Theses of the PhD dissertation

INVESTIGATION OF THE BIOLOGY AND DAMAGE OF THE PATHOGEN (*MACROPHOMINA PHASEOLINA*) CAUSING CHARCOAL ROT OF SUNFLOWER IN THE CARPATHIAN BASIN

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1. AIMS AND BACKGROUND OF THE DISSERTATION

Macrophomina phaseolina (Tassi) Goidanich can be found on more than 500 monocot and dicot species in nearly every part of the world (Ghosh et al., 2018). In Hungary, it was first identified and described by Békési and colleagues (1970) on sunflower (*Helianthus annuus* L.) plants in various parts of the country. Nowadays, the disease this fungus causes has become one of the most common and destructive diseases. The symptoms on sunflower vary greatly. Other important sunflower diseases, such as *Diaporthe helianthi* or *Alternaria* spp. cause similar symptoms to *M. phaseolina.* Leaves infected by *Alternaria* species are characterised by dark necrotic lesions with a brownish-grey centre and chlorotic outlines (Kim és Mathur, 2006). In order to differentiate between these two diseases, the stem must be cut in half and the pith must be examined under magnification.

The list of host plants in Hungary has been steadily increasing since 1970. Vörös and Manninger (1973) described the pathogen on corn (*Zea mays* L.), one of the most widely grown plants. Later, Érsek (1979) isolated the fungus from one of the most prominent plants in the world, soybean (*Glycine max* (L.) Merr.). Simay (1987) identified three new host plants of this pathogenic fungus: potato (*Solanum tuberosum* L.), Jerusalem artichoke (*Helianthus tuberosus* L.) and broad bean (*Vicia faba* L.). Three years later, Simay (1990) found further host plants, among which were two horticultural crops: he had found symptoms of wilting on bean (*Phaseolus vulgaris* L.) and garlic (*Allium sativum* L.). He also examined weed-affected areas where he found that *M. phaseolina* is able to infect buttonweed *(Malva neglecta* Wallr,), common mallow *(Malva silvestris* L.), hemlock *(Conium maculatum* L.), feral cannabis *(Cannabis sativa* L. ssp. *spontanea* Sereb.) and wild parsnip *(Pastinaca sativa* L. ssp. *pratensis* (Pers.) Celak). Koppányi and colleagues (1993) isolated the pathogen from sugarbeet, whereas Simay and Kadlicskó (1993) found the pathogen on cultivated hemp (*Cannabis sativa* L. ssp. *sativa)* and valerian (*Valeriana officinalis* L.). Before the 2000s, Fischl and associates (1995) confirmed the host-parasite relationship on pepper (*Capsicum annuum* L.), while Békési and colleagues (1995) had done the same on watermelon (*Citrullus lanatus* L.). It was first reported on ligneous plants by Vajna and Rozsnyai (1995), who were first to identify the pathogen on apricot (*Prunus armeniaca* L.) in Hungary. Fischl et al. (2008) detected the pathogen in blue spruce (*Pinus pungens* Engelm.) roots.

The pathogen has two asexual forms, the pycnidial *M. phaseolina* and the microsclerotial *Rhizoctonia bataticola* forms. Sexual reproduction of the fungus remains unknown. Microsclerotia are the primary source of infection, which can survive in the soil for up to 15 years under favourable conditions (Gupta et al., 2012). Pathogens without sexual stages may have varied genetic diversity. Parasexual reproduction is a specific case of asexual reproduction. The process

when hyphal bridges (anastomoses) form between the hyphae of two genetically compatible strains and genetically distinct nuclei become hosted in a common cytoplasm, is called heterokaryosis (Jakucs és Vajna, 2003). Stability of diploid nuclei is poor, and mitotic recombination and mitotic non-disjunction can occur during their division, resulting in recombinant haploid nuclei with new allele combinations (Pontecorvo, 1956; Strom and Bushley, 2016). Results obtained from genetic testing of *M. phaseolina* show that this pathogen exhibits a high degree of genetic diversity. The parasexual exchange of genetic information between different hyphae is thought to contribute to this high variability (Almeida et al., 2003).

The genus *Macrophomina* was considered monotypic for a long time. However, Sarr and colleagues (2014) reported two different *Macrophomina* species within the *Macrophomina* genus. In the subsequent years, further new species have been identified (Zhao et al., 2019; Kouadri et al., 2021; Sanabria-Velazquez et al., 2022). Efforts to morphologically identify species belonging to the genus *Macrophomina* have been unsuccessful due to differences in microsclerotia size, variability of pure cultures and differences in pathogenicity. According to Zhao and associates (2019), the primary factor for identification is the translation elongation factor (TEF).

