Thesis of University Doctoral (PhD) dissertation

INVESTIGATION AND IDENTIFICATION OF FUNGAL POPULATIONS OF ROTTED WALNUT USING MICROBIOLOGICAL AND MOLECULAR GENETIC METHODS

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1. THE ANTECEDENTS AND THE OBJECTIVES OF THE DOCTORAL DISSERTATION

English walnut (*Juglans regia* L.) is an ancient species, one of the 25 fruit species that were known in the Neolithic (Rapaics, 1940). Its main gene center is Persia, but it is native in Hungary, because here is one of the secondary gene centers (Vavilov, 1928). It has many beneficial effects on health, which are due to its chemical composition. The regular consumption of walnut contributes to reducing the risk of coronary heart disease (Davis et al., 2007). In addition, walnut kernel is an excellent source of essential fatty acids and tocopherols (Amaral et al., 2003; 2004). Walnut is grown in the third largest area in Hungary, but the yield has shown a continuous decrease in recent years, despite the increase in harvested areas, however the export balance is positive (FAOSTAT, 2021). In the last decade, as a result of globalization and climate changes, new pathogens and pests have appeared in walnut orchards (Voigt–Tóth, 2013; Kovács et al., 2018;). The disease caused by fungi, which affects all parts of the walnut tree, is a worldwide problem that causes significant losses in the sector (Chen et al., 2014; Meng et al., 2018; Moral et al., 2019).

Species of the Botryosphaeriaceae and Diaporthaceae families are often detected as pathogens in many woody plants, including nut trees (León et al., 2020; López-Moral et al., 2020; Luna et al., 2022). In the mid-2010s, Hungarian growers faced a serious plant protection challenge due to dark, shrivelled and moldy kernels. During a preliminary survey, we cultured a large proportion of fungi belonging to the genera *Diaporthe* and *Diplodia* from symptomatic walnuts (Kovács et al., 2018). These pathogens can survive in dormant and dead plant parts, then penetrate plant tissues through wounds (Moral et al., 2019; López-Moral et al., 2020). The injuries serve as entrance for pathogens can be caused by pruning, sunburn, hail, and animals (Michailides 1991; Michailides et al., 2014).

The spread of the Botryosphaeriaceae and Diaporthaceae fungi may be further aggravated by the acceleration of climate change, which may be a serious threat to the nut trade (Kybartaite et al., 2020; Garcia et al., 2021), as there is no effective plant protection agent approved for walnut trees against these fungi. Infections occurring in autumn and winter usually remain asymptomatic until next summer. During this period inoculum can accumulate significantly. The risk of disease outbreaks caused by Botryosphaeriaceae species peaks in seasons with abundant rainfall, as the infection is mainly spread by water-borne conidia (Ahimera et al., 2004; Michailides–Morgan, 2004). However, when a wet year is followed by a drought year, symptoms caused by Botryosphaeriaceae species are typically severe, as water stress increases the host's susceptibility to infection (Marsberg et al., 2016).

Examining the infection of different parts of the walnut tree is essential for understanding the disease and determining the timing for treatments. One of the tools for this survey is BUDMON. The microbiological state of the buds can be studied at the end of the dormancy period, thus farmers get information to make decisions about plant protection management (Moral et al., 2019). ONFIT is a technique that can be used in summer, when green walnuts are developing. After freezing the green nuts, possible latent pathogens can be detected, thus giving an idea of the expected infection at harvest and the coniditon of the orchard (Michailides et al., 2000). These methods can also help to determine the routes of infection.

Although damage of the kernels is the main problem, we have to mention the colonization of woody tissues by Botryosphaeriaceae and Diaporthaceae families (Agustí-Brisach et al., 2019), however the nuts can be considered more susceptible and sensitive from this point of view in Hungary (Zabiák et al., 2023). These fungi have already caused significant losses in this way in California (Chen et al., 2014). And the question may arise as to whether the pathogenic fungi found in infected kernels can spread to other tissues of the tree, as Moral et al. experienced in walnuts (Moral et al., 2019). Studying this can be important not only for walnuts, but also for favorable climatic conditions for pathogens, which may even results new host-pathogen relationships (Davis–Shaw, 2001).

The purposes of the experiments of my research work were (1) to culture the fungi present in different parts of the walnut tree and the determination of the pathogens using morphological and molecular biological markers, (2) identify the optimal growth temperature of the pathogenic fungi, (3) and the study of the pathogenicity of walnut pathogens on green walnut fruits and branches. In order to explore new host plant-pathogen relationships emerging as a potential consequence of climate change (4), we investigated the virulence of pathogenic species on apple branches. Furthermore, we aim to (5) investigate chemical and biological plant protection options against the strains belonging to the genera involved in the disease and (6) to study the population genetics of the isolated fungal strains.

2. MATERIAL AND METHODS

2.1. Sampling and locations

The plant parts for the tests were collected from four walnut orchards in northeastern Hungary and one from Alsószentiván (Transdanubia). One orchard is located in the University of Debrecen, Faculty of Agricultural and Food Sciences and Environmental Management, External Department of Integrated Fruit Production (AKIT, ÚJKI), while the remaining four are commercial orchards.

Sampling of the twigs was carried out in June 2018, during which symptomatic and asymptomatic samples were collected to determine pathogenic fungal genera and possible latent infection. During the survey of the four sampling sites in Eastern Hungary, 10 twigs from 10 trees, nine buds and seven green fruits were also collected.

The pathogenicity of the cultured *Diaporthe eres* and *Diplodia seriata* species was tested on green walnuts, which were collected from Újfehértó in August 2018.

The next sampling took place during the harvest. We collected both symptomatic and symptomless walnuts from three orchards (Hajdúdorog, Jánkmajtis, Tarpa), a total of 200 pieces.

