forests

MDPI

Article

Survival of European Ash Seedlings Treated with Phosphite after Infection with the *Hymenoscyphus fraxineus* and *Phytophthora* Species

Nenad Keča [1,*], Milosz Tkaczyk [2], Anna Żółciak [2], Marcin Stocki [3], Hazem M. Kalaji [4], Justyna A. Nowakowska [5] and Tomasz Oszako [2]

1 Faculty of Forestry, University of Belgrade, Kneza Višeslava 1, 11030 Belgrade, Serbia
2 Forest Research Institute, Department of Forest Protection, Sękocin Stary, ul. Braci Leśnej 3, 05-090 Raszyn, Poland; m.tkaczyk@ibles.waw.pl (M.T.); a.zolciak@ibles.waw.pl (A.Ż.); t.oszako@ibles.waw.pl (T.O.)
3 Faculty of Forestry, Białystok University of Technology, ul. Piłsudskiego 1A, 17-200 Hajnówka, Poland; m.stocki@pb.edu.pl
4 Institute of Technology and Life Sciences (ITP), Falenty, Al. Hrabska 3, 05-090 Raszyn, Poland; hazem@kalaji.pl
5 Faculty of Biology and Environmental Sciences, Cardinal Stefan Wyszynski University in Warsaw, Wóycickiego 1/3 Street, 01-938 Warsaw, Poland; j.nowakowska@uksw.edu.pl
* Correspondence: nenad.keca@sfb.bg.ac.rs; Tel.: +381-63-580-499

Received: 6 June 2018; Accepted: 10 July 2018; Published: 24 July 2018

Abstract: The European *Fraxinus* species are threatened by the alien invasive pathogen *Hymenoscyphus fraxineus*, which was introduced into Poland in the 1990s and has spread throughout the European continent, causing a large-scale decline of ash. There are no effective treatments to protect ash trees against ash dieback, which is caused by this pathogen, showing high variations in susceptibility at the individual level. Earlier studies have shown that the application of phosphites could improve the health of treated seedlings after artificial inoculation with *H. fraxineus*. Three-year-old *F. excelsior* seedlings were inoculated with the following pathogens: a *H. fraxineus*, *Phytophthora* species mixture (*P. plurivora*, *P. megasperma*, and *P.* taxon *hungarica*), in combination with two pathogens and mock-inoculated as the control, and then either watered or treated with ammonium phosphite (Actifos). Results showed significant differences in the survival of seedlings and symptoms of disease development among the treatments. Chlorophyll-*a* fluorescence parameters indicated a decrease in photosynthetic efficiency in infected plants, suggesting that they were under strong biotic stress, but none of the parameters could be used as a reliable bioindicator for ash decline disease. The application of Actifos enhanced the production of triterpenes (ursolic and oleanolic acid), and decreased the production of phenols (tyrosol) and sterols (β-sitosterol) in seedlings infected with *H. fraxineus*. Treatment with Actifos caused seedlings to enhance their response to pathogen(s) attack and increase their survival probability.

Keywords: *Fraxinus excelsior*; invasive pathogens; ash dieback; chlorophyll-*a* fluorescence; phenols; triterpenes; sterols; ammonium phosphite

1. Introduction

European ash (*Fraxinus excelsior* L.) is a valuable component of forest ecosystems where small pure or mixed stands are created with other broadleaved species in deep humid soil [1,2]. Ash as a species was for a long time considered to be free from substantial pests and disease that could threaten

the cultivation of a high-quality wood for a wide range of uses by the forestry industry. Due to fast growth and usable wood, ash for many years was the tree of choice for foresters [3].

Suddenly, about 25 years ago, reports of the decline of ash stands appeared in Poland [4]. At that time, there was not much attention directed to ash, because Europe was already facing a decline in oak [5], beech [6], spruce, and fir [7,8], which were both ecologically and economically more significant species. Approximately a decade later, the problem of ash dieback started becoming apparent, with reports not only from Poland, but also from neighboring Baltic countries, indicating that the problem with ash health was present in the wider region of the northeastern part of continental Europe [9]. Thorough research in the affected parts of Poland indicated a new fungal pathogen, which was named *Chalara fraxinea* by Kowalski [10], as a potential cause of the chronic decline of ash [11]. The species was later associated with its teleomorph [12], and it was subsequently named *Hymenoscyphus fraxineus* (Baral, Queloz, and Hosoya) [11].

Molecular studies have demonstrated that the pathogen was imported from eastern Asia, where it occurs on Manchurian ash (*Fraxinus mandshurica* Rupr.) [13]. After introduction into Europe, the pathogen was quickly established because of the abundance of a susceptible host population, and therefore spread epidemically across the European continent [14]. By 2012, the alien invasive pathogen was reported in the continental part of Europe, the Scandinavian Peninsula, the eastern part of Russia, and also in the British Isles and Ireland [15]. Currently, only countries belonging to the Mediterranean basin (Spain, Greece, Turkey, the southern part of Italy) have not yet reported the presence of the ash decline (EPPO, 2018).

In the affected regions, damage caused by the fungus *H. fraxineus* was higher in younger stands than in older ones, ranging from $\geq$60% in Germany [16], 57% up to 80% in young stands in Norway [17], or 3% to 35% in older and younger stands in France, respectively [18], but even in the devastated areas, some trees showed good crown conditions and a high survival rate during an attack [19], indicating the presence of genetic resistance existing within the populations [15,20]. Individual resistance between resilient populations of Manchurian and susceptible European ash individua was ascribed to the presence of a chemical compound such as iridoid glycoside in the leaves of the more resistant trees [21,22].

Changes in the health condition of single trees were observed yearly [23], indicating that survival is strongly influenced by environmental conditions and the genetic potential [20] of individuals to initiate the production of secondary metabolites such as iridoid glycoside, which induces plant resistance against the pathogen [15,21,24].

The complex action of phosphites (phosphonates) and their priming in plant-pathogen interactions is well known [25,26] and applied in the induction of resistance against *Phytophthora* root pathogens on beech [27] and *Eucalyptus* [28]. Phosphites are widely used in plant and nursery production as plant growth stimulators, and are available on the market under different brands, i.e., Kalex (K_3HPO_3) or Actifos ($(NH_4)_2HPO_3$) [29–31]. A study performed in a laboratory has shown that phosphites can alter the development of *H. fraxineus* colonies in laboratory conditions [32], but also plays a role in the survival of artificially inoculated seedlings [30,33].

Based on previous knowledge, we hypothesized that there would be clear differences in the tested parameters between three-year-old *F. excelsior* seedlings that were treated and not treated with ammonium phosphite (Actifos) after infection with three types of inocula (*H. fraxineus*, *Phytophthora* species mixed, or *H. fraxineus*–*Phytophthora* mixed combination). We assumed that seedlings infected with *H. fraxineus* would have the highest mortality rate, and those with the combination of pathogens would have the worst health status, according to the measured physiological parameters.

The chlorophyll fluorescence method is often used for the selection of species or varieties that are resistant to stress factors that also act on the photosynthetic apparatus as well as on the overall condition/performance of plants and their yielding. Studies based on the chlorophyll fluorescence method concern both crop plants and trees. Much research has shown that this method helps to predict the hidden changes caused by abiotic and biotic stresses in trees. Moreover, it has been proven that

measuring chlorophyll fluorescence parameters allows the non-invasive estimation of external stressor effects [34–36].

Trees have evolved an array of diverse chemical defenses to cope with pathogen attack. Among these, terpenes and phenolic metabolites are among the most studied components in the resistance of trees to pathogens [37,38]. Phenolic such as pinosylvin, pinosylvin monomethyl ether, stilbenes, and flavonoids are connected with increased resistance to *Ceratocystis polonica*, *Heterobasidion annosum* s.l., *Gremmeniella abietina*, etc. [39]. The different phenolics extracted from aspen showed antifungal activity against *Phellinus tremulae*, and catechol and salicin were found to be inhibitory to *Hypoxylon mammatum* [40]. There is a lack of information about the composition of the chemical compounds involved in resistance to pathogens in angiosperm trees and ash species. In order to obtain information about biochemical processes occurring in infected host tissues, an exploratory analysis of chemical compounds produced in inoculated seedlings versus control plants was conducted with the use of gas chromatography followed by mass spectrophotometry (GC-MS) [41,42]. Both methods should allow an insight into the physiological processes reflecting the interaction between the *H. fraxineus* and *Phytophthora* species playing a part in the dieback phenomenon of European ash.

This paper aims to present the differences in: (i) the survival of *F. excelsior* seedlings over a period of two years (2016/2017); (ii) growth parameters including root development, (iii) re-isolation and PCR-based confirmation of the pathogens from dead and living seedlings; (iv) an exploratory analysis of triterpenes, phenols, and sterols in the cortical tissue samples; and (v) the chlorophyll-*a* fluorescence (ChlF) of leaves as an indicator of the plant metabolism and health status of the seedlings [43].

2. Materials and Methods

2.1. Plant Material

One hundred and sixty three-year-old European ash seedlings were grown in a greenhouse at the Forest Research Institute (IBL, Sękocin Stary, Poland) and planted in 1-L pots filled with a 1:1 (v:v) peat:perlite mixture at the beginning of the vegetation period in May 2016. The temperature range in the greenhouse was between −5 °C and 30 °C, and the photoperiod was the same as in nature with the dormant period from December till March. Fertilization was done at the beginning of the experiment with N:P:K fertilizer with 20 g per plant. At the beginning of the experiment, *F. excelsior* seedlings had a mean stem height of 127.7 ± 2.52 mm and mean stem diameter (at soil level) of 4.14 ± 0.06 mm.

Eighty seedlings were used for treatment with water/control and Actifos ($(NH_4)_2HPO_3$—Agropak, Poland), as shown in Table 1. In the treatment variant with Actifos, seedlings were treated with a 0.6% Actifos water solution on 26 July 2016. Plants were regularly watered up to field capacity, every two to three days or daily during the summer months.

