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Effect of cadmium (Cd) and lead (Pb) soil contamination on the development of *Hymenoscyphus fraxineus* on *Fraxinus excelsior* and *F. angustifolia* seedlings

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In light of the increase of environmental pollution, we tested the effect of cadmium (Cd) and lead (Pb) soil contamination on ash dieback. The experiment included the inoculation of *Hymenoscyphus fraxineus* on *Fraxinus excelsior* and *Fraxinus angustifolia* seedlings growing on unpolluted soil, soil contaminated with cadmium (Cd), and soil contaminated with lead (Pb). At the end of the experiment, 173 days after soil contamination and 50 days since inoculation, all *F. excelsior* and *F. angustifolia* seedlings inoculated with *H. fraxineus* showed ash dieback symptoms in comparison to their control groups. However, both *F. excelsior* and *F. angustifolia* seedlings grown on contaminated soil had significantly increased necrotic lesions in comparison to the seedlings grown on uncontaminated soil. Our results showed for the first time that cadmium (Cd) and lead (Pb) soil contamination can significantly contribute to ash dieback and increase damage to *F. excelsior* and *F. angustifolia* seedlings.

Keywords: Soil Contamination, Cadmium (Cd), Lead (Pb), *Hymenoscyphus fraxineus*, *Fraxinus excelsior*, *Fraxinus angustifolia*

Introduction

Ash dieback caused by the invasive fungus *Hymenoscyphus fraxineus* Baral, Queloz, and Hosoya (Kowalski) is a main threat to the preservation of common ash (*Fraxinus excelsior* L.) and narrow-leaved ash (*Fraxinus angustifolia* Vahl) in Europe (Pautasso et al. 2013, Gross et al. 2014). Fungus forms numerous inoculum which is easily transferred between different areas and the strategies aimed at removing infected trees and forming quarantine zones do not show to be efficient in disease suppression (Baxter et al. 2023). Many studies showed that *Fraxinus angustifolia* is slightly less sensitive to dieback than *F. excelsior* (Enderle et al. 2019), while one study showed the opposite results (Adamčikova et al. 2018). *Hymenoscyphus fraxineus* pathogen is tolerant to a wide range of environmental conditions (Gross et al. 2014) whereby the fungus virulence remains high even in populations that have been present in some areas for a long time (Lygis et al. 2017). However, the influence of several environmental conditions, such as site, stand, and climate conditions, on the development or spread of this pathogen has been studied (Bakys et al. 2009, Gross et al. 2014, Pliura et al. 2015, Marçais et al. 2016, Havrdová et al. 2017, Grosdidier et al. 2018, Hietala et al. 2018, Volke et al. 2019). Understanding the machanism of plant tolerance to pathogens and other harmful factors is particularly important in the context of global warming, where the pollutant mobility in the environment is intensively increasing (Morkunas et al. 2018). For example, environmental pollution with cadmium and lead has become a global problem (Bouida et al. 2022).

The harmful effects of cadmium on plants are reflected in the disruption of stomatal closure, inhibition of nutrient uptake, reduction of enzyme activities, and disruptions in photosynthesis and respiration, which can lead to cell structure disruptions and mutagenic effects (Kiran et al. 2022). Lead has also a harmful effect on plants cells as it hinders the cell absoprtion of potassium, calcium, magnesium, copper, or iron, damages cell organelles, and causes reactive oxygen species that further damage nucleic acids, lipids, and proteins (Kiran et al. 2022). Plants have detoxification mechanisms that protect them to some extent from the negative effects of heavy metals. Indeed, the heavy metal effects on plants are related to their dose as well as to the type and developmental stage of the plant (Yadov 2010, Morkunas et al. 2018). There is a large amount of data on the detoxification mechanisms of plants from cadmium and lead (Nas & Ali 2018, Niekerk et al. 2021).

