

Ash dieback and contributing factors of forest weakening in provenance tests in the Sumy region

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Abstract

The aim of this study was to evaluate the health condition of *Fraxinus excelsior* L. in provenance tests, with special focus on ash dieback (ADB), but taking into account also other causes of its decline. The research was carried out in the provenance tests of *F. excelsior* in the forest-steppe part of the Sumy region. ADB symptoms were revealed in all provenance tests. For 2012–2019 the health condition index, ADB incidence and severity increased for all provenances except the Steppe. Collar rot was present in all ash provenances. Fungi species were isolated from the stem parts of ash at all provenances. *Hymenoscyphus fraxineus* induced longest necrotic lesions following wound inoculation of stems of 7–10-years-old plants of European ash in the forest while inoculation with both *Cytospora* sp. and *Diplodia* sp. resulted in smaller necroses. The conclusion from other regions about the coincidence the damage of European ash by ADB and collar rots as well as the coincidence the damage of European ash by collar rot and tree colonization by *Hylesinus crenatus* (Fabricius, 1787) is supported.

Key words: *Fraxinus excelsior*; collar rot; health condition; incidence; severity

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1. Introduction

Recently, in many European countries, deterioration of health condition of *Fraxinus excelsior* L. stands has been registered (Matsiakh & Kramarets 2014; Goberville et al. 2016; Enderle et al. 2017; Meshkova & Borysova 2017), which is largely due to the spread of a new invasive pathogen *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz & Hosoya (Queloz et al. 2011; Baral et al. 2014; Gross et al. 2014). The presence of this fungus in the symptomatic shoots of European ash (*Fraxinus excelsior*) from the Sumy region of Ukraine was confirmed by molecular methods (Davydenko et al. 2013b). The occurrence of this pathogen in ash shoots with pronounced symptoms of the disease (necrotic spots on the bark, discoloration) was statistically proven. For trees affected, the gradual death of crowns, the presence of necrotic spots on the bark of shoots, leaves and stems, discoloration of wood and shoots, uneven flowering, and premature fall were registered (Skovsgaard et al. 2010; Metzler et al. 2012; Davydenko & Meshkova 2017).

Provenance tests with long-term history is a very suitable approach to study many issues, among which

is the susceptibility of different provenances to some pathogens (Metzler et al. 2012; Stener 2013; McKinney et al. 2014; Enderle et al. 2015). Such provenance tests of *Fraxinus excelsior* were created in 1930 under the leadership of prof. V. M. Andreiev in the Sumy region (Davydenko et al. 2013a).

In 2012, inspection of these provenance tests was carried out with assessment of ash dieback (ADB) incidence and severity as well as the health condition for each provenance (Davydenko et al. 2013a). Over the past years, the progressive development of ash dieback has been registered in many sample plots in different regions of Ukraine (Davydenko et al. 2019). Therefore, it was the basis for repeated assessment of ash dieback parameters in the provenance tests in the Sumy region.

The aim of this study was to evaluate the health condition parameters of *Fraxinus excelsior* in provenance tests, with special focus on ash dieback, but taking into account also other causes of its decline. One aspect of our research on ash dieback was aimed at determining fungi associated with ash trees and the ability of certain fungi to cause necrosis on the shoots of European ash saplings.

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2. Material and methods

The research was carried out in the provenance tests of *Fraxinus excelsior* in the State Enterprise “Trostyanetske Forest Economy” in the forest-steppe part of the Sumy region (50°29'05" N; 34°57'56" E).

Within these stands, 5 provenance tests of *F. excelsior* were tested: Poliskyi, Western Forest-Steppe, Right-Bank Forest-Steppe, Left-Bank Forest-Steppe and Steppe.

From each provenance, 50 living trees of European ash were inspected. Besides diameter at breast height (DBH) several parameters of ash health condition were additionally assessed in each provenance test. Social position within the stand was assessed according to Kraft classification (class 1 – predominant trees, class 2 – dominant trees, class 3 – subdominant trees, class 4 – suppressed trees and class 5 – dying trees) (Lech et al. 2020).

Category of tree health condition was evaluated on a range of visual characteristics (crown density and color, the presence and proportion of dead branches in the crown, etc.) according to National scale (Davydenko et al. 2019). Each tree was referred to one of four categories of health condition (1st – healthy; 2nd – weakened; 3rd – severely weakened; 4th – drying up). Health condition index (HCI) for each plot was calculated as mean of the health condition categories of studied trees.

The incidence of ash dieback, collar rots, and *Hylesinus crenatus* (Fabricius, 1787) (Coleoptera: Curculionidae: Scolytinae) galleries was estimated as a proportion of living ash trees with characteristic symptoms within the sample from each provenance.

