Interaction between *Hymenoscyphus fraxineus* and *Phytophthora* species on young *Fraxinus excelsior* seedlings

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ABSTRACT

Common pathogenicity tests on 16-month-old ash plants were performed using isolates of *Hymenoscyphus fraxineus* and three different *Phytophthora* spp. isolated under declining ash trees in Wolica Nature reserve in Poland. There were no statistically significant differences among the different inoculation treatments and lesion lengths averaged 1.67 cm. After analyses of inoculated roots, five out of 10 root parameters showed statistically significant differences, and after a Duncan's Multiple Range test, significant differences were found with control plants while there were no differences among the treatments. The largest root loss was with the treatment inoculated with both *H. fraxineus* and *P. plurivora* (HF + PP), and both fine and total root lengths were 2.30 and 2.27 times smaller, respectively, than the non-inoculated control. The earliest mortality of young ash plants was observed also with the HF + PP treatment. Results showed high aggressiveness of *H. fraxineus* to young ash plants, with indications that root infections with *Phytophthora* spp. may contribute to greater damage, but more research is needed to clarify the interaction between *H. fraxineus* and *Phytophthora* spp. in ash dieback.

Key words: *Hymenoscyphus fraxineus* (*Chalara fraxinea*), ash dieback, root pathogens, pathogenicity, *Phytophthora* species

RÉSUMÉ

On a effectué des tests de pathogénicité combinée sur des plants âgés de 16 mois avec deux isolats d'*Hymenoscyphus fraxineus* et trois différentes espèces de *Phytophthora* spp isolées sous des frênes dépérissants dans la réserve naturelle de Wolica en Pologne. Il n'y a pas eu de différence significative entre les différents traitements d'inoculation et les lésions mesuraient en moyenne 1,67 cm. L'analyse des racines inoculées a révélé que cinq des 10 paramètres des racines montraient des différences significatives et un test de Duncan a permis de confirmer qu'il y avait aussi des différences significatives avec les plants témoins, bien qu'il n'ait pas eu de différence entre les traitements. La perte de racines la plus importante a été enregistrée dans le traitement inoculé avec une combinaison de *H. fraxineus* et *P. plurivora* (HF + PP); les racines fines et grossières se sont avérées 2,30 et 2,27 fois plus courtes respectivement que celles des plants témoins sans inoculation. Les jeunes plants de frêne sont aussi morts plus rapidement lorsqu'ils avaient reçu le traitement (HF + PP). Les résultats ont montré la grande agressivité de *H. fraxineus* pour les jeunes plants de frêne, indiquant de plus qu'une infection racinaire par *Phytophthora* spp. pouvait en accroître le dommage. Il faudra poursuivre les recherches pour mieux comprendre comment *H. fraxineus* et *Phytophthora* spp. interagissent dans le dépérissement du frêne.

Mots-clés : *Hymenoscyphus fraxineus* (*Chalara fraxinea*), dépérissement du frêne, agents pathogènes des racines, pathogénicité, espèces de *Phytophthora*

Introduction

European ash (*Fraxinus excelsior* L.) dieback is a major problem affecting plantations and natural ash stands in most European countries (Husson *et al.* 2012; Rytönen *et al.* 2011). The first symptoms of this phenomenon were observed in the early 1990s in north-eastern Poland and Lithuania (Kowalski 2006; Timmermann *et al.* 2011), and a new species, *Chalara fraxinea* Kowalski (Kowalski 2006), as the cause of mass mortality of ash trees (Kowalski and Holdenrieder 2009a, 2009b). Kowalski and Holdenrieder (2009b) also suggested *Hymenoscyphus albidus* (Gillet) W. Phillips as a teleomorph of *C. fraxinea*, while Queloz *et al.* (2011) described *H. pseudoalbidus* Queloz as closely related but dif-

ferent from *H. albidus*. Finally, Baral *et al.* (2014) suggested *Hymenoscyphus fraxineus* (Kowalski) Baral, Queloz and Hosoya as a proper scientific name for this pathogenic fungus. Based on the available literature, the most recent countries where *H. fraxineus* has been reported on European ash were Serbia (Keča *et al.* 2017) and Montenegro (Milenković *et al.* 2017). Narrow-leaved ash (*Fraxinus angustifolia* Vahl.) is also susceptible to *H. fraxineus* after European ash (Kirisits *et al.* 2010).