Although heavy metals in high concentrations have a harmful effect on all organisms, smaller doses of heavy metals can stimulate the physiological processes of pathogens (*i.e.*, show a hormetic effect), though further studies are needed to understand their effect on pathogenicity/virulence (Gajewska et al. 2022). These studies are particularly important for *Hymenoscyphus fraxineus* because little knowledge is currently available on the influence of environmental factors on the development of this pathogen, thereby improving measures to recover and protect infected *F. excelsior* and *F. angustifolia* trees in habitats contaminated with heavy metals. In most European countries, the concentration of Cd in forest floor indicates that deposition

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is the main transfer route, with average concentrations for forest topsoil ranging around 0.09 mg kg^{-1}, ranging from a minimum of 0.02 to a maximum of 3.17 mg kg^{-1} (Tóth et al. 2016, Bommarez et al. 2021). In European forest soils, lead also shows high levels of deposition, with average concentration in the forest topsoil of 15.3 mg kg^{-1} and ranging from 1.63 to 151.12 mg kg^{-1} (Tóth et al. 2016, Bommarez et al. 2021). In general, there is a great variability in concentrations of these elements among the European countries, whereby in forest floor cadmium values range from 0.40 to 0.65 mg kg^{-1} and lead from 40 to 65 mg kg^{-1} (Bommarez et al. 2021). Compared to the previously established critical values of heavy metals in soil (Tyler 1992) current average values are only few to several tens of times smaller than the critical thresholds.

Following the above-mentioned, the objectives of this research were to (i) examine the influence of soil contamination with cadmium and lead on *Hymenoscyphus fraxineus* development on two-year-old seedlings of *Fraxinus excelsior* and *Fraxinus angustifolia*; (ii) examine the difference in the influence of lead and cadmium on *Hymenoscyphus fraxineus* development on two-year seedlings of *F. excelsior* and *F. angustifolia*; (iii) examine the difference in *H. fraxineus* development on two-year-old seedlings of *F. excelsior* and *F. angustifolia* grown on soil contaminated with cadmium or lead. The tested null hypotheses were: (i) there is no difference in the development of *H. fraxineus* grown in uncontaminated soil and soil contaminated with cadmium or lead; (ii) there is no difference in the influence of cadmium and lead on the development of *H. fraxineus*; (iii) there is no difference in the development of *H. fraxineus* between *F. excelsior* and *F. angustifolia* seedlings grown on soil contaminated with cadmium or lead.

Material and method

Plant and soil material

A total of 300 seedlings of two-year-old *F. excelsior* and *F. angustifolia* were used for the experiment. Soil (3.8% coarse sand 0.2-2 mm; 25.50% fine sand 0.02-0.2 mm; 35.10% dust 0.002-0.02 mm; 35.6% clay particles < 2 µm; 29.30% total sand 0.02-2 mm; 70.70% total clay < 0.02 mm; 6.62% organic matter) was collected at the location Obrenovac (44° 39′ 58″ N, 20° 13′ 56″ E – Fig. 1). A total of 150 *F. excelsior* and 150 *F. angustifolia* seedlings were planted in plastic bags filled with 1.8 kg of the above soil at the end of the 2021 growing season. Seedlings of *F. excelsior* belonged to a Western Serbia provenance and seedlings of *F. angustifolia* were from a Central Hungary provenance. At the beginning of the experiment, the average height (H) of seedlings was 90.5 ± 17 cm and 96 ± 22 cm for *F. excelsior* and *F. angustifolia*, respectively.

The experiment was carried out under controlled conditions. The planted seedlings were kept in a closed climate chamber with a temperature range of 25-35 °C in the period before inoculation and 15-25 °C during and after inoculation.

Soil contamination

The soil was artificially contaminated with cadmium and lead during the 2022 growing season, using cadmium nitrate tetrahydrate ($Cd(NO_3)_2 \times 4H_2O$) and lead nitrate ($Pb(NO_3)_2$). Hence, treatments included uncontaminated soil, cadmium-contaminated soil, and lead-contaminated soil.