Severity of ash dieback for each ash tree was estimated by score: 0 points – no symptoms, 1 point – the presence of several indirect symptoms (uneven leafing, single drying of shoots, individual necroses on healthy shoots, drying up to 10% of branches; 2 points – the presence of these symptoms, drying of more than 10% and up to 50% of shoots and branches, formation necrosis, 3 – drying of more than 50% of the crown, discoloration of wood in the area of necroses, the presence of necroses and drying of leaves, shoots, branches, 4 – damage to 100% of the crown, the presence of necroses even on green shoots and branches, leaves, stems (Metzler et al. 2012). Severity of ash dieback for each provenance was evaluated as an arithmetic average of all trees scores.

By epicormic shoots, the trees were rated as follow: 0 – absence of epicormic shoots; 1 – sporadic epicormic shoots; 2 – multiple epicormic shoots; 3 – total stem coverage with epicormic shoots (Meshkova & Borysova 2017). Each stem collar was examined for the presence or absence of stem necroses and signs of wood decay fungi. The score of wood decay severity (collar rots) considered the part of damaged stem circumference: 0 points – absence; 1 point – up to 25% of circumference; 2 points – 26–50%; 3 points – 51–75%; 4 points – over 75% of circumference (Davydenko et al. 2019).

Fungal isolation. The wood samples were taken by Haglöf increment borer 10 cm or over in length from *F. excelsior* trees randomly. Five samples per each provenance (25 samples in total) were packed individually in Falcon tubes, marked and placed into freezer (–20 °C). After surface sterilization in flame, wood samples were placed in Petri dishes containing ca. 30 ml of 3% malt extract agar medium (MEA) and incubated at room temperature (ca. 21 °C) in the dark. Petri dishes were checked daily and outgrowing fungal mycelia were sub-cultured to new Petri dishes with MEA. Fungal cultures were divided into groups based on their morphology and for species identification representative cultures from each group were subjected to sequencing of the internal transcribed spacer of the fungal ribosomal RNA (ITS rRNA). Isolation of DNA, amplification and sequencing followed methods described by Davydenko & Meshkova (2017). The thermal cycling was carried out using an Applied Biosystems GeneAmp PCR System 2700 thermal cycler (Foster City, CA, USA): initial denaturation step at 95 °C for 5 min. was followed by 35 amplification cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s and final extension step at 72 °C for 7 min. Raw sequence data were analyzed using the SeqMan Pro version 10.0 software from DNASTAR package (DNASTAR, Madison, WI, USA). The criteria used for identification were: sequence coverage > 80%; similarity to taxon level 98–100%, similarity to genus level 94–97%. Some fungal species were detected by morphological symptoms (fruit bodies, spores, etc.).

Pathogenicity test. In the inoculation tests carried out in May 2020, the following fungi, considered as putative pathogens, were used: *Alternaria alternata* (Fr.) Keissl., *Didymella macrostoma* (Mont.) Qian Chen & L. Cai, *Hymenoscyphus fraxineus*, *Epicoccum nigrum* Link, and *Fusarium avenaceum* (Fr.) Sacc., *Cytospora sp. 15* and *Diplodia sp.* (for the last two fungi, only genera were identified). The pathogenicity of these fungi to the *F. excelsior* plants was determined in the field experiment. The stems of 7–10-year-old plants growing in the forest were each superficially wounded by removal of bark (0.5 cm diameter) with a razor blade in 8 cm from the shoot tip. A disc cut from a fungal culture was placed on each wound and then covered by Parafilm strip. In the field experiment, five trees per isolate were used (35 trees per all fungal species), whereas 10 control plants were inoculated with sterile MEA. Observations of both necrosis and tree condition (crown dieback) were made at weekly intervals for 4 months. The data were expressed as mean lesion length. Re-isolations of the test fungi from all the inoculated plants were attempted by plating material from lesions (if present) or from points of inoculation. The lesion material was taken from sections ca. 5 mm both above and below each inoculation point. Fungi growing from the material were re-isolated as described in the above procedures for isolation.

Normality tests, summary statistics, one-way analysis of variance (ANOVA), Tukey HSD test with a significance level of $p < 0.05$ were performed. Shapiro-Wilk test has proved the normality of analyzed parameters (ADB, collar rots and *H. crenatus* incidence and severity, health condition index) when comparing the provenances between each other in 2012 and 2019, and each provenance in 2012 and 2019. Microsoft Excel software and statistical software package PAST: Paleontological Statistics Software Package for Education and Data Analysis (Hammer et al. 2001) were used.

3. Results

The results of the 2012 assessment showed that the diameter (30.3–32.1 cm), Kraft class (2.3–2.6) and health condition index (1.8–2.4) of individual provenances did not have statistically significant differences (Table 1).

In 2019 assessment, the diameter (31.1–33.2 cm), Kraft class (2.2–2.6) and health condition index (2.5–2.8) of individual provenances also did not have statistically significant differences (Table 2).

Statistically significant differences were registered between the provenances in terms of branch dieback and the presence of epicormic shoots (Table 2). The branch dieback was the lowest in the Steppe provenance (5.5%), which is significantly less than in the other provenances, except for the Western forest-steppe. The highest values of branch dieback were assessed in the Poliskyi and Right-bank forest-steppe provenances (15.3%), with the maximum values reaching 50% (Poliskyi).