Due to its biology, the fungus prefers warm, dry weather and therefore causes significant economic damage in areas with dry summers (Sarr et al., 2014). As the climate continues to change, the pathogen will cause significant damage in areas where it was previously absent or present only to a small extent. The pathogen is common across Europe and causes minor to major economic damage every year. Before the 2000s, the fungus was found in Belgium (Hunt, 1952), Croatia (Acimovič, 1962), Hungary (Békési et al., 1970), Greece (Pantidou, 1973), France (Alabouvetto and Bremeersch, 1976), the United Kingdom (Scholefield and Griffin, 1979), Italy (Zazzerini, 1980), Spain (Jimenéz-Diaz et al., 1983), Portugal (De Barros, 1985), Romania (Bontea, 1985), the Netherlands (Turkensteen és Lablans, 1988), Germany (Müller and Grill, 1991), Turkey (Onan et al., 1992), Serbia (Aćimović, 1998) and Bulgaria (Alexandrov, 1999). In the 2000s, its presence was first confirmed in the Czech Republic (Kudlíková et al., 2002), then in Slovakia (Bokor, 2007). In recent years, the pathogen has also been found in Malta (Haleem et al., 2016), Slovenia (Žerjav et al., 2017), Ukraine (Tančić-Živanov et al., 2019) and Denmark (Dell'Olmo et al., 2022). Among the aforementioned countries, Denmark, the United Kingdom and the Netherlands are known for their cooler, wetter climates, but this extremely adaptable pathogen is now also present in these areas.

The pathogen can cause up to 100% yield loss (Dhingra and Sinclair, 1978). This widespread plant pathogenic fungus causes significant economic damage to a number of economically important crops. Based on the studies of Romero Luna and associates (2017),

charcoal rot was among the top five diseases causing yield loss in the United States of America between 2010 and 2013. According to Van der Waals and colleagues (2013), the main temperature will rise by 2 °C due to climate change, whereas average annual rainfall will decrease. This effect will increase the frequency of optimal conditions for charcoal rot. At present, there is limited information available on the yield loss caused by the disease in South Africa. Bioecological factors in arid and semi-arid regions of India contribute to the development of the disease. In the case of cowpea (*Vigna unguiculata* (L.) Walp.), 80% occurrence of the pathogen was reported (Lodha et al., 1986), and as for sunflower, a 30% loss of seed weight was documented (Raut, 1981). Annual yield loss in soybean can reach 80% (Gupta and Chauhan, 2005). In chickpea (*Cicer arietinum* L.), an infection rate of nearly 40% was reported (Indira and Gayatri, 2003).

Control measures against the fungus are necessary due the damage it causes, but its extreme adaptability, high genetic variability, wide host range and the viability of microsclerotia leave few available control methods. When planning the management, the effectiveness of *Trichoderma* species should be taken into account. Several *Trichoderma* species can be used as potential biocontrol agents and growth enhancers for various crops (Savazzini et al., 2009). There are several antagonist species within the genus *Trichoderma*. These species exhibit antagonism through indirect (competition for nutrients or habitat) or direct (mycoparasitism) means (Benitez et al., 2004). In addition to biological control, it is also important to study its chemical control. The European Union's continued withdrawal of active substances add to the already difficult control of this pathogen. However, there are substances available that can be used against it. Lokesh and colleagues (2020) examined different systemic (azoxystrobin, carbendazim, hexaconazole, propioconazole, tebuconazole, tiophanate methyl, difenoconazole) and contact copperoxychloride, mancozeb, propineb, thiram, chlortalonil) fungicides at different concentrations under *in vitro* conditions against *M. phaseolina*. Many of the aforementioned fungicides are still available in Hungary. Lokesh and colleagues (2020) determined that as concentrations of systemic fungicides increased, their mycelial growth inhibition efficiency also increased.

The aims of this dissertation are the following:

1. To confirm the widespread distribution of the pathogen and to accurately identify the symptoms of the disease in the Carpathian Basin.
2. To assess the economic damage caused by the disease in the years 2019 and 2020.
3. To determine the size of microsclerotia in the pith of sunflower and potato dextrose agar culture medium.
4. To examine the mycelial compatibility of the fungus in order to identify the different vegetative compatibility groups.
5. To identify the pathogen on a species-level using molecular biology techniques (PCR).
6. To investigate possible biological controls under *in vitro* conditions
7. To investigate the chemical control options against the pathogen *in vitro*, *in vivo* and under large parcel field conditions.

2. MATERIALS AND METHOD

2.1. Confirming the widespread distribution of the pathogen and accurately identifying the symptoms of the disease in the Carpathian Basin.

The distribution of the pathogen in the Carpathian Basin was studied in the years 2019 and 2020. A total of 66 areas were examined, along with one located in the Czech Republic. Within each area, 20 sunflower plants were selected randomly and were examined. The lower third of the stem was cut and if microsclerotia were detected on the inside, it was placed in a sterile box.

Symptoms on sunflower plants were examined in 2021 for a complete vegetation period. Four parcels were randomly selected in the field. 20 plants per parcel were marked and visually observable symptoms were monitored weekly. In order to determine the time of pathogen penetration, sunflower tissues were dyed with lactophenol cotton blue dye and tissue samples from the stem were placed on potato dextrose agar medium and incubated.