Thresholds of cadmium or lead-contaminated soil are 3.5 mg kg^{-1} Cd and 500 mg kg^{-1} Pb in the organic layer (Tyler 1992) or 2 mg kg^{-1} Cd and 200 mg kg^{-1} Pb for multifunctional land use (De Vries & Bakker 1998). The remediation value of heavy metals in the soil was calculated using the formula (De Vries & Bakker 1998, RS Official Gazette 2019):

$$(SW,IW)b=(SW,IW)sb \cdot \frac{A+(B\cdot\%clay)+(C\cdot\%OM)}{A+(B\cdot 25)+(c\cdot 10)} \quad (1)$$

where *(SW, IW)b* is the corrected remediation value for a specific soil, *(SW, IW)sb* is the heavy metal remediation value for a standard soil (for cadmium *(SW, IW)sb* = 12 mg kg^{-1} ; for lead *(SW, IW)sb* = 530 mg kg^{-1}); *%clay* is the measured percentage of clay in a specific soil (particle size < 2 µm); *%OM* is the measured percentage of organic matter in a specific soil; and *A, B, C* are constants depending on the type of metal (for cadmium *A* = 0.4, *B* = 0.007, *C* = 0.021; for lead *A* = 50, *B* = 1, *C* = 1).

Each plastic bag, filled with 1.8 kg of soil, was injected with an amount of 51.52 mg of cadmium nitrate tetrahydrate or 1654.4 mg of lead nitrate dissolved in 20 ml of distilled water to achieve an amount of 12.05 mg kg^{-1} (21.29 mg/1.8 kg) for cadmium, and 575.02 mg kg^{-1} (1035.03 mg/1.8kg) for lead.

After planting, the soil of all treatments, including the inoculated and control seedlings, was watered identically with distilled, demineralized water three times a week during the first three weeks after contamination. Later, until the end of the experiment, the soil was watered with tap water, two to four times a week. Watering was carefully done to avoid water drainage from the bags and leaching of heavy metals from the substrate.

Inoculation test

A test of inoculation under the bark with *Hymenoscyphus fraxineus* was carried out four months after the addition of cadmium nitrate tetrahydrate and lead nitrate to soil. Fifty seedlings from each tree species were randomly chosen for each treatment, of which 40 were inoculated with *H. fraxineus* while 10 seedlings served as control. Three-week-old cultures of HF050 strain of the fungus isolated from the National Park “Biogradska gora”, taken from the mycological collection of the University of Belgrade, Faculty of Forestry, and grown on 3% malt extract agar (Biolab, Hungary; Torlak, Serbia) were used for inoculation. Openings on the bark were made using a sterilized scalp at the same seedling height, and they were inoculated with fragments of mycelium 5×5 mm. Meanwhile, the control seedlings were inoculated with fragments of pure 3% malt extract agar. The in-

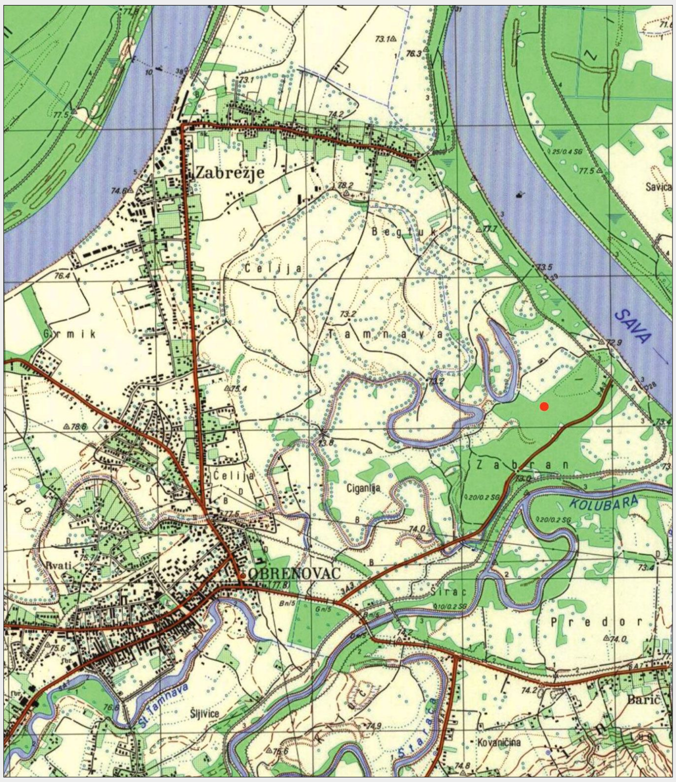

Fig. 1 - The location from which the soil was collected.

oculated point on the seedling bark were wrapped with Parafilm® and covered with aluminum foil.

