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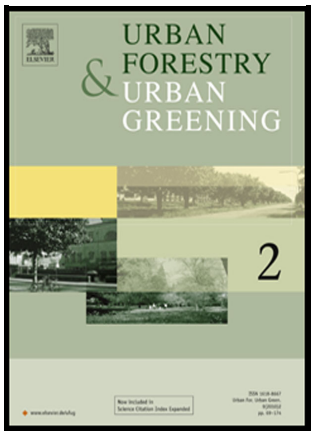

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The Protection of Ash Trees Against Ash Dieback by Tree Injections

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Abstract:

Ash dieback caused by a non-native pathogenic fungus *Hymenoscyphus fraxineus* has been decimating populations of European *Fraxinus* species for over 30 years. Nevertheless, there is still a considerable amount of valuable ashes of this species in European cities and landscapes. Although it has been shown in many studies that the effect of *H. fraxineus* on adult and senescent trees tends to be rather chronic, the infection by this fungus also causes a substantial decrease in the lifespan of these trees. This research is dealing with the control of ashes using the tree injection technology. It is focused on the development of bark necroses, as this symptom results in the highest damage to a tree. Trees were first inoculated with two different strains of *H. fraxineus*. Trees with proven positive inoculation were injected with six different fungicides. Subsequently, over the course of nine months, the area of developing bark necroses was measured using image analysis methods. After the field part of the experiment was finished, the extent of the infection by *H. fraxineus* in tissues was determined and confirmed by PCR. Three out of six tested variants (Tr. 1: 7.1% propiconazole , Tr. 3: 7% propamocarb and 2.7% fosetyl, and Tr. 6: 0.015% sodium selenite) displayed significant deceleration of the growth of bark necroses compared to control. Tr. 3 demonstrated the highest effectiveness with only a very small increment of the necrosis area during the first three months after the injection treatment and a follow-up complete cessation of growth. Reisolations confirmed high mortality of *H. fraxineus* after this treatment. Hence the presented study can contribute to substantial prolongation of the lifespan of ashes in Europe.

Keywords: bark necrosis, *Fraxinus excelsior*, *Hymenoscyphus fraxineus*, inoculation, veteran tree

1. Introduction

Since the beginning of the 1990s European populations of ashes (*Fraxinus* spp.) have been affected by the not indigene pathogenic fungus *Hymenoscyphus fraxineus* (Kowalski) Baral, Queloz & Hosoya 2014. Due to a very efficient airborne spore dispersal (Čermáková et al., 2017) it has very quickly spread all over the continent (Timmermann et al., 2011; Dal Maso et al., 2014). Ash is a key tree species in a temperate zone of Europe, especially in floodplain forest ecosystems. Therefore, Ash Dieback (hereafter ADB) endangers not only the future existence of this species but also many other organisms that are dependent on it (Mitchell et al., 2014).

Among European ash species ADB endangers mostly the common ash (*Fraxinus excelsior* L.) and the narrow-leaved ash (*F. angustifolia* Vahl.) (Gross et al., 2014; Papić et al., 2018a), the manna ash (*F. ornus* L.) is the least susceptible to the pathogen (Schwanda and Kirisits, 2016). Certain *F. excelsior* cultivars are also heavily affected, e.g. 'Westhof's Glorie', 'Raywood' a 'Atlas' (Kowalski et al., 2010; Junker et al., 2014). Outside European ash species, the infection has also been observed in *F. americana*, *F. mandshurica*, *F. nigra*, *F. ornus*, *F. pennsylvanica* and *F. sogdiana* (Drenkhan and Hanso, 2010; Kirisits and Schwanda, 2015).

ADB affects all tree age clases. It is particularly dangerous for seedlings and young trees, where it causes acute dieback during one vegetation period (Schumacher et al., 2009; Kirisits and Freinschlag, 2012). However, also in adult and senescent trees it causes serious chronic damage leading to their premature decline (Papić et al., 2018b). Hence the disease is a serious problem not only in nurseries (Schumacher et al., 2009), where it often results in significant losses in silviculture ash production, but also in urban forestry, where ashes often comprise a significant portion of species spectrum and important trees. The infestation leads to a gradual decline and secondary infections by fungi, predominantly root rots caused by species of the genus *Armillaria* sp. (Coker et al., 2019; Madsen et al., 2021).

A certain hope for the preservation of ashes in Europe is given by the fact that approximately 1% of individuals are considered resistant. (Stener, 2013; McKinney et al., 2014; Lobo et al., 2015). This resistance displays a remarkable heritability, which was shown in several studies in Austria, the Netherlands, Croatia, and Slovakia (Adamčíková et al., 2018). The resistance of the offspring is probably influenced by a combination of a genetic predisposition and environmental conditions (Muñoz et al., 2016; Wohlmuth et al., 2018).

Nowadays there are no known effective measures to cease or at least decelerate the spread and development of the disease, which is why current measures are aimed mostly at stopping the dispersal of *H. fraxineus*. Cooke et al. (2013) suggest various measures to reduce the production and dispersal of ascospores, including removal and burning of plant remnants with infected parts, in particular fallen infected leaves, or fungicide and biocide treatment of these fallen infected remnants.

An interesting and so far not tested method of control of precious ashes against the pathogenic fungus *H. fraxineus* might be tree injections. This technology started to be used during the 1970s (Reil and Beutel, 1976), when it was heavily deployed against a pathogenic fungus *Ophiostoma novo-ulmi* (Jones and Gregory, 1971; Shigo et al., 1980). The application of tree injection technology in the control of woody plants against a vast spectrum of diseases and pests has been successfully tested. In the USA this technology is also used against insect

pests causing tree decline, such as *Adelges tsugae*, *Anoplophora glabripennis*, *Agrilus planipennis* (Fernandéz-Escobar et al., 1999; McCullough, 2019).

At the turn of the millennium, this method started to be used in Europe (Portugal, Italy, France, Hungary, etc.) in the control of trees against harmful organisms and diseases (Grosman et al., 2009; Sousa et al., 2013; Kiss et al., 2020; Lee et al., 2020; Werrie et al., 2021). The benefit of tree injection is mostly its effective application that is not dependent on precipitation and weather conditions, unlike conventional spraying, thanks to a direct introduction of an active substance into the tree stem (Montecchio, 2013). The use of this method is suitable in an urban environment due to the highly reduced impact of applied substances on properties, citizens, and non-target species (Ferracini and Alma, 2008; Kobza et al., 2011). The most commonly used injectants are systemic insecticides and fungicides, biological control agents, and phosphorus-based mixtures, and a big potential lies in the use of nanotechnologies (Hao et al., 2017; Keča et al., 2018; Masikane et al., 2020; Dann and McLeod, 2021; Werrie et al., 2021).

The aim of this research was to test the efficacy of the tree injection against ADB using six fungicidal preparations. It is assumed that the treatment of the trees increases the resistance of physiologically active tissues against the pathogen growth, thanks to which physiological functions are not affected and the tree is going to be protected against the infestation for several years.

