MYCOBIOTA IN THE SEEDS OF NARROW-LEAVED ASH (FRA XinUS ANGUSTIFOLIA VAHL)

SUMMARY

Narrow-leaved ash (Fraxinus angustifolia), currently the most damaged forest tree species in the Republic of Croatia, is suffering from dieback primarily caused by pathogenic fungus Hymenoscyphus fraxineus. Since health status of seeds is very important for future seedling production, objective of this study was to screen narrow-leaved ash seeds for presence of this main pathogen and other potentially parasitic fungi. Seeds were collected from five locations and analysed using three different methods. Results revealed relatively good health status of inspected seeds, with total of 15 different fungal taxa identified in less than 40% of samples and no confirmation of Hymenoscyphus fraxineus presence. Most frequently detected fungi were various species of genus Alternaria and species Sphaerulina berberidis, while other taxa occurred rarely. Although identified fungal species haven’t caused visible symptoms on seeds after one to two months of storage, many of them are known seed pathogens or opportunistic ash (Fraxinus spp.) pathogens and could have a negative effect on seeds after longer period of storage or storage in unfavourable conditions.

KEY WORDS: fungal isolation, nested PCR, Alternaria sp., Sphaerulina berberidis

INTRODUCTION

Narrow-leaved ash (Fraxinus angustifolia Vahl), ecologically and economically very important species in lowland forests, is currently the most damaged forest tree species in the Republic of Croatia with 75% of trees having significantly defoliated crown according to the ICP Forests program data for 2017 (Potočić et al. 2018). Existing research revealed that, among other factors, there are several parasitic fungi involved in the decline in roots and stem collars of affected trees (Kranjec 2017), with pathogenic fungus Hymenoscyphus fraxineus (T. Kowalski) Baral, Queloz & Hosoya confirmed as the primary causative agent of crown dieback at multiple locations (Diminić 2015, Milotić et al. 2016). Presence of this pathogen responsible for large-scale dieback of common (Fraxinus excelsior L.) and narrow-leaved ash throughout Europe has been confirmed in roots, stems, branches, shoots, petioles and leaves of both tree species (Kowalski 2006, Gross et al. 2014, Chandelier et al. 2016), but also in the symptomatic and visually healthy seeds of common ash from Latvia and Sweden (Cleary et al. 2013, Hayatgheibi 2013, Marčiulynienė et al. 2018).

Yield and health status of narrow-leaved ash seeds are of great importance in the Republic of Croatia, since they are...
necessary for nursery production of seedlings, majority of which are further used for forest stand regeneration or afforestation. For this purpose seeds are collected from adult trees in existing natural stands selected and registered as seed sources, forest stands which are phenotypically above average and specially managed for the purpose of seed collection and thus registered as seed stands and seed orchards established also for the purpose of seed collecting, from the genetically superior individual trees (Anon 2009, 2011, 2013, 2014).

Fungal presence in the seeds of forest tree species in general is considered to be a significant cause of shortened seed longevity during storage (Sutherland et al. 2002), reduced seed germination due to embryo or endosperm deterioration and potential cause of diseases that affect other developmental stages of plants, such as increased damping-off, shoot dieback, cankers and dieback of older seedlings (Cram 2009), although number of species just act as endophytes or saprotrophs and do not adversely affect the performance of seeds sown in nurseries (Mittal and Wang 1987).

In Croatian narrow-leaved ash forest stands there was a recorded case of seedlings delivered from a nursery Zalužje, Forestry Office (FO) Vinkovci, which expressed symptoms of *Hymenoscyphus fraxineus* dieback approximately one month after being planted in the field, FO Vinkovci, Management Unit (MU) Vrbanske šume, Subcompartment (SC) 91b, although the pathogen wasn’t confirmed on older ash trees sampled in the area nearby SC 132a (MU Vrbanske šume) and SC 49a (MU Kusare) (FO Vinkovci) (Anon 2015). This finding raised a question of infection origin and possibility that pathogen spread from seeds into the plant tissue, eventually causing visible dieback symptoms in grown seedlings.