The prevalence of epicormic shoots ranged from 0.8 points in the Steppe and Left-Bank Forest-Steppe provenances to 1.7 points in the Right-Bank Forest-Steppe. However, Tukey's test showed a significant difference only between Right-Bank Forest-Steppe and two above mentioned provenances ($P < 0.05$).

In 2019 compared with 2012, the DBH increased in all provenances, and the Kraft class a bit increased only in the Left-bank Forest Steppe and Steppe provenances.

The health condition index significantly increased in 2019 compared with 2012 for all provenances except the Steppe, for average sample from 2.1 to 2.6.

In 2012, ADB incidence was the lowest in the Western Forest-Steppe provenance (25%), almost twice as high in Poliskyi and Right-bank Forest-Steppe provenance and the highest in the rest provenances (83.3 and 91.7% in the Left-bank Forest Steppe and Steppe respectively) (Table 3). The differences between these three groups of provenances are significant.

Table 3. Comparison of ADB parameters in *Fraxinus excelsior* provenance tests in 2012 and 2019.

Provenance	ADB incidence, [%]±SE		ADB severity, [points] ±SE	
	2012	2019	2012	2019
Poliskyi	58.3±6.97 a	90.0±6.71 d	0.7±0.09 b	1.8±0.12 d
Western Forest-Steppe	25.0±6.12 b	75.0±4.78 e	0.3±0.09 a	1.3±0.09 c
Right-bank Forest Steppe	58.3±6.97 a	70.0±5.12 ae	0.7±0.09 b	1.2±0.12 c
Left-bank Forest Steppe	83.3±5.27 c	100.0±0.00 d	0.8±0.05 b	1.8±0.11 d
Steppe	91.7±3.91 c	75.0±4.78 ce	1.4±0.09 c	1.3±0.09 c
Total	63.3±3.05 a	82.0±3.84 b	0.8±0.04 b	1.5±0.10 d

Note: *Means followed by different letters in each column are significantly different at the 95% confidence level.

In 2019 compared to 2012, ADB incidence increased 1.3 times in total, 3 times in the West Forest-Steppe, 1.5 times in Poliskyi and 1.2 times in the Right-bank and Left-bank Forest-Steppe provenances, however, the increase was significant only for three provenances (Table 3). Significance of increase the ADB incidence for Right-bank Forest-Steppe and of its decrease for Steppe provenance was not proved.

In 2012, ADB severity score was the lowest in the Western Forest steppe (0.3 points) and the highest in the Steppe provenance (1.4 points) (Table 3). The rest three provenances with ADB severity score 0.7–0.8 points did not differ significantly among themselves, but were significantly above the minimum and below the maximum value of this parameter.

In 2019, ADB severity score was the lowest in the Right-bank Forest steppe (1.2 points) and didn't differ statistically from Western Forest-Steppe and Steppe (1.3

Table 1. General parameters of *Fraxinus excelsior* health condition in different provenance tests (2012).

Provenance	DBH, [cm] ±SE	Kraft class, [points] ±SE	Health condition index, [points] ±SE
Poliskyi	30.3±1.21 a	2.5±0.08 a	1.8±0.09 a
Western Forest-Steppe	31.1±1.21 a	2.5±0.06 a	1.8±0.08 a
Right-bank Forest Steppe	31.3±1.30 a	2.6±0.05 a	2.0±0.10 a
Left-bank Forest Steppe	32.1±1.39 a	2.3±0.06 a	2.4±0.09 a
Steppe	31.6±1.23 a	2.5±0.07 a	2.4±0.09 a
Total sample	31.3±0.84 a	2.5±0.06 a	2.1±0.04 a

Note: *Means in each column are not significantly different at the 95% confidence level.

Table 2. General parameters of *Fraxinus excelsior* health condition in different provenance tests (2019).

Provenance	DBH, [cm] ±SE	Kraft class, [points] ±SE	Health condition index, [points] ±SE	Branch dieback, [%] ±SE	Epicormic shoots, [points] ±SE
Poliskyi	31.1±1.0 a	2.5±0.1 a	2.5±0.1 a	15.3±2.3 a	0.9±0.2 ab
Western Forest-Steppe	31.9±1.6 a	2.5±0.1 a	2.6±0.1 a	11.0±1.8 ab	1.1±0.2 ab
Right-bank Forest Steppe	32.2±1.6 a	2.6±0.1 a	2.7±0.1 a	15.3±1.5 a	1.7±0.3 b
Left-bank Forest Steppe	33.2±1.3 a	2.2±0.1 a	2.8±0.1 a	14.5±2.3 a	0.8±0.2 a
Steppe	32.2±1.6 a	2.4±0.1 a	2.6±0.1 a	5.5±1.4 b	0.8±0.2 a
Total sample	32.1±0.63	2.4±0.06	2.6±0.05	12.3±0.91	1.0±0.1

Note: *Means followed by different letters in each column are significantly different at the 95% confidence level.

points each) provenances. The ADB score in Poliskiyi and Left-bank Forest Steppe provenances was significantly higher (1.7 points each) comparing to rest provenances (Table 3).