2. Materials and Methods

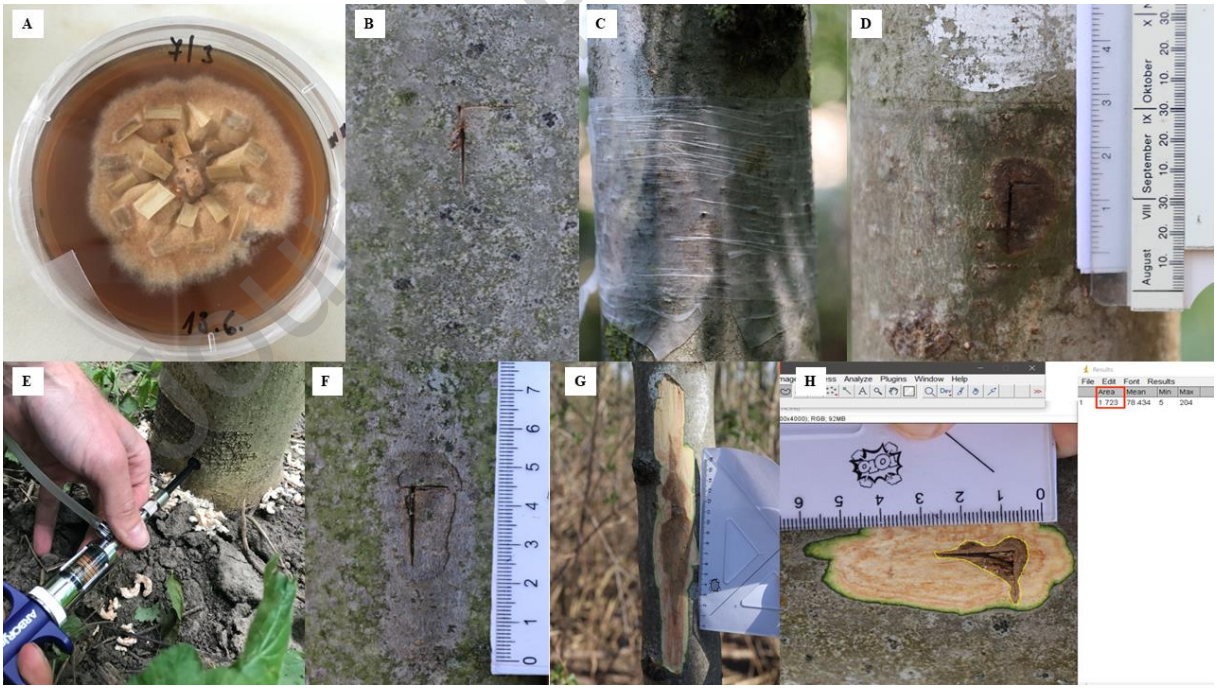

Figure 1. Methodology: (A) Inoculation media; (B) Stem after the inoculation; (C) Inoculation spot wrapped with parafilm; (D) Successfully inoculated individual; (E) Injection of successfully inoculated trees; (F) Checking of developing bark necroses; (G) The extent of xylem infection; (H) Measuring of the necrosis area in ImageJ.

2.1. Localities and tree selection

To test the efficacy of treatment of ash trees by means of tree injections, two selected forest stands were located in South Moravia, Czech Republic. Both localities have comparable

environmental conditions. They are heavily waterlogged areas near watercourses with the soils belonging to the soil group fluvisol (World Reference Base for Soil Resources 2023). Both stands are 17-year old common ash monocultures. The geographical and ecosystem characteristics of the examined localities are as follows: Vranovice: 100% presence of *F. excelsior*, GPS: N 48°56.60770', E 16°36.12318', altitude: 173 m a.s.l., Forest type: Floodplain and Křenovice: 100% presence of *F. excelsior*, GPS: N 49°9.40843', E 16°49.28553', altitude: 210 m a.s.l., Forest type: Floodplain. Both stands show clearly visible symptoms of dieback of a comparable intensity.

Individual trees were selected with the emphasis on a comparable growth (stem-base circumference 60-80 cm, circumference at breast height 30-50 cm), health condition, and vitality, intensity of *H. fraxineus* infestation and spatial distribition. Selected trees were without mechanical wounds, symptoms of infestation by other parasitic or pathogenic organisms, and without observed significant decrease in vitality. The intensity of infestation by *H. fraxineus* was assessed visually according to Lenz et al. (2012, 2016). Only the trees belonging to degree 1 reaching the canopy inside the stand were selected so the results were not affected by different microclimate and ecological conditions of the edge of the stand.

2.2. Isolation and identification of *H. fraxineus* from lesions

Isolation of *H. fraxineus* was carried out both before and after the field experiment. First, at both sites 15 ash annual shoots with typical symptoms of *H. fraxineus*, bark necroses, were collected (15 May 2019). After the transport to the laboratory, wood chips of ca. 5 × 5 × 5 mm in size were taken from each annual shoot at the boundary between the bark necrosis and healthy wood. Pieces were surface sterilized by soaking first for 4 min in a solution of 5% sodium hypochlorite, then for 1 min in 96% denatured ethanol, then again for 1 min in 96% denatured ethanol and finally for 5 min in sterilized distilled water. After drying in layers of sterile blotting paper they were placed on the surface on malt extract agar (M137 - Hi Media Laboratories, Maharashtra, India; composition: 30 g 1^{-1} malt extract, 5 g 1^{-1} mycological pepton, 15 g 1^{-1}agar with 100 mg 1^{-1} streptomycin sulphate. Petri dishes with the diameter of 90 mm were used. Five pieces were placed in one Petri dish with every piece being moderately pushed into the agar. All Petri dishes were incubated in the dark at 21°C and were examined every 2-3 days for at least 4 weeks. Outgrowing colonies were transferred to new Petri dishes with the MEA medium without streptomycin. Grown fungal cultures were subjected to DNA extraction, polymerase chain reaction (PCR), and sequence analyses and determination using GenBank database. Fresh fungal tissue was collected from plates containing pure cultures, and a Polymerase Chain Reaction (PCR) was conducted using Phire Plant Direct PCR Master Mix (F160S, Thermo Scientific®, Thermo Fisher Scientific) in accordance with the manufacturer's instructions. The Internal Transcribed Spacer (ITS) barcoding region was amplified using the primers ITS5 and ITS4 (White et al., 1990). The PCR products were visualised on a 1-1.2% agarose gel, and amplicons of the expected size were subsequently sent to Eurofins Genomics (Ebersberg, Germany) for Sanger sequencing. The species identity was verified by aligning the sequences to the reference sequences via NCBI BLAST (Altschul et al., 1990; Sayers et al., 2023).

The same procedure was repeated for sampling after the end of the field experiment and confirmation of the ADB presence. The wood chips were taken from more places – 20 mm above and under the transition zone of necrosis, from the transition zone between the necrosis

and healthy wood, and from the centre of the necrosis. This assessment was done for 50% of studied trees. Reisolations were done for three reasons: 1) To verify whether the extent of tissue discoloration at the infection point corresponds to the area affected by the infection; 2) To ascertain whether the pathogen stays active in the tissues after the injection treatment or dies; 3) To prove that the measured necroses were caused by the inoculated fungus.

2.3. Preparation of ash wood chips for inoculation

Inoculum preparation started on 13 June 2019. Two growing cultures of *H. fraxineus* were selected, one from each study site. These were subcultured to a new cultivation medium prepared analogously to the medium for initial isolations, differing only in added 50 g of frozen ash leaves to promote growth of *H. fraxineus* and not containing streptomycin. This medium is recommended by Botella et al. (2016). The medium was poured into 60 mm diameter Petri dishes with a volume of 15 ml. Wood chips of a maximum size 4 × 4 × 2 mm were cut from the healthy wood of live annual shoots of *F. excelsior* without bark. They were placed in a glass Petri dish, twice wrapped in aluminium foil and sterilized in an autoclave at 121°C for 15 min. After cooling they were placed onto a pure culture of *H. fraxineus* that grew in a Petri dish for 8 days. Wood chips were left being overgrown by the mycelium for the next 19 days. It was important that their whole surface was covered by the mycelium making then usable for the inoculation (see Fig. 1). For inoculation pure cultures no. 2093 (OR072635) and 2094 (OR072636) deposited in the collection of microorganisms of Phytopathological and mycological laboratory in the Department of Forest Protection and Wildlife Management at Mendel University in Brno.