The objective of this research was to screen narrow-leaved ash forest stands for the presence of pathogenic fungus *Hymenoscyphus fraxineus* and simultaneously detect other possible seed-borne pathogens in order to estimate the health status and suitability of seeds collected from registered seed sources and seed stands for further nursery seedling production.

**MATERIALS AND METHODS**

*Fraxinus angustifolia* seeds were collected in period from August to November 2017 from visually healthy trees in four natural forest stands registered as narrow-leaved ash seed sources and one registered narrow-leaved ash seed stand (Table 1). Seeds were examined for fungal presence after one to two months of storage at room temperature, using both classical method of mycelia isolation on artificial media and a nested PCR method to analyse DNA directly from seeds. Seeds were additionally screened for presence of pathogenic fungus *Hymenoscyphus fraxineus* using species specific primers (Johansson et al. 2010).

**Isolation of fungi from seeds – Izolacija gljiva iz sjemena**

Twelve seeds from each of five locations were used for fungal isolation on malt extract agar medium (MEA, Oxoid, Basingstoke, UK) supplemented with streptomycin sulphate (200 mg 1\(^{-1}\), Sigma-Aldrich, St. Louis, USA). Seeds were surface sterilized in a solution of sodium hypochlorite (approx. 4% active chlorine) for one minute and then rinsed three times in sterile distilled water. Seeds cut in half were plated on medium in Petri dishes (9 cm diameter) and incubated in dark at 20 °C for four weeks (Bulovec 2018). Petri dishes were checked weekly for fungal growth and emerging mycelia were subcultured to MEA medium. Pure cultures were grouped into morphotypes and at least one isolate of each morphotype group was used for molecular identification.

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**Extraction of DNA was performed according to Allemann et al. (1999) with modifications (Kranjec et al. 2017) and PCR amplification was conducted with primers ITS 1 and ITS 4 (White et al. 1990) in 25 µl reactions containing 200 µM deoxyribonucleoside triphosphates, 0.4 µM of each primer, 0.5 U of Taq DNA polymerase with reaction buffer (Sigma-Aldrich, St. Louis, USA), 1.5 mM MgCl\(_2\), and 1 µl of 100-fold diluted DNA template. Cycling conditions were as follows: an initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 45 s, extension at 72 °C for 90 s and a final

### Table 1. Locations and dates of narrow-leaved ash seed collection

<table>
<thead>
<tr>
<th>Location</th>
<th>Dates of seed collection (year 2017)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gunja</td>
<td>22 August – 6 September</td>
</tr>
<tr>
<td>Lipovljani</td>
<td>21 August – 15 September</td>
</tr>
<tr>
<td>Novoselec</td>
<td>25 August – 3 November</td>
</tr>
<tr>
<td>Vukovar</td>
<td>23 August – 30 August</td>
</tr>
<tr>
<td>Županja</td>
<td>23 August – 6 September</td>
</tr>
</tbody>
</table>
extension step at 72 °C for 5 min. The resulting PCR products were sequenced using primer ITS 4 at the DNA sequencing facility of Macrogen Europe (Amsterdam, Netherlands). After processing raw data using the BioEdit Sequence Alignment Editor v.7.2.5 software (Hall 1999), sequences were identified by comparison with reference sequences in NCBI GenBank using BLAST tool (Altschul et al. 1990). Sequences with 98 – 100% similarity were identified to the species level and with 94 – 97% of similarity to the genus level (Bakys et al. 2011).

Analysis of DNA from seeds – Analiza DNA iz sjemena

Twenty seeds from each of five locations were analyzed for fungal presence using a nested PCR method. After surface disinfection of samaras by immersing them in 35% H2O2 for three minutes, seeds were aseptically removed, cut into small pieces (1 – 2 mm long), placed in separate 2 ml centrifuge tubes and freeze-dried for 24 h (Cleary et al. 2013). Samples were homogenized in TissueLyser II (Qiagen, Hilden, Germany) at 30 Hz for two minutes. DNA was extracted following the protocol according to Minas et al. (2011). First PCR was conducted using the primers ITS1-F (Gardes and Bruns 1993) and ITS 4 (White et al. 1990) under the same cycling conditions and with same reagents concentrations as in the described PCR protocol used for DNA analysis of isolated mycelia. The PCR products were size separated by gel electrophoresis on 2% agarose gels stained with GelStar Nucleic Acid Gel Stain (Lonza, Rockland, USA) and visualised under UV light. All bands were aseptically excised from the gel, purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, USA) and re-amplified in a second PCR using the primers ITS 1 and ITS 4 (White et al. 1990) under the same cycling conditions and with same reagents concentrations as in the first one. The resulting PCR products were sequenced using primer ITS 4 at the DNA sequencing facility of Macrogen Europe (Amsterdam, Netherlands) and identified using NCBI GenBank database as already described in this paper.