ADB severity score significantly increased over the period 2012–2019 for all provenances except Steppe one.

Collar rot incidence was the lowest (20–25%) in the Western Forest-Steppe and Right-bank Forest Steppe provenances, and significantly higher (40–45%) in other provenances. Collar rot severity score varied from 1.2 to 1.8 in different provenances but the difference between them was not significant. Trees colonization by *H. crenatus* was absent in Western Forest-Steppe and Right-bank Forest Steppe provenances. It varied from 5% (Poliskiyi provenance) to 30% (Steppe provenance) but the differ-

ence between them was not significant. Severity score of trees colonization by *H. crenatus* was 0.1–0.3 points with also insignificant difference (Table 4).

The results obtained allowed pooling the data for all provenances to test the hypothesis about the possible coincidence ash dieback and collar rots, ash dieback and tree colonization by *H. crenatus*, as well as collar rots and tree colonization by *H. crenatus* (Table 5).

The χ^2 -test proved the coincidence the damage of European ash by ash dieback and collar rots as well as the coincidence the damage of European ash by collar rots and tree colonization by *H. crenatus* and doesn't support the hypothesis about the coincidence the damage of European ash by ash dieback and tree colonization by *H. crenatus* (Table 5).

Table 4. Incidence and severity of collar rots and *Hylesinus crenatus* as the most common damages of European ash in provenance tests (2019).

Provenance	Collar rots (CR)		<i>Hylesinus crenatus</i> (HC)	
	incidence, [%] ±SE	severity [points] ±SE	incidence, [%] ±SE	severity [points] ±SE
Poliskiyi	45±11.1 a	1.8±0.21 a	5±4.9 a	0.1±0.05 a
Western Forest-Steppe	20±8.9 b	1.3±0.23 a	0	0
Right-bank Forest Steppe	25±9.7 ab	1.2±0.22 a	0	0
Left-bank Forest Steppe	45±11.1 a	1.8±0.16 a	15±8.0 a	0.2±0.08 a
Steppe	40±11.0 a	1.3±0.22 a	30±10.2 a	0.3±0.11 a
Total sample	35±4.8 a	1.5±0.10 a	10±3.0 a	0.1±0.03 a

Note: *Means followed by different letters in each column are significantly different at the 95% confidence level.

Table 5. Proportion of European ash trees with mixed damage and statistical analysis of correlation between mixed forms of damage in European ash trees (2019).

Damage type A	Damage type B	Proportion of ash trees with symptoms of mixed damage, [%]	Number of inspected trees with the damage				r	χ^2_{fact}	Conclusion about correlation
			A and B	A without B	B without A	neither A nor B			
ADB	CR	35±4.8	35	47	0	18	0.34	11.8	proven
ADB	HC	10±3.0	10	72	0	18	0.16	2.4	not proven
CR	HC	8±2.7	8	27	2	67	0.32	10.6	proven

Notes: $\chi^2_{0.05} = 3.84$; N = 100; ADB – ash dieback; CR – collar rot; HC – *Hylesinus crenatus*

Table 6. Fungi identified on *Fraxinus excelsior* stem for different provenances.

Fungi	Frequency of isolation, [%]					All provenances
	Poliskiyi	Western Forest-Steppe	Right-bank Forest Steppe	Left-bank Forest Steppe	Steppe	
<i>Alternaria alternata</i> (Fr.) Keissl.	13.04	5.56	0.00	4.08	7.41	6.35
<i>Alternaria</i> sp.	8.70	0.00	18.18	2.04	7.41	4.76
<i>Armillaria</i> sp.	4.35	0.00	9.09	6.12	3.70	3.97
<i>Aureobasidium pullulans</i> (de Bary & Löwenthal) G. Arnaud	0.00	0.00	0.00	0.00	3.70	1.59
<i>Cladosporium</i> sp.	4.35	0.00	9.09	4.08	11.11	6.35
<i>Cytospora</i> sp. 15	0.00	0.00	9.09	8.16	0.00	3.97
<i>Didymella macrostoma</i> (Mont.) Qian Chen & L. Cai	8.70	16.67	0.00	4.08	3.70	5.56
<i>Diplodia</i> sp.	8.70	0.00	0.00	2.04	7.41	3.97
<i>Epicoccum nigrum</i> Link	8.70	5.56	9.09	6.12	3.70	6.35
<i>Fusarium</i> sp.	8.70	5.56	0.00	6.12	0.00	4.76
<i>Fusarium avenaceum</i> (Fr.) Sacc.	0.00	16.67	0.00	0.00	7.41	4.76
<i>Gliocladium</i> sp.	13.04	0.00	0.00	0.00	14.81	5.56
<i>Hymenoscyphus fraxineus</i> (T. Kowalski) Baral, Queloz & Hosoya	0.00	5.56	0.00	8.16	0.00	3.97
<i>Hysteroglyphium fraxini</i> (Pers.) De Not.	0.00	0.00	9.09	0.00	14.81	3.97
<i>Phialophora</i> sp.	0.00	5.56	0.00	4.08	0.00	2.38
<i>Mortierella</i> sp.	0.00	0.00	9.09	8.16	0.00	0.79
<i>Mollisia cinerea</i> (Batsch) P. Karst	0.00	0.00	0.00	6.12	0.00	2.38
<i>Penicillium</i> sp. A	0.00	11.11	0.00	8.16	7.41	7.94
<i>Penicillium</i> sp. B	0.00	0.00	9.09	0.00	3.70	0.79
<i>Pezizula cinnamomea</i> (DC.) Sacc.	4.35	0.00	0.00	4.08	0.00	2.38
<i>Phialocephala</i> sp.	0.00	0.00	0.00	2.04	0.00	0.79
<i>Sordaria fimicola</i> (Roberge ex Desm.) Ces. & De Not.	8.70	11.11	0.00	2.04	3.70	4.76
<i>Pseudocamarosporium brabeji</i> (Marinc., M.J. Wingf. & Crous) Crous	0.00	0.00	9.09	2.04	0.00	1.59
<i>Sordaria</i> sp.	0.00	11.11	0.00	4.08	0.00	3.17
<i>Trichoderma viride</i> Pers.	4.35	0.00	0.00	0.00	0.00	1.59
Unidentified sp. 51	0.00	5.56	0.00	4.08	0.00	3.17
Unidentified sp. 58	4.35	0.00	9.09	4.08	0.00	2.38
Unidentified sp. 74	4.35	0.00	9.09	2.04	2.38	2.38