2.4. Inoculation of the selected trees

Inoculation of the selected 80 and 60 trees at the locality Křenovice and Vranovice, respectively, was performed on 10 July 2019. Each tree was inoculated with two cultures at two different places 1.5 m above the ground at the opposite sides of the stem.

At the inoculation point the bark was cut out with a scalpel to the cambium (see Fig. 1). A wood chip inoculated with *H. fraxineus* was carefully inserted under the bark. Next, the detached bark was returned back and wrapped wirh Parafilm to prevent its drying out (see Fig. 1). Between individual inoculations tools were sterilized in flame and by rinsing them with denatured ethanol. Parafilm was left on the stem till the next control on 26 July 2019. Then the parafilm was removed and the successfulness of inoculations was checked. Succesfully inoculated trees – with a visually perceptible bark necrosis – were treated with injections.

2.5. The tree injection treatment

Trees were treated with the tree injection technology on 26 July 2019. In total, 48 trees were treated at both localities and divided into 8 variants with 6 individuals per variant. Trees were assigned to treatments randomly. Individual variants were marked with color and unique number on the stem. The treatment was carried out with the QUIK-jet technology (Arborjet, Inc., Woburn, 2023) (see Fig. 1).

2.6. Preparations, dosage, and concentrations

Six active ingredients were tested. Active ingredients were selected based on the literature search. Two preparations (Propizol 250 EC and Phospho-JET) are from the USA where they

are used in tree protection, but have not been tested against ADB yet. Three preparations are registered in the EU and Czech Republic (Previcur Energy, Horizon 250 EW, and Champion 50 WP). They are usually spray-applied and their efficacy against ADB has not been verified yet. In addition, a single active ingredient sodium selenite was tested. The details about used preparations, active ingredients, and dosage are shown in Table 1. Two control groups were used, one being injected with distilled water, the other not injected at all.

Table 1. Used preparations, active ingredients, concentrations, and dosages.

Treatment	Commercial name	Active ingredient	Volume	Dosage
Control	Control trees without treatment			
Control H_2O	X	Distilled H_2O	5 ml of mixture/inj. hole	inj. hole/100 mm of tree CBH
Tr. 1	Propizol 250 EC	7.1% propiconazole	5 ml of mixture/inj. hole	inj. hole/100 mm of tree CBH
Tr. 2	Phospho-JET	15.3% mono-and di-potassium salts of phosphorous acid	5 ml of mixture/inj. hole	inj. hole/100 mm of tree CBH
Tr. 3	Previcur Energy	7% propamocarb and 2.7% fosetyl	5 ml of mixture/inj. hole	inj. hole/100 mm of tree CBH
Tr. 4	Horizon 250 EW	1,7% tebuconazole	5 ml of mixture/inj. hole	inj. hole/100 mm of tree CBH
Tr. 5	Champion 50 WP	1% copper hydroxide	5 ml of mixture/inj. hole	inj. hole/100 mm of tree CBH
Tr. 6	X	0,015% sodium selenite 0,015%	5 ml of mixture/inj. hole	inj. hole/100 mm of tree CBH

2.7. Development of bark necroses surveillance

Observation of the development of bark necroses at inoculation spots was conducted at 30-day intervals from August 2019 to April 2020. During the initial check in August the area of necroses emerging after the successful inoculations was measured and was subtracted from the follow-up regular measurements to prevent the distortion of the results by the development of necroses before the injection. During the following checks each inoculation point was photographed with the attached scale (see Fig. 1). At the end of the field part of the experiment in April 2020 half of the examined trees (randomly selected 3 individuals per variant) were subjected to destructive analysis with the aim of ascertaining the extent of the infection in tissues. Bark and underbark tissues of these trees were gradually being removed up to the heartwoord. Photos of the exposed layers were taken regularly to document the extent of necrotic tissues. Photos with the largest necrosis area were used for the follow-up analysis. Reisolations were done as described in Chapter 2.2.

The area of bark necrosis was determined using image analysis in ImageJ (Schindelin et al., 2015). The total area of the necrosis (mm^2) was obtained after taking pictures of all necroses (Canon EOS 250D & Sigma 18-300 mm f/3,5-6,3 DC Macro OS HSM C, Canon, Ota City, Japan & Sigma Corporation, Kawasaki, Japan) and calibration of the software. For each photo the scale was calibrated by means of the tool "Analyze – Set scale". The outline of the necrosis was traced with the "Freehand" tool and the area delimited by the curve was measured ("Analyze – Measure") (Fig. 1). The area in pixels was converted to the area in mm^2 using the scale in the photo.

2.8. Statistical analysis

Datasets from the experiment were analysed using the software TIBCO Statistica 12 (DataFriends s.r.o., Pilsen, Czech Republic). The decision of using nonparametric methods for the analysis of variance, Kruskal–Wallis test and Dunn's multiple comparison test, was made according to the Shapiro–Wilk test of normality and Levene's test of homoscedasticity. For all analyses, the level of significance was set at $\alpha = 0.05$. TIBCO Statistica 12 was also used to make figures.

3. Results

3.1. Increase in the area of bark necroses during the experiment

During the first three months (September, October, and November) the increase of the necrosis area was observed for all the variants. However, from the beginning different growth rates were recorded. Both control variants and Treatment 2 (15.3% mono-and di-potassium salts of phosphorous acid) showed the fastest growth with the increment of 110-180 mm^2. Treatment 1 (7.1% propiconazole), 4 (1.7% tebuconazole), and 5 (1% copper hydroxide) showed the increase of 70-90 mm^2 during the same period. Treatment 3 (7% propamocarb and 2.7% fosetyl) and 6 (0.015% sodium selenite) displayed the increase of ca. 15-25 mm^2. These differences during the first three months are most likely already caused by the effect of injected active ingredients. Fig. 2 shows the gradual increase in the area of bark necrosis at the inoculation point for all studied variants at 30-day intervals.

In the course of the next three months (December, January, and February) all the variants except for Treatment 5 demonstrated a very low increase in the necrotic area tending to stagnation. In the case of Treatment 5 the necrotic area continued to increase with the growth rate being similar to the previous period. The area increased from ca. 80 mm^2 to ca. 130 mm^2 making this variant comparable to both controls.

During the last two-month period (March, April) both control variants and Treatment 2, 4, and 5 again showed the increase of the necrotic area, while in the case of Treatment 1, 3, and 6 no growth was observed.