2.2. Assessment of the economic damage caused by the infection in the growing seasons of 2019 and 2020.

The second aim of this dissertation was to examine the economic damage caused by this pathogen. In 2019-2020, NK Neoma sunflower hybrids were randomly examined for *M. phaseolina* infection in 12 areas in the eastern region of the country. In each of the two years, 100-100 randomly selected plants were examined in each selected area. The lower one third of the stems of the studied plants (up to 50-60 cm in height) were cut in half and were examined for symptoms. Since the heads are usually not the shape of a regular circle, the head diameters of the studied plants were measured along two perpendicular diagonals. The heads were individually threshed, the total seed weight and thousand seed weight were measured, and the oil content of the infected and healthy seeds were analysed separately. Losses caused by the pathogen were examined in relation to environmental factors and crop rotation. Therefore, information regarding crop rotation for the previous 4 years was requested from the farmers in the areas and data from the nearest meteorological station to the studied areas were used. The average temperature, the number of hot days and the distribution of rainfall during the vegetation period (April-September) were recorded and analysed. Following the study, data collected from the 12 study areas were analysed using Statistica 7 software. Values for infected and healthy populations were compared using a t-test if our data met the assumptions of parametric tests. The homogeneity of variance was tested using Levene's test, while the normal distribution was tested using Q-Q plots. In the other cases, a non-parametric Mann-Whitney U-test was used at $P<0.5$ significance level. The

relationship between weather parameters and disease incidence was analysed using linear regression.

2.3. Examination of microsclerotia size of the fungus and determining the compatibility groups.

In connection with the third and fourth aim of this dissertation, the average size of microsclerotia found both in the stems of the collected isolates and on the pure cultures in the laboratory was determined. Microsclerotia are never perfectly spherical in shape, thus the measurement was carried out on two perpendicular diameters using a microscope. The results were evaluated. Pure cultures of all the isolates examined were tested in Petri dishes to determine their compatibility. The tests were performed according to the method of Csöndes (2011). Subsequently, hyphal anastomoses between isolates were examined under microscope.

2.4. Identification of the pathogen on a species-level using molecular biology techniques (PCR).

The fifth aim of this dissertation was to identify the pathogen on a species-level using ITS and TEF-α primers. The large amount of mycelia required to isolate the DNA were acquired by growing mycelia in Czapek Dox Broth culture medium for 7 days in an incubator shaker at 170 rpm, under dark conditions at 30°C. Isolation of DNA was done according to the protocol of the Macherey-Nagel Nucleospin Plant II DNA isolation KIT. The primer pairs ITS1/ITS4 and EF-728/EF2 were used for the identification of the species. The PCR product was purified using the Clean-Up (Macherey-Nagel) KIT. Sequencing was done by Microsynth Austria. Sequencing for the ITS1-ITS4 primer pair was performed for the ITS1 primer, while for the TEF primers, sequencing was performed for both primers. The resulting sequences were complemented using the Chromas sequence reader, then the corrected sequences were aligned with the sequences of the other *Macrophomina* species in Clustal X. The sequences were cut to the same length in GeneDoc.

Relationships between species were determined using the Neighbour-Joining statistical method (in MEGA X) with 1000 bootstrap replicates.

2.5. Investigating possible biological controls under *in vitro* conditions

To achieve the sixth aim, 8 Trichoderma species were tested: *T. harzianum* T22, *T. asperellum* T1, *T. asperellum* T34, *T. simmonsii* and *T. afroharzianum*, *T. harzianum*, *T. gamsii*, *T. orientale*. The efficacy of the aforementioned species against *M. phaseolina* was determined using a confrontation test in Petri dishes. Inhibition was determined on the third day and biocontrol

index on days 3 and 7. Statistical evaluation was performed using Tukey test or Mann-Whitney U test in Statistica 7 software depending on the parametricity.

2.6. Investigation of the chemical control options against the pathogen *in vitro*, *in vivo* and under large parcel field conditions

The seventh aim was to find a pesticide active substance with good efficacy against the pathogen. As the first step, 8 different active ingredients were tested in Petri dishes with 5 replicates in 3 doses. The fungal growth inhibition rate was measured on day 3 for the mycelium colony and on day 5 for the microsclerotium colony. Based on the results obtained during the tests, the active substance prochloraz was selected for further testing. The effect of prochloraz on sunflower germination and on the pathogen *M. phaseolina* was first tested in Petri dishes. Based on literature sources, 4 doses were determined: 0,6-, 0,3-, 0,15- and 0,075 l t^{-1}. Fungicide treated and water treated seeds were placed on microsclerotium colonies grown for 7 days on PDA medium and the same number of Petri dishes were tested without the pathogen.

Based on the results, the two highest doses were further tested in vivo. The main objective was to determine the dose to which the plant does not react negatively but the pathogen does. The plants were tested up to 6 weeks of age. The first penetration of the pathogen into the host plant was determined. In conclusion from the results of the experiment, the dose 0.3 l t^{-1} was further tested under field conditions. The efficacy of prochloraz as seed treatment and as crop treatment was investigated. A total of 16 experimental large parcels were set up: 4 control parcels (no seed treatment); 4 control parcels (no seed treatment) but one crop treatment applied; 4 seed treated parcels; 4 seed and crop treated parcels were set up in random placement. The study was evaluated according to the methodology described in section 2.2. Statistical analysis was performed using Tukey test or Mann-Whitney U test in Statistica 7 software depending on the parametricity.

3. RESULTS

3.1. The spread of the fungus and the symptoms

The results related to the first aim showed that the pathogen is widespread in the Carpathian Basin. A total of 66 areas were studied in the Carpathian Basin, 58 of which were infested with *Macrophomina* sp. The presence of the fungus was also confirmed in the Czech Republic, as previously described (Figure 1). We were the first to detect the presence of the pathogen in Austria and were able to identify it in Slovenia on sunflower, where it is considered a new host plant for the pathogen.

