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*Dedicated to Professor Maria Ławrynowicz
on the occasion of the 45th anniversary
of her scientific activity*

# The occurrence of *Hymenoscyphus pseudoalbidus* apothecia in the leaf litter of *Fraxinus excelsior* stands with ash dieback symptoms in southern Poland

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The paper presents the results of a study performed in 28 ash stands located in Myślenice and Dynów Forest Districts in southern Poland. The intensity of *Fraxinus excelsior* disease process was estimated based on the disease symptoms analysis of 1400 trees. The amount of infectious material of *Hymenoscyphus pseudoalbidus* (anamorph *Chalara fraxinea*) was estimated by the leaf litter examination on 166 square (0.5 × 0.5 m) plots. The total number of ash leaf rachises and apothecia were counted. The analysis shows that the estimated number of *H. pseudoalbidus* apothecia may reach from 370 thousand to over 13 million per hectare at a time.

**Key words:** *Fraxinus excelsior,* ash dieback, *Hymenoscyphus pseudoalbidus*

## INTRODUCTION

For the past twenty years intense symptoms of European ash (*Fraxinus excelsior* L.) dieback could have been observed in Europe. While originally, this phenomenon was restricted to Poland and Lithuania (Sierota et al. 1993; Grzywacz 1995; Kowalski 2001; Stocki 2001; Przybył 2002; Vasiliauskas et al. 2006), the disease spread consequently to reach ashes in Scandinavia, western Europe, and some regions of southern Europe (Kirisits et al. 2009; Ioos et al. 2009; Timmermann et al. 2011; Hauptman et al. 2012). In 2012 the ash dieback symptoms were also reported from British Isles (Coghlan 2012). The fungus *Chalara fraxinea* T. Kowalski is believed to be the main causal agent of ongoing ash dieback process. This species was originally described based on cultures isolated from necrotic areas on ash shoots collected in Włoszczowa Forest District (Kowalski 2006, 2007). The artificial inoculation of ash shoots proved its high pathogenicity (Bakys et al. 2009; Kowalski, Holdenrieder 2009a). The occurrence of *Chalara fraxinea* was confirmed in more than twenty European countries where the ash dieback symptoms were observed (Halmschlager, Kirisits 2008; Ioos et al. 2009; Timmermann et al. 2011). Further studies revealed that *Chalara fraxinea* is an anamorphic state of fungus from the genus *Hymenoscyphus*. It was initially believed that it was *Hymenoscyphus albidus* (Robergere ex Desm.) W. Phillips, known in Europe since 1851 (Kowalski, Holdenrieder 2009b).

This opinion was however changed on the basis of molecular analyses which showed that this species is comprised of two, genetically distinct, subpopulations. They are hardly distinguishable morphologically, but they differ significantly in their potential to generate disease symptoms. Eventually the highly virulent subpopulation of *H. albidus* was recognized to be the teleomorphic state of *Chalara fraxinea* and described as a new cryptic species *Hymenoscyphus pseudoalbidus* Queloz et al. (Queloz et al. 2011). It produce the apothecia on previous year ash leaf rachises in the stand floor. The molecular studies based on two-hundred and thirty apothecia-derived cultures originating from four regions of Poland, confirmed the presence of highly virulent *H. pseudoalbidus* in declining ash stands while *H. albidus* was not found (Kraj, Kowalski 2013). The apothecia of *H. pseudoalbidus* are produced mainly from early July to the end of September (Kowalski 2012). This is also the season when apothecia release the ascospores that infect ashes and initiate their disease process. The number of *H. pseudoalbidus* apothecia produced in the stand floor indicates the amount of infectious material and consequently the risk level for *Fraxinus excelsior* stands.

The primary goal of the study was the estimation of the overall number of *H. pseudoalbidus* apothecia that may be produced on ash rachises in the leaf litter in the selected stands in southern Poland. Additionally, in order to correlate their presence with the stand health, the intensity of the selected disease symptoms for this stands was estimated.