Table 1. Experimental design of three-year-old European ash seedlings.

	Mock Inoculation and Control	***Hymenoscyphus fraxineus* (Hf)**	***Phytophthora* Mix ***	***H. fraxineus* + *Phytophthora* Mix**	**Total**
Water	20	20	20	20	80
Actifos	20	20	20	20	80
Inoculation time	No inoculation	26 September 2016	10 July 2016	10 July 2016 26 September 2016	

* *Phytophthora* mix—*P. plurivora*, *P. hungarica*, *P. megasperma*.

The seedlings were measured for root collar diameter (mm—at the soil level) and stem height (mm) at the beginning of the experiment, in November 2016 and at the end of the experiment in September 2017. The plants' shoots and roots were dried in an oven at 65 °C for 48 h to obtain a total dry weight measured with 10^{-3} g accuracy. Twenty seedlings per treatment were analyzed.

At the end of the experiment, the ash seedlings were removed from the soil and thoroughly washed before scanning with an Epson Perfection V700 Photo Scanner. Subsequently, root morphology

was assessed with WinRhizo Pro (Regent Instruments Inc., Quebec City, QC, Canada). After assessment, the roots were separated into fine roots (<2 mm) per length of mother roots (2–5 mm) and oven-dried and weighed [44].

2.2. Fungal Inoculum

Seedlings in each of the treatments were separated into four groups of 20 plants, which were inoculated with (i) *Hymenoscyphus fraxineus* (Hf); (ii) *Phytophthora* mix (Phy) (mixture of three species *P. plurivora* T. Jung and T.I. Burgess, *P. megasperma* Drechsler, *P.* taxon *hungarica*—which were isolated from ash stands in Poland [9]; (iii) *H. fraxineus* + *Phytophthora* mix (Hf + Phy) and (iv) mock inoculation as control (Mi) (Table 1).

Pathogenicity tests with *Phytophthora* species were performed using a soil infestation test according to Jung et al. [45], and inoculum consisted of four to six-week-old cultures of mix isolates of *P. plurivora*, *P. megasperma*, and *P. hungarica* grown at 20 °C in 500-mL Erlenmeyer flasks on an autoclaved mixture of 250 cm^3 of fine vermiculite (Agra vermiculite® RHP, Rhenen, The Netherlands) and 20 cm^3 of whole millet seeds thoroughly moistened with 175 mL of vegetable juice broth (200 mL 1-1 of vegetable juice (Fortuna®, Agros-Novasoki, Warsaw, Poland), 800 mL 1-1 distilled water amended with 3 g 1-1 $CaCO_3$). After this period, the inoculum was put into the soil at a ratio of 20 cm^3 to 25 cm^3 of inoculum per 1000 cm^3 of the soil mixture. Control groups of plants were inoculated only with rinsed sterile vermiculite-vegetable juice mixture at the same ratio. Each box with pots was flooded immediately after inoculation for 72 h. The inoculation with *Phytophthora* mix was performed on 12 July 2016, which was two months before the *H. fraxineus* inoculation.

A three-week-old culture of *H. fraxineus* (strain KY613994, Sękocin Stary, Poland), growing on 2% malt extract agar (MEA) (Merck, Darmstadt, Germany) in Petri dishes, was used for the stem inoculation in a small cut in the bark made by a scalpel sterilized in 95% ethanol. A plug of bark was removed, and a 3-mm disk of mycelium from the margin of a colony was placed in the wound. All of the wounding control was inoculated with sterile agar plugs. After inoculation, the stems were sealed with Parafilm (Sigma-Aldrich, Taufkirchen, Germany). Inoculation with *H. fraxineus* was carried out on 26 September 2016.

Stem lesion lengths were measured upward and downward from the inoculation point successively after the death of the seedlings.

2.3. Re-Isolation and Confirmation of H. fraxineus and Phytophthora spp.

Re-isolation of the pathogen was carried out by taking small fragments (2 mm × 2 mm) of wood surface sterilization in a solution of 1% NaOCl and placing them in 90-mm Petri plates containing 2% MEA. The dishes were incubated at 4 °C for 20 days and checked regularly.

The fragments of wood and bark (approx. 50 mg) were taken from four inoculation points at the stem as marked from one to four (Figure 1) and ground to powder in liquid nitrogen. The sampling point (1) was at the place of artificial inoculation, while points two and three were usually at the margin or outside of the cankers. Point four was 1 cm above point three, and outside the range of the cankers. DNA was extracted using the Genomic Mini AX Plant kit and cleaned with Anti-Inhibitor Kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's instructions. *H. fraxineus* was confirmed with species-specific primers HFrax-F—5′CTTTAGCAGGTCGCCCTCT 3′ and HFrax-R—5′TGCTGGCAAGACACCGCAA 3′ to amplify a 389-bp fragment of the ribosomal DNA [45].

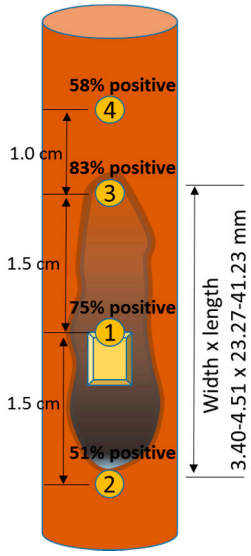

Figure 1. Sampling points (1–4) for PCR confirmation of *H. fraxineus* pathogen in the seedlings. Point 1 refers to the inoculation place; points 2 and 3 were 1.5 cm below and above sampling point 1, respectively; point 4 was 1 cm and 2.5 cm above points 3 and 1, respectively.

The 25-µL PCR mixture consisted of 2.5 µL of 10× Buffer (GenoPlats, Rokocin, Poland), 2 µL of $MgCl_2$ (25 mM), 0.5 µL of dNTP's, 0.25 µL Taq (5 U/µL)(GenoPlats, Rokocin, Poland), 1.0 µL of each primer (40 nM final conc.), 1 µL of DNA (ca. 100 nM), and up to 25 µL of Milli-Q water. Cycling conditions were slightly changed from Drenkhan et al. (2016) with initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 68 °C for 30 s, elongation at 72 °C for 1 min, and a final extension at 72 °C for 10 min. PCR products were electrophoresed on 1.5% agarose gels and visualized under UV light using a GelDoc XR + gel documenting system (BioRad, Hercules, CA, USA).

To confirm the presence of Phytophthora species in the root tissue, five samples (around 2 mm in length) were collected from rotten parts of the roots. Tissue samples were surface-sterilized with 1% sodium hypochlorite. Afterwards, small fragments (approximately 3 mm × 3 mm) were cut out with a scalpel and placed on PARP (Pimaricin, Ampicillin, Ryfampicin and PCNB) selective media. Additionally, the tissue fragments extracted from the uninfected plants were placed on a medium. Petri dishes were incubated at 22 °C for one week. The growth of mycelium was monitored each day [46].

2.4. Chlorophyll-a Fluorescence Measurements

Chlorophyll-*a* fluorescence (ChlF) measurements of leaves were performed using a Handy PEA fluorimeter (Hansatech Instruments, King's Lynn, Norfolk, UK). Measurements were performed after 20 min of dark adaptation of leaves using leaf clips [36,41,43]. An excitation red light (emitted from three diodes with a wavelength peak of 650 nm and intensity of 3500 µmol m^{-1} s^{-1}) was used for the induction of chlorophyll fluorescence, and 1 s of transient fluorescence was measured [43,47]. ChlF transients were used for calculation of an OJIP test (major phases of fluorescence rise from O to P with two intermediate steps J and I) and basic parameters. One measurement per plant was taken in each seedling on 9 September 2016 and 27 July 2017.

2.5. Analyses of Chemical Composition of Seedlings

Forty-two seedlings from eight treatments were randomly selected from the total of 160 seedlings at the end of the experiment in September 2017. In the treatment with *H. fraxineus* infection, only dead plants were the subject of analyses, while in other treatments, living plants were analyzed.

Diethyl ether and pyridine were purchased from POCH S.A. (Gliwice, Poland), whereas *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) and a standard mixture of *n*-alkanes (C_{10}–C_{40}) were purchased from Sigma-Aldrich (Poznań, Poland).

The chemical composition of 42 ash seedlings was analyzed according to the method of Stocki et al. [48]. Ash shoots up to 5 mm in thickness were selected, milled into a 0.5-mm fraction, and dried for 48 h at 50 °C. Raw materials (1 g) were extracted three times with 25 mL of diethyl ether. Extracts were filtered through paper filters. The solvent was removed using a rotor evaporator (Büchi, Switzerland) [49,50]. Dry residues of diethyl ether extracts (10 mg) were dissolved with 1 mL of pyridine, and 100 µL of BSTFA was added. Mixtures were heated for 30 min at 60 °C and analyzed by gas chromatography-mass spectrometry (GC-MS) using an Agilent 7890A gas chromatograph (USA) equipped with an Agilent 5975C mass selective detector (USA). An injection of a 1-µL sample was performed using an Agilent 7693A autosampler (USA). The separation was performed on an HP-5MS (30 m × 0.25 mm × 0.25 µm film thickness) fused silica column at a helium flow rate of 1 mL/min. The injector worked in a split (1:50) mode at an injector temperature of 300 °C. The initial column temperature was 50 °C, rising to 320 °C, at 3 °C/min; the final temperature was held for 10 min. The ion source and quadrupole temperatures were 230 °C and 150 °C, respectively. Electron ionization mass spectral (EIMS) was obtained at an ionization energy of 70 eV. The detection was performed in a full scan mode from 41 a.m.u. to 600 a.m.u. After integration, the percentage of each component in the total ion current (TIC) was calculated. Mass spectral data and calculated retention indices were used to identify compounds. Mass spectrometric identification was carried out with an automatic system of GC-MS data processing supplied by the National Institute of Standards and Technology (NIST) and a library of mass spectra [51]. Retention indices of analyses were determined, taking into account C_{10}–C_{40} *n*-alkanes retention times and comparing them with the NIST and databases [51].

