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HEALTH CONDITION AND ASH DIEBACK SYMPTOMS DEVELOPMENT IN *FRAXINUS EXCELSIOR* INOCULATED WITH *HYMENOSCYPHUS FRAXINEUS*

Davydenko K. V., Meshkova V. L. Health condition and ash dieback symptoms development in Fraxinus excelsior inoculated with Hymenoscyphus fraxineus. *The specificities of ash dieback development on ash seedlings and 20-years-old ash trees after inoculation with H. fraxineus have been evaluated. During four years after inoculation ash seedlings and 20-years old trees demonstrated the worsening of the health condition. This process was more active in the year with higher precipitation of vegetation period. The length of necrotic lesions on infected 20-year-old trees increased more intensively than on seedlings. However, no 20-year-old tree died during four years of experiment, while seedlings mortality exceeded 80 % in the 3rd year after inoculation. Re-isolation of Hymenoscyphus fraxineus from bordering necrotic/visually healthy tissue from inoculated plants confirms responsibility for ash dieback in the experiment……15 Ref.*
Key words: European ash (Fraxinus excelsior L.), Hymenoscyphus fraxineus, health condition, ash dieback, lesion length.

Давиденко К. В., Мешкова В. Л. Санітарний стан і розвиток симптомів халарового некрозу ясенів Fraxinus excelsior, інокульованих Hymenoscyphus fraxineus. *Вивчали особливості розвитку халарового некрозу сіянців і 20-річних дерев ясена після інокуляції H. fraxineus. Упродовж чотирьох років після інокуляції цим грибом стан сіянців і 20-річних дерев погіршувався. Цей процес відбувався інтенсивніше у рік із більшою кількістю опадів упродовж вегетаційного періоду. Довжина некротичних плям у заболоні інфікованих 20-річних дерев зростала інтенсивніше, ніж у заболоні сіянців. Водночас жодне 20-річне дерево не загинуло за чотири роки експерименту, тоді як відпад сіянців перевершив 80 % у третій рік після інокуляції. Повторне виділення H. fraxineus із некрозів у заболоні інокульованих рослин підтвердило роль цього гриба у розвитку халарового некрозу у досліді……15 назв.*
Ключові слова: ясен звичайний (*Fraxinus excelsior* L.), *Hymenoscyphus fraxineus*, санітарний стан, халаровий некроз, довжина некротичних плям.

Давиденко К. В., Мешкова В. Л. Санитарное состояние и развитие симптомов халарового некроза ясеней Fraxinus excelsior, инокулированных Hymenoscyphus fraxineus. *Изучали особенности развития халарового некроза сеянцев и 20-летних деревьев ясеня после инокуляции H. fraxineus. В течение четырех лет после инокуляции этим грибом состояние сеянцев и 20-летних деревьев ухудшалось. Этот процесс происходил более интенсивно в год с большим количеством осадков в течение вегетационного периода. Длина некротических пятен в заболони инфицированных 20-летних деревьев нарастала интенсивней, чем в заболони сеянцев. Однако ни одно 20-летнее дерево не погибло за четыре года эксперимента, в то время как отпад сеянцев превысил 80 % на третий год после инокуляции. Повторное выделения H. fraxineus из некрозов в заболони инокулированных растений подтвердило роль этого гриба в развитии халарового некроза в опыте……15 назв.*
Ключевые слова: ясень обыкновенный (*Fraxinus excelsior* L.), *Hymenoscyphus fraxineus*, санитарное состояние, халаровый некроз, длина некротических пятен.

Introduction. Health condition of ash stands in Ukraine has become worse since 2006 [1, 6]. One of the main causes of European ash (*Fraxinus excelsior*) dieback was alien invasive fungus *Hymenoscyphus fraxineus*, which was firstly found in Ukraine in 2011 [7]. Perhaps the

first symptoms of disease appeared more earlier in western Ukraine which borders Poland where the disease was reported in the 1990s and spread fast across Europe [9, 14].