The 25 wood samples from the ash stem yielded a total of 126 fungal isolates (23 from provenance Poliskyi (Pol), 18 from provenance Western Forest-Steppe (WF), 11 from provenance Right-bank Forest Steppe (RBFS), 47 from provenance Left-bank Forest Steppe (LBFS) and 27 from provenance Steppe (S) respectively), representing 28 taxons, although only 12 of them could be identified to species level (Table 6).

Only *Epicoccum nigrum* was isolated from all five provenances. From among all fungi, *Penicillium* sp. A, *Epicoccum nigrum*, *Cladosporium* sp. and *A. alternata* occurred most frequently.

Four of the fungal species were isolated only from a single provenance: *Aureobasidium pullulans* (Steppe), *Mollisia cinerea*, *Phialocephala* sp. (Left-bank Forest Steppe), *Trichoderma viride* (Poliskyi). Each provenance has a large number of unique species but also a number of shared species. The highest diversity in terms of species richness and Shannon's index was found for the Left-Bank Forest Steppe (Fig. 1).

Relatively high species richness was also found for the Poliskyi and Steppe provenances. Lowest species richness was found in the Right-bank Forest Steppe. However, all groups had relatively higher Shannon index values (comparable to samples from Right-bank Forest Steppe). For species richness values, all pairwise comparisons are significantly different ($p < 0.05$) with Tukey tests except Western Forest-Steppe and Right-bank Forest Steppe; and for Wilcoxon test all are significantly different ($p < 0.05$) except Western Forest-Steppe and Right-bank Forest Steppe. For Shannon index values no pairwise comparisons are significantly different with either the Tukey or Wilcoxon test.

Beta diversity statistics/comparisons among the samples were performed with non-metric multi-dimensional scaling (NMDS) analysis (Fig. 2). Figure 2 shows a map for beta diversity with unweighted unifrac distances among groups.

The highest beta diversity levels are found between Western Forest-Steppe and Right-bank Forest Steppe groups as well as Poliskyi and Right-bank Forest Steppe groups. NMDS picture groups samples into rough clusters corresponding to either their geography (Poliskyi, Western Forest-Steppe, Right-bank Forest Steppe, etc.). A similar pattern can be seen in the plot (Fig. 2). The Right-bank Forest Steppe sample is a diverse set of fungal species in comparison to the Poliskyi and Western Forest-Steppe samples that are only a few close associated fungi with *F. excelsior*. The fungi set from Poliskyi samples groups more closely with Steppe samples than the Western Forest-Steppe, Right-bank Forest Steppe and Left-bank Forest Steppe samples (despite the close geographical proximity of Right-bank Forest Steppe and Left-bank Forest Steppe), thus there seems to be strong influence of provenance on fungal community composition. Left-bank Forest Steppe is the sample with much higher species richness than the others.

Pathogenicity tests. Inoculation with *H. fraxineus*, *Cytospora* sp. and *Diplodia* sp. isolates was followed by the development of brownish lesions on all stems of 7–10-year-old plants. The lesions were already obvious after 4 months and significantly longer than control and other isolates. The inoculation trials showed that there were significant differences in the length of necroses ($F = 5.60$, $p = 0.018$) due to the *H. fraxineus* between the three inoculated species. The mean wood necrosis length on the branches of *H. fraxineus* was 25.6 ± 1.5 mm (mean \pm standard error) while *Cytospora* sp. and *Diplodia* sp. 11.7 ± 1.1 mm and 6.7 ± 1.3 mm, respectively. Moreover, among both the upper and lower lesions, some showed a superficial splitting of the bark. None of the inoculated plants died within 4 months. In re-isolation, *Cytospora* sp. and *Diplodia* sp. were obtained from necrotic sections of all inoculated stems of *H. fraxineus*.