The experiment was completed 50 days after inoculation and 173 days after soil contamination, when 70% of all ash seedlings showed clearly visible symptoms of ash dieback. Seedlings were cut at the level of root collar and the occurrence of dead top and necrotic lesions has been recorded. Dieback was quantified visually based on the occurrence of withered leaves or premature leaf shedding, as well as declined and deformed branches or rachises in the upper third of the crown. Necrotic lesions were measured immediately after peeling of the bark based on discoloration of the cambium. The length of necrotic lesions was measured vertically in accordance with the height of the seedling, while the width of necrotic lesions was measured as the circumference at the largest visible spot of discoloration. The necrotic area was calculated as the area of the ellipse. Re-isolation of the pathogen was attempted from all inoculated seedlings. The seedlings were cut into round sections at several heights along the stem and sections with fresh necrosis and visible difference between necrotic and healthy tissue were used for re-isolations. These parts were first sterilised on the outer surface by wiping with 96% alcohol and 3-5 seconds of exposure to flame of tissue. Then the bark was completely peeled off and the outer tissues were wiped again with 96% alcohol, exposed to flame for a few seconds and placed on 3% malt extract agar.

Statistical methods

Testing the difference in proportions of dieback, the occurrence of necrosis, and re-isolation between different groups of seedlings was performed using the Z-test for proportions. Normality of distribution of necrotic lesion size was tested by Kolmogorov-Smirnov test and homogeneity of variance of the dimensions of necrotic lesions among various soil treatments was tested using Levene's test. Since the conditions for the application of parametric tests or models were not met, non-parametric tests were applied for further analyses. The Kruskal-Wallis test and Dunn's *post hoc* test were used to test for differences in length and width of necrotic lesions caused by *H. fraxineus* between different soil treatments. The Mann-Whitney U test was used to test for differences in length and width of necrotic lesions between inoculated and control seedlings, as well as between *F. excelsior* and *F. angustifolia* seedlings on certain soil treatments. Statistical tests were performed using the software packages SPSS® ver. 26 (IBM, Armonk, NY, USA) and Excel® 2016 (Microsoft Corp., Redwoods, WA, USA).

Results

Effect of soil contamination with cadmium and lead on F. excelsior dieback

The results of Z-test showed that the proportion of *F. excelsior* seedlings showing dead top and necrotic lesions, and successful re-isolations of *H. fraxineus* was not significantly different among different soil treatments (Tab. 1, Fig. 2a-f). The average necrotic area of the inoculated seedlings of *F. excelsior* was 1023.02 mm^2 in uncontaminated soil, 1736.84 mm^2 in cadmium-contaminated soil and 1552.24 mm^2 in lead-contaminated soil. While the average necrotic area was 143.25, 136.59 and 160.14 mm^2 for control seedlings grown in uncontaminated, Cd-contaminated and Pb-contaminated soil, respectively.

The Mann-Whitney U test showed significant differences both in the length ($U = 2.500$, $p < 0.001$) and width ($U = 141.500$, $p < 0.001$) of necrotic lesions between inoculated and control seedlings. The Kuskal-Wallis test revealed significant differences in the length of necrotic lesions ($H = 15.414$, $p < 0.001$) and the width of necrotic lesions ($H = 11.915$, $p = 0.003$) caused by *H. fraxineus* among *F. excelsior* seedlings grown under different treatments. Similarly, Dunn's *post hoc* test showed significant differences in the length of necrotic lesions between different treatments on inoculated *F. excelsior* seedlings (Tab. 1).