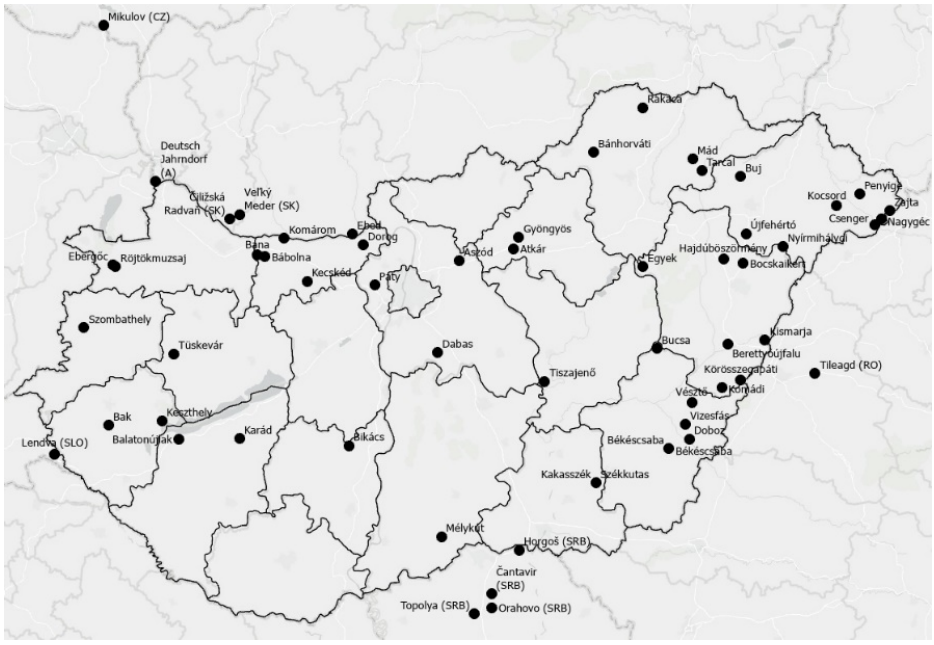

Figure 1. *Macrophomina phaseolina* occurrences confirmed in the study

Although the pathogen infects the seedlings, in Hungary, the soil temperature is low when sunflower is sown. The infection was first detected in samples taken in the second week of June. Symptoms were first visible in July, the lower leaves had lost turgor. By the end of July, wilting plants were observed in patches across the field. It was found that the spotted black discoloration on the outside of the stem is not always visible. In August, the epidermal layer separating from the lower third of the stem and the appearance of microsclerotia on the root collar were common symptoms. It was determined that the most reliable factor for identifying the pathogen was the mass presence of microsclerotia within the stem, from the root collar up to 40-50 cm height. Microsclerotia can also be present in green stems under extreme weather conditions (year 2022).

3.2. Assessment of the economic damage caused by the disease in the years 2019 and 2020.

M. phaseolina was present in all sampling sites in both years studied. It was found that crop rotation can have a strong influence on the amount of inoculum in the soil. The occurrence of the pathogen and crop rotation are closely related. In areas where 2 years had passed between sunflower sowings, 77.34% of the plants were showing symptoms of the disease, compared to 67.00% in areas where 3 years had passed between sowings of sunflower crops.

Disease incidence was examined in connection with the weather conditions. The weather conditions considered were the number of hot days, the amount of precipitation during the vegetation period and the average temperature on a monthly basis (Table 1). There was a wide variation in weather conditions both between the sites examined and between the years studied, so that neither precipitation nor temperature data could be used to draw clear conclusions.

Table 1. Disease incidence, number of hot days and the average temperature during the vegetation period in the two years studied

	Area	Disease incidence %	Number of hot days	Average temperature during the vegetation period (°C)
2019	Székkutas 1	73	32	18.3
	Békéscsaba 1	77	42	18.1
	Vésztő 1	78	35	18.8
	Bucsa 1	70	45	17.8
	Hajdúdorog 1	83	46	17.6
	Buj 1	82	46	17.6
2020	Székkutas 2	71	43	17.8
	Békéscsaba 2	68	31	17.4
	Vésztő 2	68	49	19.4
	Bucsa 2	65	20	17.1
	Hajdúdorog 2	64	25	17.1
	Buj 2	9	25	17.1

M. phaseolina infection caused significant yield loss due to reduction in head diameter, seed weight and thousand seed weight of the examined plants (Table 2). The lowest impact was observed for head diameters. Loss of total seed weight was the highest. The decrease in thousand seed weight showed the same trend in the two years studied.