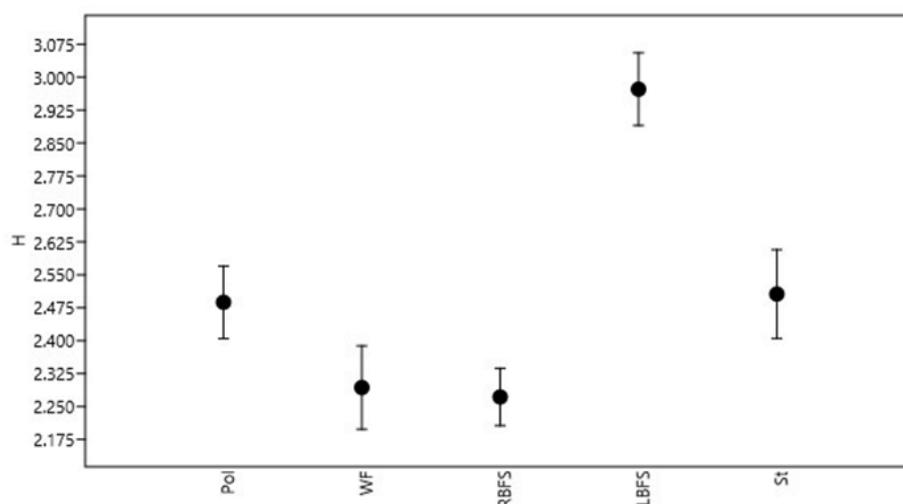


Fig. 1. Box plots of Shannon indices in the five different groups (Pol – Poliskyi, WF – Western Forest Steppe, RBFS – Right-bank Forest Steppe, LBFS – Left-bank Forest Steppe, St – Steppe).

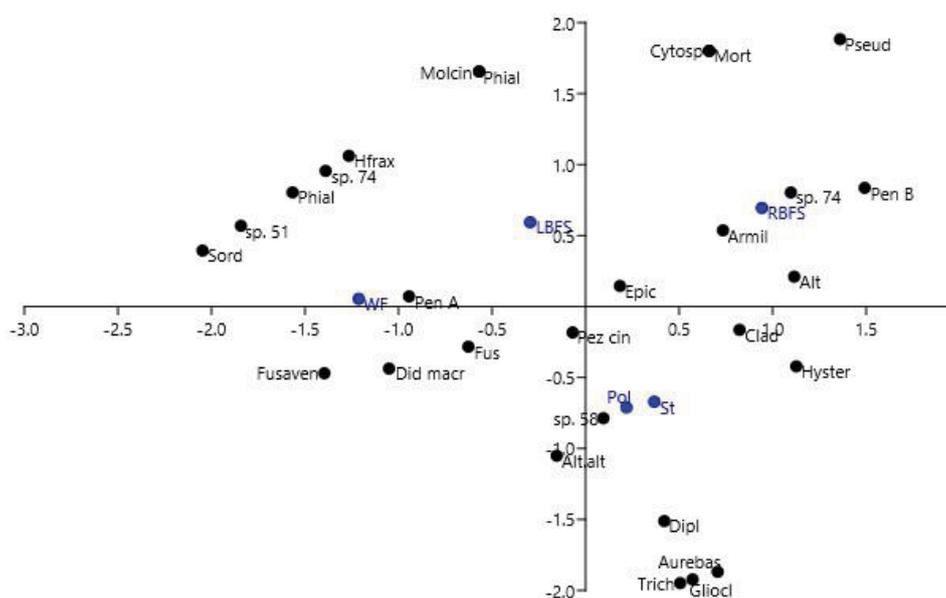


Fig. 2. NMDS plot of fungal species from Pol – Poliskyi, WF – Western Forest Steppe, RBFS – Right-bank Forest Steppe, LBFS – Left-bank Forest Steppe, St – Steppe.

Legends on the plot corresponds to Alt.alt – *Alternaria alternata*; Alt – *Alternaria* sp.; Armil – *Armillaria* sp.; Aurebas – *Aureobasidium pullulans*; Clad – *Cladosporium* sp.; Cytosp – *Cytospora* sp. 15; Did macr – *Didymella macrostoma*; Dipl – *Diplodia* sp.; Epic – *Epicoccum nigrum*; Fus – *Fusarium* sp.; Fusaven – *Fusarium avenaceum*; Gliocl – *Gliocladium* sp.; Hfrax – *Hymenoscyphus fraxineus*; Hyster – *Hysteroglyphium fraxini*; Phial – *Phialophora* sp.; Mort – *Mortierella* sp.; Molsin – *Mollisia cinerea*; Pen A – *Penicillium* sp. A; Pen B – *Penicillium* sp. B; Pez cin – *Pezizella cinnamomea*; Phialc – *Phialocephala* sp.; Sord – *Sordaria fimicola*; Pseud – *Pseudocamarosporium brabeji*; Trich – *Trichoderma viride*; sp 51 – Unidentified sp. 51; sp 58 – Unidentified sp. 58; sp 74 – Unidentified sp. 74.