We found significant differences in the length and width of necrotic lesions between *F. excelsior* seedlings grown on non-contaminated soil and cadmium-contaminated soil (Tab. 1, Fig. 2d, Fig. 2e). Also, there was a significant difference in the length of necrotic lesions while no significant differences were found in the width of necrotic lesions between seedlings grown in uncontaminated soil and lead-contaminated soil (Tab. 1, Fig. 2d, Fig. 2f). Meanwhile, *F. excelsior* seedlings grown on soils contaminated with cadmium and lead showed no significant differences neither in length nor width of necrotic lesions (Tab. 1, Fig. 2e, Fig. 2f).

Effect of soil contamination with cadmium and lead on F. angustifolia dieback

The Z-test showed that the proportion of *F. angustifolia* seedlings showing dead top and necrotic lesions, and successful re-isolations of *H. fraxineus* was not significantly different among different soil treatments (Tab. 2, Fig. 3a-f). In *Fraxinus angustifolia* seedlings inoculated with *Hymenoscyphus fraxineus*,

The average necrotic area was 785.14, 2044.69 and 1514.27 mm^2 for *F. angustifolia* seedlings inoculated with *H. fraxineus* and grown in uncontaminated, Cd-contaminated and Pb-contaminated soil, respectively. As for control seedlings, average necrotic area was 118.54 mm^2 in uncontaminated soil, 131.94 mm^2 in Cd-contaminated soil and 130.62 mm^2 on lead-contaminated soil.

The Mann-Whitney U test showed that significant differences both in the length ($U = 0.000$, $p < 0.001$) and width ($U = 255.000$ 180.000, $p < 0.001$) of necrotic lesions between inoculated and control seedlings. Significant differences were also found in the length ($H = 41.292$, $p < 0.001$) and width ($H = 40.709$, $p < 0.001$) of necrotic lesions on narrow-leaved ash seedlings (*F. angustifolia*) grown on different soil treatments after Kruskal-Wallis test. Similarly, Dunn's *post hoc* test showed the presence of significant difference in the length of necrotic lesions between different treatments of inoculated seedlings (Tab. 2).

Similarly to *F. excelsior*, we found signifi-

Tab. 1 - Incidence and severity of symptoms caused by *Hymenoscyphus fraxineus* depending on soil contamination by Cd and Pb on 2-year-old *Fraxinus excelsior* after 50 days of inoculation. Different letters indicate significant differences (p<0.05) among treatments after Dunn's *post-hoc* test.

Treatments	Seedlings (n)			Mean lesion (mm)		Re-isolations freq. (%)
	Inoculated	With dieback	With necrotic lesions	Length	Width	
Hymenoscyphus fraxineus	40	37 [a]	40 [a]	34.78 ± 22.25 [a]	8.99 ± 1.50 [a]	75 [a]
Hymenoscyphus fraxineus + Cd	40	32 [a]	40 [a]	48.10 ± 21.67 [b]	11.16 ± 3.02 [b]	85 [a]
Hymenoscyphus fraxineus + Pb	40	34 [a]	40 [a]	47.82 ± 25.21 [b]	9.88 ± 2.50 [ab]	85 [a]
Control clean soil	10	0	0	7.11 ± 4.71 [c]	5.10 ± 2.73 [c]	0
Control Cd	10	0	0	6.60 ± 4.70 [c]	5.30 ± 2.87 [c]	0
Control Pb	10	0	0	8.00 ± 4.94 [c]	5.90 ± 2.18 [c]	0

Fig. 2 – Example of symptoms caused by *Hymenoscyphus fraxineus* on two-year-old *Fraxinus excelsior* seedlings: (a) dieback on uncontaminated soil; (b) dieback on cadmium contaminated soil; (c) dieback on lead contaminated soil; (d) necrotic lesions on uncontaminated soil; (e) necrotic lesions on cadmium contaminated soil; (f) necrotic lesions on lead contaminated soil; (g, j) control on uncontaminated soil; (h, k) control on cadmium contaminated soil; (i, l) control on lead contaminated soil.