The presence of pathogens from the genus *Phytophthora* under declining ash trees has been connected with the dieback. Several studies during the last two decades reported the occurrence of these pathogenic organisms in rhizosphere

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soils and necrotic tissues associated with severe damage of significant forest tree species (Brasier *et al.* 2005; Jung *et al.* 2013), including ash (Orlikowski *et al.* 2011; Akilli *et al.* 2013). Tkaczyk *et al.* (2016) also reported isolation of several different *Phytophthora* species from ash in Białowieża Forest Nature Reserve in Poland. Nevertheless, our understanding of *Phytophthora* species associated with ash is still very limited, as is information on the interaction between these two pathogenic organisms in the ash dieback phenomenon.

During the studies of ash dieback in the Wolica Nature Reserve, three different *Phytophthora* species were isolated from rhizosphere soil of declining ash trees, including *P. megasperma* Drechsler, *P. plurivora* Jung & Burgess, and *P. sp. hungarica* Bakonyi *et al.* (Pacia *et al.* 2017). In addition, high disease severity and different *H. fraxineus* symptoms were recorded. Due to lack of the data about the interaction of *H. fraxineus* and *Phytophthora* species on ash, a pathogenicity test of 16-month-old *Fraxinus excelsior* seedlings with *H. fraxineus* and *Phytophthora* species was performed to: i) test whether root loss caused by the activity of *Phytophthora* species influenced the extent of necrosis caused by *H. fraxineus*; ii) to test whether the infections with *H. fraxineus* influenced root loss caused by *Phytophthora* species; and, ii) to clarify the possible interaction of *H. fraxineus* and *Phytophthora* species.

Materials and Methods

Sampling, isolation, and identification of *Hymenoscyphus fraxineus*

Sampling and isolation of *H. fraxineus* were carried out according to Kowalski (2006). Samples were collected from symptomatic parts of the stems, branches and shoots in Wolica Nature Reserve (Forest District Chojnów (RDLP) in Warsaw, Poland; coordinates: 51.1° N, 10.5° E). Samples 3–4 mm were taken from the transition zone between necrotic and healthy phloem tissues using a flamed scalpel. Tissue surface sterilization was performed in 1% sodium hypochlorite, with 4% active hypochlorite for three minutes. The samples were then washed three times in distilled water and plated on 18 g l⁻¹ of Malt Extract medium (MEA, Germany) and 18 g l⁻¹ of agar (BTL, Poland). Incubation was at 18–20°C in the dark. After the appearance of the first hyphae, the samples were transferred onto fresh MEA medium and incubated at 20°C in the dark.

Four-week-old cultures were observed under a light microscope (ZEISS Axioskop 2) equipped with a Nikon Ds-fi1 camera and NIS Elements AR4¹ software. The identification of isolates as *Hymenoscyphus fraxineus* was based on disease symptoms, on the colony shape of pure cultures incubated for four weeks in the dark at 20°C, and on the shape and size of phialides observed in the pure cultures and compared with previously identified isolates.

Isolates of *Phytophthora* species used in the experiment

Isolates were taken from the Forest Research Institute-IBL collection of isolates, all originating from declining ash stands in Wolica Nature Reserve and previously morphologically and molecularly identified. Three different *Phytophthora*

species included isolate IBL/2012/8/1 of *P. plurivora* (JX274424), IBL/2012/9/3 of *P. megasperma* (JX274423), and IBL/2012/9/8 of *P. sp. hungarica* (JX274428).