Detection of Hymenoscyphus fraxineus in seeds – Utvrđivanje prisutnosti gljive Hymenoscyphus fraxineus u sjemenu

DNA extracted from seeds, as previously described, was additionally checked for the presence of Hymenoscyphus fraxineus in a PCR reaction with species specific primers: forward (5’AGCTGGGGAAACCTGACTG) and reverse (5’ACACCGCAAGGCACCTATC) (Johansson et al. 2010), and with same reagents concentrations as in previous analysis. The thermal cycling was carried out as follows: an initial denaturation step at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 60 s, extension at 72 °C for 30 s and a final extension step at 72 °C for 7 min (Hayatgheibi 2013). DNA of confirmed Hymenoscyphus fraxineus isolate obtained from earlier research (isolated from Fraxinus angustifolia stem collar, Kranjec 2017) was used as a positive control in each PCR reaction. PCR products were run on 1% agarose gels stained with GelStar Nucleic Acid Gel Stain (Lonza, Rockland, USA) and visualised under UV light.

RESULTS

REZULTATI

Analysis of Fraxinus angustifolia seeds by mycelia isolation on MEA medium and nested PCR revealed fungal presence in 20 – 58% of screened seeds, depending on the method used and location they originated from (Table 2). Isolation of mycelia on MEA medium resulted in growth of 26 fungal isolates belonging to 15 different taxa, 10 of which were identified to the species level (Table 3). The nested PCR analysis resulted in identification of 19 different fungal taxa, 10 of which were identified to the species level (Table 4).

Most frequently detected taxa were Sphaerulina berberidis and Alternaria sp. with Alternaria alternata and A. tenuissima identified to the species level. Among the most frequently detected were also seven sequences obtained in nested PCR which corresponded to Fungal endophyte isolate 4480 according to NCBI GenBank and might be a species of genus Sphaerulina, which is next closest match in the given database. Species of Alternaria occurred in the seeds from all five locations included in this research and Sphaerulina berberidis occurred in seeds from four of those locations (not confirmed only in seeds from stand HR-FAN-SI-111/030 in Vukovar).

Neither of sequences obtained by first two described methods belonged to Hymenoscyphus fraxineus. Presence of this pathogenic fungus in seeds was not confirmed by using
Table 3. Identified fungal taxa in narrow-leaved ash seeds by mycelia isolation on MEA medium

<table>
<thead>
<tr>
<th>Fungal taxa identified according to NCBI GenBank</th>
<th>Accession number in NCBI GenBank</th>
<th>Percentage of Fraxinus angustifolia seeds where fungus is present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alternaria sp.</td>
<td>MH137756</td>
<td>13,3%</td>
</tr>
<tr>
<td>Alternaria tenuissima (Kunze) Wiltshire</td>
<td>MH137745</td>
<td>3,3%</td>
</tr>
<tr>
<td>Cercospora beticola Sacc.</td>
<td>MH137755</td>
<td>1,6%</td>
</tr>
<tr>
<td>Cladosporium cladosporioides (Fresen.) G.A. de Vries</td>
<td>MH137753</td>
<td>1,6%</td>
</tr>
<tr>
<td>Cladosporium herbarum (Pers.) Link</td>
<td>MH137759</td>
<td>1,6%</td>
</tr>
<tr>
<td>Cladosporium sp.</td>
<td>MH137748</td>
<td>1,6%</td>
</tr>
<tr>
<td>Colletotrichum sp.</td>
<td>MH137751</td>
<td>1,6%</td>
</tr>
<tr>
<td>Phomopsis velata (Sacc.) Traverso</td>
<td>MH137754</td>
<td>1,6%</td>
</tr>
<tr>
<td>Phomopsis cucurbitae McKeen 1957</td>
<td>MH137752</td>
<td>1,6%</td>
</tr>
<tr>
<td>Botryosphaeria stevensii Shoemaker</td>
<td>MH137758</td>
<td>1,6%</td>
</tr>
<tr>
<td>Fusarium oxysporum Schltld.</td>
<td>MH137749</td>
<td>1,6%</td>
</tr>
<tr>
<td>Lophiotoma sp.</td>
<td>MH137750</td>
<td>1,6%</td>
</tr>
<tr>
<td>Penicillium sp.</td>
<td>MH137760</td>
<td>1,6%</td>
</tr>
<tr>
<td>Sphaerulina berberidis (Niessl) Quaedvl., Verkley &amp; Crous</td>
<td>MH137747</td>
<td>6,6%</td>
</tr>
<tr>
<td>Venturia fraxini Aderh.</td>
<td>MH137761</td>
<td>1,6%</td>
</tr>
</tbody>
</table>