cant differences in length and width of necrotic lesions between *F. angustifolia* seedlings grown on uncontaminated soil and cadmium-contaminated soil (Tab. 2, Fig. 3d, Fig. 3e). Likewise, *F. angustifolia* seedlings grown in uncontaminated soil showed significant differences in length and width of necrotic lesions compared with those grown in lead-contaminated soil (Tab. 2, Fig. 3d, Fig. 3f). In contrast, no significant difference were detected in the length of necrotic lesions between seedlings grown in soils contaminated with cadmium and lead, while they showed significant differences in the width of necrotic lesions (Tab. 2, Fig. 3e, Fig. 3f).

Comparison of F. excelsior and F. angustifolia dieback on uncontaminated and contaminated soil

We did not found any significant differences in the proportion of seedlings with dieback symptoms ($z = 1.6233$, $p = 0.10524$), necrotic lesions ($z = 0.000$, $p = 1.000$), and re-isolation of the fungus ($z = 0.5008$, $p = 0.61708$) between *F. excelsior* and *F. angustifolia* seedlings grown in uncontaminated soil after Z-test. The Mann-Whitney U test showed that there was no significant difference in the length of necrotic lesions ($U = 741.000$, $p = 0.570$), while there was a significant difference in the width of necrotic lesions ($U = 493.000$, $p = 0.003$) between the two species for seedlings grown on uncontaminated soil (Tab. 1, Tab. 2, Fig. 2d, Fig. 3d).

Likewise, we found no differences in the proportion of seedlings with dieback symptoms ($z = 0.2864$, $p = 0.77182$), necrotic lesions ($z = 0.000$, $p = 1.000$), and re-isolation of the fungus ($z = 0.5885$, $p = 0.5552$) between the two species grown in soil contaminated with cadmium after Z-test. Similarly, no significant differences in the length (Mann-Whitney $U = 715.000$, $p = 0.413$) and width ($U = 715.500$, $p = 0.412$) of necrotic lesions were found between *F. excelsior* and *F. angustifolia* seedlings grown on cadmium-contaminated soil (Tab. 1, Tab. 2, Fig. 2e, Fig. 3e).

The Z-test showed that there is no statistically significant difference between the

Tab. 2 - Incidence and severity of symptoms caused by *Hymenoscyphus fraxineus* depending on soil contamination by Cd and Pb on 2-year-old *Fraxinus angustifolia* after 50 days of inoculation. Different letters indicate significant differences ($p<0.05$) among treatments after Dunn's *post-hoc* test.

Treatment	Seedlings (n)			Mean Lesion (mm)		Re-isolation freq. (%)
	Inoculated	With dieback	With necrotic lesions	Length	Width	
Hymenoscyphus fraxineus	40	32 ^{a}	40 ^{a}	29.70 ± 13.52 ^{a}	8.08 ± 1.70 ^{a}	70 ^{a}
Hymenoscyphus fraxineus + Cd	40	33 ^{a}	40 ^{a}	53.69 ± 25.10 ^{b}	11.63 ± 2.98 ^{b}	80 ^{a}
Hymenoscyphus fraxineus + Pb	40	28 ^{a}	40 ^{a}	49.18 ± 16.92 ^{b}	9.60 ± 1.79 ^{c}	80 ^{a}
Control clean soil	10	0	0	6.40 ± 4.42 ^{c}	4.65 ± 3.27 ^{d}	0
Control Cd	10	0	0	6.36 ± 4.90 ^{c}	5.20 ± 2.86 ^{d}	0
Control Pb	10	0	0	6.30 ± 3.53 ^{c}	5.90 ± 2.18 ^{d}	0

two *Fraxinus* species in the proportion of seedlings showing dieback symptoms (z = 1.6064, p = 0.1074), necrotic lesions (z = 0, p = 1), and re-isolation of the fungus (z = 0.5885, p = 0.5552) on lead-contaminated soil. Further, no significant differences in the length (Mann-Whitney U = 691.500, p = 0.296) and width (U = 774.000, p = 0.800) of necrotic lesions were found between *F. excelsior* and *F. angustifolia* on lead-contaminated soil (Tab. 1, Tab. 2, Fig. 2f, Fig. 3f).