## MATERIALS AND METHODS

The study was performed in August 2011 in 14 stands of Myślenice and 14 stands of Dynów Forest Districts. The selected stands represented mostly fresh upland broadleaved forest habitat, but they varied in age. The percentage of ash ranged from 40 to 90% for most stands, as only a few of them were characterized by lower proportion of ashes (Tabs 1, 2). The stands were selected randomly using information from forest management plans. Fifty centrally located neighboring trees of *Fraxinus excelsior* were examined for their health condition in each stand. Each examined tree was categorized as: i/ dead, ii/ with visible disease symptoms, or iii/ with no macroscopic disease symptoms. Furthermore, additional data were recorded for all trees classified as type ii: the presence of local trunk necroses, the presence of dead top, and the crown condition measured by the percentage of dead branches ranked as: below 25%, 26-50%, 51-75% and above 76% (Fig. 1). In total, the health condition evaluation was carried out for 1400 trees, 700 per each of two forest districts (Tabs 1, 2).

In order to evaluate the frequency of *H. pseudoalbidus* apothecia, 6 experimental plots were designated per stand (or 5 for two smallest stands). The plots, 0.5 × 0.5 m (0.25 m$^2$) each, were located in 50 m intervals along the diagonal of the stand. All previous year ash leaf rachises from each plot were collected, packed in separate, carefully described envelopes and stored at approx. 20°C in a laboratory. For each plot the rachises were sorted as those harboring *H. pseudoalbidus* apothecia or apothecia free ones and the overall number of rachises for both groups was counted. Subsequently, the number of *H. pseudoalbidus* apothecia was estimated, while the apothecia of initial state, that were hardly visible with naked eye, were not counted (see Fig. 6). Apart from the overall number of rachises and apothecia, the parallel number per 1 m$^2$ was calculated for each stand. In total, the estimation of apothecia frequency was carried out in 82 plots in Myślenice and 84 plots in Dynów Forest Districts.

## RESULTS

The ash dieback symptoms were present in all analyzed stands (Tabs 1, 2). The following disease signs could be observed: dead branches or branch tops, dead tree tops, local trunk necroses associated occasionally with brown exudates, and epicormic shoots developing on trunks and at the base of living branches. The frequency of these symptoms varied between the analyzed stands. Although dead branches could be observed for 70.4% trees in Myślenice (Tab. 1) and 79.9% trees in Dynów (Tab. 2) Forest Districts, the branch dieback process was not very advanced as it did not exceeded 25% of the crown for most individuals (Fig. 1). Some trees however were characterized by more than 50% or even 75% proportion of dead branches. These trees were twice more frequent in Myślenice Forest District (Fig. 1). On the

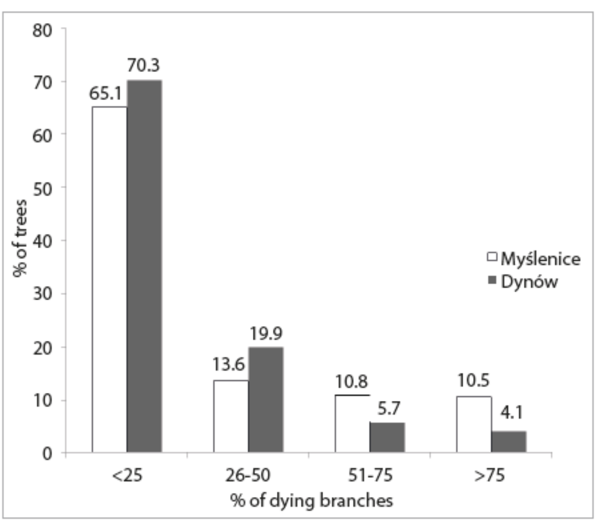


Fig. 1. The intensity of branch dieback of *Fraxinus excelsior* in stands of Myślenice and Dynów Forest Districts.