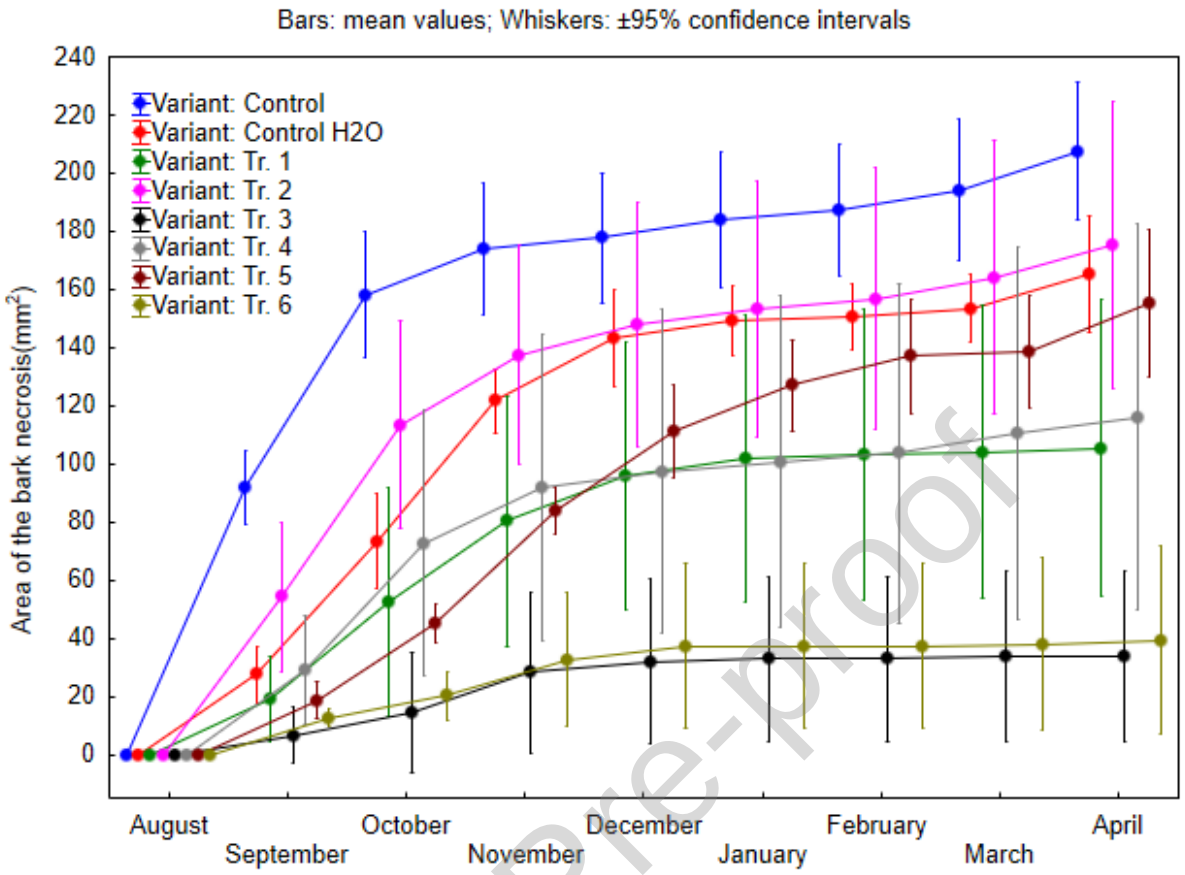

Figure 2. Growth of the bark necrosis area during the field experiment.

3.2. Final area of the bark necroses

Fig. 3 displays differences in the bark necrosis size at inoculation points at the end of the field part of the experiment (April 2020). Treatment 3 (7% propamocarb and 2.7% fosetyl) apparently shows the highest effectivity with the mean increase in the necrotic area since the injection being 33.9 mm^2. It significantly differed (K–W test: H (7; 111) = 38.236, $p < 0.001$) from the non-injected control (Dunn's test: $p < 0.001$; 207.4 mm^2) and Treatment 2 (15.3% mono-and di-potassium salts of phosphorous acid) (Dunn's test: $p < 0.001$; 175.5 mm^2). No other significant differences were detected (Dunn's test: $p > 0.061$;). Treatment 6 (0,015% sodium selenite) would very likely show significant differences from control variants and Treatment 2 if the sample size was larger. The increase in the necrosis area was only 39.3 mm^2.

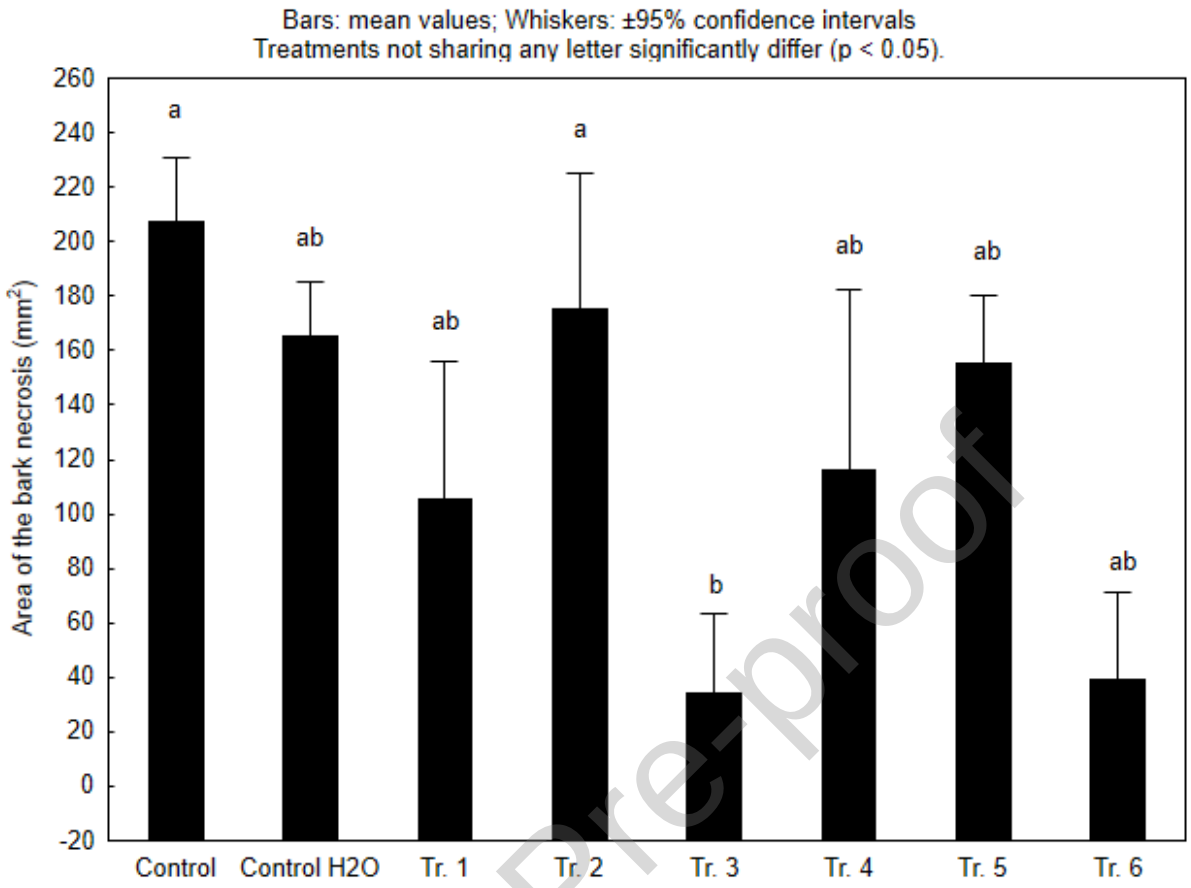

Figure 3. The bark necrotic area at the end of the field experiment. Bars (mean values) and whiskers (±95% confidence interval) with all letters being different differ significantly according to Dunn's test of multiple comparisons.

3.3. Underbark tissue area affected by the infection

The maximum area of tissues inside the stem affected by the infection is presented in Fig. 4. This analysis confirmed difference (K–W test: H (7; 95) = 47.640, $p < 0.001$) and the high efficacy of Treatment 3 (7% propamocarb and 2.7% fosetyl) with the mean area 88.1 mm^2, which was significantly lower value compared with both control variants (Dunn's test: $p < 0.001$) and Treatment 5 (1% copper hydroxide) (Dunn's test: $p < 0.003$). In the case of the non-injected and water-injected control variant the mean area was 907.1 mm^2 and 803.0 mm^2, respectively. Treatment 5 showed the value of 642 mm^2. Treatment 1 (7.1% propiconazole) and 6 (0,015% sodium selenite) also showed significantly lower mean areas of affected tissues compared to both control variants (Dunn's test: $p < 0.004$). The mean areas were 173.8 mm^2 for Treatment 1 and 150,5 mm^2 for Treatment 6. Treatments 2, 4, and 5 did not significantly differ from the control variants (Dunn's test: $p > 0.533$).