At the end of the dormant period, we collected 150 symptom-free catkins and 10 female buds from Jánkmajtis. The purpose of the study was to determine the latent infection and to assess the level of infection with BUDMON method (Michailides et al., 2014).

In June 2019, green fruits were collected from Alsószentiván (50 pcs) and Jánkmajtis (70 pcs) for the ONFIT examination of immature fruits.

In 2019, during the harvest, we sampled walnuts from commercial orchards located in Alsószentiván (43 pcs), Tarpa (42 pcs) and Jánkmajtis (50 pcs). Based on the experience gained during our tests, extended plant protection treatments with Folicur Solo were applied this year without experimental setup.

2.2. Isolation of the microbial population of different plant parts of walnut

We examined the pathogenic microflora of different plant parts of the walnut tree in order to study the presence of Botryosphaeriaceae and *Diaporthe* pathogens associated with nut rot.

The outer layer of the plant samples was removed with a sterile scalpel, then the tissue was cut into four parts and placed in a Falkon tube. Then the plant parts were soaked for one minute in 10% chlorogenic sesquihydrate (Neomagnol, Parma Produkt Ltd.) and 0.1 % in Tween20 solution (Merck KGaA), and for buds in 5% NaClO solution. The disinfectant was washed off the surface of the samples by double rinsing with sterile water.

2.3. Identification of cultures based on morphological and molecular characters

During morphological identification, the characteristics of colonies were observed. Microscopic characteristics were analysed with optical microscope (Zeiss AxioImager phase-contrast microscope, equipped with AxioCam MRc5 camera) at 400x magnification (Jacobs–Rehner 1998; Andersen et al., 2002; Summerell et al., 2003; Gomes et al., 2013; Visagie et al., 2014).

Genomic DNA was extracted with NucleoSpin Plant II Kit (Macherey-Nagel) following the manufacturer's protocol. Mycelia of pathogens were placed into tubes that contained bashing beads (Zymo Research Corp) and lysis buffer. It was followed by PCR reaction to amplify the ITS region and tef1 gene, using primer pairs ITS1 and ITS4 (IDT) (White et al., 1990), and EF1-728F and EF1-986R (IDT), respectively (Carbone-Kohn, 1999). The final volume of PCR mixture was 25 µL, and contained 12,5 µL DreamTaq Green Master Mix (Thermo Fisher Scientific, Germany), 0.5 µL of each primer (10 pmol µL-1), 10.5 µL nuclease-free water, and 1 µL of DNA solution (10 ng µL-1).

Amplification started with an initial denaturation step of 3 min at 95 ˚C, followed by 35 cycles of 30 s at 95 ˚C, 45 s at 56 ˚C for ITS or 55 ˚C for tef1 products, 60 s at 72 ˚C and a final extension at 72 ˚C for 5 min.

PCR products were separated in 1% agarose gel (Bioline) stained with GelRed (Biotium). PCR fragment purification was performed with NucleoSpin Gel, PCR Clean Up Kit (Macherey-Nagel). Isolated fragments were sent for sequencing to the Microsynth GmbH (Vienna, Austria), then the sequences were analysed using the National Center for Biotechnology Information BLAST tool (http://ncbi.nlm.nih.gov/blast/).

Sequences were aligned first with Clustal X 2.1 (Larkin et al., 2007) and adjusted using Genedoc (Nicholas et al., 1997), if it was necessary. Phylogenetic tree was created with the MEGA 7.0 software (Kumar et al., 2016) and nearest-neighbor interchange (NNI) method with 1000 bootstrapped replications. The maximum likelihood method, based on the cpREV+F model was used for all sequences. Positions containing gaps and missing data were not considered. For maximum likelihood analyses, the nearest-neighbor interchange was used as the heuristic method for tree inference. Support for internal branches was assessed by 1000 bootstrapped pseudoreplicates of data.

2.4. Detection of latent infecion by ONFIT

Immature walnuts were studied by ONFIT (Overnight Freezing-Incubation Technique) method (Michailides et al., 2010) to detect latent infection of walnuts. In early June, 38

symptomatic and 32 asymptomatic walnuts were collected from Jánkmajtis and 50 symptomatic green walnuts from Alsószentiván. Symptoms included deep brown lesions and spots on the surface of the green husks.

Samples were disinfected, then the green walnuts were incubated at -16 °C for 15 hours. After freezing, the nuts were placed in plastic containers, and incubated under 95% relative humidity for 14 days at 25 °C. Specific mycelia and pycnidia were observed on the surface of the husks and were isolated for further analysis.

2.5. Growth temperature test

The optimal temperature for growth of four *Diaporthe eres* (J2034, J2028, JT2024, JT2050) and two *Botryospaheria dothidea* (JT2015, T2016) isolates was determined. 10 mm mycelial plugs were cut with sterile cork borer from fungal colonies. Plugs of each isolate were plated on PDA in three replicates and incubated at 15, 20, 25, 30, and 35°C. Colony diameters were measured for seven days.

2.6. Pathogenicity of *Diplodia seriata* and *Diaporthe eres* isolates on green walnuts

Pathogenicity tests were carried out to analyze the ability of *Diplodia seriata* and *Diaporthe eres* isolates to infect green walnut husks and their pathogenic capacity. Fungi were isolated from twigs, buds, and green fruits. Five *D. eres* (J1004, T1010, U1001, U1003, and U1008) and three *D. seriata* (D1012, U1012, and U1013 isolates were selected for the inoculation (Chen et al, 2014).