Table 1
The occurrence of disease symptoms on *Fraxinus excelsior* and the occurrence of the *H. pseudoalbidus* apothecia on ash leaf rachises in the litter in Myślenice Forest District

| Age class (years) | Compartment subcompart-ment | Site type *1 | Ash percentage | Number of analyzed trees | Number (%) of trees | | | | Number of plots | Number of rachises*3 | | Number of apothecia*3 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | dead | with dead top | with dead branches | symptom-less | | on plots | per 1 m$^2$ | on plots | per 1 m$^2$ |
| < 20 | 3 g | La | 60 | 50 | 23 | - | 25 | 2 | 5 | 23 | 18 | 168 | 134 |
| | 26 f | La | 10 | 50 | 18 | - | 23 | 9 | 5 | 57 | 46 | 280 | 224 |
| Σ | | | | 100 | 41(41.0) | - | 48(48.0) | 11(11.0) | 10 | 80 | 32 | 448 | 179 |
| 21-40 | Tp 1*2 | Lc | 50 | 50 | 8 | - | 40 | 2 | 6 | 43 | 29 | 748 | 499 |
| | 48 b | Lb | 60 | 50 | 18 | - | 28 | 4 | 6 | 21 | 14 | 330 | 220 |
| Σ | | | | 100 | 26(26.0) | - | 68(68.0) | 6(6.0) | 12 | 64 | 21 | 1078 | 359 |
| 41-60 | 9 d | La | 40 | 50 | 12 | - | 35 | 3 | 6 | 92 | 61 | 571 | 381 |
| | 29 h | La | 50 | 50 | 9 | - | 37 | 4 | 6 | 100 | 67 | 998 | 665 |
| | 99 d | Lc | 80 | 50 | 6 | - | 41 | 3 | 6 | 82 | 55 | 899 | 599 |
| | 304 a | Lc | 70 | 50 | 8 | - | 41 | 1 | 6 | 87 | 58 | 2003 | 1335 |
| Σ | | | | 200 | 35(17.5) | - | 154(77.0) | 11(5.5) | 24 | 361 | 60 | 4471 | 744 |
| > 60 | 27 a | Lc | 10 | 50 | 30 | - | 19 | 1 | 6 | 55 | 37 | 690 | 460 |
| | 136 d | Ld | 40 | 50 | 9 | - | 40 | 1 | 6 | 96 | 64 | 1357 | 905 |
| | 279 b | La | 60 | 50 | 5 | 2 | 41 | 2 | 6 | 67 | 45 | 1195 | 797 |
| | 279 c | La | 50 | 50 | 9 | 2 | 38 | 1 | 6 | 91 | 61 | 1330 | 887 |
| | 279 d | La | 60 | 50 | 1 | 1 | 42 | 6 | 6 | 134 | 89 | 1305 | 870 |
| | 280 g | La | 80 | 50 | 4 | - | 43 | 3 | 6 | 98 | 65 | 603 | 402 |
| Σ | | | | 300 | 58(19.3) | 5(1.7) | 223(74.3) | 14(4.7) | 36 | 541 | 60 | 6480 | 720 |
| Total | | | | 700 | 160(22.9) | 5(0.7) | 493(70.4) | 42(6.0) | 82 | 1046 | 51 | 12477 | 609 |

*1 La = fresh upland deciduous forest, Lb = wet upland deciduous forest, Lc = riparian upland deciduous forest, Ld = wet mountain deciduous forest

*2 Tp 1 – the stand established on former agricultural land

*3 the average values were rounded to full number

Table 2
The occurrence of disease symptoms on *Fraxinus excelsior* and the occurrence of the *H. pseudoalbidus* apothecia on ash leaf rachises in the litter in Dynów Forest District