Disease-causing agent of ash dieback was described by T. Kowalski in 2006 as a new fungal species, *Chalara fraxinea* based on conidia and conidiophore morphology of the vegetative stage, since no sexual stage of the fungus was known at the time [12]. In 2010 detailed molecular investigations showed, that ash dieback pathogen is a species entirely new to Europe and is identical to previously described Japanese Lambertella albida [15]. Since 2014, in accordance with rules of fungi nomenclature, this fungus name is *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz, Hosoya [4].

Symptoms of the ash dieback include wilting as a result of dying branches and shoots and discoloration of different parts of the tree: brown spots on buds, leaves and petioles; dead shoots; and brown lesions on branches and stems, epicormic shoots [11].

H. fraxineus reproduces sexually on overwintered petioles from the previous years in the leaf litter [3, 5, 9]. Germ tube formation and development of appressorium facilitate the progress of spores penetration to leaf cuticle [5]. Necroses extend proximally along leaf veins to rachises and shoots and result in typical ash dieback symptoms as wilting of shoots and bark cankers [3], root collar necrosis and stem lesions [5, 14]. The presence of necrotic lesions on the stem or root collar may worsen of ash tree condition and promote to attack by *Armillaria* species [13].

Ash dieback in Kharkiv region revealed crown dieback, presence of pseudosclerotia on ash petioles, and abundant apothecia production. *H. fraxineus* was frequently and consistently isolated in all of the symptom categories [1, 6, 8].

To evaluate potential risks for Ukrainian ash stands and natural regeneration pathogenicity tests on material of F. excelsior had to be carried out.

The aim of study was to investigate the specificities of ash dieback development on ash seedlings and 20-years-old ash trees after inoculation with *Hymenoscyphus fraxineus*.

Materials and methods. *H. fraxineus* was isolated on MEA (3 % malt extract agar) from necrotic lesions on symptomatic bark and leaves of European ash collected in forest stands of Kharkiv region. To confirm *H. fraxineus* presence fungal cultures were used for DNA extraction using PCR-based techniques and species — specific primers [10].

Inoculation experiments on ash seedlings and trees were carried out in the forest stands of the State Forest Enterprise "Zhovtneve Forest Economy" in Kharkiv region.

In May 2014 and May 2015, ash 5-years-old seedlings and 20-years-old trees were inoculated with *H. fraxineus* (30 seedlings and 12 ash trees every year).

Inoculations were done by cutting out a piece (ca. 5×10 mm in size) of the bark on the stem ca. 80–100 cm for seedling and 120 cm for young tree above the root collar with a sterile scalpel, placing fungal inoculum on the exposed sapwood and covering it up with the bark and wrapping in Parafilm [11]. As a control, 10 seedlings and 10 trees of the same age were inoculated with sterile 3 % MEA.

Inoculated ashes were inspected in 2014–2017. Category of health condition, ash dieback score, mortality of seedlings or trees and the length of necrotic lesions were evaluated simultaneously in all variants in May and in September.

Category of 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 "Sanitary rules in the forests of Ukraine" [2]. Each tree was referred to one of six categories of health condition (1^{st} — healthy; 2^{nd} — weakened; 3^{rd} — severely weakened; 4^{th} — drying; 5^{th} — recently died; 6^{th} — died over year ago). Index of health condition for forest stand was calculated as mean weighted from trees number of each category of health condition.

Ash dieback score was assessed according to scale of possible symptoms for ash dieback [6, 8], where 0 — no ash dieback symptoms (ADB), 1 — minor and indirect symptoms (uneven foliage expansion, necrotic lesions in healthy shoots and dead branches in 10 % of crown size;

2 — dead shoots and branches with necrotic lesions formation in 10–50 % of crown size; 3 — death of 50 % of crown size, wood discoloration, necrotic lesions, dead leaves, shoots, branches, 4 — total crown damage, necrotic lesions on green shoots, branches, stem and butt.