Table 2. The influence of infection on the parameters studied in sunflower

	2019	2020	2019	2020
Head diameter (cm±SH)			**yield loss (%)**	
Healthy	25.07 (±0.20)a	24.27 (±0.14)a		
Infected	18.11 (±0.10)b	20.42 (±0.10)b	-27.76	-15.86
Seed weight (g±SH)				
Healthy	133.85 (±1.50)a	128.41 (±1.08)a		
Infected	77.15 (±1.00)b	95.59 (±0.90)b	-42.36	-25.56
Thousand seed weight (g±SH)				
Healthy	84.44 (±0.66)a	82.38 (±0.40)a		
Infected	58.64 (±0.66)b	68.30 (±0.55)b	-30.55	-17.09
Oil content (%)				
Healthy	42.67 (±0.24)a	41.89 (±0,24)a		
Infected	44.98 (±0.22)b	44.57 (±0,19)b	5.41	6.40

Source: own construction

3.3. Examination of the microsclerotia size of the fungus and determining the compatibility groups.

The average diameter of microsclerotia found within the sunflower stem was between 60-90 µm. According to our measurements, the average diameter of microsclerotia grown on culture media were between 108-145 µm.

In the compatibility testing of the isolates, 5133 pairings were performed. Of the total number of possible pairings (1711), no anastomosis was observed in 42 cases. The isolate with the identifier DENI_MacPha054, registered in the culture collection of the Plant Protection Institute at the University of Debrecen, did not form anastomoses with 7 other isolates and was therefore considered the isolate with the highest number of barrier zone formations. The isolates with the highest number of anastomoses formed a barrier zone between one isolate and another.

Based on the visually assessed results of anastomosis formation between the 59 isolates tested, the isolates cannot be divided into compatibility groups. Of the total number of possible pairings (1711), 97.55% showed anastomosis formation between two isolates. The results show that the studied isolates belong to the same compatibility group.

3.4. Identification of the pathogen on a species-level using molecular biology techniques

Species belonging to the genus *Macrophomina* are difficult to distinguish using solely morphological parameters. For this reason, the only certain method for identification is molecular identification, during which the samples were first tested using the primer pair ITS1-ITS4. The sequences were submitted to the NCBI gene bank. The ITS phylogenetic tree was created using the identifiers of the sequences deposited in the NCBI gene bank and based on literature sources.

The evaluation of the phylogenetic tree clearly shows that the ITS sequence is not sufficient for species-level identification. Species were not clearly grouped by the program, therefore, the samples were also sequenced with TEF-α primers. The sequences obtained from the TEF-α sequencing were grouped by species by the MEGA X program. In this grouping, the program assigned the samples isolated to *Macrophomina phaseolina* species. It can be concluded that all isolates collected are confirmed to be *Macrophomina phaseolina*.

3.5. Investigating possible biological controls under *in vitro* conditions

On the third day of the experiment, the tested species belonging to the genus *Trichoderma* showed the following results regarding the inhibition percentage against *M. phaseolina*: *T. afroharzianum* 60%, *T. asperellum* T1 strain 56%, *T. asperellum* T34 strain 54%, *T. harzianum* T22 strain 62%, *T. gamsii* 66%, *T. harzianum* 61%, *T. orientale* 59% and *T. simmonsii* 64%. On the third day of the experiment, the biocontrol index (%) was also determined. Among the tested antagonist species, *T. afroharzianum* showed 59%, *T. asperellum* T1 strain 67%, *T. asperellum* T34 strain 64%, *T. harzianum* T22 strain 62%, *T. gamsii* 64%, *T. harzianum* 53%, *T. orientale* 59%, *T. simmonsii* 64% biocontrol activity against the pathogen. From the fourth day of measurement onwards, the confrontation zone shifted continuously towards the pathogen in all cases, however, none of the antagonists sporulated on the pathogen. By the fifth day, *T. harzianum* T22 and *T. gamsii* formed an abundance of aerial mycelia and sporulated on the pathogen. On the sixth day of the experiment, *T. afroharzianum* and *T. simmonsii* completely covered the Petri dish. By the seventh day, only *T. orientale* had not completely covered the plate, its biocontrol index was 57%. The other tested *Trichoderma* species have covered the entire Petri dish, thus showing a 100% biocontrol index (%).

3.6. Investigation of the chemical control options against the pathogen *in vitro*, *in vivo* and under large parcel field conditions

3.6.1. Testing fungicide active substances under in vitro conditions

The 8 tested active substances can be divided into three groups by mode of action: QoI (azoxystrobin, pyraclostrobin), SDHI (boscalid, benzovindiflupyr, fluopyram) and DMI (prochloraz, prothioconazole, tebuconazole).

On the third day, mycelium colonies were measurable in the medium treated with azoxystrobin (34.1 mm), boscalid (42.5 mm), fluopyram (18 mm) and pyraclostrobin (11.2 mm) even at the lowest dose. No mycelial colonies were observed on the medium containing active substances belonging to the DMI group. The results of the experiment using half of the maximum doses of the tested active substances as indicated in their respective authorisation documents showed that the pathogen was able to grow in the media treated with azoxystrobin (26,9 mm), boscalid (38 mm) és a fluopyram (16,8 mm). Benzovindiflupyr also belonging to the SDHI group, completely inhibited the growth of the pathogen. The other active substances have exhibited total inhibition of *M.phaseolina* mycelial growth on the third day. Similarly to the medium dose results, the results of the maximum dose test showed that the pathogen was only able to grow on medium treated with azoxystrobin (21.3 mm), boscalid (28.3 mm) and fluopyram (15.4 mm).