| Age class (years) | Compartment subcompartment | Site type *1 | Ash percentage | Number of analyzed trees | Number (%) of trees | | | | Number of plots | Number of rachises*2 | | Number of apothecia*2 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | dead | with dead top | with dead branches | symptomless | | on plots | per 1 $m^2$ | on plots | per 1 $m^2$ |
| < 20 | 57 d | La | 50 | 50 | 5 | - | 45 | - | 6 | 42 | 28 | 240 | 160 |
| | 95 b | La | 70 | 50 | 8 | 1 | 41 | - | 6 | 36 | 24 | 220 | 147 |
| | 95 c | La | 80 | 50 | 11 | 2 | 37 | - | 6 | 31 | 21 | 154 | 103 |
| | 96 c | La | 80 | 50 | - | - | 40 | 10 | 6 | 26 | 17 | 169 | 113 |
| | 125 n | Lc | 50 | 50 | 4 | 5 | 41 | - | 6 | 8 | 5 | 25 | 17 |
| Σ | | | | 250 | 28(11.2) | 8(3.2) | 204(81.6) | 10(4.0) | 30 | 143 | 19 | 808 | 108 |
| 21-40 | 68 a | La | 10 | 50 | 1 | 1 | 48 | - | 6 | 77 | 51 | 464 | 309 |
| | 97 d | Lc | 20 | 50 | - | 15 | 32 | 3 | 6 | 38 | 25 | 288 | 192 |
| Σ | | | | 100 | 1(1.0) | 16(16.0) | 80(80.0) | 3(3.0) | 12 | 115 | 38 | 752 | 250 |
| 41-60 | 98 i | La | 30 | 50 | - | 2 | 46 | 2 | 6 | 89 | 59 | 561 | 374 |
| | 107 a | Lc | 60 | 50 | - | 3 | 47 | - | 6 | 40 | 27 | 258 | 172 |
| | 107 d | La | 90 | 50 | 3 | 5 | 42 | - | 6 | 55 | 37 | 466 | 311 |
| | 108 a | La | 50 | 50 | 4 | 7 | 39 | - | 6 | 65 | 43 | 326 | 217 |
| | 109 a | Lc | 50 | 50 | - | 18 | 32 | - | 6 | 81 | 54 | 420 | 280 |
| Σ | | | | 250 | 7(2.8) | 35(14.0) | 206(82.4) | 2(0.8) | 30 | 330 | 44 | 2031 | 271 |
| >60 | 110 a | Lc | 80 | 50 | - | 24 | 26 | - | 6 | 41 | 27 | 260 | 173 |
| | 125 t | Lc | 10 | 50 | 1 | 6 | 43 | - | 6 | 10 | 7 | 55 | 37 |
| Σ | | | | 100 | 1(1.0) | 30(30.0) | 69(69.0) | - | 12 | 51 | 17 | 315 | 105 |
| Total | | | | 700 | 37(5.3) | 89(12.7) | 559(79.9) | 15(2.1) | 84 | 680 | 32 | 4166 | 198 |

*1 La = fresh upland deciduous forest, Lc = riparian upland deciduous forest

*2 the average values were rounded to full number

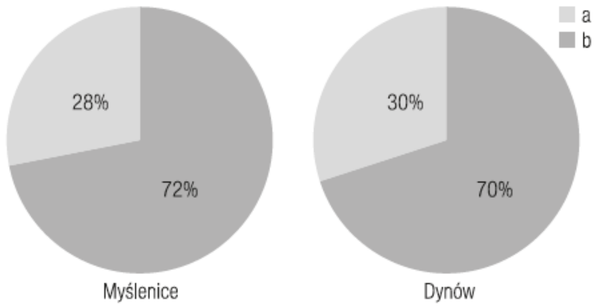


Fig. 2. The occurrence of necrotic areas on trunks of *Fraxinus excelsior* in Myślenice and Dynów Forest Districts; a – trunks with necrotic areas rachises with apothecia, b – trunks without necrotic areas rachises without apothecia.