The length of the necrotic lesions on the sapwood was measured to estimate the area colonised by fungus.

To confirm *H. fraxineus* presence in necrotic lesions, two pieces of wood tissue ca. 1 cm length from each side of boundary necrotic/visually healthy zone were removed and plated on MEA to re-isolate the inoculated fungi.

Data on precipitation for 2014–2015 were taken from meteorological station Kharkiv (49°90' N; 36°30' E).

The statistical analysis included one-way analysis of variance (ANOVA) (StatSoft Software, Ver. 7).

Results and discussion. Health condition of ash seedlings inoculated with *H. fraxineus* worsened during the years of experiment (Fig. 1). For the seedlings inoculated in 2014 health condition score got worse changing from 1 to 2.7 points in the 1st year, the next spring has reached 3.8 points and then the process slowed down. However, in the 4th year the most of inoculated seedlings were dead, and health condition score has reached 4.7 points in September.

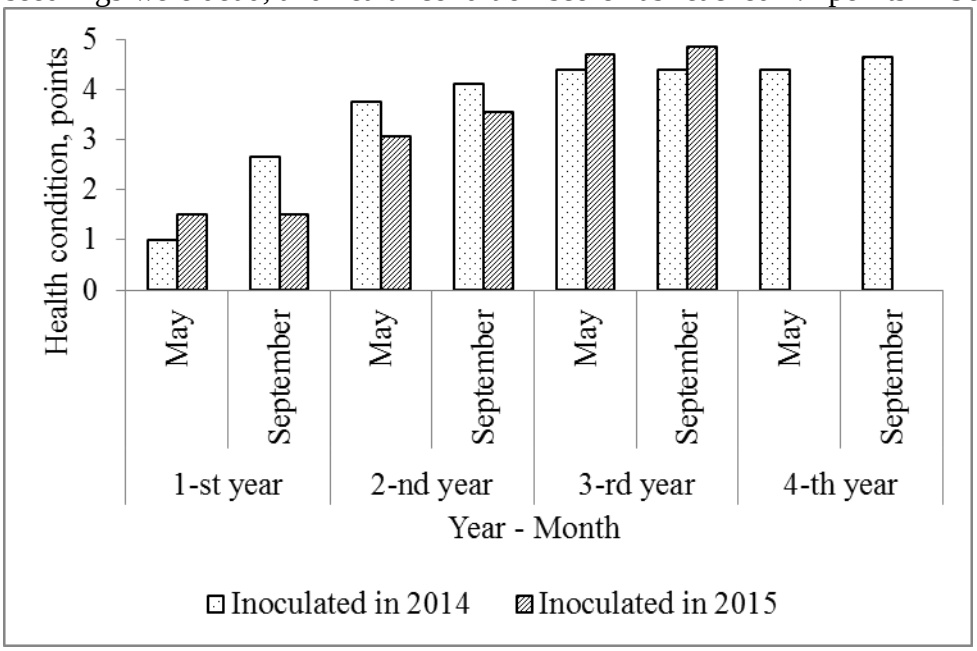

Fig. 1. Dynamics of health condition score for ash seedlings after inoculation with *H. fraxineus*

For the seedlings inoculated in 2015 health condition score did not change significantly in the 1st year, which can be explained by some differences in weather conditions. For example, precipitation for the most months of vegetation in 2014 was considerably higher, than in 2015 (Fig. 2). However health condition score has reached 3.1 and 4.9 points in May of the 2nd and 3rd year after inoculation (see Fig. 1). Health condition of control seedlings did not worsen during our research and health condition score did not exceed 1.2 points.