Plant preparation and experiment design

Fraxinus excelsior seedlings 16-months-old were planted in individual 1 L pots containing 1 L of non-infected, autoclaved substrate mixture of perlite (Agra perlite[®], RHP, Netherlands), humus (Agrohum, Lomianki, Poland) and sand, in volume proportion 1:1:1. In addition, to avoid excessive root disturbance and wounding at the time of inoculation, two sterile 15-ml glass tubes were placed in each pot to a depth of 15 cm at 5-cm from the stem base to provide a cavity for inoculation.

Five different combinations of pathogenicity treatments with *H. fraxineus* and *Phytophthora* spp. were included and 10 plants per combination were used; the controls were the sixth treatment (Table 1). Since *P. plurivora* was most often isolated under ash trees in the study area, one combination included an inoculum of only *P. plurivora* while the second included a mixture of all the isolated species, *P. plurivora* + *P. megasperma* + *P. sp. hungarica* in inoculum proportions of 1:1:1. The temperature in the greenhouse ranged between 22 °C and 25 °C and the day length was set at 14 hr using an artificial light system.

Pathogenicity tests with *Phytophthora* species

Pathogenicity tests were done using the soil infestation test according to Jung *et al.* (1996), and the inoculum consisted of 4- to 6-week-old cultures of individual isolates of *Phytophthora* spp. grown at 20°C in 500 ml Erlenmeyer flasks on an autoclaved mixture of 250 cm³ of fine vermiculite (Agra vermiculite[®] RHP, Netherlands) and 20 cm³ of whole millet seeds thoroughly moistened with 175 ml of vegetable juice broth (200 ml l⁻¹ of vegetable juice (Tymbark, Poland), 800 ml l⁻¹ distilled water amended with 3 g l⁻¹ CaCO₃). Plants were placed into 80 L boxes (10 pots in each) and glass tubes

Table 1. Size of the plants and necrotic lesion lengths (mean and standard error) a month after ash shoots were inoculated with *Hymenoscyphus fraxineus* and four months after *Phytophthora* inoculation

Treatments	Height (cm)	Collar diameter (mm)	Lesion length (mm)
Control	15.2 ± 4.3	4.5 ± 0.8	0.00
<i>H. fraxineus</i>	14.3 ± 3.4	4.5 ± 0.8	1.7 ± 0.4 ²
<i>H. fraxineus</i> + Mix ¹	14.6 ± 3	4.5 ± 1	1.6 ± 0.3 ²
<i>H. fraxineus</i> + <i>P. plurivora</i>	15.7 ± 3.6	4.4 ± 0.7	1.7 ± 0.4 ³
Mix	14.6 ± 1.3	3.3 ± 0.3	0.00
<i>P. plurivora</i>	17.7 ± 1.3	4 ± 0.3	0.00

¹Mix of *Phytophthora* species included *P. plurivora* + *P. megasperma* + *P. sp. hungarica* in inoculum volume proportion 1:1:1;

²Dieback of one plant a month after ash shoots were inoculated;

³Dieback of three plants a month after ash shoots were inoculated.

removed. Prior to inoculation, the inoculum was rinsed under distilled water. Cavities were filled with inoculum at a ratio of 20 to 25 cm³ of inoculum per 1000 cm³ of the soil mixture. Controls were inoculated only with rinsed, sterile vermiculite-vegetable juice mixture at the same ratio. Each box was flooded immediately after inoculation for 72 hr and flooding repeated once every three weeks for 72 hr. During the second and every other flooding, re-isolations from the infested substrates were made by the leaf baiting method using young beech and oak leaves to determine the ability of the isolates to spread, survive and form sporangia with zoospores in the soil. After every flooding, discarded water was sterilized with sodium hypochlorite.