Species of the most frequently detected genus in this research, Alternaria sp., haven’t caused visible symptoms on seeds although the identified Alternaria alternata and Alternaria tenuissima are reported as seed pathogens on Betula spp. and Robinia pseudoacacia L. (Lilja 1979, Sunita 1998) and causative agents of Malus spp. and Punica grata num L. fruit rot during storage (Zambounis et al. 2015). Alternaria alternata has also been found in cryptomycotic bark, wood and buds of declining Fraxinus excelsior (Pukacki and Przybyl 2005, Davydenko et al. 2013, Kowalski et al. 2016), indicating that it can act as an opportunistic pathogen in already declining ash tissue, possibly in the narrow-leaved ash seeds as well if they are under the influence of negative biotic and abiotic factors while on a tree or stored in unfavourable conditions after the harvest. Other frequently detected species, Sphaerulina berberidis, has so far been reported only as leaf endophyte of several tree species (Eo et al. 2014) and most probably has the same role in the narrow-leaved ash seeds since it hasn’t induced any visible symptoms in the analysed samples.

The remainder of identified species in narrow-leaved ash seeds were present in only one to three samples, but included some of the well known tree pathogens such as Phomopsis velata (synonym Diaporthe eres) and Botryosphaeria stevensii (synonym Diplodia mutila), which were also found in Fraxinus excelsior seeds in Latvia and Sweden (Cleary et al. 2013). Former is known for causing stem canker and dieback of several tree species (Quaroni et al. 1980, Anagnostakis 2007, Thomidis and Michailides 2009), fruit deterioration (Ristić et al. 2016) and being present in necrotic tissue and collar rots of Fraxinus excelsior (Kowalski et al. 2016, Langer 2017). Latter is known as a parasite involved in bark necrosis, canker formation and dieback of Fraxinus excelsior and Fraxinus ornus L., Quercus spp. and other tree species (Ragazzi et al. 1999, Przybyl 2002, Sidoti and Gramata 2004, Sims et al. 2016). Some of identified species are reported to be seed or fruit pathogens on other plant species, like Fusarium oxysporum on Robinia pseudoacacia seeds (Sunita 1999), Cladosporium cladosporioides on tobacco seeds (Nicotiana tabacum L.) (Wang et al. 2014) and stored hazelnuts (Corylus avellana L.) (Mohaddam and Taherzadeh 2007), and Cladosporium herbarum on stored figs (Ficus carica L.) (Montalegre et al. 2000) and Prunus spp. fruits (Tonini and Capriotti 1996). Venturia fraxini, known primarily as endophyte (Schlegel et al. 2016), but...
also confirmed in leaf blotches and other necrotic tissue on *Fraxinus* spp. (Anselmi 2001, Bakys et al. 2009), is first time reported in ash seeds. For the rest of the identified species there is no documented evidence of their presence in trees. Instead they are known for being pathogens of agricultural plants (*Cercospora beticola*, *Phomopsis cucurbitae*) (Bertetti et al. 2012, Vaghefi et al. 2017), pathogens of stored *Pisum sativum* L. seeds (*Aspergillus ruber*) (Harman et al. 1972), saprotrophs in forest soil (*Vishniacozyma tephrensis*) (Mašínová et al. 2017), parasites of nematodes (*Didymella heteroderae*) (Chen et al. 1996) and leaf spot causing agents on *Coprosma robusta* Raoul (*Mycosphaerella coacervata*) (Hood 1985). Since most of the described species were present at low frequencies their effect on general health status of seeds cannot be very significant, but ability of those known as potential parasites to induce symptoms and decline of seeds and later seedlings under the unfavourable conditions remains possible.