The efficacy of the tested substances on microsclerotium formation was evaluated on day 5. Azoxystrobin had the weakest effect on the pathogen. The microsclerotium colony formed on the medium treated with the lowest dose was 36 mm in diameter (57% inhibition). In addition to azoxystrobin, only the medium treated with boscalid produced microsclerotium colony (40.9 mm) on day 5, corresponding to 51% inhibition. Of the other active substances used in the experiment (regardless of grouping), no microsclerotium colonies were formed at the doses tested. In the case of azoxystrobin at medium dose, a colony of 27.6 mm in diameter formed, which corresponds to 67% inhibition. As for the highest doses, the percentage of inhibition was 71% in this case.

3.6.2. Testing the effect of prochloraz as seed treatment in vitro in Petri dishes

Syngenta Ltd. provided untreated NK Neoma seeds for the experiment, which were used to carry out the experiment. The pathogen infects the seedlings (under adequate conditions), inhibiting their growth which this experiment also confirmed. Based on the results of the treated seeds inoculated with the pathogen that were incubated in Petri dishes and results from the control seeds, it can be established that seed treatment had a positive effect on seedling size, while the

pathogen had a negative effect on seedlings grown from seeds not treated with prochloraz (Figure 2).

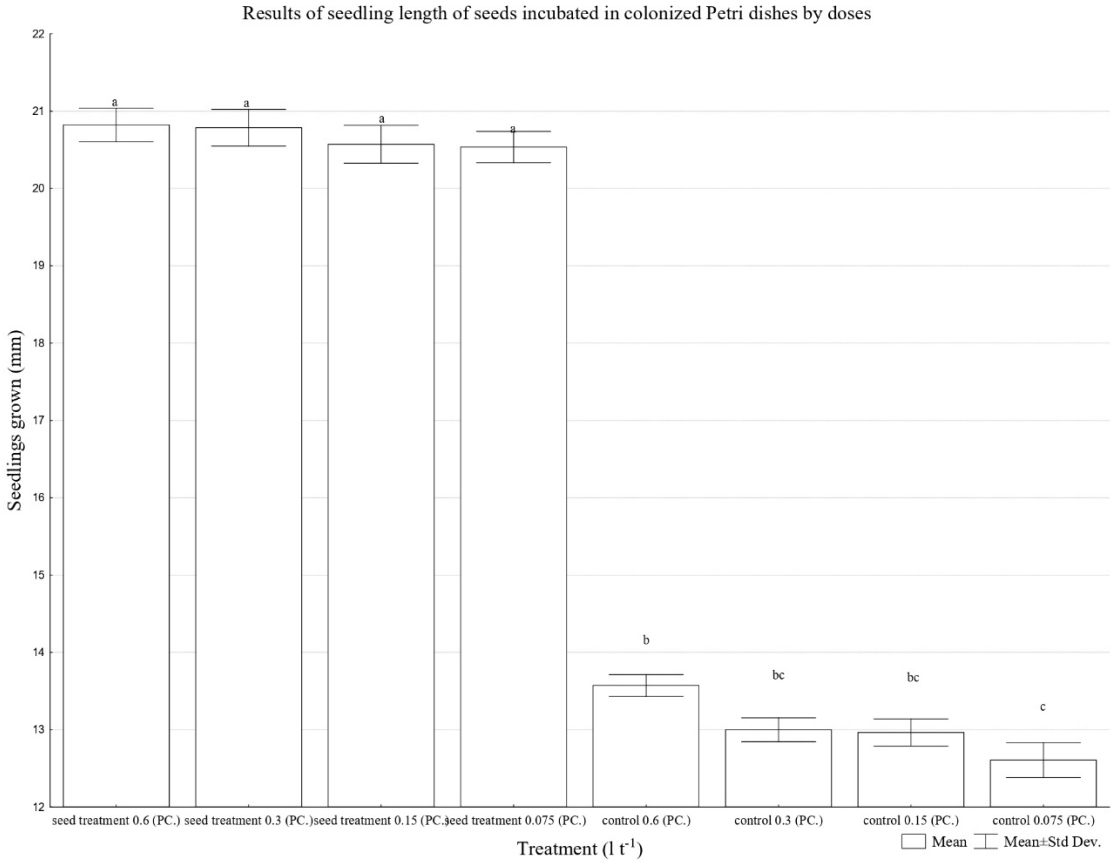

Figure 2. Results of seedling length of seeds incubated in colonized Petri dishes by doses (seed treatment: prochloraz-treated; control: water-treated; PC.: pathogen-colonized growing medium)

By analysing the phytotoxicity of prochloraz, it can be determined that seed treatment was not only effective for preventing early infection by *M. phaseolina*, but also had no phytotoxic effect on the seedlings, as the seedlings of the colonized and non-colonized Petri dishes were nearly the same size on day 10. The visual results showed that the pathogen had infiltrated the majority of the control seeds and formed microsclerotia on their surfaces. In the case of prochloraz-treated seeds, no mycelial colonization was observed on the surface of the seeds at doses 0.6 l t^{-1} and 0.3 l t^{-1}, whereas at doses 0.15 l t^{-1} and 0.075 l t^{-1}, some mycelial colonization was observed, but no microsclerotia. Therefore, the former two doses were further examined.