Green walnuts from a "Milotai 10" cultivar without visible symptoms were collected from the Újfehértó site. First, the walnuts were submerged for one minute in 10% chlorogenic sesquihydrate (Neomagnol, Parma Produkt Ltd.) and 0.1 % in Tween20 solution (Merck KGaA). After inoculation and incubation, both the husks and the kernels were analyzed and classified according to the severity of symptoms and the McKinney index (Imc%), a measure of disease severity, was calculated both for the walnut husk and the kernel (McKinney, 1923) (**Figure 1**).

Figure 1: Walnut kernels defected differenctly after artificial inoculation

2.7. Pathogenicity test of walnut and apple branches

The pathogenicity test was carried out to determine whether the pathogenic isolates from the rotted nut can also cause symptoms in the tissues of the walnut tree branches. Healthy branch samples were collected in the Demonstration Garden of the University of Debrecen. The inoculation was performed with three *Botryosphaeria dothidea* (JT2015, JT2035, T2016) and six *Diaporthe eres* (J2012, J2023, J2024, J2028, J2034, JT2050) isolates based on the description by Tang et al. (2011). After four weeks of incubation symptoms were observed and the extent of the lesions was measured.

The susceptibility of other plant species to fungal infections and the infection possibilities were also examined during the research work. For this purpose, we infected apple branches *in vitro* with isolates of *Diplodia seriata* (D1011) isolated from walnut twig, *Botryosphaeria dothidea* (JT2015) and *D. eres* (JT2036) isolated from rotted walnut kernels (Tang et al, 2011). The healthy apple branches were collected from the Horticultural Experimentation Site of Pallag.

2.8. Investigation of the fungicide sensitivity of walnut pathogenic fungi

The inhibitory effect of plant protection products was investigated using poisoned media technique. We tested the sensitivity of two *Diaporthe eres* (J2028, J2034) and one *Botryosphaeria dothidea* (JT2015) isolates *in vitro.*

The sensitivity of the three pathogenic isolates was examined against four active agents or combinations [ciprodinil 37,5% + fludioxonil 25% (Switch), fluopiram 17,7% + tebukonazol 17,7% (Luna Experience), tebukonazol 25% (Folicur Solo), valamint fluopiram 21,3% + trifloxistrobin 21,3% (Luna Sensation)]. The preparations were mixed in PDA media (Biolab), and then a 10-mm mycelium plug was placed on the solid agar. They were incubated at room temperature for seven days, then colony diameters were recorded, and inhibition index was calculated (Pandey et al., 1982).

To calculate the EC50 value for tebuconazole, it was used in the lowest concentration indicated in the license document, and in its 1-10.000-fold dilution. After the incubation period, the colony diameters were measured and the mycelial growth inhibition (I%) of the fungicides was determined. A linear regression analysis was performed to calculate the EC50 (Pasche et al., 2004).

2.9. Study of the inhibitory effect of *Epicoccum nigrum* and *Trichoderma gamsii*

The antagonistic effect of *Epicoccum nigrum* (Accession number: MT111108) isolated from walnut tree and *Trichoderma gamsii* TR08 (OK560831) isolated by Kovács et al. (2021) was studied in direct confrontation. During the experiment, the inhibitory effect of the antagonists was analyzed against two *Diaporthe eres* (J2028, J2034), one *Botryosphaeria dothidea* (JT2015) and one *Diplodia seriata* isolate (D1011). Mycelium plug from the active growth zone of the cultures was placed on a PDA plate. After the incubation period, mycelial inhibition of the antagonists was determined by calculating Biocontrol-Index (Szekeres et al., 2006).

2.10. Population genetics study based on microsatellite markers

Genetic diversity was studied among 34 isolates grown from walnuts using microsatellite markers. The three examined species were *Botryosphaeria dothidea*, *Diaporthe eres* and *Diplodia seriata*. Amplification of DNA segments was performed using four primers: (ACAC)5, (GGA)7, (GTG)5, M13 (IDT). The PCR mixture contained 2 µl of primer, 7 µl of nuclease-free water, 11 µl of DreamTaq Green Master Mix, and 2 µl of DNA. Before the molecular biological tests, we optimized the annealing temperature of the PCR reaction and the dNTP concentration of the reaction mixture, as well as the composition of the agarose gel (SeaKem or Bioline, 1% and 2%). Amplification began with an initial denaturation cycle of 3 min at 95 ˚C, followed by 40 cycles of 20 sec at 95 ˚C, 60 sec at 56 ˚C, and then 2 min at 72 ˚C. The last phase of the program was the final chain extension at 72 ˚C for 10 minutes. After the gel electrophoresis, the gel photos were taken with a FlourChem M (ProteinSimple) machine, and the AlphaView Stand Alone program (ProteinSimple) was used to evaluate the results.

To analyze the PCR data, individual isolates were evaluated for the presence or absence of different amplicons. The dendrograms of the three investigated fungal species were then prepared using the UPGMA software. The resulting phylogenetic trees were divided into clades based on genetic distances.

2.11. Statistical analysis

To evaluate the data obtained during the experiments, we used the non-parametric Mann–Whitney U-test for pairwise comparison, because the values did not fulfill the conditions imposed on parametric tests (normal distribution, homogeneity of variances by Levene test).

The significance level used in the analyzes was 5%. StatSoft Statistica 7 software and MS Excel 2016 were used for the analysis and the creation of diagrams.

3. RESULTS

3.1. Microbial population of different plant parts of walnut in early summer

One of the most significant objectives of the research was the isolation of fungi from different parts of the walnut tree and the morphological and molecular biological determination of the pathogens.