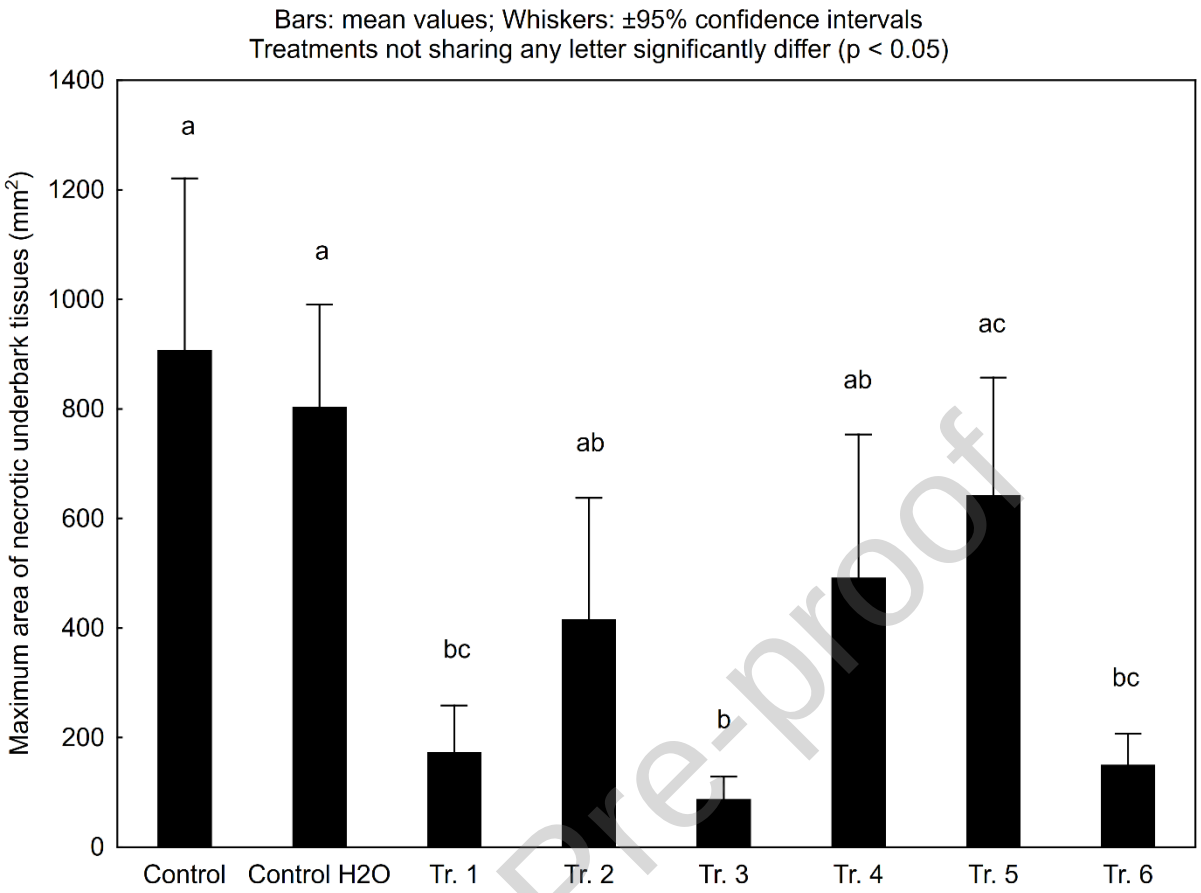

Figure 4. Maximum underbark tissue area affected by the infection. Bars (mean values) and whiskers (±0.95 confidence interval) with all letters being different differ significantly according to Dunn's test of multiple comparisons.

3.4. Reisolations

It was confirmed that the extent of tissue discoloration corresponds to the area affected by the pathogenic fungus *H. fraxineus*. After reisolations from 48 inoculation points on 24 trees there was not a single case of successfully isolated *H. fraxineus* from 20 mm above or below the transition zone of the discoloration. All successful inoculations were done from lesions or the transition zone of the lesion and healthy wood.

The success of reisolations for all variants is presented in supplementary data. The reisolation was considered successful when at least one *H. fraxineus* culture was obtained from the inoculation point. In total 3 trees with 6 inoculation points were tested per variant. In the case of both control variants and Treatment 5 (1% copper hydroxide) the pathogen remained active in the tissues. All reisolations were successful in these cases. On the contrary, for Treatment 1 (7.1% propiconazole) and 3 (7% propamocarb and 2.7% fosetyl) the reisolation success rate was extremely low, 15-30%. For the other variants the reisolation success rate ranged from 65% to 85%.

4. Discussion

Three tested preparations successfully suppressed ash dieback fungus in the infected ash tissues, suggesting high potential to use injection method in control of this pathogen in urban areas. Considering the global impact of ADB on ashes throughout Europe, a question suggests itself,

how to protect important ash trees in an urban environemt against this pathogen. Previous research and studies were rather concerned with the reduction of infection pressure on trees, e.g. by raking fallen leaves and their composting, burrying, or burning and subsequent adding of a substrate that promotes faster degratation of the infectious material (Kirisits and Freinschlag, 2012). Turczański et al. (2021) presented the idea of applying biocidal and fungicidal preparations directly onto the infectious material. The temperature increase is assumed to have a negative effect on the spread of *H. fraxineus* due to the accompanying drought, during which the infection spreads slower (Kowalski and Bartnik, 2010). In most cases it is only an imperfect solution, because spores in the infectious material can spread over long distances (Giongo et al., 2017). Tree injections may thus be a more effective method. If some of the tested preparations ensure a significant increase of resistance of the treated trees, it will present the first possibility of treatment of important ash trees. Our results suggest that this phytosanitary protection of individual trees is more of a long-term character with the tree being able to resist the pathogen longer. In Europe this method has so far been studied only by Dal Maso et al. (2014). They tested 6 commercial fungicidal preparations on 5 different *H. fraxineus* cultures in vitro. Two of them that were most effective, allicin and thiabendazole, were injected to inoculated ash trees. Their results show the effectiveness of these two compounds, with the 55.8% and 67.2% reduction of bark necrosis growth for allicin and thiabendazole, respectively. The necrosis stopped growing after approximately 5-7 months after the application in spring 2013. However, no checking was done in the next vegetation period that would confirm the efficacy of these compounds after the dormancy period.

Because of the vast amount of preparations on the market it would be valuable to experimentally test also others. In this study six preparations were tested with these active ingredients: 7.1% propiconazole, 15.3% mono- and di-potassium salts of phosphorous acid, 7% propamocarb and 2.7% fosetyl, 1.7% tebuconazole, 1% copper hydroxide, and 0.015% sodium selenite. The growth of bark necroses was checked also in the next vegetation period (March and April 2020) and confirmed that the use of three of these compounds led to a complete suppression of the necrosis growth, e.g. the presence of no growth rate and extremely low reisolation success rate in the case of Treatment 1, 3, and 6 during the last two-month experimental period (see Fig. 2 and "supplementary data", respectively) may explain the lethal influence of 7.1% propiconazole, 7% propamocarb and 2.7% fosetyl, and 0.015% sodium selenite to *H. fraxineus* in tissues of the host plants. This finding is a confirmation of the high efficacy of these three variants against this pathogen.