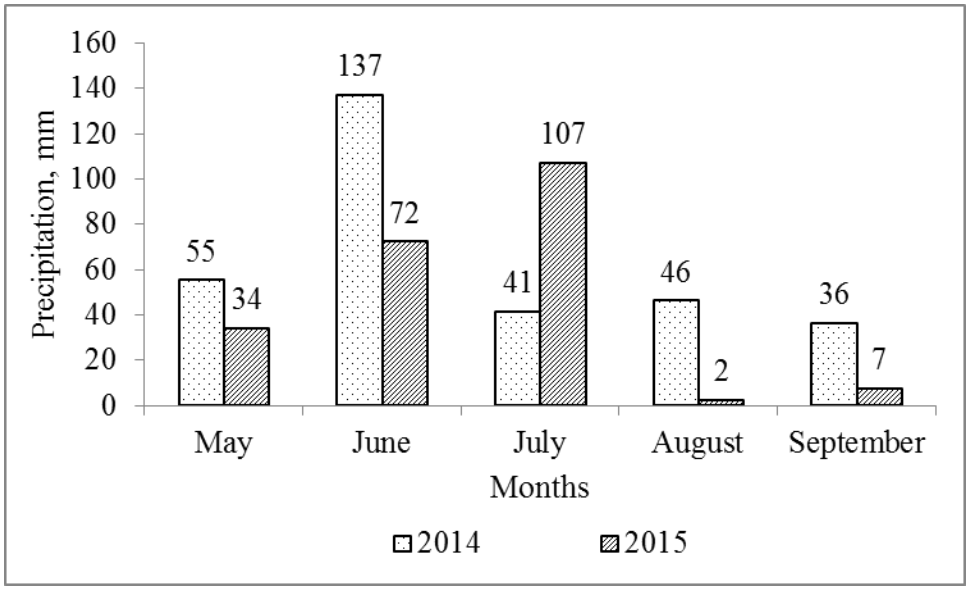

Fig. 2. Precipitation in the years of ash seedlings inoculation with *H. fraxineus*

Ash dieback symptoms developed almost at the same rate (Fig. 3). For the seedlings inoculated in 2014 ash dieback symptoms score increased up to 3.3 points in September in the year of inoculation. Over the next two years this index increased rather slowly and has reached 3.8 points in September of the 4th year after inoculation.

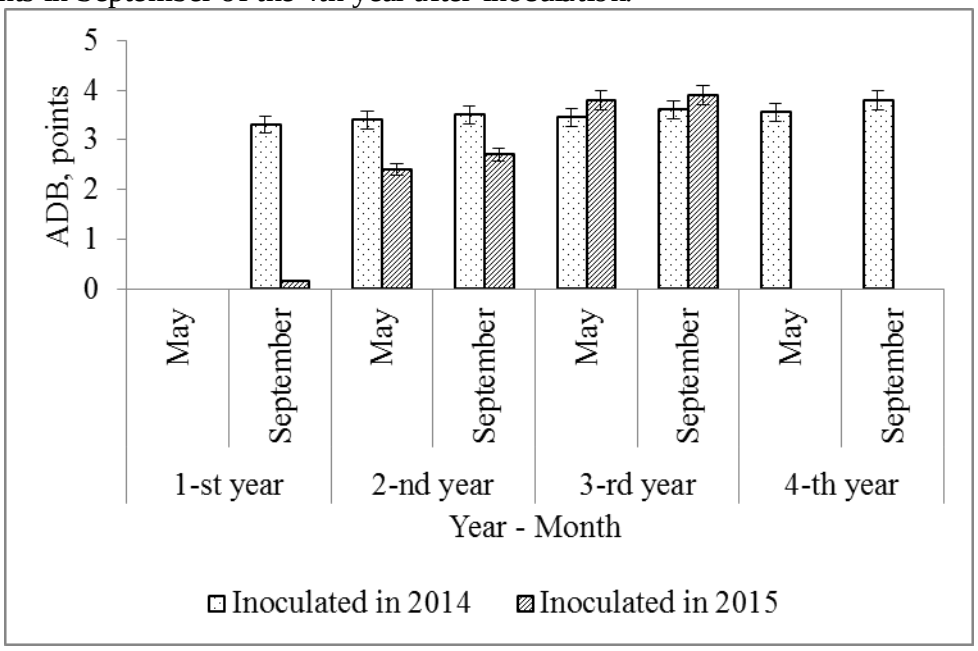

Fig. 3. Dynamics of ash dieback symptoms (ADB) score for ash seedlings after inoculation with *H. fraxineus*

For the seedlings inoculated in 2015 ash dieback symptoms has been developed in September. Ash dieback symptoms score for this group of plants was considerably lower, than for those inoculated in 2014 (3.3 and 0.2 points for seedlings inoculated in 2014 and 2015 respectively), which can also be explained by rather dry weather in 2015 (see Fig. 3).