*Hymenoscyphus fraxineus* was not found in the seeds of *Fraxinus angustifolia* analysed in this research performed.

### Table 4. Identified fungal taxa in narrow-leaved ash seeds by nested polymerase chain reaction (PCR)

<table>
<thead>
<tr>
<th>Closest sequence match in NCBI GenBank</th>
<th>Fungal taxa identified</th>
<th>Accession number in NCBI GenBank</th>
<th>Percentage of <em>Fraxinus angustifolia</em> seeds where fungus is present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alternaria alternata</td>
<td><em>Alternaria alternata</em> (Fr.) Keissl.</td>
<td>MH137762, MH137763, MH137764, MH137765</td>
<td>4%</td>
</tr>
<tr>
<td>Alternaria brassicicola/A. alternata</td>
<td><em>Alternaria</em> sp. FA_N0V7</td>
<td>MH137766, MH137767</td>
<td>2%</td>
</tr>
<tr>
<td>Alternaria sp. isolate B6-25</td>
<td><em>Alternaria</em> sp. FA_L9</td>
<td>MH137768</td>
<td>1%</td>
</tr>
<tr>
<td>Alternaria alternata/A. porri/A. gaisen/A. tenuissima/A. brassicæ/A. mali/A. ochroleuca</td>
<td><em>Alternaria</em> sp. FA_Z11</td>
<td>MH137769</td>
<td>1%</td>
</tr>
<tr>
<td>Alternaria sp./Phoma sp./Talaromyces sp.</td>
<td><em>Ascomycota</em> sp. FA_L18</td>
<td>MH137770</td>
<td>1%</td>
</tr>
<tr>
<td>Aspergillus ruber</td>
<td><em>Aspergillus ruber</em> (Jos. König, Spieck. &amp; W. Bremer) Thom &amp; Church</td>
<td>MH137771</td>
<td>1%</td>
</tr>
<tr>
<td>Cladosporium A144</td>
<td><em>Cladosporium</em> sp. FA_V9</td>
<td>MH137772</td>
<td>1%</td>
</tr>
<tr>
<td>Cladosporium cladosporioides</td>
<td><em>Cladosporium</em> cladosporioides (Fresen.) G.A. de Vries</td>
<td>MH137773</td>
<td>1%</td>
</tr>
<tr>
<td>Cladosporium herbarum</td>
<td><em>Cladosporium</em> herbarum (Pers.) Link</td>
<td>MH137774</td>
<td>1%</td>
</tr>
<tr>
<td>Cryptococcus tephrensis</td>
<td><em>Vishniacozyma</em> tephrensis *Vishniac ex Xin Zhan Liu, F.Y. Bai, M. Groenew. &amp; Boekhout</td>
<td>MH137775</td>
<td>1%</td>
</tr>
<tr>
<td>Diaporthe eres</td>
<td><em>Phomopsis velata</em> (Sacc.) Traverso</td>
<td>MH137776</td>
<td>1%</td>
</tr>
<tr>
<td>Didymella heteroderae</td>
<td><em>Didymella heteroderae</em> (Sen Y. Chen, D.W. Dicks. &amp; Kimbr.) Qian Chen &amp; L. Cai</td>
<td>MH137777</td>
<td>1%</td>
</tr>
<tr>
<td>Dipodia mutila</td>
<td><em>Botryosphaeria stevensii</em> Shoemaker</td>
<td>MH137778, MH137779</td>