We found that the orchards that suffered significant crop losses in previous years, had good general condition, however there were also young plant parts showing brown lesions in the early summer. During the tests, fungi belonging to the genera *Diaporthe* and *Diplodia* were isolated from symptomatic branches and fruits, as well as from asymptomatic buds. More than one third (35%) of the 40 examined walnut trees were infected with any of these pathogens, which were isolated from young tissues. Based on the ITS and *tef1* marker sequences, the isolated strains belong to the species *Diplodia seriata* and *Diaporthe eres*. These species are worldwide pathogens that can colonize several woody hosts, including walnut (Phillips et al., 2007; Gomes et al., 2013; Abramczyk et al., 2018; Fan et al., 2018). Both species were previously isolated from grapes in Hungary (Kovács et al., 2014; 2017), but in this study we were the first to isolate and identify them from English walnut using molecular markers. Infection caused by *D. seriata* has been observed in several cases in woody parts of fruit trees in areas with a Mediterranean climate (Chen et al., 2014; Sohrabi et al., 2020), and in recent years also in Central Europe (Eichmeier et al., 2020). *Diaporthe* species can also colonize walnut trees. Several members of the genus have been identified in walnut in Chile (Luna et al., 2022), California (Agustí-Brisach et al., 2019), Spain (López-Moral et al., 2020) and China (Fan et al., 2018). The species *D. eres* caused severe nut damage in hazelnut plantations (Battilani et al., 2018; Eichmeier et al., 2020).

Walnut twigs can be infected through pruning wounds, which remain susceptible to infection for at least four months after the cutting in October and February, while in other woody cultures, pruning wounds are susceptible to necrotizing fungi for only a few weeks (Michailides et al., 2012). Fungi can enter the host through cracks caused by growth, wounds caused by pruning, animals and weather conditions. Rainy weather increases the development

and spread of fungal spores, which can cause a large-scale proliferation of pathogens (Moral et al., 2019; López-Moral et al., 2020).

3.2. Fungal population of walnuts in 2018

During the research, 200 walnuts were collected in the fall of 2018. 68% of the samples were inedible. Their proportion was similar for the three sampled locations, as well as for the young plantation in Hajdúdorog. *Alternaria* spp., *Botryosphaeria* spp., *Diaporthe* spp., *Fusarium* spp. and *Penicillium* spp. were cultured in a considerable ratio. In California, a similar microbiome was cultured from walnut kernels (Michailides et al., 2012). The McKinney index of the genera was the highest for the isolates classified in the genera *Botryosphaeria* (74%) and *Diaporthe* (62%), which indicates the relationship between the rotten nuts and the two pathogenic genera. The average scale values were also the highest when these genera were present alone and together (**Figure 2**).

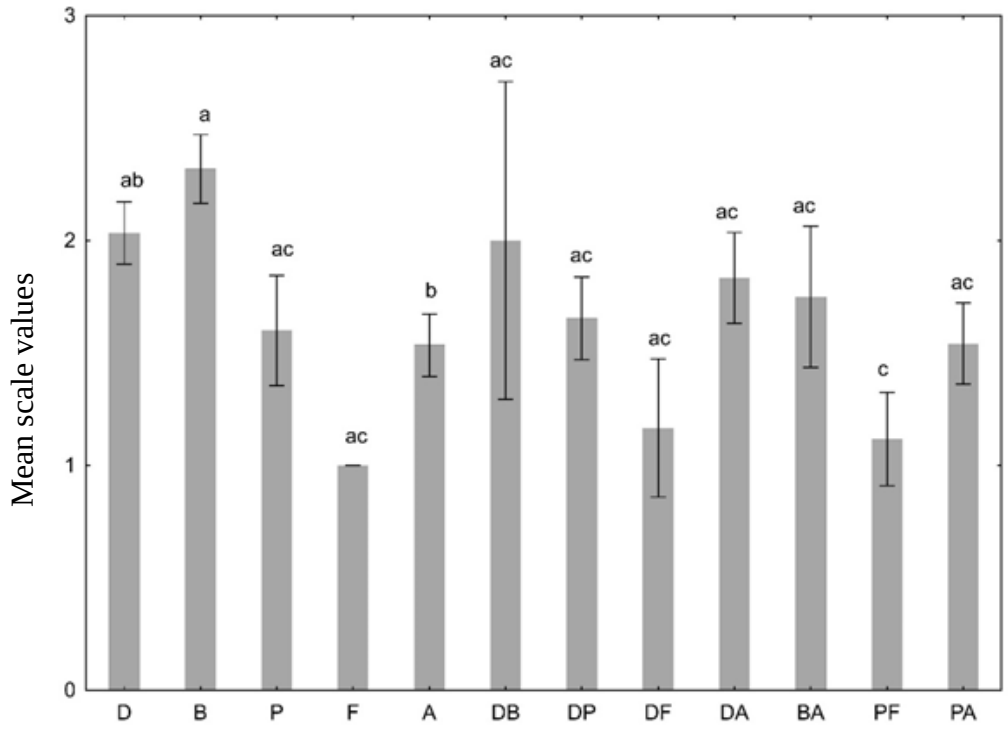

Figure 2: Genera isolated from walnut kernels and their scale values. D=*Diaporthe* spp., B=*Botryosphaeria* spp., P=*Penicillium* spp., F=*Fusarium* spp., A=*Alternaria* spp. Letter above the columns mark the result of a Mann-Whitney U test. Vertical lines mark standard error.

Walnuts from Jánkmajtis were the most infected with *Botryosphaeria* (29%) and *Diaporthe* (48%) fungi, followed by Tarpa (*Botryosphaeria* spp. 10%, *Diaporthe* spp. 28%), and Hajdúdorog (*Botryosphaeria* spp. 2%, *Diaporthe* spp. 18%) (**Figure 3**).