2.6. Statistical Analysis

Differences in mean parameters measured for the seedlings were detected by analysis of variance with one-way ANOVA followed by Tukey tests. Homogeneity of variance was confirmed by Levene's test.

Wood chemical component, ChlF parameters, and root morphology data were not normally distributed (checked by means of the Kolmogorov–Smirnov test), so they were processed with the Kruskal–Wallis test, followed by the Mann–Whitney U test for multiple comparisons, as the data were not normally distributed after the log transformation.

The effects of treatments on chlorophyll-*a* fluorescence parameters were calculated using one-way ANOVA, with statistically significant differences being determined by the LSD Fisher post-hoc test with significance $p < 0.05$.

The Spearman rank correlation was used to measure the strength of association and the direction of the relationship between selected parameters of chlorophyll fluorescence, survival of seedlings, growth parameters, and chemical component analyses.

Statistical analyses were performed using SPSS ver. 20 (IBM Corp., Armonk, NY, USA).

3. Results

At the control, in April 2017, and six months after inoculation, 100% of the seedlings inoculated with *H. fraxineus* (Hf) and 55% of those inoculated with *H. fraxineus* + *Phytophthora* mix were dead in water treatment (Figure 2). In Actifos treatments, all of the *H. fraxineus* infected plants were alive, and in the joint infection with *Phytophthora* (Phy), only three seedlings died (Figure 2).

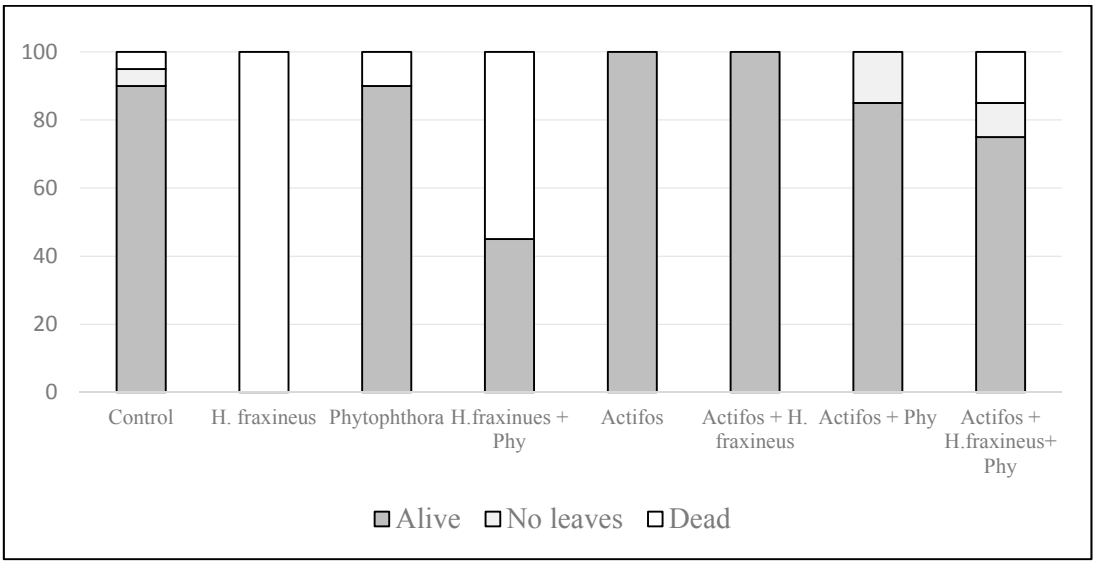

Figure 2. Survival of ash seedlings vs. mortality rate at the end of experiment (September 2017).

Dead seedlings were examined for extension of necrotic lesions and wood discoloration. The lesion length and width values were 23.27–41.23 mm and 3.40–4.51 mm, respectively, without significant differences among treatments ($p > 0.05$).

During re-isolation of *H. fraxineus* from the tissues of dead seedlings, the species was identified in only 2.4% of all of the cultures. Among the obtained isolates, *Fusarium* spp. dominated (38.8%), followed by *Alternaria* spp. (28.7%) and *Phomopsis* spp. (17.7%) (data not presented), suggesting the succession of fungal communities on dead ash tissues.

Molecular identification of the pathogen from necrotic tissues at four sampling points (Figure 1) was successful in 66% of the samples. *H. fraxineus* DNA was isolated and amplified most often from the point that was 1.5 cm above the inoculation place (point 3) in 83% of samples, followed by 75% at inoculation point (1), and 58% and 51% at the upper and lower outermost points of sampling, respectively (Figure 1). In order to confirm the presence of the pathogen's DNA in living plants, from the treatment with Actifos (Act + Hf, Act + Hf + Phy) samples of tissues from the margin of the callus were taken, but the PCR product was obtained in only 8% (3/37) of the reactions.

One year after the start of the experiment, small but significant variations in the diameter and height of the seedlings were observed between eight treatments (Table 2). According to the analyses, these differences resulted from both the individual variability of the seedlings and the type of treatment applied.

Table 2. *Fraxinus excelsior* seedling growth characteristics (mean ± SE) for eight treatments at the end of the experiment (September 2017).

	Diameter (D)	Diff in D	Height (H)	Diff in H	Shoot/Root Dry Weight Ratio	Leaf Area
		10^{-3} m			g	cm^2
Water	±	0.41 ± 0.06 b	114.50 ± 7.01 a	22.70 ± 5.93 a	0.29 ± 0.26 a	106.47 ± 12.04 a
Hf	6.07 ± 0.17 **b	0.08 ± 0.01 d	174.04 ± 6.69 c	0.71 ± 0.07 d	0.55 ± 0.47 b	0.00 ± 0.00 c
Phy	6.26 ± 0.23 a	0.57 ± 0.14 a	151.36 ± 6.93 c	11.79 ± 2.38 b	0.35 ± 0.19 ab	65.91 ± 8.91 b
Hf + Phy	6.25 ± 0.21 a	0.16 ± 0.08 c	171.54 ± 7.98 c	9.29 ± 4.45 c	0.53 ± 0.67 b	101.09 ± 27.41 b
Actifos	5.38 ± 0.19 c	0.19 ± 0.05 c	134.39 ± 7.46 b	23.11 ± 7.56 a	0.34 ± 0.17 ab	111.31 ± 17.28 a
Act + Hf	6.06 ± 0.16 b	0.39 ± 0.07 b	183.20 ± 8.84 d	14.05 ± 3.17 b	0.36 ± 0.29 ab	91.22 ± 24.24 a
Act + Phy	6.08 ± 0.20 b	0.26 ± 0.04 b	136.89 ± 5.65 b	11.97 ± 1.82 b	0.29 ± 0.21 a	40.35 ± 9.48 b
Act + Hf + Phy	5.93 ± 0.10 b	0.40 ± 0.15 b	150.13 ± 8.75 c	8.65 ± 3.43 c	0.29 ± 0.31 a	62.76 ± 17.21 b

* mean ± SE. ** Different letters behind values indicate significant differences obtained by Tukey's post hoc tests ($p < 0.05$).

The smallest values for the diameter (D) were observed for treatment with Actifos, while the highest were measured for *Phytophthora* and combined *Phytophthora* and *H. fraxineus* treatments, respectively (Table 2). Differences in the diameter between the 2016 and 2017 measurements (annual plant radial increment) (diff. in D) were significant between the water and Actifos treatments, but not for the other treatments with Actifos. Reduction of the height increment (diff. in H) was significant for all of the infestation treatments, while the values for water and Actifos treatments were highest, and did not differ from each other. The leaf area at the end of the experiment was reduced for all of the *Phytophthora* treatments (Table 2).

The nine tested root parameters showed a significant difference between treatments and allowed division into three groups (Table 3). If we compare with the water (control) treatment, treatment, total root length (total and fine root) and surface area were significantly different only for Hf + Phy and Act + Phy treatments. In the Actifos treatments, there were fewer root tips—both in the total number of tips (NoT) and fine root tips (FRT)—compared to the water treatment (Table 3). Seedlings in the Actifos treatments produced fewer fine roots—as measured by fine root length (FRL) versus mother root length (MRL) and fine root tips (FRT/MRL) in relation to the length of mother roots compared to the water treatment (Table 3).

During 2016, the photosynthetic efficiency (based on chlorophyll fluorescence signals analysis) of the studied ash seedlings showed that the combinations of Actifos + *Phytophthora* mix and *H. fraxineus* + *Phytophthora* mix were the most similar to the control treatment (water). This was expressed by showing less loss of absorbed light energy as heat dissipation (DI parameters), a higher level of reaction center reduction in photosystem II (PSII) (Phi and Psi parameters), better electron transport (ET parameters), and reduction of the first acceptors of photosystem I (PSI) (RE parameters) (Figure 3). The total positive response of the photosynthetic machinery for these two combinations can also be seen by the values of the performance index parameters (PI). On the other hand, worse functioning of the photosynthesis apparatus was noted in the case of *H. fraxineus* (Hf) alone (Figure 3). During the following year (2017), we were not able to measure chlorophyll fluorescence signals for the latter due the loss of leaves. Plant treatment with Actifos showed a very positive response of the photosynthetic efficiency of the tested tree seedlings that were not affected by *H. fraxineus*. Moreover, some enhancements at the level of PSII functioning (performance index, or PI) that were expressed as an increase in the absorbed and trapped light energy and accelerated electron transport rate were observed (Figure 3). A similar response was observed when the trees were treated with an Actifos + *Phytophthora* mix. The application of the *Phytophthora* mix, whether alone or with Actifos, maximally reduced the effect of *H. fraxineus* (Hf) on the trees' photosynthetic efficiency. The application of Actifos by itself on the trees affected by *H. fraxineus* did not help improve this efficiency (Actifos + Hf). In both years of the study, the maximal photosynthetic efficiency of the PSII (Fv/Fm) parameter did not show significant changes (Figure 3).