Pathogenicity test with *H. fraxineus*

Inoculum for *H. fraxineus* infections was prepared by transferring pure cultures of the pathogen onto MEA medium and growing for 3-4 weeks at 20 °C in the dark. Young shoots of healthy ash trees were collected and strips 2-mm wide were cut tangentially into 4-5 mm long pieces, placed on to Petri plates and sterilized. The sterilized pieces were plated on the surface of pure cultures and left to be overgrown by *H. fraxineus* mycelium for four weeks.

Inoculation of ash with *H. fraxineus* followed Bakys *et al.* (2009) and Kowalski and Holdenrieder (2009a). The bark of young stems was cleaned and surface sterilized with cotton moistened in 70% ethanol. At 5-7 cm above the soil level, 4-5 mm pieces of bark were removed with a flamed sterile scalpel. Prepared pieces of inoculum were placed onto the wounded tissue and sealed with Parafilm "M" (Bemis, USA) and aluminum foil. The control group was inoculated with sterile pieces of autoclaved wood. Inoculation with *H. fraxineus* was performed three months after the inoculations with *Phytophthora* species.

Re-isolation and examination

Four months after inoculation with *Phytophthora* species and a month after inoculation with *H. fraxineus*, plants were removed from the substrate and roots washed under running water. After washing, re-isolations were done by plating necrotic parts and pieces of fine roots onto selective agar media (V8-PARPNH) (Jung *et al.* 1996). Pieces of fine roots from control groups, as well as from the group inoculated only with *H. fraxineus*, were also plated onto selective agar media.

All roots were scanned using WinRhizo® software (Regent Instruments, Canada), and EPSON Perfection V700 Photo Scanner. Roots scanned with WinRhizo software were dried at 65 °C in a drying oven until constant weight (Termaks Series 2000, Norway), and dry biomass was measured of fine and coarse roots using the Sartorius analytic A200S scale (GMBH, Germany).

Sealed plants, inoculated with *H. fraxineus* were opened and analyzed for the presence of any necrosis and after measurement of necrotic lengths in the axial direction above the inoculation points, bark tissues were surface sterilized and re-isolations were attempted from necrotic edges by placing small necrotic pieces onto MEA amended with streptomycin. Pieces from the control group were also plated.

Statistical analyses

Lesion lengths as well as measurements of root parameters were tested using one-way analyses of variance at $p = 0.05$. Differences between mean necrotic length were analyzed using the Kruskal-Wallis test while differences between the other root parameter means were examined with the Duncan's Multiple Range post hoc test ($\alpha = 0.05$). All the analyses were performed with STATISTICA® (ver. 12).

Results

Hymenoscyphus fraxineus necrotic lesion lengths

Four months after inoculation with *Phytophthora* species and a month after inoculation with *H. fraxineus*, the first plants inoculated with *H. fraxineus* started to decline and the field experiment was ended. Re-isolations were successful in 83% of attempted cases. Lesion lengths ranged from 0.9 to 2.4 cm, with a mean value of 1.7 cm for all plants (Table 1). The results show that these treatments did not differ: *H. fraxineus*, *H. fraxineus* + Mix, and *H. fraxineus* + *P. plurivora* ($p < 0.97$) in necrotic lesion lengths. However, these treatments were different from the controls and other treatments based on standard error. The results also show that different treatments did not influence the necrosis length.

Results of soil infestation test

Re-isolations were successful in 100% of plated necrotic parts, and in 87.5% of plated root pieces. Root pieces from controls and treatment with only *H. fraxineus* inoculation were negative after the re-isolations. For specific fine root length, specific root tip density, fine root length per main root length, number of fine root tips per main root length and fine root length per dry weight of main root, there were no statistically significant differences. For tests where significantly significant treatment differences were found by ANOVA ($p < 0.05$), then Duncan's Multiple Range test was performed (Table 2).