The ash dieback symptoms score for seedlings inoculated in 2014 have been developed gradually and ADB score has reached 3.6 and 3.8 points in the 3rd and 4th years after inoculation. Ash dieback symptoms score in seedlings inoculated in 2015 exceeded that of seedlings inoculated in 2015 in the 3rd year after inoculation and reached 3.9 points in September (see Fig. 3).

Any dead ash seedling was found in the year of their inoculation with *H. fraxineus* (Fig. 4). The second year after inoculation 55 % of seedlings inoculated in 2014 and 55 % of seedlings inoculated in 2015 have died in May. In September of the 2nd year after inoculation seedlings mortality had reached 55 and 40 %, and in May of the 3rd year it was 80 and 85 % respectively.

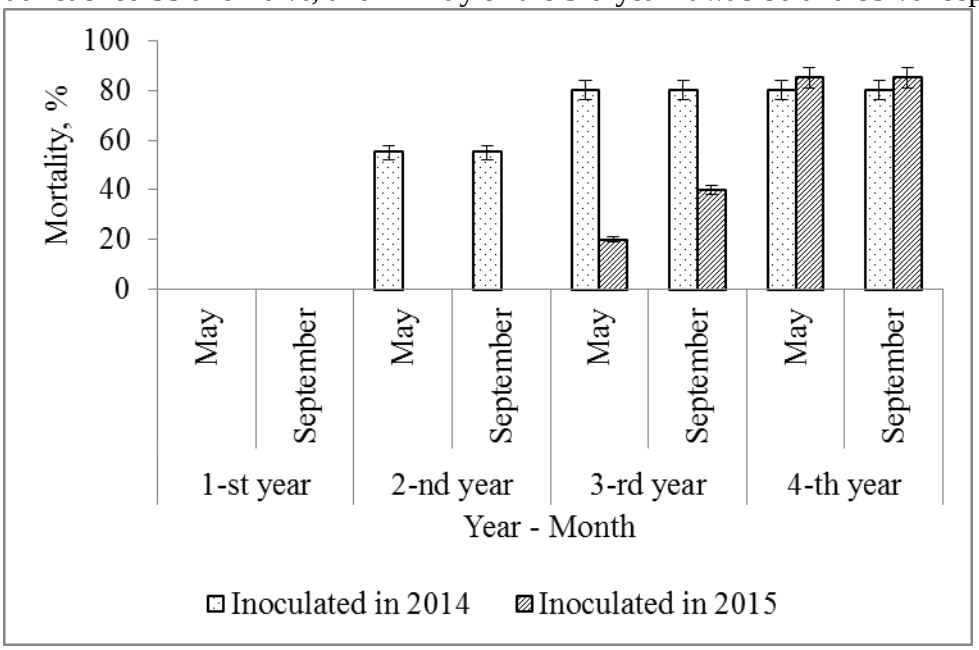

Fig. 4. Cumulative total rate of ash seedlings mortality after inoculation with *H. fraxineus*

Typical formation of necrotic lesions was a common response to inoculation ash trees with the H. fraxineus and no significant difference between treatments was recorded in first three months after inoculation.