In the water treatments, there was no significant difference in the content of triterpenes between the infected and control seedlings. The total amount of sterols was significantly lower in the Hf inoculation type, while the highest value was held by the combined Hf + Phy inoculation. *H. fraxineus*-infected plants had a significantly higher content of phenolic compounds compared to other water treatments (Figure 4).

The Actifos treatments showed differences in the chemical composition of seedlings, but only the reduced amount of total triterpenes in the Hf + Phy inoculation type was significant. The measured amounts of sterols and phenols in Actifos treatments were not significantly different; however, the content of phenols in Hf + Phy-infected seedlings was four times higher compared to other Actifos variants (Figure 4).

Table 3. Mean values and results of Tukey test for eight root morphology parameters of common ash seedlings observed in eight treatments.

	Water	Hf	Phy	Hf + Phy	Actifos	Act + Hf	Act + Phy	Act + Hf + Phy
TRL (cm)	1014.83 ± 76.44 a*	846.93 ± 71.98 a	1065.22 ± 61.93 a	1541.93 ± 90.66 b	1061.89 ± 60.16 a	1346.74 ± 58.42 b	941.83 ± 36.91 a	889.81 ± 64.57 a
FRL (cm)	978.85 ± 75.05 a	793.48 ± 69.36 a	1012.55 ± 59.24 a	1474.27 ± 92.32 b	1012.31 ± 58.89 a	1268.28 ± 54.86 b	893.9 ± 34.87 a	842.42 ± 62.38 a
SA (cm^2)	173.07 ± 12.83 a	208.34 ± 18.21 a	217.88 ± 12.69 a	347.37 ± 28.9 b	207.8 ± 10.26 a	320.72 ± 17.44 b	194.8 ± 11.14 a	187.12 ± 13.91 a
FRSA (cm^2)	97.99 ± 7.9 a	97.49 ± 9.74 a	113.09 ± 7.24 a	174.16 ± 9.87 b	115.71 ± 6.83 a	161.98 ± 8.21 b	105.21 ± 4.5 a	101.32 ± 8.32 a
NoT (n)	5387.95 ± 612.69 a	3500 ± 480.15 abc	5093.2 ± 637.52 ab	7340.44 ± 2510.38 a	2084.7 ± 305.99 c	5178 ± 628.92 ab	4016.3 ± 662.69 abc	2494.05 ± 361.36 bc
FRT (n)	5384.7 ± 612.65 a	3496.29 ± 479.88 abc	5087.6 ± 637.19 ab	7334.22 ± 2510.51 a	2081.55 ± 305.97 c	5172.75 ± 628.81 ab	4012.25 ± 662.24 abc	2489.5 ± 361.08 bc
FRL/MRL	43.1 ± 4.27 b	30.56 ± 5.46 ab	33.84 ± 2.39 ab	32.69 ± 4.78 ab	29.32 ± 2.53 ab	24.73 ± 1.79 a	24.19 ± 1.68 a	30.3 ± 6.91 ab
FRT/MRL	273.09 ± 55.7 b	158.2 ± 39.07 ab	165.8 ± 23.42 ab	196.08 ± 75.8 ab	57.08 ± 7.74 a	105.44 ± 18.16 a	100.4 ± 15.59 a	84.81 ± 13.91 a

TRL—Total Root Length; **SA**—Surface Area; **NoT**—Number of Tips; **FRL**—Fine Root Length; **MRL**—Mother Root Length; **FRL/MRL**—Fine Root Length/Mother Root Length; **FRT/MRL**—Fine Root Tips/Mother Root Length; **FRSA**—Fine Root Surface Area; **FRT**—Fine Root Tips; * different letters in the row indicate significant difference according to Tukey test ($p < 0.05$).

Figure 3. Comparison of JIP parameters for selected treatments. All of the elements are normalized to the control treatment. Asterisks (*, **, ***) denote significant differences between control and treated plants according to the Fisher LSD test at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively.

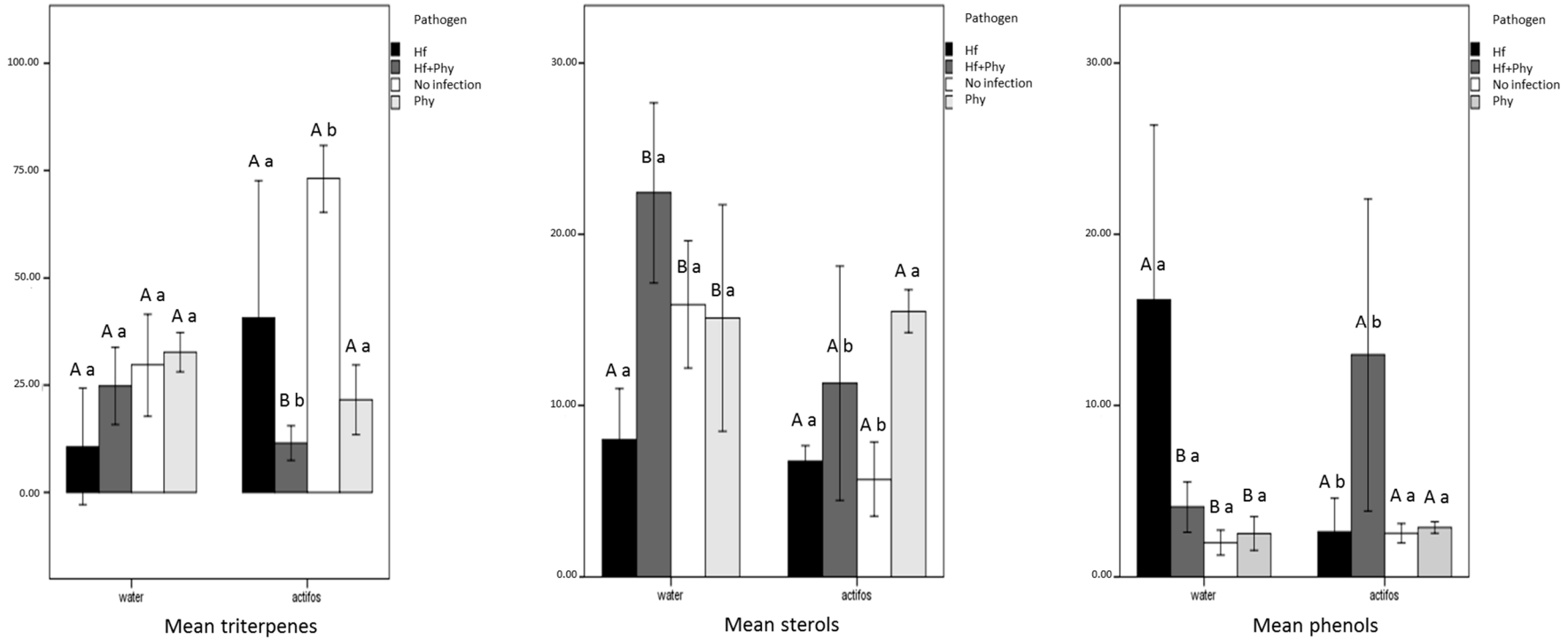

Figure 4. Content (%) of triterpenes, sterols, and phenols of cortical tissue samples of *F. excelsior* seedlings subjected to ammonium phosphite treatment (water and Actifos) after control, Hf, Phy, Hf + Phy infection. Uppercase letters indicate significant differences ($p < 0.05$ Kruskal–Wallis and Mann–Whitney *U*-test) between mock-inoculated and fungal inoculated plants within the same treatment (water and Actifos). Lowercase letters indicate significant differences ($p < 0.05$ Mann–Whitney *U*-test) between plants subjected to different treatments and the same inoculation type.

Differences between the treatments (water and Actifos) within the same inoculation type were significant for the total triterpene, sterol, and phenol amounts. The content of the triterpenes and sterols was significantly varied for uninfected as well as Hf + Phy-infected seedlings. The amount of sterols was significantly lower in Actifos-treated seedlings infected with Hf + Phy, while the amounts of phenols within the same combination of treatment/infection type showed an opposite trend (Figure 4).

In the water treatments, there was no significant difference in the content of main triterpenes, sterols, and phenols between the infected and control seedlings. However, *H. fraxineus*-infected plants had a higher amount of tyrosol and lower content of ursolic acid and β-sitosterol compared to other water treatments (Figure 5).