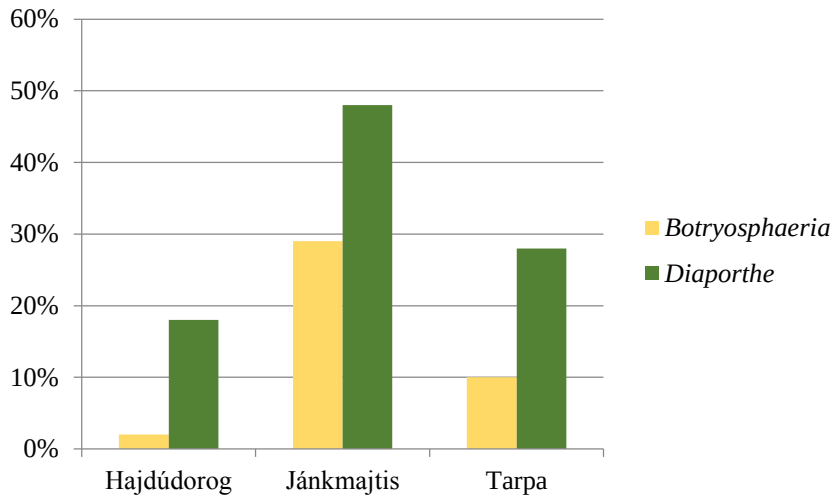

Figure 3: Ratio of *Botryosphaeria* and *Diaporthe* genera in the kernels originated from the sampled areas.

After the sequence analysis of the ITS and *tef1* markers, isolates were classified in the species *Botryosphaeria dothidea* and *Diaporthe eres*. These two species were isolated from rotten chestnuts in the northern part of Croatia (Ivić–Novak, 2018). In Chinese walnut plantations, *B. dothidea* caused yield losses of 20%-50% (Li et al., 2023), and more than 50% in California (Moral et al., 2019).

3.3. Monitoring of buds

The following year, at the end of winter and dormancy, *Diaporthe* spp. was found in 44% of the monitored catkins in the plantation that suffered the most severe crop damage at the previous year (Jánkmajtis). Isolates were classified as *D. eres* based on genetic methods. Pathogens belonging to the genus *Botryosphaeria* were isolated from only four buds. Both groups of fungi were present in 40-40% of the collected female buds. Based on these results, the method (BUDMON) can be used for early monitoring of the fungal walnut disease and for estimation of plant protection risks in Hungary. This information can give professionals a clue in estimating plant protection risks and in determining the method and timing of necessary treatments. The colonization of male buds by pathogenic fungi can cause infection during pollination, and in the case of female buds, the creation of latently diseased, symptomatic

nuts. Michailides and Morgan (2004) experienced something similar when studying the disease of pistachios caused by *Botryosphaeria dothidea*, which was explained by the fact that the overwintering of the fruiting bodies on the branches could be the result of the infection of the buds, as the spores can reach the newly formed plant parts with the precipitation. Although the technique may be suitable for estimating the infestation of plantations, it does not take into account the weather conditions after bud break (Morgan et al., 2009).

3.4. Detection of latent infecion by ONFIT

The ONFIT analysis of the immature fruits from Jánkmajtis, Tarpa and Alsószentiván excluded the latent infection at the beginning of June in. However, *Botryosphaeria* and *Diaporthe* species were present in 49% of the symptomatic samples. *Botryosphaeria* spp. was observed on 34 green fruits, while *Diaporthe* species appeared on 50 samples, and both genera were observed on the same fruit in 25 cases. Consequently, the symptoms caused by these two genera may develop already in the middle of fruit development, before the damage of walnut husk fly. Since symptoms associated with *Botryosphaeria* and *Diaporthe* fungi were already observed at the beginning of June, it is recommended to apply ONFIT at an earlier time, when potential pathogens are still present in the crop in an inactive form. In this study, BUDMON and ONFIT successfully tested for the first time in Hungary to survey the walnuts threatening disease complex.

3.5. Examination of the fungal community of mature nuts in 2019

The result of assessment the condition of the nuts was completely different in the fall of 2019. Taking into account the areas sampled in both years (Tarpa, Jánkmajtis), the proportion of inedible kernels was 33%, while the previous year it was 61%. The difference was also evident in terms of the isolated genera. Walnut pathogen *Botryosphaeria* and *Diaporthe* spp. were present in 6% (4-4 pcs.), while this value was 64% in 2018 (**Figure 4**). In 2019, *Pencillium* species were primarily responsible for the nuts becoming inedible, which could be grown in the largest quantities from samples classified in class 2 and class 3, i.e. partially dried or severely rotten walnuts, but they were also often present in fruits registered in class 1. As in 2018, the Imc value of the genera *Botryosphaeria* and *Diaporthe* was the highest (67-67%).