In total, inoculation of all ash seedlings and trees with H. fraxineus resulted in necrotic dark-brown submerged lesions of different size. The lesions were generally extended in both proximal and distal directions from the wounded area. But lesion lengths were not significantly different by Fishers test ($p \leq 0.05$) following ANOVA ($n = 6$) for both ash seedlings and trees. Furthermore, no significant differences between wood and bark necroses lengths ($p < 0.01$) were observed on ash seedlings in both experiments. The control treatment resulted in very small dark area around inoculation place. In all experiments fungus induced lesions length on the inoculated ash seedlings was significantly different from the control ($p < 0.001$).

Lesion lengths in seedlings inoculated both in 2014 and 2015 has increased almost the same during two years after inoculation. In September of the 1st year lesion length was 11.4 and 12.1 mm respectively, in September of the 2nd year it was 24.4 and 29.1 mm respectively (Fig. 5). Since that date lesion lengths in seedlings inoculated in 2015 exceeded those of seedlings inoculated in 2014, and in September of the 3rd year lesion lengths have reached 25.3 and 34.5 mm respectively.

Health condition of inoculated 20-years-old ash trees gradually worsened during experiment (Fig. 6). Score of health condition for trees inoculated in 2014 has increased from 1.3 points in the first year to 2.8 points in the third year and reached up to the 3.2 points in the fourth year of experiment. Health condition of ash trees inoculated in 2015 has been worsened steadily also and has reached 3.2 points in three years (see Fig. 6).

Ash dieback score for 20-years-old trees inoculated in 2014 and 2015 increased about twice (1.7 times) for two and three years after inoculation (Fig. 7). Probably, more rapid disease development in 20-years-old ash trees inoculated in 2015 can be explained by higher precipitation in the year of inoculation (see Fig. 2).

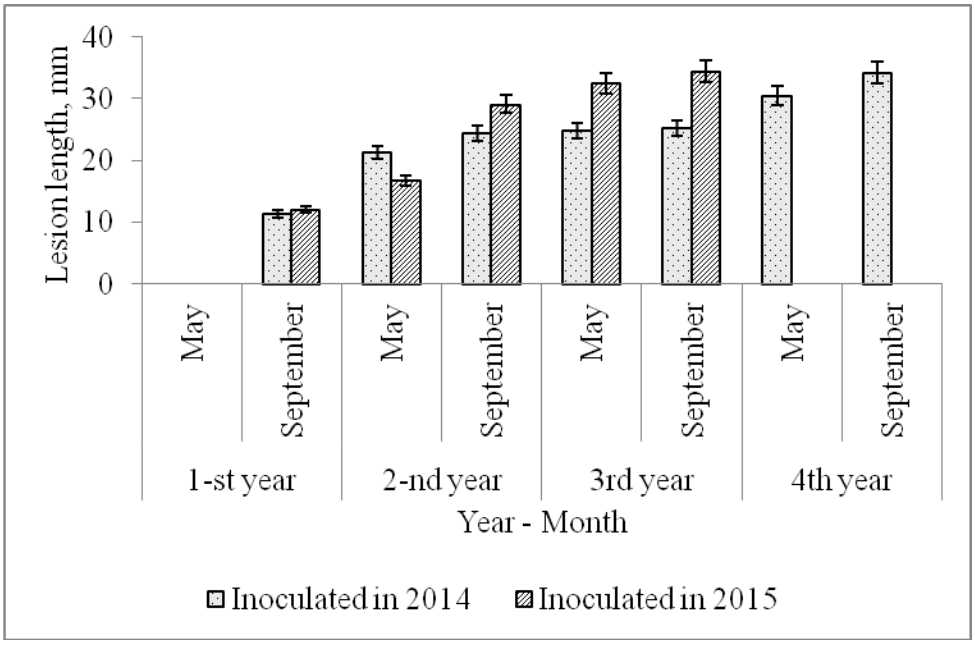

Fig. 5. Development of necrotic lesion length in ash seedlings after inoculation with *H. fraxineus*