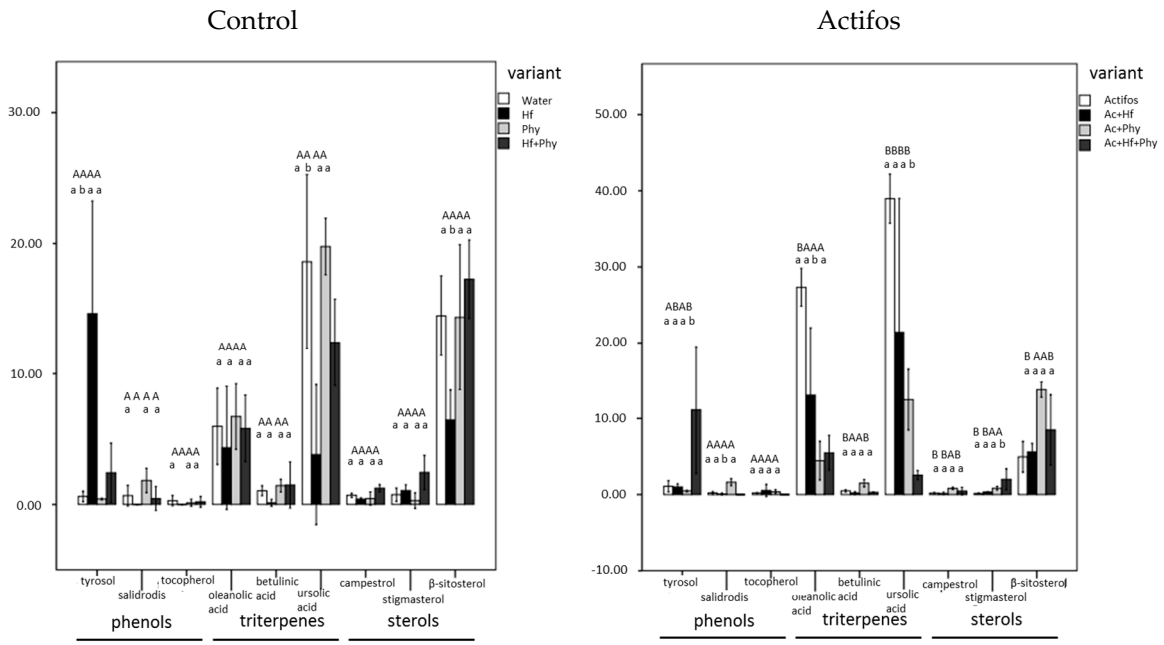

Figure 5. Content (%) of selected compounds in extract from the shoots of *F. excelsior* subjected to ammonium phosphite treatment (water and Actifos) after control for Hf, Phy, and Hf + Phy infection. Lowercase letters indicate significant differences ($p < 0.05$ Kruskal–Wallis and Mann–Whitney *U*-test) between mock-inoculated and fungal inoculated plants within the same treatment (water and Actifos). Uppercase letters indicate significant differences ($p < 0.05$ Mann–Whitney *U*-test) between plants subjected to different treatments and the same inoculation type.

The Actifos treatment showed significant differences in the amount of all sterols, as well as tyrosol, oleanolic, and betulinic acids (Figure 5).

The profiles of the main triterpenes, sterols, and phenols showed a significant difference between treatments (water and Actifos) and infection type. The amount of tyrosol was significantly decreased for seedlings infected with Hf, and increased for those infected with Hf + Phy. The content of ursolic acid within the same combination of treatment/infection type showed opposite trends. Some infected seedlings treated using Actifos had a significantly lower content of salidroside, oleanolic acid, stigmasterol, and β-sitosterol (Figure 5).

A strong correlation that was both positive and negative, ranging from 0.46 to 0.81, was observed for the tested chlorophyll fluorescence (Chlf) parameters (Table 4). The negative correlation was for both performance indexes and energy dissipation (DI/CSo). There were no statistically significant differences in correlations between the survival of seedlings and Chlf parameters. A positive correlation was observed between the survival and height and diameter of seedlings. The diameter of the tested plants strongly correlated with the height of the tested seedlings. Phenols were negatively correlated to triterpenes and sterols.

Table 4. Spearman rank-based correlation matrix for the interaction of the 11 selected parameters.

	Parameters	Mean	s.d.	1	2	3	4	5	6	7	8	9	10
Chlorophyll fluorescence (159)	(1) PIabs	0.743	0.065	—									
	(2) PItot	1.230	0.893	**0.60** **	—								
	(3) DI/CSo	67.476	21.529	**−0.81** **	**−0.46** **	—							
	(4) ET/CSo	89.500	24.877	**0.53** **	**0.68** **	−0.05	–						
No. of living plants (8)	(5) Survival	14.875	7.060	−0.39	**−0.53**	0.02	−0.53	–					
Growth parameters (187)	(6) Height	141.30	38.94	−0.03	**−0.16** *	−0.04	−0.09	0.58	—				
	(7) Diameter	5.79	1.044	−0.12	**−0.21** **	0.10	−0.09	0.65	**0.57** **	—			
	(8) Leaf area	88.52	82.303	−0.02	**−0.04**	0.07	0.08	0.34	0.11	**0.19** *	—		
Chemical analysis (24)	(9) Phenols	5.714	6.400	**−0.50** *	−0.11	**0.43** *	**−0.23**	**0.39**	0.18	0.16	−0.09	—	
	(10) Triterpenes	30.615	21.612	0.24	0.04	−0.25	−0.01	−0.05	0.05	0.13	0.31	**−0.50** *	—
	(11) Sterols	12.590	6.260	0.14	−0.12	−0.04	0.05	−0.32	−0.16	−0.12	−0.03	−0.32	−0.28

* $p < 0.05$ level (2-tailed); ** $p < 0.01$ level (2-tailed).

4. Discussion

The mortality of seedlings in ash stands infected with *H. fraxineus* constitutes major damage and a threat to the existence of the species on the sites [16,52]. The regeneration of ash on heavily infested sites is endangered both by infestation and a lower production of seed [53], and by a high infection rate of seedlings [54]. The survival rate of young trees is very low because of the small crown volume and girdling of the main stem [18,55], while in older trees, this process is slower and takes time to be observed while the disease progressively develops on the shoots [56–58].

The high pathogenicity of *H. fraxineus* was demonstrated in this study. The mortality rate in the water treatment after the first experimental period on three-year-old seedlings was 100%. None of the infected seedlings survived the winter 2016/17. This confirms earlier findings that the disease progresses during the winter months, and that ash trees with later flushing and longer dormancy suffer from greater damage [59]. Molecular confirmation of the pathogen spread in the bark tissues, and wood showed the development of lesions above and below the point of inoculation (Figure 1) [10,12,60]. The pathogen preferentially followed the acropetal mode of transport in the plant, progressing faster and further up to 2.5 cm in the direction of the movement of water and mineral material rather than in the opposite direction, which is typical for vascular pathogens [61–63]. All of the plants treated with ammonium phosphite—Actifos before infection with *H. fraxineus*—survived until the end of the experiments. Even though some authors speculate that this pathogen can behave as an endophyte in vigorous plants, it was not possible to prove the presence of fungal DNA in the transient zones surrounding the callus tissues [64].

Although we hypothesized that the seedlings infected with *Phytophthora* mix through the soil and *H. fraxineus* in the stem would have the highest mortality rate, the two-year experiment showed that nine and 17 of 20 seedlings survived in the control and Actifos treatment groups, respectively. The efficiency of phosphites against the *Phytophthora* species is well documented [27,28,65], and it seems that the application of Actifos prevented the development of *Phytophthora* and reduced damage in roots [30]. Also, there is a possibility that the *Phytophthora* species in the mix for soil infestation contained less pathogenic species, but we cannot neglect that the survival of seedlings infected with *H. fraxineus* in these variants was much higher than in the water/control groups infected with the fungus. The results presented allow us to hypothesize that two months earlier, the soil infection with the *Phytophthora* mixture triggered still unknown resistance mechanisms, and ensured the higher survival of seedlings infected with *H. fraxineus.*

Seedlings showed differences in development, especially in the second year of the experiment. Reduction in height increment was significant in the infections with *H. fraxineus,* suggesting a decrease in the vitality of seedlings infected with *Phytophthora* species [30]. A similar conclusion could be drawn for the leaf area of plants infected with the *Phytophthora* mixture, which had obviously smaller leaves. This situation is well known for root system infections with pathogens such as *Phytophthora* spp., *Armillaria* spp., and *Phellinus weirii* [66–69]. Regarding Actifos-treated seedlings infected with *H. fraxineus,* there were no differences compared with both the control and Actifos, while differences in height growth were significantly smaller from the control, but still higher than in all of the other inoculation variants (Table 2).

Root analyses were not correlated with the seedlings' above-ground development. No differences existed among variants and treatments for total root length (TRL) and surface area (SA), suggesting that *Phytophthora* did not cause a notable destruction of the root system. The number of root tips decreased in the treatments with phosphites [28,70], and a similar situation was noted with number of tips (NoT) and fine root tips (FRT). The toxicity of phosphites to roots probably triggers a resistance mechanism of the plants against pathogens. The differences observed in the seedlings' above and below-ground development point to the importance of the individual vulnerability of ash trees to both pathogens [15,71].

Chlorophyll fluorescence enables a fast and non-invasive assessment of the photosynthetic apparatus function of any photosynthesizing organisms, so it has recently become a very popular

method for the detection of any plant stressors [42,72,73]. It also allows changes in the tested material (mostly leaves) to be predicted before any visible changes can be seen [36,74,75]. However, studies on trees and specialized host–pathogen interactions are still scarce [76]. The analyzed chlorophyll fluorescence parameters showed a clear difference between the control variants, Actifos-treated plants, and inoculated seedlings before any visible symptoms could be detected by the naked eye (at the end of the first study period). The worst photosynthetic performance was observed for infections by *H. fraxineus*. As a result, in the conditions of the experiment, none of the seedlings survived until spring. Also, autumn 2016 measurements showed that seedlings sprayed with Actifos suffered from the treatments, which is common for trees under stress [77]. This was not the case in the second year, where Actifos-treated plants showed chlorophyll fluorescence parameters that were quite similar to the control plants.

Plants infected with *H. fraxineus* showed the lowest level of photosynthetic performance and the highest "cost" of photosynthetic machinery survival, which was expressed as a loss of the absorbed light as heat energy. This is a standard response when plants deal with any biotic or abiotic stress [36].

Treatment of healthy seedlings with Actifos had a slightly positive effect on the photosynthetic efficiency, maintaining a similar level as in the control, but the application of Actifos did not help improve this efficiency when the plants were infected by *H. fraxineus*, suggesting that some other host–pathogen interactions occur in asymptomatic plants [78]. The photosynthetic efficiency of seedlings was reduced when plants were treated with the *Phytophthora* mix alone. However, its application helped the tree seedlings infected by *H. fraxineus* to maintain photosynthetic performance at an adequate level. The application of the *Phytophthora* mix in combination with Actifos did not have a significant effect on the photosynthetic apparatus status in seedlings both infected and uninfected by *H. fraxineus*. This result suggests that resistance reactions are not connected with the changes in biochemical energy consumption (boost of photosynthesis) to protect plants [44], but with the production chemical compounds (phenols, tannins, etc.) that can kill or delay fungal development in the infected tissues [79].