other hand, 12.5% of ashes in Dynów Forest District were characterized by dead tree top. These trees were especially frequent in compartments 110a, 109a and 97d (Tab. 2). For Myślenice Forest District, dead tops were observed for only 0.7% of ashes. The disease process has led to the death of some trees. In Myślenice Forest District 22.7% of overall 700 analyzed trees were found to be dead. Those trees were especially frequent it two young stands less than 20 years old and one 73 years old in compartment 27a (Tab. 1). The percentage of dead trees in Dynów Forest District has reached the level of 5.3%. These trees were especially frequent here in young stands i.e. less than 20 years old (Tab. 2). The local trunk necroses were observed on 28 - 30% of trees (Fig. 2). Among analyzed trees, 6.0% in Myślenice and 2.1% in Dynów Forest Districts did not show any macroscopic disease symptoms on trunk or in the crown (Tabs 1, 2).

The 82, 0.5 × 0.5 m sized, experimental plots located in Myślenice Forest District contained 1046 previous year ash leaf rachises (Tab. 1). There was between 21 and

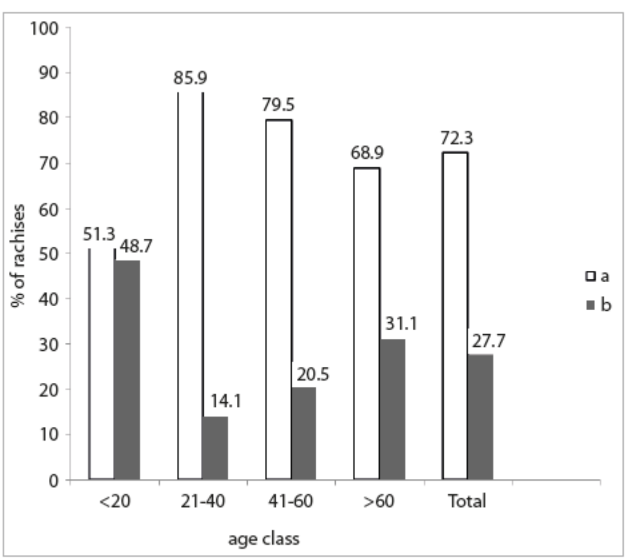


Fig. 3. The proportion of ash leaf rachises according to the presence of *Hymenoscyphus pseudoalbidus* apothecia in the Myślenice Forest District stands.

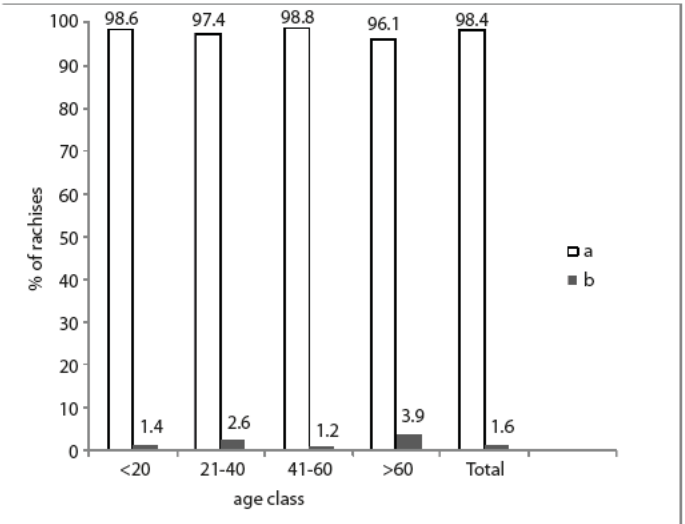


Fig. 4. The proportion of ash leaf rachises according to the presence of *Hymenoscyphus pseudoalbidus* apothecia in the Dynów Forest District stands.

134 rachises in 6 plots of a single stand. The approximate number of rachises per 1 m$^2$ in over 40 years old stands exceeded twice to threefold their number in stands of lower age classes (Tab. 1). At the time of the analysis, in August 2011, 72.3% of rachises harbored the *H. pseudoalbidus* apothecia (Fig. 3). The lowest proportion of apothecia-carrying rachises was observed in stands younger than 20 years old (Fig. 3). The overall number of *H. pseudoalbidus* apothecia reached 12477. Approximately the highest number of apothecia per 1 m$^2$ was recorded in 41-60 years old stands, but the lowest – in the young, less than 20 years old, stands (Tab. 1). The number of apothecia per 1 m$^2$ in particular stands varied from 134 (compartment 3g) to 1335 (compartment 304a). While extrapolating to a greater scale, these data show that 1 hectare may contain from 1 340 000 to even 13 350 000 apothecia at a time.

