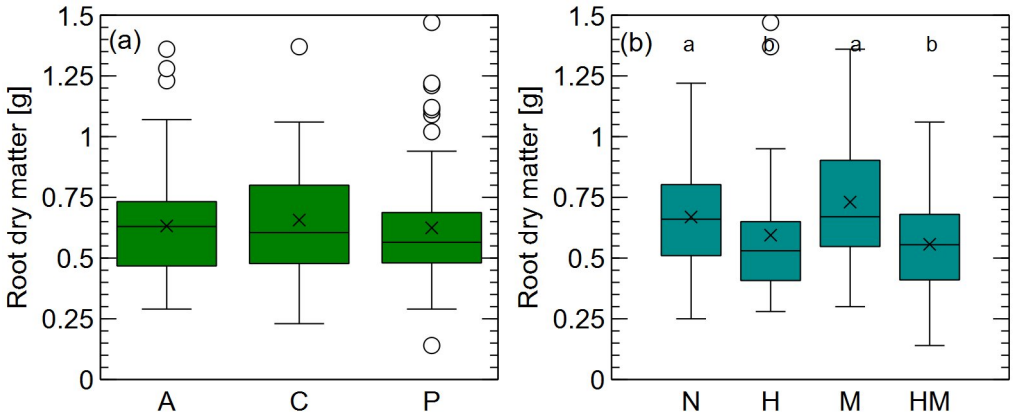

Figure 10. Root dry matter depending on the application of (**a**) phosphites, (**b**) pathogens. Different lowercase characters at the top of the figure (**b**) indicate statistical significance of the difference between the treatments, according to the post-hoc Tukey test. No statistically significant difference at the $p < 0.05$ level was found for the main effect of phosphites application—subfigure (**a**).

3.3. Result of Pathogen Re-Isolation

The DNA test carried out confirmed the initial results based on morphology. The re-isolation activities carried out confirmed that the two pathogen species used in the experiment were the fungus *H. fraxineus* and mixtures of pathogenic oomycetes of the genus *Phytophthora* present in the tissues of the inoculated ash trees and in the soil of the rhizosphere.

4. Discussion

4.1. Possible Use of Phosphites in Plant Protection

Research into the use of phosphoric acid (H_3PO_3) and its salts in the 1950s was part of the post-war search for alternatives to phosphate fertilizers. These studies showed that phosphites were not as effective as phosphoric acid (H_3PO_4) and its derivatives in crop protection, although positive differences were found when comparing phosphite-treated plants to control plants. Over the next 30 years, the use of phosphoric acid as a fertilizer in agriculture was not further investigated, but the focus was mainly on research into its effects on plant disease development [15].

In 1974, Rhone-Poulenc filed a provisional patent application for the chemical preparation Fosetyl-Al (fosetyl aluminium, a salt of orthophosphoric acid (III)), which was not finally granted until February 1979. The formulation has found application in wine protec-

tion. Thanks to its properties, it spread quickly through the plant and also reached the roots, so that they could be protected. Another important feature of the patented formulation is the fact that it converts slowly to orthophosphoric acid (III), which reduces the risk of phytotoxicity and prolongs the duration of action [15].

The need for a new formulation to reduce the impact of *Phytophthora* pathogens is explained by the fact that the diseases caused by these pathogens caused huge financial losses to potato, tomato and vine growers worldwide in the late 1970s. Aluminium phosphetyl was used as a foliar fungicide, and in the early 1980s it was found that phosphites were more effective on woody plants through their direct application (injection) into the tree trunks. This form very quickly became the favored approach for woody plants. In the 1980s, aluminum fosetyl was also found to protect plants against pathogens such as *Pythium*, *Venturia inaequalis*, *Erwina amylovora*, *Rhizoctonia solani* and some *Fusarium* species. At the time, scientists believed that phosphites interacted directly with the pathogens to be controlled and therefore had to be present in sufficiently high concentrations at the site of infection [24,25]. However, later studies have shown that phosphite concentration is not directly related to efficacy and that higher concentrations were sometimes nontoxic to fungi [26]. Other studies have shown that phosphites have a spore-limiting effect on *Phytophthora* pathogens, cause changes in the cell wall structure of plants, and reduce the number of pathogen suppressors that mask the disease. This discovery has contributed to the development of new phosphite-based preparations and popularized their use in crop protection [15].