The very well-known and most used chlorophyll fluorescence (ChlF) parameter (Fv/Fm or Phi_{po}) related to the maximal efficiency of the PS-II photosynthesis system (data not shown) did show significant changes, which means that it cannot be recommended as a reliable bioindicator [36].

Based on the correlation analysis, it seems that there is a strong and significantly different correlation between ChlF parameters. The survival of the seedlings seems to be negatively correlated with the tested performance indexes (PI_{abs}, PI_{tot}) and electron transport (EI/CSo). The significant correlation between PIabs and DI/CSo suggests their relatedness to phenol production, but it is not possible to make further conclusions based on the knowledge presented.

Stronger plants had an improved chance of surviving either an *H. fraxineus* or *Phytophthora* attack (Table 3), which correlates with studies showing that damage to developed trees is less frequent [16,18]. A positive correlation was observed between the height and diameter of seedlings and their survival, and also the height/diameter interaction was strong and significant.

Trees have developed a wide range of defense mechanisms that can help them survive interactions with forest pathogens and insects [79]. Resistance to pests can be constitutive or induced, and has the potential to physically or chemically inhibit or stop pathogens or insects [80,81]. After physical barriers have been bypassed, multiple mechanisms are induced to produce chemical compounds such as phenols, terpenes, PR proteins, and secondary resins in localized or systemic induced resistance [79,82]. Induced resistance can be activated by biotic (pathogens, endophytes) [83] or abiotic compounds such as phosphites [28].

In the variant where plants were infected with Hf (only watered), the disease developed and seedlings died, while the content of phenolic compounds amounted to almost 800% compared to the control (=100%). The application of Actifos increased the amount of phenolics by 27%, which was the same as in the variant when the seedlings were sprayed with Actifos and infected with Hf (~32%). This level prevented lesion development and activated callus formation. When the infection

combined Hf and *Phytophthora,* the ash trees defended themselves by increasing their content of phenolic compounds by over six times (>650%) after treatment with Actifos, and in this case, some of them did not die. Changes in terpene and sterol amounts were notable, too. The seedlings treated with Actifos showed a notable increase in terpenes both in the control and Hf treatments, while a decrease was observed for infections with the *Phytophthora* mix. The total amounts of sterols were suppressed in the Actifos treatments. A few compounds originating from the different chemical groups that were suppressed by the application of Actifos may be of interest for further analyses. Nevertheless, the observed differences in total amounts indicate great individual variation between plants. The interaction between phenols and triterpenes was negative (Table 3), suggesting that an increase of phenols induces a significant reduction in triterpene production, but the role of these and other components involved in host–pathogen interaction and the survival of ash seedlings/trees under *H. fraxineus* attack needs further clarification.

5. Conclusions

F. excelsior seedlings inoculated with *H. fraxineus* showed significant differences in survival, lesion development, the production of phenols, terpenes, and sterols, and ChlF responses in plants sprayed with ammonium nitrate in the Actifos compared to water (control) treatment. Seedlings treated with Actifos prior to inoculation with *H. fraxineus* managed to survive the pathogen attack and prevent the development of the disease. It is not possible to be certain whether the pathogen is eradicated from the plant, as it was not possible to confirm this with species-specific primers, or whether it was merely suppressed in a latent phase of development, which is in line with the parameters obtained for the ChlF response. However, several attempts to re-isolate the pathogen on artificial media failed.

The combination of the possibility to manipulate plant vigor and individual genetically conditioned resistance, which was earlier reported, could be a method to ensure the improved survival of seedlings in the first years after their establishment until they move to higher social status/DBH (Diameter at breast height) classes, where they will have a greater chance of avoiding lethal damage from pathogens.

Author Contributions: Conceptualization, N.K. and T.O.; Funding acquisition T.O., N.K.; Methodology M.T., A.Z., H.M.K., M.S., J.A.N., N.K., T.O.; M.T., A.Z., N.K. data collection. N.K., M.T., H.M.K., M.S. analyzed the data. N.K., H.M.K., T.O. wrote the paper.

Funding: The research was supported by the Ministry of Science and Higher Education of Poland as the part of statutory activities "Udział obcych, inwazyjnych gatunków *Phytophthora* w zamieraniu korzeni jesionów w interakcjach pędów przez nowo odkryty grzyb *Chalara fraxinea*"; Project No 240325. This work received partial funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No. 635646, POnTE ("Pest Organisms Threatening Europe").

Acknowledgments: The authors thank the IBL funds supporting the 3-month stay of Nenad Keča in the Department of Forest Protection in Sękocin Stary.

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

References

1. Dobrowolska, D.; Hein, S.; Oosterbaan, A.; Wagner, S.; Clark, J.; Skovsgaard, J.P. A review of European ash (*Fraxinus excelsior* L.): Implications for silviculture. *Forestry* **2011**, *84*, 133–148. [CrossRef]
2. Kerr, G.; Cahalan, C. A review of site factors affecting the early growth of ash (*Fraxinus excelsior* L.). *For. Ecol. Manag.* **2004**, *188*, 225–234. [CrossRef]
3. Heinze, B.; Tiefenbacher, H.; Litschauer, R.; Kirisits, T. Ash dieback in Austria—History, current situation and outlook. In *Dieback of European Ash (Fraxinus spp.): Consequences and Guidelines for Sustainable Management*; Vasaitis, R., Enderle, R., Eds.; SLU Service/Repro: Uppsala, Sweden, 2017; pp. 33–53, ISBN (print version) 978-91-576-8696-1; ISBN (electronic version) 978-91-576-8697-8.
4. Przybył, K. Fungi associated with necrotic apical parts of *Fraxinus excelsior* shoots. *For. Pathol.* **2002**, *32*, 387–394. [CrossRef]

5. Thomas, F.M.; Blank, R.; Hartmann, G. Abiotic and biotic factors and their interactions as causes of oak decline in Central Europe. *For. Pathol.* **2002**, *32*, 277–307. [CrossRef]
6. Scharnweber, T.; Manthey, M.; Criegee, C.; Bauwe, A.; Schröder, C.; Wilmking, M. Drought matters—Declining precipitation influences growth of *Fagus sylvatica* L. and *Quercus robur* L. in north-eastern Germany. *For. Ecol. Manag.* **2011**, *262*, 947–961. [CrossRef]
7. Diaci, J.; Rozenbergar, D.; Anic, I.; Mikac, S.; Saniga, M.; Kucbel, S.; Visnjic, C.; Ballian, D. Structural dynamics and synchronous silver fir decline in mixed old-growth mountain forests in Eastern and Southeastern Europe. *Forestry* **2011**, *84*, 479–491. [CrossRef]
8. Oszako, T.; Delatour, C. *Recent Advances on Oak Health in Europe*; Forest Research Institute: Warsaw, Poland, 2000; p. 281, ISBN 83-87647-20-9.
9. Pacia, A.; Nowakowska, J.A.; Tkaczyk, M.; Sikora, K.; Tereba, A.; Borys, M.; Milenković, I.; Pszczółkowska, A.; Okorski, A.; Oszako, T. Common Ash Stand Affected by Ash Dieback in the Wolica Nature Reserve in Poland. *Baltic For.* **2017**, *23*, 183–197.
10. Kowalski, T. *Chalara fraxinea* sp. nov. associated with dieback of ash (*Fraxinus excelsior*) in Poland. *For. Pathol.* **2006**, *36*, 264–270. [CrossRef]
11. Baral, H.-O.; Queloz, V.; Hosoya, T. *Hymenoscyphus fraxineus*, the correct scientific name for the fungus causing ash dieback in Europe. *IMA Fungus* **2014**, *5*, 79–80. [CrossRef] [PubMed]
12. Kowalski, T.; Holdenrieder, O. The teleomorph of *Chalara fraxinea*, the causal agent of ash dieback. *For. Pathol.* **2009**, *39*, 304–308. [CrossRef]
13. Zhao, Y.-J.; Hosoya, T.; Baral, H.-O.; Hosaka, K.; Kakishima, M. *Hymenoscyphus pseudoalbidus*, the correct name for *Lambertella albida* reported from Japan. *Mycotaxon* **2012**, *122*, 25–41. [CrossRef]
14. Timmermann, V.; Borja, I.; Hietala, A.M.; Kirisits, T.; Solheim, H. Ash dieback: Pathogen spread and diurnal patterns of ascospore dispersal, with special emphasis on Norway. *Bull. OEPP/EPPO Bull.* **2011**, *41*, 14–20. [CrossRef]
15. McKinney, L.V.; Nielsen, L.R.; Collinge, D.B.; Thomsen, I.M.; Hansen, J.K.; Kjær, E.D. The ash dieback crisis: Genetic variation in resistance can prove a long-term solution. *Plant Pathol.* **2014**, *63*, 485–499. [CrossRef]
16. Enderle, R.; Metzler, B.; Riemer, U.; Kändler, G. Ash dieback on sample points of the National forest inventory in south-western Germany. *Forests* **2018**, *9*, 25. [CrossRef]
17. Timmermann, V.; Nagy, N.E.; Hietala, A.M.; Børja, I.; Solheim, H. Progression of Ash Dieback in Norway Related to Tree Age, Disease History and Regional Aspects. *Baltic For.* **2017**, *23*, 150–158.
18. Marçais, B.; Husson, C.; Caël, O.; Collet, C.; Dowkiw, A.; Saintonge, F.-X.; Delahaze, L.; Chandelier, A. Estimation of Ash Mortality Induced by *Hymenoscyphus fraxineus* in France and Belgium. *Baltic For.* **2017**, *23*, 159–167.
19. Stocks, J.J.; Buggs, R.J.A.; Lee, S.J. A first assessment of *Fraxinus excelsior* (common ash) susceptibility to *Hymenoscyphus fraxineus* (ash dieback) throughout the British Isles. *Sci. Rep.* **2017**, *7*, 16546. [CrossRef] [PubMed]
20. Enderle, R.; Nakou, A.; Thomas, K.; Metzler, B. Susceptibility of autochthonous German *Fraxinus excelsior* clones to *Hymenscyphus pseudoalbidus* is genetically determined. *Ann. For. Sci.* **2015**, *72*, 183–193. [CrossRef]
21. Sollars, E.S.A.; Harper, A.L.; Kelly, L.J.; Sambles, C.M.; Ramirez-Gonzalez, R.H.; Swarbreck, D.; Kaithakottil, G.; Cooper, E.D.; Uauy, C.; Havlickova, L.; et al. Genome sequence and genetic diversity of European ash trees. *Nature* **2016**, *541*, 212–216. [CrossRef] [PubMed]
22. Sambles, C.M.; Salmon, D.L.; Florance, H.; Howard, T.P.; Smirnoff, N.; Nielsen, L.R.; McKinney, L.V.; Kjær, E.D.; Buggs, R.J.A.; Studholme, D.J.; Grant, M. Ash leaf metabolomes reveal differences between trees tolerant and susceptible to ash dieback disease. *Sci. Data* **2017**, *4*, 170190. [CrossRef] [PubMed]
23. Marciulyniene, D.; Davydenko, K.; Stenlid, J.; Cleary, M. Can pruning help maintain vitality of ash trees affected by ash dieback in urban landscapes? *Urban For. Urban Green.* **2017**, *27*, 69–75. [CrossRef]
24. Harper, A.L.; McKinney, L.V.; Nielsen, L.R.; Havlickova, L.; Li, Y.; Trick, M.; Fraser, F.; Wang, L.; Fellgett, A.; Sollars, E.S.A.; et al. Molecular markers for tolerance of European ash (*Fraxinus excelsior*) to dieback disease identified using Associative Transcriptomics. *Sci. Rep.* **2016**, *6*, 19335. [CrossRef] [PubMed]
25. Guest, D.; Grant, B. The complex action of phosphonates as antifungal agents. *Biol. Rev. Camb. Philos. Soc.* **1991**, *66*, 159–187. [CrossRef]
26. Conrath, U.; Pieterse, C.M.J.; Mauch-Mani, B. Priming in plant-pathogen interactions. *Trends Plant Sci.* **2002**, *7*, 210–216. [CrossRef]