3.6.3. Results of the pot experiment

Among the doses of the active substance tested in Petri dishes, 0.6 l t^{-1} and 0.3 l t^{-1} were tested in the pot experiment. The results showed that the pathogen was detected in the root collar

tissue of infected sunflower plants on the third week of the experiment. The pathogen stunted the root growth of the untreated sunflower plants. At the same time, there was no significant difference in root length between plants grown in the pathogen-colonized medium and plants grown in the non-colonized medium. Therefore, the seed treatment protected the plants from damage. Seed treatment did not affect plant height. However, presence of the pathogen resulted in stunted control plants on the third day.

In the case of plants evaluated on the sixth week, the lowest root length was observed in control plants grown in colonized medium. Root lengths of untreated plants in the pathogen-colonized medium were found to be significantly smaller than root lengths of control and treated plants grown in non-colonized medium and also that of the treated plants grown in the colonized medium. The largest root lengths were measured for the control non-colonized plants.

In terms of plant height, the lowest plant height was recorded for control plants grown in pots inoculated with the pathogen. The presence of the pathogen strongly influenced plant growth. No significant difference was observed between untreated plants grown in pathogen-free medium and plants grown in pathogen-colonized medium that were treated with 100% dose. Therefore, it can be determined that the seed treatment effectively prevented plant damage by the pathogen. Phytotoxicity was also taken into account when choosing the effective dose. Plants treated at 0.3 l t^{-1} did not suffer the negative effect of the pathogen nor the phytotoxicity of the seed treatment, whereas at 0.6 l t^{-1}, plants showed symptoms of phytotoxicity. Consequently, the 0.3 l t^{-1} dose was further tested.

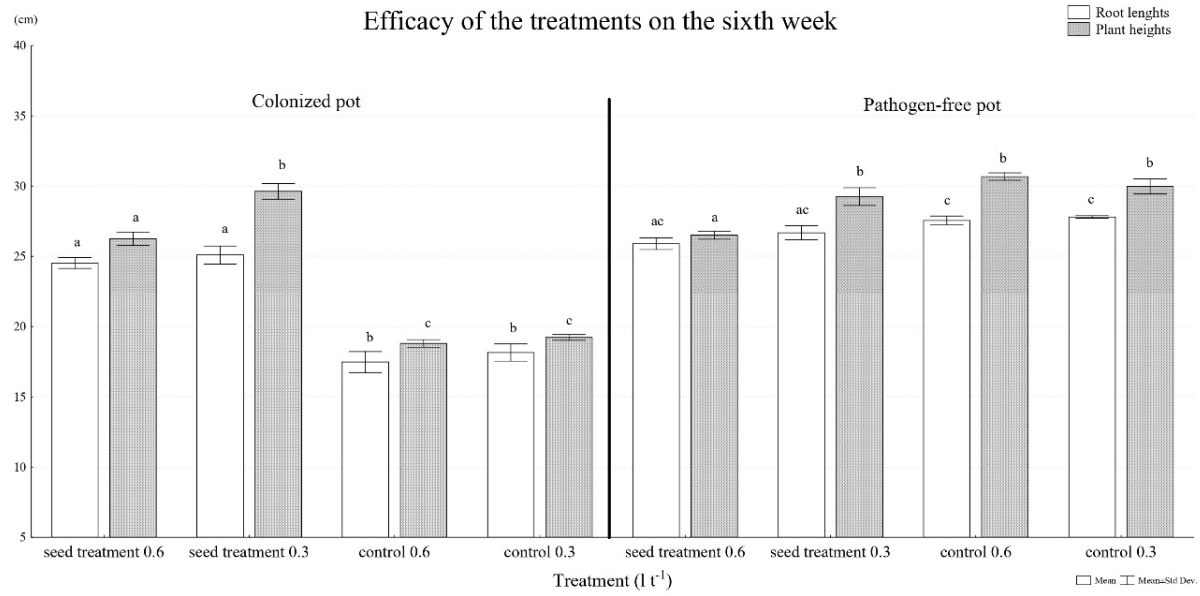

Figure 3. Efficacy of the treatments on the sixth week
seed treatment: prochloraz-treated; control: water-treated

3.6.4. Testing prochloraz under field conditions

Efficacy of prochloraz was tested in relation to value indicator parameters of sunflower (head diameter, total seed weight, thousand seed weight, oil content). It was found that seed treatment protected the plants from infection. In parcels where treated seeds were sown, but no crop treatment was applied, the average disease incidence was 8.26%, whereas it was 6.52% in the case of parcels where a single crop treatment was applied. There was no statistically significant difference between the two treatments. Infection rates were significantly higher in control parcels (46%, 54%), but no significant difference was observed between the two treatments. It was found that crop treatment had no effect on infection rates in either control or seed treated conditions.