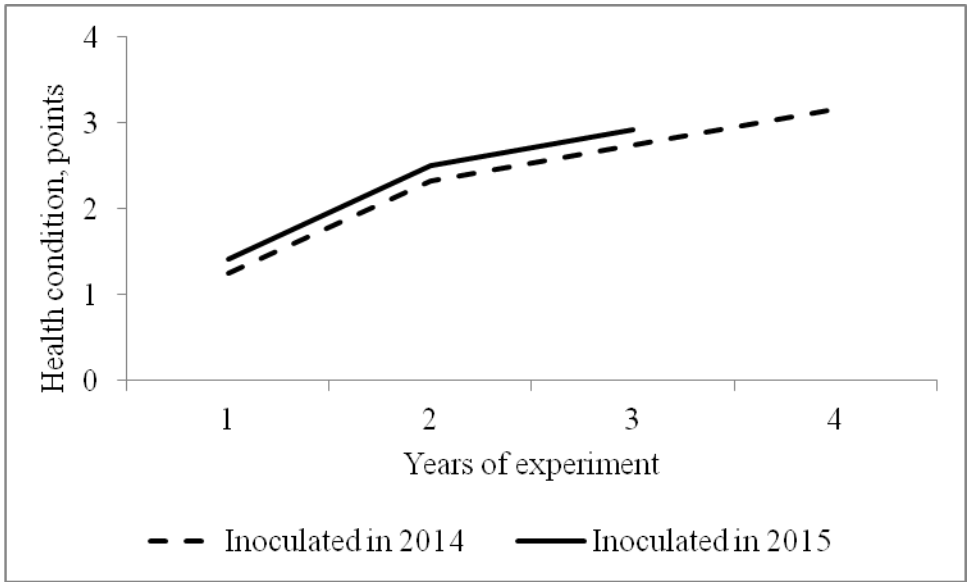

Fig. 6. Health condition score for 20-year-old ash trees after inoculation with *H. fraxineus*

Despite of severe dieback and typical symptoms of ash dieback such as stem necroses, wood discoloration and wilt of leaves and branches above inoculation place, no 20-year-old tree mortality have been observed during experiment. None of ash dieback symptoms or tree death was observed for control trees.

Lesion length on 20-year-old trees increased more intensively, than in seedlings (Fig. 8).

In September of the year of inoculation lesion length has reached 62.7 and 60.3 mm on on 20-year-old trees inoculated in 2014 and 2015 respectively. Lesion length continued to grow during all years of research and has reached 179.1 mm in May of the 4th year (see Fig. 6).

Moreover, despite the formation of typical symptoms of ash dieback during three years, single inoculation did not cause high tree mortality but only brought to health condition worsening with score from 1.25 points in 2014 to 3.2 points in 2017.