The question is whether the lesions are a viable indicator according to which it is possible to assess the successfulness of the treatment. This symptom was selected mainly because it is the most destructive symptom of the disease with its progress easily measurable by means of image analysis. This decision was based on the work by Bengtsson et al. (2014) who showed that the extent of tissue discoloration (lesion) roughly corresponds to the extent of infection. This study also showed similar results in terms of the speed of the lesion development – fast lesion growth during the summer period and its subsequent slowdown from November to March. This experiment was finished in April when re-acceleration of the lesion growth was observed, especially in control variants. These results together with the results of the above-cited study imply that the most suitable time for the treatment is the beginning of the vegetation period. During this time, tree injections are very effective due to high sap flow rate that speeds up the

distribution of active ingredients and it is also the time before the growth of lesions. The treatment at this time may thus prevent their emergence.

Keča et al. (2018) found a positive effect of ammonium phosphite water solution treatment on survival of ash seedlings infected by *H. fraxineus*. Successful usage of phosphonate fungicides against pathogens from the genus *Phytophthora*, including tree injections, has been reported by many authors from all over the world (Masikane et al., 2020; Solla et al., 2021, etc.). In contrast, in our research the application of mono-and di-potassium salts of phosphorous acid via tree injection showed to be one of the not very effective variants. This discrepancy may be explained by the mode of action of these compounds – they stimulate the natural defence capacity of a tree and do not act directly on the pathogenic organism. The mechanism of action of this group of compounds when used against pathogenic agents is described in detail by Dann and McLeod (2021).

Considering the fact that this experiment was performed only once and lacked repeated application, it would be reasonable to continue with the next measurements during the next years using the active ingredients that proved to lead to a suppression of the necrosis growth in the tissues. This would eliminate a potential bias of the results that can be effected by specific climate conditions during a single vegetation period. Follow-up research should also focus on the question how long the tree is effectively protected against the pathogen. These results would enable to determine the needed time interval between individual tree injection treatments with the aim of ensuring maximum and long-term protection of ashes against the pathogen. As another example, the use of tree injections against the emerald ash borer (*Agrilus planipennis*) at 2 to 3 year intervals, that have already been used in the USA for a longer time, are even mentioned in the strategy of Department for Enviroment, Food and Rular Affairs (2019).

It is also certainly desirable to search for other compounds that would show similar efficacy. An interesting possibility would be the use of fungicidally effective nanomaterials or novel biocontrol preparations (Hao et al., 2017; Brühwiler and Sieber, 2021; Werrie et al., 2021).

Very interesting is using of the injection of endophytic fungi or bacteria species with an antagonistic relationship to the pathogenic fungus *H. fraxineus*, or which are directly linked to the tolerance of some ash trees to infection of *H. fraxineus*. For example, Schlegel et al. (2016) determined the use of *Boeremia exigua* as a potential defence against *H. fraxineus*. Also, Nawrot-Chorabik et al. (2021) presented the finding of two potentially interesting endophytes significantly retarding the progression of *H. fraxineus* infection in *F. excelsior* and *F. pensylvanica* and Bilański et al. (2022) observed a significant reduction in the fructification of *H. fraxineus* on petioles in which the endophytic fungus *Pyrenochaeta fraxiniana* was present. These authors also confirmed the antagonistic relationship of *P. fraxiniana* to *H. fraxineus* in live petioles, where the presence of this fungus slowed the passage of infection through the petiole into the tissues. Barta et al. (2022) inoculated four endophyte species directly into ash stems at the site of *H. fraxineus* infestation and observed significant retardation in the growth of the underbark necrotic area. The spectrum of bacteria occurring in tolerant ash trees to *H. fraxineus* was studied by Ulrich et al. (2022). These authors found a correlation between the occurrence of a previously undescribed bacterium (*Luteimonas fraxinea*) and ash tree resistance. They also found that this bacterium can actively prevent the penetration of *H. fraxineus* infection into the leaves of the host plants in which it occurs. In a field experiment, they applied this bacterium by spraying it on the leaves of *F. excelsior* seedlings and observed

that it was able to protect the seedlings from infection for three years. The results of our study suggest that this could be a very interesting line of research in the coming years. A combination of tree injection technology and the results of the research mentioned above provide an effective and very nature-friendly way to protect ash trees against *H. fraxineus* infection. These results might be potentially used in the management of other causal agents of bark cankers on ash trees, e.g. *Armillaria* sp. and *Neonectria punicea* (Meyn et al. 2019, Karadzic et al. 2020), but this needs other research.

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References

Adamčíková, K., Pažitný, J., Pastirčáková, K., 2018. Individual resistance of Fraxinus angustifolia and F. excelsior clones to Hymenoscyphus fraxineus. J. Plant Prot. Res. Vol. 58, 227–233. https://doi.org/10.24425/122937

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–10. https://doi.org/…0-2

Barta, M., Pastirčáková, K., Ostrovský, R., Kobza, M., Kádasi Horáková, M., 2022. Culturable Endophytic Fungi in *Fraxinus excelsior* and Their Interactions with *Hymenoscyphus fraxineus*. Forests 13, 1098. https://doi.org/…098

Bengtsson, S.B.K., Barklund, P., von Brömssen, C., Stenlid, J., 2014. Seasonal Pattern of Lesion Development in Diseased Fraxinus excelsior Infected by Hymenoscyphus pseudoalbidus. PLoS ONE 9, e76429. https://doi.org/10.1371/journal.pone.0076429

Bilański, P., Grad, B., Kowalski, T., 2022. *Pyrenochaeta fraxinina* as colonizer of ash and sycamore petioles, its morphology, ecology, and phylogenetic connections. Mycol. Prog. 21, 74. https://doi.org/…7-8

Botella, L., Čermáková, V., Bačová, A., Dvořák, M., 2016. ADA, a fast-growth medium for *Hymenoscyphus fraxineus*. For. Pathol. 46, 85–87. https://doi.org/10.1111/efp.12251

Brühwiler, V., Sieber, T.N., 2021. Aqueous leaf extract of *Ligustrum vulgare* inhibits ascospore germination and mycelial growth of *Hymenoscyphus fraxineus*. For. Pathol. 51. https://doi.org/10.1111/efp.12657

Čermáková, V., Kudláček, T., Rotková, G., Rozsypálek, J., Botella, L., 2017. Hymenoscyphus fraxineus mitovirus 1 naturally disperses through the airborne inoculum of its host,

Hymenoscyphus fraxineus, in the Czech Republic. Biocontrol Sci. Technol. 27, 992–1008. https://doi.org/10.1080/09583157.2017.1368455

Coker, T.L.R., Rozsypálek, J., Edwards, A., Harwood, T.P., Butfoy, L., Buggs, R.J.A., 2019. Estimating mortality rates of European ash (*Fraxinus excelsior*) under the ash dieback (*Hymenoscyphus fraxineus*) epidemic. PLANTS PEOPLE PLANET 1, 48–58. https://doi.org/10.1002/ppp3.11

Cooke, L., Fleming, C., McCracken, A., 2013. DARD E&I project 12/3/S7: Efficacy of biocides, disinfectants and other treatments to limit the spread of ash dieback caused by Chalara fraxinea.

Dal Maso, E., Cocking, J., Montecchio, L., 2014. Efficacy tests on commercial fungicides against ash dieback in vitro and by trunk injection. Urban For. Urban Green. 13, 697–703. https://doi.org/10.1016/j.ufug.2014.07.005

Dann, E., McLeod, A., 2021. Phosphonic acid: a long-standing and versatile crop protectant. Pest Manag. Sci. 77, 2197–2208. https://doi.org/10.1002/ps.6156

Department for Enviroment, Food And Rural Affairs, 2019. Conserving our ash trees and mitigating the impacts of pests and diseases of ash: A vision and high-level strategy for ash research.