<td>2%</td>
</tr>
<tr>
<td>Mycosphaerella coacervata</td>
<td><em>Mycosphaerella coacervata</em> Syd.</td>
<td>MH137780</td>
<td>1%</td>
</tr>
<tr>
<td>Fungal endophyte isolate 4480</td>
<td><em>Fungal endophyte</em> FA_2017</td>
<td>MH137781, MH137782, MH137783, MH137784, MH137785, MH137786, MH137787</td>
<td>7%</td>
</tr>
<tr>
<td>Uncultured Ascomycota isolate FL7.5</td>
<td><em>Ascomycota</em> sp. FA_Z19</td>
<td>MH137788</td>
<td>1%</td>
</tr>
<tr>
<td>Phoma sp. ZP-40</td>
<td><em>Phoma</em> sp. FA_G14</td>
<td>MH137789</td>
<td>1%</td>
</tr>
<tr>
<td>Phomopsis sp. RJ-2015 isolate 310Jb14</td>
<td><em>Phomopsis</em> sp. FA_N6</td>
<td>MH137790</td>
<td>1%</td>
</tr>
<tr>
<td>Sphaerulina berberidis</td>
<td><em>Sphaerulina berberidis</em> (Niessl) Quaedvl., Verkley &amp; Crous</td>
<td>MH137791, MH137792, MH137793, MH137794</td>
<td>4%</td>
</tr>
</tbody>
</table>
by applied methodology, thus not supporting the hypothesis that fungus has spread from infected seeds to seedlings planted in the field from the local nursery. Still, these findings do not exclude the possibility that the fungus could be present and thus spread on the surface of samaras, since this aspect of transmission was not investigated. The fact that this pathogen has been confirmed in both symptomatic and visually healthy seeds from trees of various levels of susceptibility to the fungus in similar research conducted on *Fraxinus excelsior* (Cleary et al. 2013, Hayatgheibi 2013, Marčiulyniéné et al. 2018) and not in the *Fraxinus angustifolia* seeds analysed in this research, could be due to high summer temperatures (July and August 2017 maximum > 35 °C) (DHMZ 2017b, a) characteristic for the narrow-leaved ash distribution area in the Republic of Croatia, which seems to be a limiting factor for the spread of pathogen (Hauptman et al. 2013, Grosdidier et al. 2018) or due to seed collection method, where only seeds from visually healthy narrow-leaved ash trees from registered seed stands and natural stands registered as seed sources are collected for further purpose of nursery seedling production. In addition, recent surveys conducted by Marčiulyniéné et al. (2018) found no evidence of fungus being able to spread from infected seeds to grown plants, which still doesn't exclude this possibility in the opinion of authors.