4.2. Ash Dieback and Overcoming Its Problem with the Use of Phosphites

The following research hypotheses were formulated in this study to better understand interactions among pathogens and ash seedlings:

A The inoculation of ash shoots with the fungus *Hymenoscyphus fraxineus* (T. Kowalski) [6] leads to the death of the ash tree;

B The inoculation of ash roots by introducing a mixture of three *Phytophthora* species into the soil (*P. plurivora* [27], *P.* taxon *hungarica* [28], and *P. megasperma* [29] leads to the death of ash trees;

C The simultaneus inoculation of ash trees with two pathogens (*H. fraxineus* and *Phytophthora* spp.) leads to synergistic effects and accelerated disease symptoms leading to tree death;

D The use of preparations with added phosphite reduces the negative effects of *Phytophthora* pathogens and the fungus *H. fraxineus*, and has a positive effect on the health of ash seedlings.

One of the few experiments that used a very similar methodology was a study conducted in 2017 [30]. The main difference in this experiment was that an inoculum consisting of a mixture of three of the same *Phytophthora* species was introduced into the soil 2 months before the inoculation of the ash shoots using the fungus *H. fraxineus*. In addition, the plants were sprayed with Phos60 and Actifos at the time of inoculation. The protective effect of Actifos was very pronounced in this case. The foliar application of Actifos resulted in 100% survival or the ash trees inoculated with the fungus *H. fraxineus*, whereas the ash trees without Actifos protection died completely. The phenomenon of ash dieback, which has persisted for years, has led to the cessation of sowing the species in nurseries (as a forest-forming species), and the intensity of infection of seedlings still observed in experimental plantations suggests that this situation will continue in the coming years [31,32]. The results obtained in the present study confirm the validity of this position, i.e., ash is not a forest-forming species and should continue to be introduced into forest plantations. However, it is useful to continuously monitor the disease status of young ash trees, e.g., by growing them in forest nurseries on a small area (but also on small forest cultures mixed with other species), as practiced in the Chojnów Forest District (near Warsaw, Poland). If an improvement in the health status of ash trees is observed, this solution will enable an early response and appropriate changes to the current approach.

The positive results of the experiment in question also justify the continuation of research into the use of phosphite fertilizer to protect ash trees. The study confirmed that the use of preparations with the addition of phosphites reduces the unfavourable effects of the interaction between pathogens of the genus *Phytophthora* and the fungus *H. fraxineus* by adjusting four analysed traits (FRS, fine root surface, FRV, fine root volume, TRV, total root volume, and SL, length of seedlings) to the level of seedling controls, confirming the fourth hypothesis of the positive effect of the tested phosphite preparations on the health status of ash trees. The saved fine roots are important for the efficiency of water uptake, especially if the next growing season is dry. Seedlings with damaged fine roots cannot take up enough water to ensure good photosynthetic efficiency in the leaves. The ability to produce immune substances and, thus, the ability to survive under high pressure from pathogenic fungi and oomycetes depends on the amount of assimilates obtained.

The experiment also confirmed the first hypothesis, assuming that ash seedlings infected with the fungus *Hymenoscyphus fraxineus* die.

The second hypothesis, which was that soil inoculation by using a mixture of three *Phytophthora* species would lead to the death of the fine roots of ash trees, was not confirmed. This is probably related to secondary metabolites created by plants that are toxic to pathogens [33].

The simultaneous inoculation of ash trees with fungal pathogens and oomycetes led to a statistically significant reduction in fine root growth parameters (in particular FRS, fine root surface area, FRV, fine root volume, and TRV, total root volume), but it is not possible to say conclusively whether this mechanism is a synergistic or additive effect. Phosphite preparations are an alternative to the use of fungicides, e.g., based on fosetyl aluminum (Aliette, Mancozeb). However, they can only be used in tree nurseries. There is also a risk that the fungicides mask the disease without the seedlings recovering. The pathogens then escape from the nurseries together with asymptomatic plants, e.g., into the forest, where they start the disease process in their hosts under favorable conditions. Not only can the pathogens become resistant to chemical preparations, but the preparations used may also prove ineffective in reducing their population. This is because fungicides are designed to inhibit the synthesis of ergosterols, which is effective against fungi but not against oomycetes, which produce cholesterol.