In the cases where there were significant treatment effects, the non-inoculated control differed significantly from the remaining treatments. As well, the lowest values in general in terms of area, weight or length was after inoculation with both *H. fraxineus* and *P. plurivora*, but this was significantly different only from the non-inoculated control (Table 2). In the case of main root length, there was no statistically significant difference between the non-inoculated control and the treatments with inoculation using a mix of *Phytophthora* species or the single *P. plurivora* (Table 2). In the remaining cases, a significant difference was found only in comparison to the non-inoculated control with no statistically significant difference among the treatments.

Discussion

The results show the ability of *Phytophthora* spp. and *Hymenoscyphus fraxineus* to infect root systems of young ash seedlings and to cause damage and different root losses. However, there were generally no significant differences between root inoculation with *Phytophthora* spp. on lengths of necrotic lesions caused by *H. fraxineus*. Moreover, significant root losses were recorded compared to the non-inoculated, but not among treatments including different combinations of *Phytophthora* spp., *H. fraxineus*, and with both *Phytoph-*

Table 2. Mean values and the results of Duncan's test for five root parameters of common ash after the pathogenicity soil infestation test

Parameter	Treatments						(p)
	Control	<i>H. fraxineus</i> + Mix	<i>H. fraxineus</i> + <i>P. plurivora</i>	<i>H. fraxineus</i>	Mix	<i>P. plurivora</i>	
Total root length (cm)	768 a	415 b	337 b	523 b	417 b	416 b	0.005
Fine root length (cm)	755 a	406 b	328 b	513 b	408 b	403 b	0.006
Mother root length (cm)	13 a	8.7 b	8.8 b	9.3 b	9 b	13 a	0.019
Number of Tips (n)	1426 a	590 b	439 b	624 b	508 b	585 b	0.001
Fine Root Surface Area (cm ²)	131 a	74 b	61 b	81 b	72 b	66 b	0.002

thorae and *H. fraxineus*. Out of 10 root parameters, five had significant effects and means were separated with Duncan's Multiple Range test. The lowest value of fine root length, as one of the most important parameters reflecting root condition, was recorded in the case of dual inoculation with *P. plurivora* and *H. fraxineus*. This value was 2.3 times smaller and statistically significantly different only with the non-inoculated control, while total root length was 2.3 times smaller than the non-inoculated control. Compared to the *H. fraxineus* treatment, fine root lengths and mother root lengths were 1.6 times smaller but not statistically significant. The decline of the first three ash plants was recorded in this treatment which suggests that mortality was driven by root infection after infection of the shoot tissue. However, further large-scale field and pathogenicity studies are required to support this conclusion. Similar trends were found with other root parameters, but they were not statistically significantly different from other treatments including the non-inoculated control.

The results in this study are in agreement with previous studies on the high aggressiveness of *H. fraxineus* (e.g., Kowalski 2006; Kowalski and Holdenrieder 2009a, 2009b). Ash dieback is a serious problem affecting most European countries and one of the main goals in management strategies is to determine all the contributing factors of ash dieback in Europe. In addition to infections by *H. fraxineus* as a main cause of ash dieback, other factors may possibly play role in this phenomenon, including infection with *Phytophthora* species (Orlikowski *et al.* 2011; Akilli *et al.* 2013) and *Armillaria* species (Skovsgaard *et al.* 2010; Enderle *et al.* 2013). Results showed high aggressiveness of *H. fraxineus* to young ash plants, with indications that root infections with *Phytophthora* spp. may contribute to greater damage, but more research is needed to clarify the interaction between *H. fraxineus* and *Phytophthora* spp. in ash dieback.

Acknowledgements

We thank FRAXBACK COST Action FP1003, for giving the STSM to Ivan Milenković. We are also grateful to the project TR37008 financed by the Ministry of Education, Science and Technological Development, the Republic of Serbia, and IBL grant 240325 for material support during this work, and to IBL Grant for the Scholarship to Ivan Milenković. We are also very grateful to Mr. Artur Pacia and Malgorzata Borys for help in the laboratory work.

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