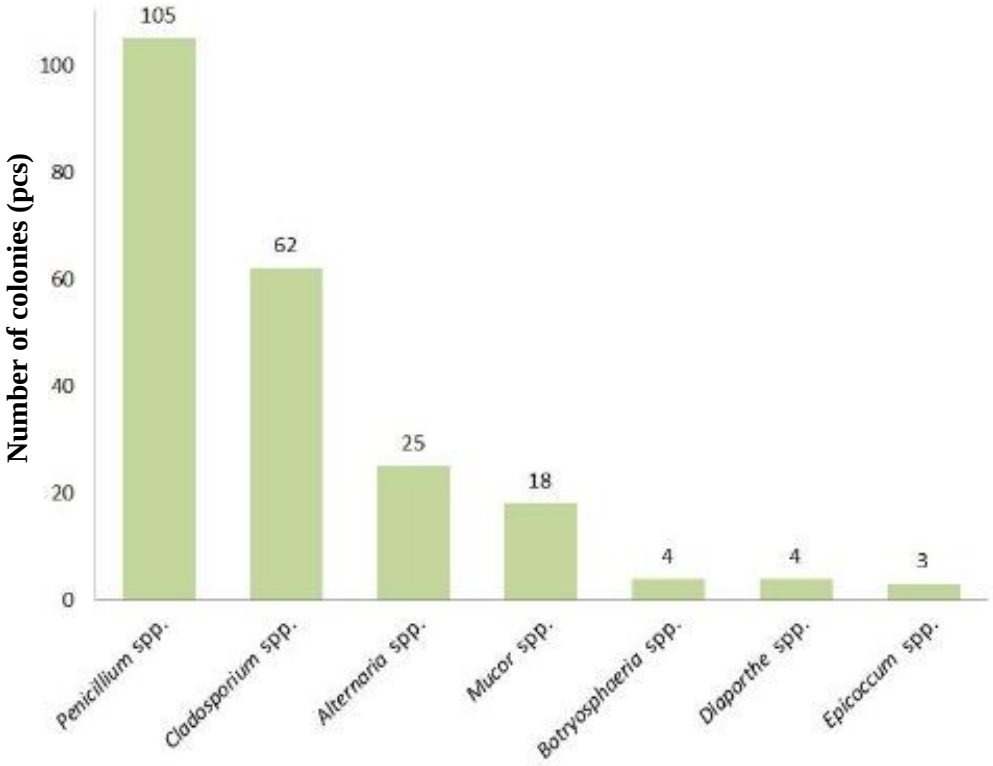

Figure 4: Number of colonies belonging to different genera isolated from walnuts in 2019

As a result of our data, treatment with tested, effective fungicide was used in extended time span under non-experimental conditions in Jánkmajtis in 2019. The technology was proved to be effective in controlling the disease. The proportion of inedible kernels decreased from 77% to 33%, and the walnut pathogenic *Botryosphaeria* and *Diaporthe* genera were cultured from 6% of the fruits. The early and repeatedly application of tebucanozole in the second half of spring could contribute to the control of the fungal disease complex of walnuts. However, the proportion of fruits unsuitable for consumption remains higher, than the infection with those two genera. *Penicillium* spp. were present in the diseased nuts in high proportion, indicating additional food safety risks (Fung–Clark, 2004). *Penicillium crustosum*, identified in our examination, caused mycotoxicosis in a dog, which ate infected nuts that had been lying on the ground for five months (Eriksen et al., 2010).

3.6. Growth temperature test

During determining the optimal growth temperature, the heat tolerance of the two tested *Botryosphaeria dothidea* was verified, and the 15 mm daily growth at 30 °C also supports the higher temperature preference of the species. *Diaporthe eres* isolates did not tolerate high temperature, but their sensitivity to rising temperatures was different. This characteristic may

contribute to the effective growth and colonization of the walnut tree by the *D. eres* at wide temperature range. Vegetative survival of *D. eres* isolates is possible, based on research results of Abramczyk et al. (2020). The average daily growth of *B. dothidea* isolates was significantly lower than experienced Sánchez et al. (2003), where this value exceeded 25 mm at the optimal temperature. The adaptation of fungi to elevated temperatures has been observed by several researchers (Michailides–Hasey, 2010; Agustí-Brisach et al., 2019). Overall, it can be said that the optimal growth conditions of the tested *Diaporthe* and *Botryosphaeria* isolates are the same as the usual temperature of 20-35 °C during nut development.

3.7. Pathogenicity of *Diplodia seriata* and *Diaporthe eres* isolates on green walnuts

When green fruits were inoculated *in vitro* with *Diaporthe eres* and *Diplodia seriata* fungi from branches, buds and fruits, 81% of the husk of the fruits rotted after three weeks. *D. eres* isolates caused more severe symptoms compared to *D. seriata* isolates. Based on the symptomatic kernels (89%) and the successful re-isolation of the pathogens, the inoculum penetrated in the internal tissues, so late infection may result defected fruits, and the appearance of pathogens may cause problems until September. López-Moral et al. (2020) carried out *in vitro* artificial inoculation in a more immature phase compared to our inoculated fruits, before the development of the hard shell, involving several fungi species in the test, during which *D. seriata* and *Diaporthe* spp. caused significantly less symptoms on the green husk, however, browning was observed on the kernels.

3.8. Pathogenicity test of walnut branches

The inoculation of *Botryosphaeria dothidea* and *Diaporthe eres* strains isolated from symptomatic nuts on walnut tree branches proved their pathogenicity. *D. eres* caused the largest lesions (170 mm). The largest mean lesion length caused by *B. dothidea* was 99 mm, so the infection potential of this species was considered to be medium on the woody tissues of walnut (**Figure 5**). The significant virulence of *D. eres* on the branches and the wide range of optimal temperature for growing may contribute to the infection of the buds and fruits either through the internal tissues of the plant or by the spread of spores formed on the outer bark.

Our results differ from those described in the literature, but other research has also shown the infecting ability of these genera on walnut trees. López-Moral et al. (2020) made *in vitro* inoculations, when the species *B. dothidea* (130 mm) was found to be more virulent compared to the genus *Diaporthe* (23 mm). An *in vivo* pathogenicity test in California also showed that *B. dothidea* was more virulent (35 mm) than *D. eres* (17 mm) (Chen et al., 2014).