4.3. Experiments with Phosphites to Protect Other Woody Plants

In one such study [21] conducted in Poland and Spain, the efficacy of phosphite was tested using potassium phosphite applied by spraying oak leaves and trunks. Particularly positive effects were observed in an experiment with English oak *Quercus robur* L. in Poland, in which 35% potassium phosphite was sprayed from the air and onto the trunks. In this experiment, which was performed in an oak forest, a 50% concentration of the preparation for aircraft spraying was used, whereas in the greenhouse experiment, the concentration was only 0.6%, and this was applied only once. Perhaps if the concentration was higher (the content of phosphite ca. 40%) and if the treatment was repeated (at least twice), the results obtained could be more spectacular. The reason for the recommended lower concentrations was the possibility of phytotoxicity. The NH4 ions present in Actifos could cause such a phenomenon at higher temperatures and in sunlight. In this case, the potassium forms of phosphite seem to be safer (e.g., Phos or Kalex). Higher concentrations (6 and 11%) of Actifos were tested in forest nurseries and caused brown spots on oak leaves [34]. However, they did not cause seedling mortality; rather, they mobilized the plants to defend themselves, putting them in a "priming" state. Foresters who used them observed effective protection against the oak powdery mildew *Erisiphe aphitoides* and even against leaf-eating insects. However, these issues require separate research. A slowdown in the growth of root systems was also observed after spraying them with phosphite [34]. In the case of oaks, this is a beneficial phenomenon because when they grow deep into the soil, there is considerable damage to the root systems when they are dug out of the nurseries after 3–4 years. They are a gateway for micro-organisms, but seedlings planted in plantations wilt in

dry conditions, and there is a lack of moisture in the soil. However, these problems need to be investigated separately. Nevertheless, the above treatment significantly improved the condition of the crowns of diseased oaks and, at the same time, confirmed that it had no negative impact on the environment, as it did not affect the composition of edaphonic bacteria in the soil. Other studies have also examined the effective use of phosphites to protect oaks [35]. In this case, a positive effect of phosphites on oaks was also observed, manifesting itself as an increase in their length and the number of root tips. Oaks that develop more fine roots have a greater chance of survival because they can resist abiotic factors, such as drought, or biotic factors, such as pathogenic oomycetes. The latter damage to fine roots mimics the consequences of a lack of moisture in the soil, as is the case in long-term drought (or physiological drought caused by excessive soil salinity), which also destroys fine plant roots.

A study carried out in Poland on the premises of seven commercial forest nurseries confirmed that the use of phosphite preparations improves the condition of young trees [34]. The studies involved five forest-forming species: pine, spruce, English oak, European beech and black alder. As a result of the tests, Actifos was found to effectively influence the growth of both shoots and root systems, making it a valuable addition to the integrated plant protection system in forest nurseries. Plants that are better supplied with nutrients and minerals (especially microelements) grow better and are more resistant to diseases.

An important element in popularizing the use of phosphite preparations has been their effective use in natural plant communities in the south-western parts of Western Australia, which are heavily affected by *Phytophthora cinnamomi* [36], a widespread and destructive plant pathogen with significant impacts on horticulture, mining, forestry and natural plant communities. Research carried out in Australia has shown, among other things, that a low concentration of phosphite in the roots influences the pathogen at the point of entry and stimulates the defense enzymes of the plant as the host.

The use of phosphites has been shown to be effective in reducing the negative impact on plants and the spread of *P. cinnamomi* in natural plant communities [37]. In western and southern Poland, where the most valuable oak stands grow, the presence of *P. cinnamomi* has been detected using molecular biological methods (data not yet published). At the same time, the death of trees and damage to the fine roots are observed. This mainly occurs during periods of high precipitation. Other oomycete species such as *P. quercina* and *P. plurivora*, have also been found, so an effective method of limiting root damage has been applied by spraying the trees with phosphites from airplanes (older stands) or by spraying the trunks of younger trees [21]. In the latter case, the phosphites penetrate the green parts of the tissue (the cracks in the bark).

5. Conclusions

- The study confirms that the use of phosphite preparations reduces the negative effects of the interaction between pathogenic oomycetes of the genus *Phytophthora* and the fungus *H. fraxineus* by protecting the fine roots; in particular, their surface and the volume.
- An improvement in the condition of the root systems (e.g., total root volume and root volume/length) leads to more efficient water uptake, and this, in turn, improves the health status of the tested ash seedlings.
- Without any intervention (protective measures), ash seedlings infected with the fungus *Hymenoscyphus fraxineus* die quickly.
- Soil pathogens of the genus *Phytophthora* do not cause serious damage to the fine roots of ash seedlings and their mortality.
- The simultaneous occurrence of shoot and root pathogens in the environment causes the deterioration of root growth parameters (especially their surface area and volume).
- Positive experimental results justify continuing research on the use of phosphites as resistance elicitors to protect ash seedlings in nurseries.

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