**CONCLUSION**

Analysed narrow-leaved ash seeds collected from visually healthy trees from registered seed sources and seed stand revealed relatively low level fungal presence in comparison to other similar studies, indicating good health status and usability for further nursery seedling production regarding this particular aspect. Identified fungal species haven’t caused visible symptoms on seeds after one to two months of storage, not excluding their possible negative effect on seeds after longer period of storage or storage in unfavourable conditions, since some of them are known as seed pathogens and some are reported as opportunistic parasites in necrotic tissues of *Fraxinus* spp. Presence of pathogenic fungus *Hymenoscyphus fraxineus* in seeds was not confirmed, so it can be concluded that potential dieback of seedlings caused by this pathogen in nurseries or in the field is a consequence of infections from affected narrow-leaved ash stands in the vicinity rather than spread of fungus from infected seeds.

**ACKNOWLEDGEMENT**

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Poljski jasen (Fraxinus angustifolia Vahl) je u Republici Hrvatskoj trenutno najoštećenija šumska vrsta drveća, sa 75 % stabala značajno osute krošnje prema podacima međunarodnog programa ICP Forests iz 2017. godine. Dosadašnja su istraživanja potvrdila patogenu gljivu Hymenoscyphus fraxineus kao primarnog uzročnika odumiranja krošanja poljskoga jasena na više lokacija te utvrdila njenu prisutnost u listovima, izbojcima, granama, bazi debla te korijenu stabala. Cilj ovog istraživanja bio je ispitati prisutnost navedenog patogena u sjemenu poljskoga jasena, a također i identificirati ostale vrste potencijalno parazitskih gljiva, kako bi se s navedenog aspekta moglo procijeniti zdravstveno stanje vrste i utvrđeno njeno zdravstveno stanje. Sjeme je prikupljeno na pet lokacija u sastojinama kategoriziranim kao sjemenski izvor ili sjemenska sastojina na području šumarija Novoselec, Lipovljani, Gunja, Županja i Vukovar. Za analizu sjemena skladištenog jedan do dva mjeseca korištene su tri različite metode, uključujući klasičnu metodu izolacije gljiva iz tkiva na hranjive podloge te molekularne metode izolacije ukupne stanične DNK iz sjemena i umnažanja ciljanih sekvenci u lančanoj reakciji polimerazom korištenjem univerzalnih početnica (ITS 1, ITS 1 – F, ITS 4) i početnica specifičnih za gljivu Hymenoscyphus fraxineus.

Analizom utvrđeno ukupno 15 različitih taksona gljiva u manje od 40 % ispitivanog sjemena, ukazujući na njegovu relativno dobro zdravstveno stanje. Najčešće su identificirani pripadnici roda Alternaria, od kojih su A. alternata i A. tenuissima identificirane do razine vrste, te vrsta Sphaerulina berberidis. Ostali identificirani taksoni zabilježeni su na svega jednoj do tri sjemenke. Iako utvrđeni taksoni gljiva nisu uzrokovali vidljive simptome ili propadanje sjemena nakon jednog do dva mjeseca skladištenja, velik broj njih se u literaturi navodi kao patogeni sjemena i plodova različitih vrsta drveća, a dio i kao oportunistički paraziti prisutni u nekrotičnom tkivu jasena (Fraxinus spp.), zbog čega se ne može u potpunosti isključiti njihov negativan utjecaj na sjeme tijekom duljih perioda skladištenja ili izlaganja nepovoljnim uvjetima. Vrsta Hymenoscyphus fraxineus niti jednom korištenom metodom nije utvrđena u analiziranom sjemenu, te nije dokazana mogućnost njena širenja na uzgojene sadnice ovim putem. Time nije isključena mogućnost njene prisutnosti na površini plodova, tj. perutki, koje su u ovom istraživanju površinski sterilizirane kako bi se smanjio utjecaj uobičajenih prisutnih epifitnih gljiva na rezultate.

**SAŽETAK**

Poljski jasen (Fraxinus angustifolia Vahl) je u Republici Hrvatskoj trenutno najoštećenija šumska vrsta drveća, sa 75 % stabala značajno osute krošnje prema podacima međunarodnog programa ICP Forests iz 2017. godine. Dosadašnja su istraživanja potvrdila patogenu gljivu Hymenoscyphus fraxineus kao primarnog uzročnika odumiranja krošanja poljskoga jasena na više lokacija te utvrdila njenu prisutnost u listovima, izbojcima, granama, bazi debla te korijenu stabala. Cilj ovog istraživanja bio je ispitati prisutnost navedenog patogena u sjemenu poljskoga jasena, a također i identificirati ostale vrste potencijalno parazitskih gljiva, kako bi se s navedenog aspekta moglo procijeniti zdravstveno stanje vrste i utvrđeno njeno zdravstveno stanje. Sjeme je prikupljeno na pet lokacija u sastojinama kategoriziranim kao sjemenski izvor ili sjemenska sastojina na području šumarija Novoselec, Lipovljani, Gunja, Županja i Vukovar. Za analizu sjemena skladištenog jedan do dva mjeseca korištene su tri različite metode, uključujući klasičnu metodu izolacije gljiva iz tkiva na hranjive podloge te molekularne metode izolacije ukupne stanične DNK iz sjemena i umnažanja ciljanih sekvenci u lančanoj reakciji polimerazom korištenjem univerzalnih početnica (ITS 1, ITS 1 – F, ITS 4) i početnica specifičnih za gljivu Hymenoscyphus fraxineus.

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**KLJUČNE RIJEČI:** izolacija gljiva, ugniježdeni PCR, Alternaria sp., Sphaerulina berberidis