In other cases, the lesion extension was not observed; however, the slight yellowing of leaves was noted after 4 months from inoculation. *A. alternata*, *Didymella macrostoma* and *Fusarium avenaceum* produced no symptoms on stems of plants in experiments although all these species induced brown discoloration (ca. 1 mm) around the inoculation points on 7–10-year-old plants. These fungi were re-isolated from the points of inoculation. No lesions developed on any of the control plants and the wounds healed within 4 months after inoculation.

4. Discussion

Invasive pathogen *Hymenoscyphus fraxineus* spread in Europe over 20 years ago (Baral et al. 2014; Gross et al. 2014; Enderle et al. 2015). Its presence in the east of Ukraine, in particular, in the Sumy region, has been confirmed by molecular methods (Davydenko et al. 2013b). In contrast to the western regions (Matsiakh & Kramarets 2014), the disease in the east develops more slowly and does not often lead to the death of trees (Davydenko & Meshkova 2017), which may be due to the features of the climate, in particular, with low precipitation (Hlásny et al. 2014; Krakovska et al. 2017). According to another hypothesis, the origin of ash plays a role in the susceptibility of trees to fungal infection (Metzler et al. 2012; Stener 2013; McKinney et al. 2014). Our

research attempted to test this hypothesis in the plots of provenance tests of *Fraxinus excelsior* which were created in 1930 under the leadership of prof. V.M. Andreiev in Sumy region (Davydenko et al. 2013a).

Provenance tests as objects of tree breeding were created primarily to determine the most adapted and productive provenances for specific growing conditions in order to regionalize the supply of seeds for reforestation. The provenances originate from seeds collected from identified stands or regions. However, nowadays they are the only natural models for predicting the impact of climate change consequences (Matyas 1996) as well as to study the tree resistance to new pathogens (Pliura et al. 2011; Metzler et al. 2012). Sometimes, the data from clonal plantations are also considered in the study of resistance (Enderle et al. 2013, 2015). To study the resistance to ash dieback, a provenance trial was established in 2005 in southwest Germany with plots at four sites and eight provenances of *Fraxinus excelsior* (Enderle et al. 2013, 2015). The intensity of the disease in single trees within the provenances varied constantly between completely unaffected trees and their mortality. The study highlights a high genetic variation in susceptibility and considerable genetic potential for resistance breeding in provenances from southwestern Germany.

In a Danish clonal trial comprising 39 *F. excelsior* clones established in 2007 with grafts at two sites, mortality was about 40% in 2013. The average percentage

of crown damage increased from 30% in 2007 to over 60% in 2013, with substantial variation among clones (McKinney et al. 2014). In France, only 8% healthy trees and 88% of the trees with more than 5% crown decline were observed. Collar lesions were observed on 33% of the trees and were present in almost all plots (Husson et al. 2011).

Genetic studies of *F. excelsior* clones in Denmark and Sweden did not reveal any differences among populations in resistance to ADB (McKinney et al. 2014). However, in Lithuania progeny trials established in 2005 at three different sites showed significant differences in resistance to ADB among populations (Pliura et al. 2011). This provides good possibilities for the selection of resistant populations. The health condition of trees was influenced also by site conditions and infection pressure from the surrounding stands.

We compared the health scores of five provenances in 2012 and 2019 by different parameters of growth and health condition. Both in 2012 and in 2019, statistically significant differences in DBH, Kraft class, and health condition index between provenances were not found (Table 1, 2). However, in 2019 compared to 2012, the health condition index significantly increased for all provenances except the Steppe. According to National scale (Davydenko et al. 2019), all provenances in 2012 could be considered as weakened ($1.5 < \text{HCI} < 2.5$), and in 2019 they all were severely weakened ($2.5 < \text{HCI} < 3.5$).

In 2019, a provenance from Steppe had significantly the lowest branch dieback (average proportion of dry branches in the crowns) (Table 2).

At the same time, ADB incidence was the lowest in the Western Forest-Steppe provenance in 2012 and this parameter did not differ from Steppe in 2019 (Table 3). ADB severity was the highest in Steppe in 2012 but was among the lowest values in 2019.

In 2019 compared to 2012, ADB incidence and severity increased for all provenances except Steppe, however maximal values (1.8 points) were assessed for Poliskyi and Left-bank Forest Steppe provenances (Table 3). So the data obtained do not allow us to draw a conclusion about the different susceptibility of individual ash provenances to the ADB. It may be connected with rather high age of trees (about 90 years old).