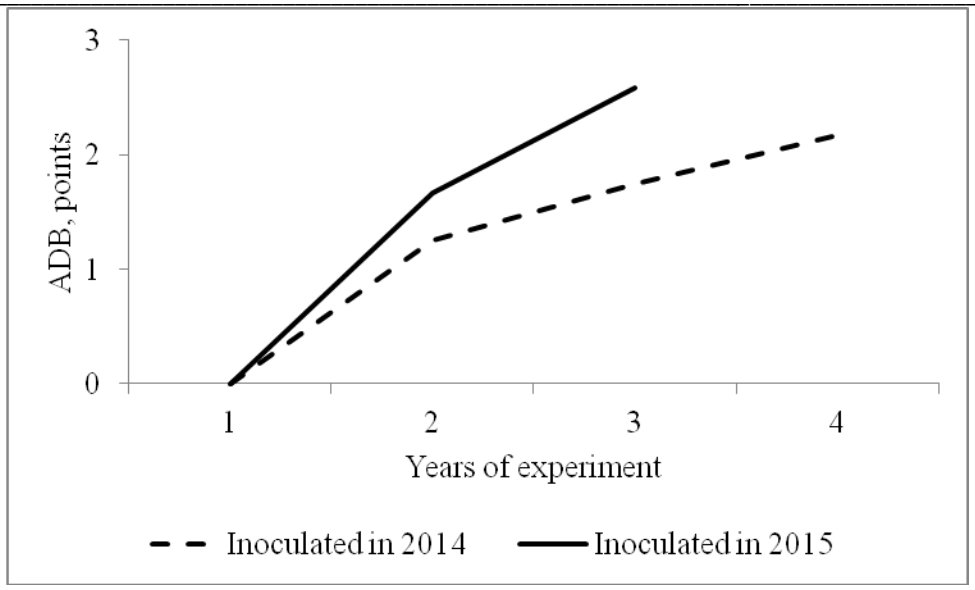

Fig. 7. Ash dieback symptoms (ADB) score for 20-year-old ash trees after inoculation with *H. fraxineus*

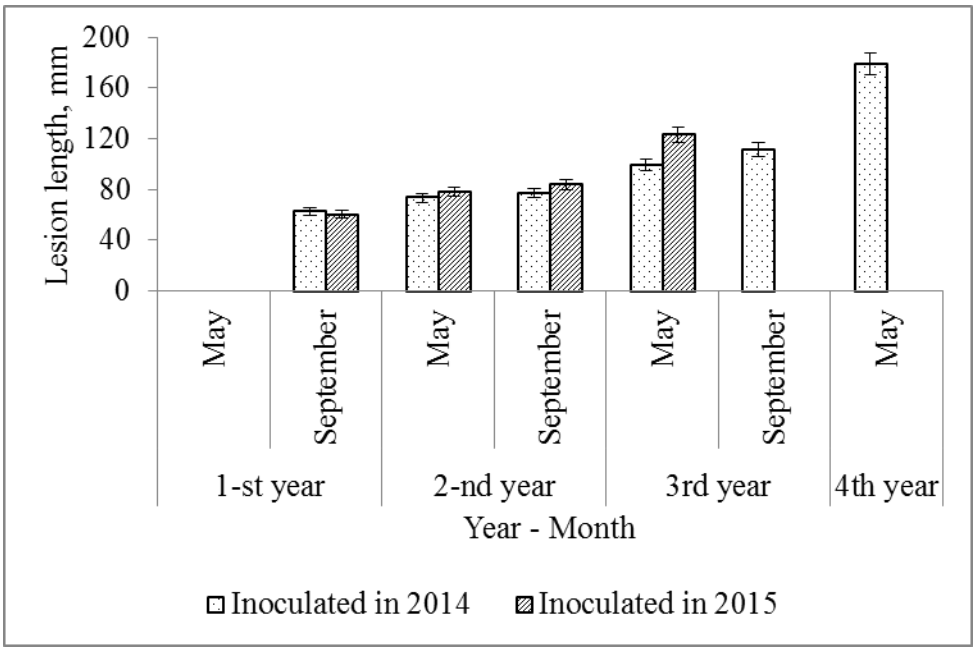

Fig. 8. Dynamics of necrotic lesion length in 20-year-old ash trees after inoculation with *H. fraxineus*

H. fraxineus was confirmed to be the main cause of necrotic lesions by re-isolation of this fungus from the margin of necroses with success rate of 98 % and 96 % for ash seedling and 20-years-old ash trees respectively. Branches inoculated with sterile MEA showed no typical ash dieback necroses, and no H. fraxineus was re-isolated from control trees.

Our results confirm that natural regeneration of ash in diseased forests can be damaged by ash dieback, which will bring to a major degradation of ash stands and other associated species [13].

Conclusions. Ash seedlings and 20-years old trees worsened health condition during four years after inoculation with H. fraxineus. Health condition score and ash dieback symptoms score increased more intensive in the year with higher pprecipitation of vegetation period. Any 20-year-old tree died during four years of experiment, but seedlings mortality had exceeded

80 % in May of the 3rd year after inoculation. The length of necrotic dark-brown submerged lesions on infected 20-year-old trees increased more intensively, than on seedlings. H. fraxineus was re-isolated from necroses in inoculated plants that confirms its responsibility for ash dieback in the experiment.

Acknowledgements

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