Discussion

This study showed the effect of increased cadmium and lead soil contamination on the development of *Hymenoscyphus fraxineus* in *Fraxinus excelsior* and *Fraxinus angustifolia*. The obtained results showed that the dimensions of necrotic lesions significantly increased in the seedlings of both species grown for a short time on soils contaminated with cadmium and lead, as compared to control seedlings grown on uncontaminated soil. Based on the above evidence, the first null hypothesis was rejected, thus supporting the alternative hypothesis of a different development of *H. fraxineus* between uncontaminated soil and soil contaminated with heavy metals (cadmium or lead).

The uptake of heavy metals from the soil is intensive in *F. excelsior* to the extent that it can serve as bioindicator of pollution (Aksoy & Demirezen 2006). The relationships among the host, pathogen and stress caused by heavy metals are complex and involve many different processes which include plant and pathogen mechanisms of tolerance to heavy metals (Gajewska et al. 2022). In the specific case, the drastic increase of necrotic lesions on seedlings grown on contaminated soil might support the hypothesis that the pathogen is more tolerant to heavy metals than the host, in that heavy metals can provoke a disturbance in the functioning of the plants and a simultaneous stimulation of the fungus. The disturbances could include changes in metabolism and a reduced defence responses of seedlings. On the other hand, the stimulation of the pathogen might be a consequence of the hormetic effect, either due to a milder stress caused by heavy metals or to the larger amount of nutrients obtained from damaged cells. Specific research is needed aimed at elucidating the role played by heavy metals in damaging the physiological processes of ash, thereby resulting in the greater pathogenicity of *H. fraxineus*.

It has been reported that cadmium and lead show similar negative effects on callus tissue cultures and seedlings of common ash (*F. excelsior*) in laboratory conditions, and the toxic effect of lead on seedlings is slightly weaker than the toxic effect of cadmium (Nawrot-Chorabik et al. 2022). Although there are no similar studies on narrow-leaved ash (*F. angustifolia*), it can be reasonably assumed that the effects of both heavy metals would be similar. The present study on the harmful effects of the interaction of *H. fraxineus* and heavy metals (cadmium and lead) on seedlings of both ash species showed that cadmium had a slightly greater effect than lead on the expansion of necrotic lesions, although both elements favor the increase of necrosis compared to control soil. Therefore, the second null hypothesis was rejected, and the alternative hypothesis that there is a difference in the effect of cadmium and lead on the *H. fraxineus* development was accepted.

On the contaminated soil, we found no significant differences in the sensitivity to dieback of these two ash species during the experiment. Thus, the third null hypothesis that there is no differences in the development of *H. fraxineus* between *F. excelsior* and *F. angustifolia* grown on soil contaminated with cadmium or lead was accepted. On uncontaminated soil, the development of *H. fraxineus* was consistent with most previous studies reporting a high sensitivity to dieback of both ash species (Kowalski & Holdenrieder 2009, Kirisits et al. 2010, Hauptman et al. 2016, Pastirčáková et al. 2020, Vemić et al. 2021), with *F. angustifolia* being slightly less sensitive (Schwanda & Kirisits 2016, Diminić et al. 2017, Nielsen et al. 2017, Papić et al. 2018, Enderle et al. 2019).

Ash trees growing in forest ecosystems are thought to be more sensitive to dieback than individual trees growing in isola-

Fig. 3 - Example of symptoms caused by *Hymenoscyphus fraxineus* on two-year-old *Fraxinus angustifolia* seedlings: (a) dieback on uncontaminated soil; (b) dieback on cadmium contaminated soil; (c) dieback on lead contaminated soil; (d) necrotic lesions on uncontaminated soil; (e) necrotic lesions on cadmium contaminated soil; (f) necrotic lesions on lead contaminated soil; (g, j) control on uncontaminated soil; (h, k) control on cadmium contaminated soil; (i, l) control on lead contaminated soil.

tion (Heinze et al. 2017). Also, in the case of *F. excelsior*, higher tree mortality was recorded in plantations than in natural stands (Coker et al. 2019). An increase in the content of cadmium and lead in uncontaminated forests and plantations, as well as the planting of seedlings on soil contaminated with these heavy metals can significantly accelerate ash dieback and thus make it even more difficult to recover ash stands and make it impossible to grow long-term plantations.