27. Dalio, R.J.D.; Fleischmann, F.; Humez, M.; Osswald, W. Phosphite Protects *Fagus sylvatica* Seedlings towards *Phytophthora plurivora* via Local Toxicity, Priming and Facilitation of Pathogen Recognition. *PLoS ONE* **2014**, *9*, e87860. [CrossRef] [PubMed]
28. Hardy, G.E.S.; Barrett, S.; Shearer, B.L. The future of phosphite as a fungicide to control the soilborne plant pathogen *Phytophthora cinnamomi* in natural ecosystems. *Australas. Plant Pathol.* **2001**, *30*, 133–139. [CrossRef]
29. Thao, H.T.B.; Yamakawa, T. Phosphite (phosphorous acid): Fungicide, fertilizer or bio-stimulator? *Soil Sci. Plant Nutr.* **2009**, *55*, 228–234. [CrossRef]
30. Tkaczyk, M.; Nowakowska, J.A.; Oszako, T. Phosphite fertilizers as a plant growth stimulators in forest nurseries (in Polish with English summary). *Sylwan* **2014**, *158*, 3–9.
31. Gómez-Merino, F.C.; Trejo-Téllez, L.I. Biostimulant activity of phosphite in horticulture. *Sci. Hortic.* **2015**, *196*, 82–90. [CrossRef]
32. Tkaczyk, M.; Pacia, A.; Siebyła, M.; Oszako, T. Phosphite fertilisers as inhibitors of *Hymenoscyphus fraxineus* (anamorph *Chalara fraxinea*) growth in tests in vitro. *Fol. For. Pol. Ser. A For.* **2017**, *59*, 79–81. [CrossRef]
33. Milosz, T.; Kubiak, K.A.; Jacek, S.; Nowakowska, J.A.; Tomasz, O. The use of phosphates in forestry. *For. Res. Pap.* **2016**, *77*, 76–81. [CrossRef]
34. Swoczyna, T.; Kalaji, H.M.; Pietkiewicz, S.; Borowski, J.; Zaraś-Januszkiewicz, E. Photosynthetic apparatus efficiency of eight tree taxa as an indicator of their tolerance to urban environments. *Dendrobiology* **2010**, *63*, 65–75.
35. Baeten, L.; Verheyen, K.; Wirth, C.; Bruelheide, H.; Bussotti, F.; Finér, L.; Jaroszewicz, B.; Selvi, F.; Valladares, F.; Allan, E.; et al. A novel comparative research platform designed to determine the functional significance of tree species diversity in European forests. *Perspect. Plant Ecol. Evol. Syst.* **2013**, *15*, 281–291. [CrossRef]
36. Kalaji, H.M.; Goltsev, V.N.; Żuk-Gołaszewska, K.; Zivcak, M.; Brestic, M. *Chlorophyll Fluorescence: Understanding Crop Performance—Basics and Applications*; CRC Press: Boca Raton, FL, USA, 2017; p. 222, ISBN 978-14-9876-449-0.
37. Himejima, M.; Hobson, K.R.; Otsuka, T.; Wood, D.L.; Kubo, I. Antimicrobial terpenes from oleoresin of ponderosa pine tree *Pinus ponderosa*: A defense mechanism against microbial invasion. *J. Chem. Ecol.* **1992**, *18*, 1809–1818. [CrossRef] [PubMed]
38. Klepzig, K.D.; Kruger, E.L.; Smalley, E.B.; Raffa, K.F. Effects of biotic and abiotic stress on induced accumulation of terpenes and phenolics in red pines inoculated with bark beetle-vectored fungus. *J. Chem. Ecol.* **1995**, *21*, 601–626. [CrossRef] [PubMed]
39. Witzell, J.; Martin, J.A. Phenolic metabolites in the resistance of northern forest trees to pathogens—Past experiences and future prospects. *Can. J. For. Res.* **2008**, *38*, 2711–2727. [CrossRef]
40. Kruger, B.M.; Manion, P.D. Antifungal compounds in aspen: Effect of water stress. *Can. J. Bot.* **1994**, *72*, 454–460. [CrossRef]
41. Fernández de Simón, B.; Esteruelas, E.; Muñoz, A.M.; Cadahia, E.; Sanz, M. Volatile Compounds in Acacia, Chestnut, Cherry, Ash, and Oak Woods, with a View to Their Use in Cooperage. *J. Agric. Food Chem.* **2009**, *57*, 3217–3227. [CrossRef] [PubMed]
42. Caligiani, A.; Tonelli, L.; Palla, G.; Marseglia, A.; Rossi, D.; Bruni, R. Looking beyond sugars: Phytochemical profiling and standardization of manna exudates from Sicilian *Fraxinus excelsior* L. *Fitoterapia* **2013**, *90*, 65–72. [CrossRef] [PubMed]
43. Kalaji, H.M.; Schansker, G.; Ladle, R.J.; Goltsev, V.; Bosa, K.; Allakhverdiev, S.I.; Brestic, M.; Bussotti, F.; Calatayud, A.; Dąbrowski, P.; et al. Frequently asked questions about in vivo chlorophyll fluorescence: Practical issues. *Photosynth. Res.* **2014**, *122*, 121–158. [CrossRef] [PubMed]
44. Angay, O.; Fralischmann, F.; Recht, S.; Herrmann, S.; Matyssek, R.; Oβwald, W.; Buscot, F.; Grams, T.E. Sweets for the foe-effects of nonstructural carbohydrates on the susceptibility of *Quercus robur* against *Phytophthora quercina*. *New Phytol.* **2014**, *203*, 1282–1290. [CrossRef] [PubMed]
45. Drenkhan, R.; Adamson, K.; Hanso, M. The earliest samples of *Hymenoscyphus albidus* vs. *H. fraxineus* in Estonian mycological herbaria. *Mycol. Prog.* **2016**, *15*, 835–844. [CrossRef]
46. Jung, T.; Blaschke, H.; Neumann, P. Isolation, identification and pathogenicity of *Phytophthora* species from declining oak stands. *Eur. J. For. Pathol.* **1996**, *26*, 253–272. [CrossRef]
47. Kalaji, H.M.; Račkovà, L.; Paganovà, V.; Swoczyna, T.; Rusinowski, S.; Sitko, K. Can chlorophyll-*a* fluorescence parametres be used as bio-indicators to distinguish between drought and salinity stress in *Tilia cordata* Mill? *Environ. Exp. Bot.* **2018**, *152*, 149–157. [CrossRef]