Drenkhan R., Hanso M., 2010. New host species for *Chalara fraxinea*. New Dis. Rep. 22, 16–16. https://doi.org/10.5197/j.2044-0588.2010.022.016

Fernandéz-Escobar, R., Gallego F. J., Benlloch, M., Membrillo, J., Infante, J., Perez De Algaba A., 1999. Treatment of oak decline using pressurized injection capsules of antifungal materials. Eur. J. For. Pathol. Volume 29, Pages1-88.

Ferracini, C., Alma, A., 2008. How to preserve horse chestnut trees from Cameraria ohridella in the urban environment. Crop Prot. 27, 1251–1255. https://doi.org/10.1016/j.cropro.2008.03.009

Giongo, S., Oliveira Longa, C., Dal Maso, E., Montecchio, L., Maresi, G., 2017. Evaluating the impact of Hymenoscyphus fraxineus in Trentino (Alps, Northern Italy): first investigations. IForest - Biogeosciences For. 10, 871–878. https://doi.org/10.3832/ifor2486-010

Grosman, D.M., Clarke, S.R., Upton, W.W., 2009. Efficacy of Two Systemic Insecticides Injected into Loblolly Pine for Protection Against Southern Pine Bark Beetles (Coleoptera: Curculionidae). J. Econ. Entomol. 102, 1062–1069. https://doi.org/10.1603/029.102.0326

Gross, A., Holdenrieder, O., Pautasso, M., Queloz, V., Sieber, T.N., 2014. *Hymenoscyphus pseudoalbidus* , the causal agent of E uropean ash dieback. Mol. Plant Pathol. 15, 5–21. https://doi.org/10.1111/mpp.12073

Hao, Y., Cao, X., Ma, C., Zhang, Zetian, Zhao, N., Ali, A., Hou, T., Xiang, Z., Zhuang, J., Wu, S., Xing, B., Zhang, Zhao, Rui, Y., 2017. Potential Applications and Antifungal Activities of Engineered Nanomaterials against Gray Mold Disease Agent Botrytis cinerea on Rose Petals. Front. Plant Sci. 8, 1332. https://doi.org/10.3389/fpls.2017.01332

Jones, T., Gregory, G., 1971. An Apparatus for Pressure Inject ion of Solutions into Trees. USDA For. Serv. Res. Pap. 7 pp.

Junker, C., Mandey, F., Pais, A., Ebel, R., Schulz, B., 2014. *Hymenoscyphus pseudoalbidus* and *Hymenoscyphus albidus*: viridiol concentration and virulence do not correlate. For. Pathol. 44, 39–44. https://doi.org/10.1111/efp.12066

Karadžić, D., Stanivuković, Z., Milanović, S., Sikora, K., Radulović, Z., Račko, V., Kardošová, M., Ďurkovič, J., Milenković, I., 2020. Development of Neonectria punicea Pathogenic Symptoms in Juvenile Fraxinus excelsior Trees. Front. Plant Sci. 11, 592260. https://doi.org/…260

Keča, N., Tkaczyk, M., Żółciak, A., Stocki, M., Kalaji, H., Nowakowska, J., Oszako, T., 2018. Survival of European Ash Seedlings Treated with Phosphite after Infection with the Hymenoscyphus fraxineus and Phytophthora Species. Forests 9, 442. https://doi.org/10.3390/f9080442

Kirisits, T., Freinschlag, C., 2012. Ash dieback caused by Hymenoscyphus pseudoalbidus in a seed plantation of Fraxinus excelsior in Austria. J. Agric. Ext. Rural Dev. 4, 184–191.

Kirisits, T., Schwanda, K., 2015. First definite report of natural infection of *Fraxinus ornus* by *Hymenoscyphus fraxineus*. For. Pathol. 45, 430–432. https://doi.org/10.1111/efp.12211

Kiss, M., Hachoumi, I., Nagy, V., Ladányi, M., Gutermuth, Á., Szabó, Á., Sörös, C., 2020. Preliminary results about the efficacy of abamectin trunk injection against the walnut husk fly (*Rhagoletis completa*). J. Plant Dis. Prot. 128, 333–338. https://doi.org/10.1007/s41348-020-00393-z

Kobza, M., Juhásová, G., Adamčíková, K., Onrušková, E., 2011. Tree Injection in the Management of Horse-Chestnut Leaf Miner. Cameraria ohridella (Lepidoptera: Gracillariidae). Gesunde Pflanz. 62, 139–143. https://doi.org/10.1007/s10343-011-0236-z

Kowalski, T., Schumacher, J., Kehr, R., 2010. Das Eschensterben in Europa – Symptome, Erreger und Empfehlungen für die Praxis.

Kowalski Tadeusz, Bartnik Czesław, 2010. Morphologial VariaTioN iN coloNies of Chalara fraxinea isolaTeD froM ash (Fraxinus excelsior l.) sTeMs WiTh sYMpToMs of DiebacK aND effecTs of TeMperaTUre oN coloNY groWTh aND sTrUcTUre. ACTA Agrobot. Vol. 63, pp 99-106.

Lee, S.-C., Lee, H.-R., Kim, D.-S., Kwon, J.-H., Huh, M.-J., Park, I.-K., 2020. Emamectin benzoate 9.7% SL as a new formulation for a trunk-injections against pine wood nematode, Bursaphelenchus xylophilus. J. For. Res. 31, 1399–1403. https://doi.org/10.1007/s11676-018-00873-x

Lenz, H., Bartha, B., Straßer, L., Lemme, H., 2016. Development of Ash Dieback in South-Eastern Germany and the Increasing Occurrence of Secondary Pathogens. Forests 7, 41. https://doi.org/10.3390/f7020041

Lenz, H., Straßer, L., Baumann, M., Baier, U., 2012. Boniturschlüssel zur Einstufung der Vitalität von Alteschen. AFZ-DerWald 2.

Lobo, A., McKinney, L.V., Hansen, J.K., Kjaer, E.D., Nielsen, L.R., 2015. Genetic variation in dieback resistance in *Fraxinus excelsior* confirmed by progeny inoculation assay. For. Pathol. 45, 379–387. https://doi.org/10.1111/efp.12179

Madsen, C.L., Kosawang, C., Thomsen, I.M., Hansen, L.N., Nielsen, L.R., Kjær, E.D., 2021. Combined progress in symptoms caused by Hymenoscyphus fraxineus and Armillaria species, and corresponding mortality in young and old ash trees. For. Ecol. Manag. 2021, 119177. https://doi.org/10.1016/j.foreco.2021.119177

Masikane, S.L., Novela, P., Mohale, P., McLeod, A., 2020. Effect of phosphonate application timing and -strategy on phosphite fruit and root residues of avocado. Crop Prot. 128, 105008. https://doi.org/10.1016/j.cropro.2019.105008

McCullough, D.G., 2019. Challenges, tactics and integrated management of emerald ash borer in North America. For. Int. J. For. Res. cpz049. https://doi.org/10.1093/forestry/cpz049

McKinney, L.V., Nielsen, L.R., Collinge, D.B., Thomsen, I.M., Hansen, J.K., Kjaer, E.D., 2014. The ash dieback crisis: genetic variation in resistance can prove a long-term solution. Plant Pathol. 63, 485–499. https://doi.org/10.1111/ppa.12196

Meyn, R., Langer, G.J., Gross, A., Langer, E.J., 2019. Fungal colonization patterns in necrotic rootstocks and stem bases of dieback-affected Fraxinus excelsior L. For. Pathol. 49, e12520.