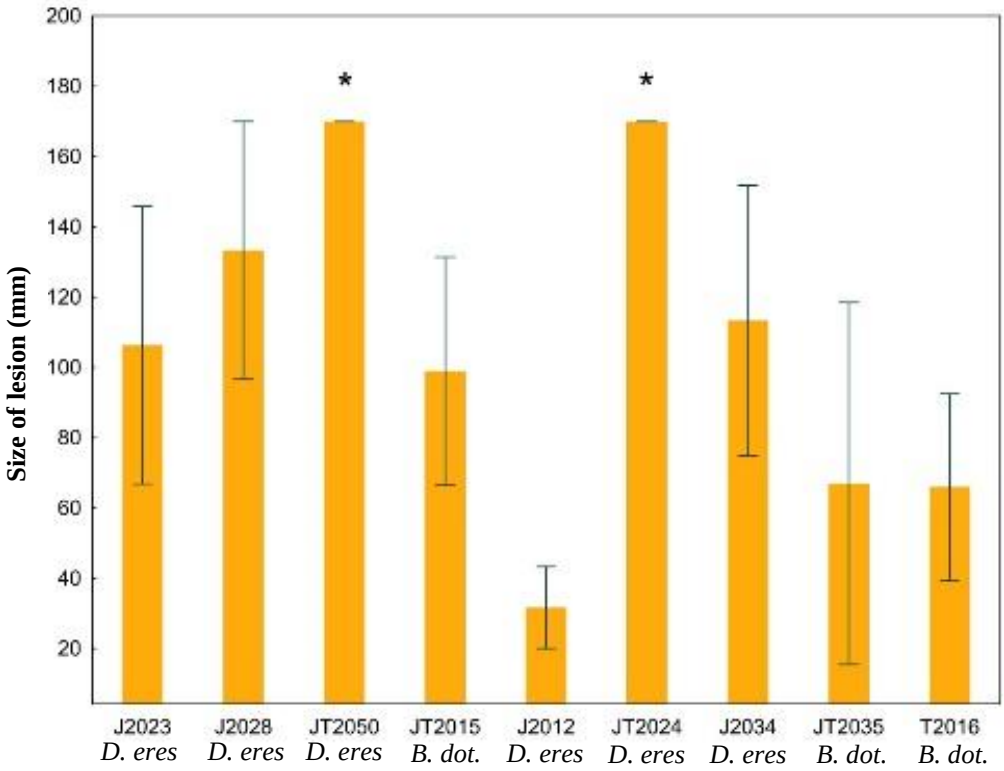

Figure 5: The extent of lesions caused by *D. eres* and *B. dothidea* in inoculated walnut twigs

Columns with asterisk mark significantly differences from control ($p<0,05$)

3.9. Pathogenicity test of apple branches

After artificial inoculation, the pathogenic isolates colonized the tissues of the apple tree branches and caused lesions. The two isolates belonging to the Botryosphaeriaceae family (*Botryosphaeria dothidea* and *Diplodia seriata*) had a similar effect, while *Diaporthe eres* caused milder symptoms. Based on the results of the pathogenicity test, the three tested species from walnuts can be considered a new potential host-pathogen interaction in Hungary, which can later lead to devastating diseases, knowing their virulence. This supports the importance of monitoring the symptoms of neighboring plant cultures to detect cross-infections.

B. dothidea is the causal agent of apple fruit damage called white rot, which causes apple pre- and postharvest rot. This disease is not widespread in Europe, but it has already been detected in Serbia (Vasić et al., 2013), and last year the same symptoms were caused by the co-infection of *B. dothidea* and *D. eres* (Vučković et al., 2022). *D. seriata* also caused fruit damage in many apple orchards in the last decade (Cácerez et al., 2016; Crespo et al., 2018).

3.10. Investigation of the fungicide sensitivity of walnut pathogenic fungi

Three of the four formulation used during the poisoned plates almost completely inhibited the growth of *Botryosphaeria dothidea* and *Diaporthe eres* isolates (cyprodinil 37.5% + fludioxonil 25%, fluopyram 17.7% + tebuconazole 17.7%, tebuconazole 25%). Among these, Folicur Solo, with the active ingredient tebuconazole was further investigated to determine the EC50 values. The obtained concentrations (0.11-0.29 mg/l) were significantly lower than the recommended one in the license document (46.88 mg/l). These fungicides can control *B. dothidea* and *D. eres* and reduce the percentage of fruit rot caused by them.

3.11. Study of the inhibitory effect of *Epicoccum nigrum* and *Trichoderma gamsii*

Using of biocontrol agents can be a solution in the management of walnut disease. Antagonistic ability of *Epicoccum nigrum* isolated from walnuts and *Trichoderma gamsii* TR08 were tested against *Botryosphaeria dothidea*, *Diaporthe eres* and *Diplodia seriata* isolates in a direct confrontation test. *E. nigrum* inhibited the growth of all four pathogenic strains included in the study (BCI=47-53%), but *E. nigrum* was not able to grow on pathogenic cultures. This inhibitory potential is not adequate for the effective use of *E. nigrum* against fungal diseases of walnuts However, the 100% Biocontrol-Index of *T. gamsii* TR08 indicating promising alternatives to fungicides in the control of fungal infections associated with nut damage. The destructive effect of *T. gamsii* was observed with under microscope, during which we observed the wrapping of the hyphae of TR08 on the pathogenic isolate.

3.12. Population genetics study based on microsatellite markers

Our strains classified as pathogenic species *Botryosphaeria dothidea*, *Diaporthe eres* and *Diplodia seriata* were successfully separated based on their genetic polymorphisms, and intra-species differences were also detected. According to the analysis of the phylogenetic trees, certain genotypes colonized only certain parts of the plant in terms of *D. eres* isolates. In several cases, the strains found in the clusters came from different plant parts, which may indicate that the infection could have originated from several sources in the plantation. No correlation was observed between geographical location and colonized plant tissues. The strains isolated from nuts and buds with a very similar genetic profile confirm the hypothesis that the infection may originate from the catkins, fungi can colonize the flowers and the fruits, but a far-reaching conclusion can be drawn after further investigations. The genetic variability of the tested pathogenic fungi was high, with low genetic distances.

4. NEW CONTRIBUTIONS TO ACADEMIC KNOWLEDGE

1. *Diplodia seriata*, *Diaporthe eres*, and *Botryosphaeria dothidea* species are present in walnut orchards, which were isolated and identified for the first time in this study using ITS and *tef1* molecular marker sequences from symptomatic plant tissues of English walnut trees, and *D. eres* and *B. dothidea* were isolated from walnuts as pathogens first time.