In different regions, it was previously found out that collar rots (Langer 2017; Davydenko et al. 2019) and stem colonization with bark beetles (Meshkova & Borysova 2017) often accompany infestations of ash with ADB. Therefore, in a survey of provenance tests in 2019, the incidence and severity of collar rots and *H. crenatus* was assessed (Table 4). Collar rot presence was revealed in all ash provenances with the lowest values in Western Forest-Steppe and Right-bank Forest Steppe. Differences between provenances in disease severity according to visual assessment were not significant. Trees colonization by *H. crenatus* was absent in Western Forest-Steppe and Right-bank Forest Steppe provenances, and the dif-

ferences between other provenances were not significant both by the incidence and severity (Table 4).

Statistical analysis supported the conclusion from other regions about the coincidence the damage of European ash by ash dieback and collar rots (Langer 2017; Davydenko et al. 2019) as well as the coincidence the damage of European ash by collar rots and tree colonization by *H. crenatus* (Meshkova & Borysova 2017) (Table 5). However, the absence of coincidence of ADB and tree colonization by *H. crenatus* may be explained by relatively low incidence of the trees inhabited by *H. crenatus* and the features of this insect to colonize the lowest part of stem with thick bark (Okolow 1970) while ADB reveals first in the crowns (Skovsgaard et al. 2010). We can suppose that the incidence of other ash bark beetles inhabiting upper stem and branches, especially *H. fraxini* (Panzer 1779) (Pedrosa-Macedo 1979) and *H. toranio* (Danthoine, 1788) (Graf 1977), can be more closely associated with ADB. However, the assessment of these insects' galleries is possible only after tree felling.

In the present study, the detected fungal communities were largely composed by the generalist saprotrophic and pathogenic fungal taxa (Table 6). Despite that several pathogenic taxa including *H. fraxineus*, *Cytospora* sp., *H. fraxini*, etc., the fungal community of all provenances showed preference towards saprotrophic species. One to three pathogenic taxa (including *A. alternata*, *E. nigrum*, *Cytospora* sp., *H. fraxineus* and *H. fraxini*) were found in all provenances. Among these, *A. alternata* and *E. nigrum* are known as opportunistic weak pathogens or even saprophytes with a worldwide distribution. *Cytophora* sp. is known as the causative agent of *Cytospora* canker. Other pathogenic fungi were *H. fraxineus* and *H. fraxini* which both are known as a widespread ash dieback-fungi causing massive ash decline. So, the pathogenic fungi were likely the primary cause of disease symptoms. Therefore, we suggest that the fungus *H. fraxineus* is a primary pathogen killing healthy ash tissues. However, later the fungus is losing the competition with other decomposing and saprotrophic fungi being more successful and taking over their domination in dead tissues. So, it explains why necrotic ash shoots usually are colonized by other plant pathogens, endophytes and saprophytes.

Our results for the study of fungal community structure in declining shoots of ash trees in the different geographic provenances as well as inoculation experiment revealed 28 fungal taxa associated with ash trees. Among them, at least three fungal species showed the ability to be pathogens that is the valuable point of this research. Next to *Hymenoscyphus fraxineus* and *Hysteroglyphium fraxini*, *Cytospora* sp. and *Diplodia* sp. have been considered to be involved in serious decline, in combination with abiotic factors. Our study confirmed previous results published. Przybył (2002), Kowalski & Łukomska (2005), Lygis et al. (2006) presented data of endophytic fungi on ash branches with the most characteristic fungi

as we did like *Alternaria alternata*, *Diplodia mutila*, *Phomopsis* sp., *Cladosporium cladosporioides*, *Cytospora ambiens* and *Phomopsis controversa*. Fungi belonging to *Phomopsis* and *Cytospora* genera are pathogens of weak trees and are often present in dying shoots (Przybył 2002; Kowalski & Łukomska 2005).

From this point of view, the knowledge of the distribution of pathogenic fungal species associated with *F. excelsior* suffering from ash-dieback phenomenon can be very useful in forest management and diversity conservation programs.

5. Conclusion

Provenance tests of European ash of about 90 years old in Sumy region (Eastern Ukraine) are affected by ash dieback (ADB) caused by pathogen *Hymenoscyphus fraxineus* for at least 10 years.

For 2012 and 2019 the health condition index, ADB incidence and severity increased for all provenances except the Steppe. However, the data obtained do not allow us to draw a conclusion about the different susceptibility of individual ash provenances to the ADB.

Collar rot was present in all ash provenances with the lowest incidence in provenances from Western Forest-Steppe and Right-bank Forest Steppe and the absence of significant differences between provenances in disease severity.

Hylesinus crenatus was not revealed in Western Forest-Steppe and Right-bank Forest Steppe provenances, and the differences between other provenances were not significant both by the incidence and severity.

Fungi isolated from stem of ash trees with varying degrees of decline were identified and the pathogenicity of some of them was determined. Among them, *Hymenoscyphus fraxineus*, *Cytospora* sp. and *Diplodia* sp. induced different necrotic lesions.

The conclusion from other regions about the coincidence the damage of European ash by ash dieback and collar rots (Matsiakh & Kramarets 2014; Langer 2017) as well as the coincidence the damage of European ash by collar rots and tree colonization by *H. crenatus* (Davydenko et al. 2019) is supported.

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