Mitchell, R.J., Beaton, J.K., Bellamy, P.E., Broome, A., Chetcuti, J., Eaton, S., Ellis, C.J., Gimona, A., Harmer, R., Hester, A.J., Hewison, R.L., Hodgetts, N.G., Iason, G.R., Kerr, G., Littlewood, N.A., Newey, S., Potts, J.M., Pozsgai, G., Ray, D., Sim, D.A., Stockan, J.A., Taylor, A.F.S., Woodward, S., 2014. Ash dieback in the UK: A review of the ecological and conservation implications and potential management options. Biol. Conserv. 175, 95–109. https://doi.org/10.1016/j.biocon.2014.04.019

Montecchio, L., 2013. A Venturi Effect Can Help Cure Our Trees. J. Vis. Exp. 51199. https://doi.org/10.3791/51199

Muñoz, F., Marçais, B., Dufour, J., Dowkiw, A., 2016. Rising Out of the Ashes: Additive Genetic Variation for Crown and Collar Resistance to *Hymenoscyphus fraxineus* in *Fraxinus excelsior*. Phytopathology® 106, 1535–1543. https://doi.org/10.1094/PHYTO-11-15-0284-R

Nawrot-Chorabik, K., Marcol-Rumak, N., Latowski, D., 2021. Investigation of the Biocontrol Potential of Two Ash Endophytes against Hymenoscyphus fraxineus Using In Vitro Plant–Fungus Dual Cultures. Forests 12, 1750. https://doi.org/…750

Papić, S., Buriánek, V., Longauer, R., Kudláček, T., Rozsypálek, J., 2018. Phenotypic variability of *Fraxinus excelsior* L. and *Fraxinus angustifolia* Vahl under the ash dieback disease in the Czech Republic. J. For. Sci. 64, 279–288. https://doi.org/10.17221/30/2018-JFS

Papic, S., Longauer, R., Milenkovic, I., Rozsypálek, J., 2018. Genetic predispositions of common ash to the ash dieback caused by ash dieback fungus. Genetika 50, 221–229. https://doi.org/10.2298/GENSR1801221P

QUIK-jet Kit, n.d. . Arborjet. URL https://arborjet.com/product/quik-jet-kit/ (accessed 3.1.23).

Reil, W. O., Beutel, J.A., 1976. A pressure machine for injecting trees. Calif. Agric.

Sayers, E.W., Bolton, E.E., Brister, J.R., Canese, K., Chan, J., Comeau, D.C., Farrell, C.M., Feldgarden, M., Fine, A.M., Funk, K., Hatcher, E., Kannan, S., Kelly, C., Kim, S., Klimke, W., Landrum, M.J., Lathrop, S., Lu, Z., Madden, T.L., Malheiro, A., Marchler-Bauer, A., Murphy, T.D., Phan, L., Pujar, S., Rangwala, S.H., Schneider, V.A., Tse, T., Wang, J., Ye, J., Trawick, B.W., Pruitt, K.D., Sherry, S.T. 2023. Database resources of the National Center for Biotechnology Information in 2023. Nucleic Acids Res. 2023 Jan 6;51(D1):D29-D38. doi: 10.1093/nar/gkac1032. PMID: 36370100; PMCID: PMC9825438

Schindelin, J., Rueden, C.T., Hiner, M.C., Eliceiri, K.W., 2015. The ImageJ ecosystem: An open platform for biomedical image analysis: T HE I MAGEJ E COSYSTEM. Mol. Reprod. Dev. 82, 518–529. https://doi.org/10.1002/mrd.22489

Schlegel, M., Dubach, V., von Buol, L., Sieber, T.N., 2016. Effects of endophytic fungi on the ash dieback pathogen. FEMS Microbiol. Ecol. 92, fiw142.

Schumacher, J., Kehr, R., Leonhard, S., 2009. Mycological and histological investigations of *Fraxinus excelsior* nursery saplings naturally infected by *Chalara fraxinea*: Investigations of *C. fraxinea* infected ash saplings. For. Pathol. 40, 419–429. https://doi.org/10.1111/j.1439-0329.2009.00615.x

Schwanda, K., Kirisits, T., 2016. Pathogenicity of *Hymenoscyphus fraxineus* towards leaves of three European ash species: *Fraxinus excelsior*, *F. angustifolia* and *F. ornus*. Plant Pathol. 65, 1071–1083. https://doi.org/10.1111/ppa.12499

Shigo, Campana, Hyland, Anderson, 1980. Anatomy of elms injected to control Dutch elm disease. J. Arboric. Vol.6, pp.96-100.

Solla, A., Moreno, G., Malewski, T., Jung, T., Klisz, M., Tkaczyk, M., Siebyla, M., Pérez, A., Cubera, E., Hrynyk, H., Szulc, W., Rutkowska, B., Martín, J.A., Belbahri, L., Oszako, T., 2021. Phosphite spray for the control of oak decline induced by Phytophthora in Europe. For. Ecol. Manag. 485, 118938. https://doi.org/10.1016/j.foreco.2021.118938

Sousa, E., Naves, P., Vieira, M., 2013. Prevention of pine wilt disease induced by *Bursaphelenchus xylophilus* and *Monochamus galloprovincialis* by trunk injection of emamectin benzoate. Phytoparasitica 41, 143–148. https://doi.org/10.1007/s12600-012-0272-y

Stener, L.-G., 2013. Clonal differences in susceptibility to the dieback of *Fraxinus excelsior* in southern Sweden. Scand. J. For. Res. 28, 205–216. https://doi.org/10.1080/02827581.2012.735699

Timmermann, V., Børja, I., Hietala, A.M., Kirisits, T., Solheim, H., 2011. Ash dieback: pathogen spread and diurnal patterns of ascospore dispersal, with special emphasis on Norway. EPPO Bull.

Turczański, K., Bełka, M., Kukawka, R., Spychalski, M., Smiglak, M., 2021. A Novel Plant Resistance Inducer for the Protection of European Ash (*Fraxinus excelsior* L.) against *Hymenoscyphus fraxineus* - Preliminary Studies. Forests 12, 1072. https://doi.org/10.3390/f12081072

Ulrich, K., Becker, R., Behrendt, U., Kube, M., Schneck, V., Ulrich, A., 2022. Physiological and genomic characterisation of Luteimonas fraxinea sp. nov., a bacterial species associated with trees tolerant to ash dieback. Syst. Appl. Microbiol. 45, 126333. https://doi.org/…333

Werrie, P.-Y., Burgeon, C., Le Goff, G.J., Hance, T., Fauconnier, M.-L., 2021. Biopesticide Trunk Injection into Apple Trees: A Proof of Concept for the Systemic Movement of Mint and Cinnamon Essential Oils. Front. Plant Sci. 12, 650132. https://doi.org/10.3389/fpls.2021.650132

White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and Direct Sequencing of Fungal Ribosomal Rna Genes for Phylogenetics, in: PCR Protocols. Elsevier, pp. 315–322. https://doi.org/…2-1

Wohlmuth, A., Essl, F., Heinze, B., 2018. Genetic analysis of inherited reduced susceptibility of Fraxinus excelsior L. seedlings in Austria to ash dieback. For. Int. J. For. Res. Forestry 2018, 514–525.

World Reference Base | FAO SOILS PORTAL | Food and Agriculture Organization of the United Nations [WWW Document], n.d. URL https://www.fao.org/soils-portal/data-hub/soil-classification/world-reference-base/en/ (accessed 3.1.23).

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Highlights

- Tree injection is an effective protection of the ash against *Hymenoscyphus fraxineus*
- Three preparations showed to be effective, greatly decelerating the lesion growth
- A mixture of 7% propamocarb and 2.7% fosetyl can eliminate the pathogen in tissues