The 84, 0.5 × 0.5 m sized, experimental plots located in Dynów Forest District contained 680 previous year ash leaf rachises (Tab. 2). There was between 8 and 89 rachises in 6 plots of a single stand. Approximately the number of rachises per 1 m$^2$ in 41-60 years old stands exceeded more than twice their number in younger than 20 years old stands (Tab. 2). At the time of the analysis, in August 2011, 98.4% of rachises harbored the *H. pseudoalbidus* apothecia (Fig. 4). Only slight differences in proportion of apothecia-carrying rachises were observed between the stands (Fig. 4). The overall number of *H. pseudoalbidus* apothecia reached 4166. Approximately, 41-60 years old stands had the highest number of apothecia per 1 m$^2$ (Tab. 2). The number of apothecia per 1 m$^2$ in particular stands varied from 37 (compartment 125t) to 311 (compartment 107d). Therefore, 1 hectare may contain from 370 000 to 3 110 000 apothecia at a time.

All apothecia-carrying and some of apothecia-free rachises were covered with blackened stroma-like mycelium (Figs 5, 6). The freshly developed apothecia were white while the dry ones become light cinnamon. The stipes were blackened at the base (Fig. 6). The apothecia diameter usually ranged from 2 to 5 mm, but as large as 7 mm ones were also observed. With stereomicroscope, it was also possible to spot the initial states of apothecia (Fig. 6).

Fig. 5. The *Hymenoscyphus pseudoalbidus* apothecia on previous year ash rachises in the litter (rachises covered by black fungal plectenchyma).

Fig. 6. Fragment of ash leaf rachis with one mature apothecium and several apothecia in the initial stage of development.

## DISCUSSION

The characteristic symptoms of ash dieback were observed in all analyzed stands of Myślenice and Dynów Forest Districts. The nature of these symptoms was the same as the ones observed in the other parts of Poland (Kowalski, Czekaj 2010), or in other European countries (Ioos et al. 2009; Kirisits et al. 2009; Hauptman et al. 2012). However, the intensity of disease process, measured by the number of trees with high proportion of dead branches, trees with dead tops, or entirely dead trees varied significantly between two forest districts or even between stands of particular forest districts. These differences result from the age of trees, habitat properties, and the ash susceptibility (Gil et al. 2006; Jaworski 2011; McKinney et al. 2011; Kowalski et al. 2012; Stener 2013). Local conditions are also an important factor that affects the formation of the infectious material reservoirs of *H. pseudoalbidus* (anamorph: *Chalara fraxinea*), the ash dieback causal agent (Kirisits et al. 2009; Holdenrieder 2012; Kowalski et al. 2012).

According to previous research, the *Chalara fraxinea* conidia cannot germinate and the *F. excelsior* trees are infected exclusively by wind-borne ascospores, that are produced in apothecia on ash leaf residues in the litter (Kirisits et al. 2009; Kowalski 2012; Gross, Holdenrieder 2013). Only occasionally apothecia may develop on dead ash shoots (Kowalski et al. 2010; Holdenrieder 2012). Beside shoots, also leaves are infected by ascospores. The *H. pseudoalbidus* infected rachises of fallen leaves darken due to formation of melanized, stroma-like plectenchyma that functions as protection against competing microorganisms, and against unsuitable environmental conditions (Gross, Holdenrieder 2013). All *H. pseudoalbidus* apothecia-carrying rachises, as well as many apothecia-free ones, were covered by plectenchyma. The total numbers of ash rachises on experimental plots of particular analyzed stands were varied. This resulted mainly from the various proportion of ash in the species composition of the stand, but also from the health condition of trees, especially from the level of foliage reduction. On the other hand, the relationship between the total number of rachises on plots and the number of apothecia-carrying rachises may be affected by many factors.