Various symptoms are associated to the occurrence of necrosis, such as dieback wilt and cancer (Skovsgaard et al. 2010). The greatest activity of necrotic lesions occurs until August (Matisone et al. 2018), namely, at the time when trees actively absorb heavy metals from the soil. However, the growth of necrotic lesions is active throughout the year (Bengtsson et al. 2014), which means that necrotic lesions continue to develop even after the vegetation period, when heavy metals are no longer absorbed from the soil. The occurrence of increased necrotic lesions on contaminated soils suggests that a faster mortality of seedlings is expected in longer periods compared to infected seedlings growing on clean soil.

The recovery of plants after stress caused by heavy metals depends on the type and concentration of the elements as well as the length of exposure, but it can be very successful even after severe stress (Chmielowska-Bak & Deckert 2021). For improving the plant recovery process, it is necessary to better understand the relationships between heavy metals (Cd and Pb) and pathogen stimulation. Based on our results, we can hypothesize that the relationship between Cd and Pb concentration and ash dieback symptoms could be linear. Modelling damages in forest ecosystems depending on the increase in concentration of heavy metals Cd and Pb could be a desirable goal. However, in cases of extreme soil pollution, heavy metal remediation measures in ash habitats can mitigate the harmful effect of ash dieback, mainly during regeneration and the initial spread of the pathogen.

Genetic improvement through the selection of genotypes more tolerant to H. fraxineus is a viable way to protect ash tree stands (McKinney et al. 2014, Enderle et al. 2019, Skovsgaard et al. 2017). Nevertheless, protective measures aimed at directly combating *H. fraxineus* and/or indirectly at reducing or eliminating the conditions favorable to its diffusion are still necessary (Semizer-Cuming et al. 2019). Based on our results, it is considered that the decontamination of the soil from cadmium and lead can significantly contribute to the survival of ash seedlings in case of infections by *H. fraxineus*. In particular, soil decontamination is necessary for more sensitive trees and plantings, or where ash dieback shows a faster development. To this end, remediation of soil polluted with cadmium and lead could be carried out by planting understory species having high phytoremediation potential, e.g., *Limonium sinuatum* (Sheikh-Assadi et al. 2015), as well as by the adoption of different management strategies aimed at removing the factors that favor ash dieback (Mitchell et al. 2014). Moreover, soil composting has been proven helpful to prevent and reduce the intensity of infection (Noble et al. 2019). Therefore, we recommend the above measures to be combined with soil phytoremediation in order to contrast the rapid development of the infection and mitigate its harmful effects in ash trees.

Conclusions

In this study, the effect of soil contamination with cadmium and lead on the development of ash dieback symptoms caused by the fungus *Hymenoscyphus fraxineus* on two-year-old *Fraxinus excelsior* and *Fraxinus angustifolia* seedlings has been investigated for the first time. Soil contamination resulted in an increased size of necrotic lesions on seedlings of both ash species. However, the width of necrotic lesions in *F. angustifolia* seedlings was higher on cadmium-contaminated soil, though on uncontaminated soil it was slightly smaller than that recorded in *F. excelsior*.

In summary, the short-term effects of soil contamination with cadmium and lead significantly affect the development of dieback in both ash species and can accelerate the dieback process of infected seedlings. Anthropogenic activities that contribute to the occurrence of Cd and Pb in the soil must be limited in the vicinity of ash habitats and measures aimed at remediation of heavy metals in soil are necessary during regeneration and reforestation in order to reduce future damage caused by infection with *H. fraxineus*.

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