48. Stocki, M.; Zapora, E.; Rój, E.; Bakier, S. Recovering biologically active compounds from logging residue of birch (*Betula* spp.) with supercritical carbon dioxide. *Przem. Chem.* **2018**, *97*, 1000–1004.
49. Laska, G.; Kiercul, S.; Stocki, M.; Bajguz, A.; Pasco, D. Cancer-chemopreventive activity of secondary metabolites isolated from *Xanthoparmelia conspersa* lichen. *Planta Med.* **2015**, *81*, PM_59. [CrossRef]
50. Laska, G.; Sienkiewicz, A.; Stocki, M.; Sharma, V.; Zjawiony, J.; Jacob, M. Secondary metabolites from *Pulsatilla patens* and *Pulsatilla vulgaris* and their biological activity. *Planta Med.* **2015**, *81*, PM_122. [CrossRef]
51. Isidorov, V. *Identification of Biologically and Environmentally Significant Organic Compounds Mass Spectra and Retention Indices Library of Trimethylsilyl Derivatives*; PWN: Warszawa, Poland, 2015; p. 429, ISBN 9788301182571.
52. Pliura, A.; Lygis, V.; Suchockas, Y.; Bartevicius, E. Performance of twenty four European *Fraxinus excelsior* popualtion in three Lithauanian progeny trials with a species emphasis on resistance to *Chalara fraxinea*. *Baltic For.* **2011**, *17*, 17–33.
53. Cleary, M.R.; Arhipova, N.; Gaitnieks, T.; Stenlid, J.; Vasaitis, R. Natural infection of *Fraxinus excelsior* seeds by *Chalara fraxinea*. *For. Pathol.* **2013**, *43*, 83–85. [CrossRef]
54. Pušpure, I.; Matisons, R.; Laviniņš, M.; Gaitnieks, T.; Jansons, J. Natural regeneration of common ash in young stands in Latvia. *Baltic For.* **2017**, *23*, 209–217.
55. Tulik, M.; Zakrzewski, J.; Adamczyk, J.; Tereba, A.; Yaman, B.; Nowakowska, J.A. Anatomical and genetic aspects of ash dieback: A look at the wood structure. *iForest* **2017**, *10*, 522–528. [CrossRef]
56. Husson, C.; Caël, O.; Grandjean, J.P.; Nageleisen, L.; Marçais, B. Occurrence of *Hymenoscyphus pseudoalbidus* on infected ash logs. *Plant Pathol.* **2012**, *61*, 889–895. [CrossRef]
57. Lenz, H.D.; Bartha, B.; Straβer, L.; Lemme, H. Development of Ash Dieback in South-Eastern Germany and the Increasing Occurrence of Secondary Pathogens. *Forests* **2016**, *7*, 41. [CrossRef]
58. Giongo, S.; Oliveira Longa, C.M.; Dal Maso, E.; Montecchio, L.; Maresi, G. Evaluating the impact of *Hymenoscyphus fraxineus* in Trentino (Alps, Northern Italy): First investigations. *iForest* **2017**, *10*, 871–878. [CrossRef]
59. Bakys, R.; Vasaitis, R.; Skovsgaard, J.P. Patterns and severity of crown dieback in young even-aged stands of european ash (*Fraxinus excelsior* L.) in relation to stand density, bud flushing phenotype, and season. *Plant Protect. Sci.* **2013**, *49*, 120–126. [CrossRef]
60. Kirisits, T.; Cech, T.L. Beobachtungen zum sexuellen Stadium des Eschentriebsterben-Erregers *Chalara fraxinea* in Österreich [Observations on the sexual stage of the ash dieback pathogen *Chalara fraxinea* in Austria]. *Forstschutz Aktuell* **2009**, *48*, 21–25.
61. Wingfield, M.J.; Seifert, K.A.; Webber, J. (Eds.) *Ceratocystis and Ophiostoma: Taxonomy, Ecology, and Pathogenicity*; APS Press: St. Paul, MN, USA, 1993; p. 293, ISBN 10: 0890541566; ISBN 13: 9780890541562.
62. Ouellette, G.B.; Rioux, D.; Simard, M.; Cherif, M. Ultrastuctural and cytochemical studies of host and pathogens in some fungal wilt diseases: Retro- and introspection towards a better understanding of DED. *For. Syst.* **2004**, *13*, 119–145.
63. Urban, J.; Dvořák, M. Sap flow-based quantitative indication of progression of Dutch elm disease after inoculation with *Ophiostoma novo-ulmi*. *Trees-Struct. Funct.* **2014**, *28*, 1599–1605. [CrossRef]
64. Bakys, R.; Vasaitis, R.; Barklund, P.; Ihrmark, K.; Stenlid, J. Investigations concerning the role of *Chalara fraxinea* in declining *Fraxinus excelsior*. *Plant Pathol.* **2009**, *58*, 284–292. [CrossRef]
65. Smillie, R.; Grant, B.R.; Guest, D. The mode of action of phosphite: Evidence for both direct and indirect modes of action on three *Phytophthora* spp. in plants. *Phytopathology* **1989**, *79*, 921–926. [CrossRef]
66. Erwin, D.C.; Ribeiro, O.K. *Phytophthora Diseases Worldwide*; APS Press: St. Paul, MN, USA, 1996; p. 562, ISBN 0-89054-212-0.
67. Hagle, S.K. Management Guide for Laminated Root Rot. In *Forest Insect and Disease Management Guide for the Northern and Central Rocky Mountains*; In cooperation with the Idaho Department of Lands and the Montana Department of Natural Resources and Conservation; Non-standard pagination; USDA Forest Service, Northern and Intermountain Regions, State and Private Forestry, Forest Health Protection: Missoula, MT, USA; Boise, ID, USA; Idaho Department of Lands, Forestry Division, Insects and Disease: Coeur d'Alene, ID, USA; Montana DNRC, Forestry Division, Forest Pest Management: Missoula, MT, USA, 2009; Chapter 11.2; p. 19.
68. Keča, N.; Karadžić, D.; Woodward, S. Ecology of *Armillaria* species in managed forests and plantations in Serbia. *For. Pathol.* **2009**, *39*, 217–231. [CrossRef]

69. Bakys, R.; Vasiliauskas, A.; Ihrmark, K.; Stenlid, J.; Menkis, A.; Vasaitis, R. Root rot, associated fungi and their impact on health condition of declining *Fraxinus excelsior* stands in Lithuania. *Scand. J. For. Res.* **2011**, *26*, 128–135. [CrossRef]
70. Jost, R.; Pharmawati, M.; Lapis-Gaza, R.H.; Rossig, C.; Berkowitz, O.; Lambers, H.; Finnegan, P.M. Differentiating phosphate-dependent and phosphate-independent systemic phosphate-starvation response networks in Arabidopsis thaliana through the application of phosphite. *J. Exp. Bot.* **2015**, *66*, 2501–2514. [CrossRef] [PubMed]
71. Muñoz, F.; Marçais, B.; Dufour, J.; Dowkiw, A. Rising out of the ashes: Additive genetic variation for crown and collar resistance to *Hymenoscyphus fraxineus* in *Fraxinus excelsior*. *Phytopathology* **2016**, *106*, 1535–1543. [CrossRef] [PubMed]
72. Percival, G.C. The use of chlorophyll fluorescence to identify chemical and environmental stress in leaf tissue of three oak (*Quercus*) species. *J. Arboric.* **2005**, *31*, 215–227.
73. Percival, G.C.; Keary, I.P.; Al-Habsi, S. An assessment of the drought tolerance of *Fraxinus* genotypes for urban landscape plantings. *Urban For. Urban Green.* **2006**, *5*, 17–27. [CrossRef]
74. Paunov, M.; Dankov, K.; Dimitrova, S.; Velikova, V.; Tsonev, T.; Strasser, R.; Kalaji, H.M.; Goltsev, V. Effect of water stress on photosynthetic light phase in leaves of two ecotypes of *Platanus orientalis* L. plants. *J. Biosci. Biotechnol.* **2015**, *SE/ONLINE*, 15–23.
75. Pšidová, E.; Živčák, M.; Stojnić, S.; Orlović, S.; Gömöry, D.; Kučerová, J.; Ditmarová, L.; Střelcová, K.; Brestič, M.; Kalaji, H.M. Altitude of origin influences the responses of PSII photochemistry to heat waves in European beech (*Fagus sylvatica* L.). *Environ. Exp. Bot.* **2017**, *152*, 97–106. [CrossRef]
76. Pollastrini, M.; Luchi, N.; Michelozzi, M.; Gerosa, G.; Marzuoli, R.; Bussotti, F.; Capretti, P. Early physiological responses of *Pinus pinea* L. seedlings infected by *Heterobasidion* sp. in an ozone-enriched atmospheric environment. *Tree Physiol.* **2015**, *35*, 331–340. [CrossRef] [PubMed]
77. Fini, A.; Ferrini, F.; Frangi, P.; Amoroso, G.; Piatti, R. Withholding irrigation during the establishment phase affected growth and physiology of Norway maple (*Acer platanoides*) and linden (*Tillia* spp.). *Arboric. Urban For.* **2009**, *35*, 241–251.
78. Steinböck, S.; Hietala, A.; Solheim, H.; Kräutler, K.; Kirisits, T. Association of *Hymenoscyphus pseudoalbidus* with leaf symptoms on *Fraxinus excelsior*: Phenology and pathogen colonization profile. In Proceedings of the COST ACTION FP1103 FRAXBACK 4th Management Committee meeting and workshop 'Frontiers in ash dieback research', Malmö, Sweden, Belgium, 4–6 September 2013; Vasaitis, R., Cleary, M.R., Eds.; [Poster]Meeting Program and Abstracts. COST: Brussels, Belgium; pp. 15–16.
79. Bonello, P.; Gordon, T.R.; Herms, D.A.; Wood, D.L.; Erbilgin, N. Nature and ecological implications of pathogen-induced systemic resistance in conifers: A novel hypothesis. *Physiol. Mol. Plant Pathol.* **2006**, *68*, 95–104. [CrossRef]
80. Cobb, F.W., Jr.; Krstić, M.; Zavarin, E.; Barber, H.W., Jr. Inhibitory effecets of volatile oleoresin components on *Fomes annosus* and four *Ceratocystis* species. *Phytopathology* **1968**, *58*, 1327–1335.
81. Eyles, A.; Bonello, P.; Ganley, R.; Mohammed, C. Induced resistance to pests and pathogens in trees. *New Phytol.* **2010**, *185*, 893–908. [CrossRef] [PubMed]
82. Reglinski, T.; Dann, E.; Deverall, B. Implementation of Induced Resistance for Crop Protection. In *Induced Resistance for Plant Defense*; Wiley Online Library: Hoboken, NJ, USA, 2014; pp. 249–299, Print ISBN 978-11-183-7183-1; Online ISBN 978-11-183-7184-8. [CrossRef]
83. Frey-Klett, P.; Garbaye, J. Mycorrhiza helper bacteria: A promising model for the genomic analysis of fungal-bacterial interactions. *New Phytol.* **2005**, *168*, 4–8. [CrossRef] [PubMed]