The development of apothecia may be delayed or even prevented by excessively dry conditions (Holdenrieder 2012; Kowalski et al. 2012). Furthermore, *H. pseudoalbidus* is a heterothalic fungal species, thus the apothecia formation requires the infected rachis to contain both mating types (Gross et al. 2012). Finally, the proportion of apothecia-carrying rachises depends on the on-tree leaf infection frequency by this fungus. If the leaves do not fall due to disease process but naturally, or the premature leaf falling is the result of the trunk- or the basal-branch-part necrosis, the proportion of *H. pseudoalbidus* infected rachises is lower. The above factors were most probably the reasons why the total number of apothecia counted on 82 plots in Myślenice Forest Districts exceeded threefold their number on 84 plots in Dynów Forest District. Those factors probably affected also the number of apothecia per 1 $m^2$ estimated for various stands, as they varied significantly in both forest districts. These differences are evidenced by data showed in tables 1 and 2. The data, when extrapolated to the area of 1 hectare, show even more explicitly that the amount of infectious material in stands with the presence of *F. excelsior* is huge. In fact, the number of the apothecia produced in a given stand is even greater, as the apothecia formation on the particular rachis is not a one-off exercise but a continuous process. While some apothecia are fully developed and release ascospores, some others are in initial, hardly visible, stage that can be seen on Figure 6. In Poland, the *H. pseudolabidus* apothecia are produced for about 3 months usually from July to September.

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# Występowanie miseczek *Hymenoscyphus pseudoalbidus* w ściole w zamierajacych drzewostanach jesionowych w południowej Polsce

## Streszczenie

Badania prowadzono w sierpniu 2011 roku w 14 drzewostanach jesionowych o różnym wieku na terenie Nadl. Myślenice oraz w 14 takich drzewostanach w Nadl. Dynów. W każdym drzewostanie, w jego środkowej części, dokonano oceny nasilenia występowania wybranych symptomów chorobowych u 50 rosnących bezpośrednio obok siebie drzew *Fraxinus excelsior.* Ogółem analizie stanu zdrowotnego poddano 1400 drzew. Dla oceny częstości występowania miseczek *Hymenoscyphus pseudoalbidus* w każdym drzewostanie zaznaczono 6 (w dwu drzewostanach o małej powierzchni po 5) poletek o wymiarach 0.5 × 0.5 m, w odstępie, co 50 m wzdłuż przekątnej drzewostanu. Z poletek zebrano i policzono wszystkie ubiegłoroczne nerwy liściowe i wykształcone na nich miseczki *H. pseudoalbidus*. Ogółem założono 82 poletka w drzewostanach Nadl. Myślenice i 84 poletka w drzewostanach Nadl. Dynów.

We wszystkich analizowanych drzewostanach jesion wykazywał różnego typu objawy chorobowe: nekrozy na pniach, zamieranie gałęzi, zamieranie wierzchołków oraz całych drzew. Na 82 poletkach o wymiarach 0.5 × 0.5 m w Nadl. Myślenice stwierdzono 1046 nerwów liściowych jesionu. Na 72.3% z nich było wykształconych 12477 miseczek *H. pseudoalbidus.* Na 84 poletkach w Nadl. Dynów stwierdzono 680 nerwów liściowych jesionu. Na 98.4% z nich było wykształconych 4166 miseczek tego patogenu. Uzyskane dane wskazują na ogromny rezer-

wuar materiału infekcyjnego *H. pseudoalbidus* wykształcający się w ściole w drzewostanach jesionowych. Z szacunkowych przeliczeń wynika, że w analizowanych drzewostanach na powierzchni 1 hektara może dojrzewać w tym samym czasie pomiędzy 370 000 a 13 350 000 miseczek.