Among the value indicator traits of sunflower, the head diameter was investigated in relation to infection. A significant difference was observed between all treatments. In both cases, crop treatment resulted in a higher average head diameter. The largest average head diameter of 26.6 cm was recorded in the seed treated + crop treated parcels. The smallest average head diameter was measured in parcels that received no seed treatment (21.56 cm). In parcels that received no seed treatment but one crop treatment, an average head diameter of 22.18 cm was measured, while in the seed treated but not crop treated parcels, an average head diameter of 25.3 cm was measured. Both seed treatment and crop treatment had a significant effect on average head diameters in treated parcels. Furthermore, the effectiveness of the treatments on the total weight of the seeds threshed from the plates was investigated. In the parcels that received no seed treatment, seeds weighing between 110-115 g were threshed from the heads. In parcels that received no seed treatment and one crop treatment, the seeds weighed between 120-125 g. In parcels where only seed treatment and seed treatment + crop treatment was applied, the recorded seed weight was ~140g per head. There was a significant difference between parcels that received seed treatment and those that did not, with no applied crop treatment, as there was an average of ~28 g difference between the two treatments. Afterwards, the thousand seed weight was also examined. Treatments had a significant effect on thousand seed weight. The highest value was recorded in the case of parcels where seed treatment and one crop treatment was applied (83 g), whereas parcels that received no seed treatment nor crop treatment showed the lowest value (57 g). The last parameter, oil content, was not significantly affected by the treatments. There was a significant increase in oil content as a result of infection, but this value was only 2%. This small increase does not compensate for the quantitative losses mentioned above.

4. NEW SCIENTIFIC EVIDENCE

1. It was confirmed that the pathogen *Macrophomina phaseolina* is widely distributed in Hungary. We founded presence of the pathogen in sunflower plants in Austria and Slovenia, where it is considered a new host plant.
2. It was determined that increased inoculum in the soil due to lack of crop rotation has a greater influence on the prevalence of the disease than environmental factors. In the case of the three year crop rotation, there were notably less infected plants in the studied fields compared to the two year crop rotation. During the study it was determined that the pathogen caused significant yield loss. Although oil content was 2% higher on average for the infected plants, this increase does not compensate for the loss of up to 42% of the total seed weight per head.
3. It was confirmed that isolates from Hungary form microsclerotia of similar size both in the host plant and on PDA medium, compared to isolates from Slovakia and isolates examined in North Dakota described in the literature.
4. When examining the formation of the anastomoses necessary for the parasexual reproduction of the pathogen, our results show that it is not possible to establish compatibility groups for the isolates collected from the Carpathian Basin. The isolates showed a high degree of compatibility (97.2%) among themselves. The DENI-MacPha054 isolate from Serbia did not form anastomoses with the other studied isolates in 7 cases, whereas the DENI_MacPha56 isolate, also from Serbia, formed anastomoses with all isolates. Geographical separation is not a reliable indicator regarding the compatibility of isolates.
5. It was confirmed that analysis using the primer pair ITS1-ITS4 is not sufficient for species-level identification. For accurate species-level identification, the use of the tefα primer pair EF728-EF2 is required.
6. It was found that of the currently known 4 *Macrophomina* species, only *Macrophomina phaseolina* is present in Hungary.
7. In the experiment aimed to investigate biological control, it was found that *T. simmonsii* has an excellent biocontrol activity against the pathogen. *T. orientale* is a potential human pathogen and its BCI was the lowest among the tested species, thus it will be excluded from future studies. Based on the results of this experiment, the other tested *Trichoderma* species parasitized the pathogen *M. phaseolina* effectively under *in vitro* conditions.
8. It was confirmed that active substances belonging to the DMI group (prochloraz, prothioconazole, tebuconazole) can be used effectively against the pathogen. The active substances could not be clearly grouped according to their mechanisms of action, because azoxystrobin, belonging to the QoI group, did not inhibit the formation of microsclerotia even

at maximum dose, whereas pyraclostrobin, also belonging to this group, was effective even at the lowest dose.

5. RESULTS APPLICABLE IN PRACTICE

1. In sunflower fields, the presence of the fungus in the host can only be clearly confirmed after cutting the lower third of the stem, because the external symptoms caused by the pathogen are often not obvious. Even plants that appear healthy on the outside may be infected on the inside. The tests confirmed that common symptoms include reduced head diameter, browned stem, wilting leaves, separation of the epidermis from the stem and the appearance of microsclerotia on the outside of the stem.
2. We confirmed that the main value measuring properties of sunflower adversely affect the infection. Due to the reduction of the thousand seed weight, the cleaning loss and scattering of the combine also increase, which can lead to significant volunteer.
3. In order to identify *Macrophomina phaseolina* on a species-level, the use of the primer pair EF-728/EF2 is necessary in addition to the ITS primer pair.
4. Products containing *Trichoderma* species are effective against the pathogen under *in vitro* conditions.
5. Seed treatment with prochloraz was significantly effective in terms of the infection index and other value indicator traits of sunflower. It was found that seed treatment with prochloraz could be effective against the pathogen *in vitro*, in pot tests and under field conditions as well. Under *in vitro* conditions, the 0,3 l t^{-1} and 0,6 l t^{-1} doses have proven to be most effective against the pathogen, though it was found that the higher dose is phytotoxic. The field experiment clearly shows the superior efficacy of seed treatment over crop treatment with regards to disease incidence and other value indicators of sunflower
6. There was no significant difference between seed treatment and crop treatment regarding infection rate and therefore it can be concluded that seed treatment is sufficient to prevent infection. Crop treatment had a positive effect on the head diameter, total seed weight and thousand seed weight of sunflower. Therefore, in practice, both seed treatment and crop treatment are important controls, neither of which can be neglected.

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7. PUBLICATIONS IN THE TOPIC OF THE DISSERTATION

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List of publications related to the dissertation

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