2. Based on the McKinney index, the genera *Botryosphaeria* (Imc=74%) and *Diaporthe* (Imc=62%) cause the most serious damage in the harvested walnuts, either alone or in combination.

3. *Diaporthe* and *Botryosphaeria* species are present in the catkins and female buds. These fungi are not present in immature, asymptomatic fruits (no latent infection), however, the genera *Botryosphaeria* and *Diaporthe* were present in 49% of symptomatic samples. Therefore, the symptoms caused by the two genera can develop already in the middle of fruit development.

4. *B. dothidea* strains isolated from walnuts are heat-loving, grow even at 35 °C, and are characterized by rapid (15 mm/day) mycelial growth at 30 °C. The sensitivity of *D. eres* isolates to temperature was different, which may contribute to the severity of the disease, since the plant is thus exposed to *D. eres* pathogens that multiply and colonize under optimal conditions in a wider temperature range.

5. *B. dothidea* and *D. eres* from symptomatic kernels cause symptoms in woody tissues of walnut. *D. eres* caused more severe symptoms (170 mm mean lesion length) than *B. dothidea* (99 mm mean lesion length).

6. The species *D. eres* and *D. seriata* can also cause damage to the nuts at the end of summer, which, entering through wounds from the infected branch, bud and fruit, cause rotting of the green husk and symptoms in the nut.

7. Isolates classified as *B. dothidea*, *D. eres* and *D. seriata* from walnuts are able to colonize and cause lesions in the woody tissues of appletrees.

8. The TR08 strain of *Trichoderma gamsii* is an effective antagonist (100% Biocontrol-Index) of the walnut pathogenic *B. dothidea*, *D. eres* and *D. seriata* strains.

5. PRACTICAL USE OF THE RESULTS

1. *Botryosphaeria* and *Diaporthe* species cause significant yield damage in mature, harvested nuts.

2. Fungi that cause walnut damage (*Diaporthe* and *Botryosphaeria* species) can infect through buds. Therefore, it is recommended to use BUDMON to assess bud infection for early monitoring of the pathogenic fungi present and to estimate plant protection risks.

3. The *Botryosphaeria* and *Diaporthe* species can be detected even in the immature fruits, before possible damage by walnut husk fly in the middle of fruit development. The ONFIT test of immature fruits can assess the fungal infection of the plantation during fruit development.

4. Infection by the genus *Pencillium* is significant in Hungarian plantations, even in the case of symptomless nuts.

5. The use of the endophytic *Trichoderma* strains (e.g. *Trichoderma gamsii* TR08) is recommended in the control of the walnut pathogenic *Botryosphaeria dothidea*, *Diaporthe eres* and *Diplodia seriata*.

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7. LIST OF PUBLICATIONS RELATED TO THE DISSERTATION

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List of publications related to the dissertation

Hungarian scientific articles in Hungarian journals (1)

1. **Zabiák, A.**, Csótó, A., Takács, F., Karaffa, E. M.: A dió terméskárosodásának etiológiája és a védekezés lehetőségei.
   Növényvédelem. 84 (5), 193-200, 2023. ISSN: 0133-0829.

Foreign language scientific articles in Hungarian journals (2)

2. **Zabiák, A.**, Csótó, A., Tóth, B., Takács, F., Pál, K., Karaffa, E. M.: Identification of Botryosphaeria dothidea and Diaporthe eres from Rotted Walnut Fruits and Other Plant Parts in Different Phenological Stages.
   Acta Phytopathol. Entomol. Hung. "Accepted by Publisher" (-), 1-20, 2023. ISSN: 0238-1249.
3. **Zabiák, A.**, Károlyi, G. M., Karaffa, E. M.: Investigation of host-specificity of phytopathogenic fungi isolated from woody plants.
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Foreign language scientific articles in international journals (1)

4. **Zabiák, A.**, Kovács, C., Takács, F., Pál, K., Peles, F., Fekete, E., Karaffa, L., Mihály, K., Flipphi, M., Karaffa, E. M.: Diaporthe and Diplodia species associated with walnut (Juglans regia L.) in Hungarian orchards.
   Horticulturae. 9 (2), 1-15, 2023. EISSN: 2311-7524.
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Hungarian abstracts (3)

5. **Zabiák, A.**, Károlyi, G. M., Karaffa, E. M.: Dióból izolált Botryosphaeriaceae fajok patogenitása az almafa fás szöveteiben.
   In: 66. Növényvédelmi Tudományos Napok 2020. Szerk.: Haltrich Attila, Varga Ákos, Magyar Növényvédelmi Társaság, Budapest, 97, 2020.

6. **Zabiák, A.**, Takács, F., Karaffa, E. M.: Diótermések endofiton gombapopulációjának meghatározása.
In: Magyar Táplálkozástudományi Társaság XLIV. Vándorgyűlése programja és az előadások összefoglalói. Szerk.: Antal Emese, Biró Lajos, Gelencsér Éva, Lugasi Andrea, Rurik Imre, Magyar Táplálkozástudományi Társaság, Budapest, 57, 2019. ISBN: 9786155606090

7. **Zabiák, A.**, Karaffa, E. M.: Diótermések endofiton gombapopulációjának vizsgálata és a fertőzöttség mértékének meghatározása.
In: 65. Növényvédelmi Tudományos Napok: Absztrakt és poszter kötet. Szerk.: Haltrich Attila, Varga Ákos, Magyar Növényvédelmi Társaság, Budapest, 66, 2019.

Foreign language abstracts (2)

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Total IF of journals (all publications): 3,1
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