HUNGARIAN UNIVERSITY OF AGRICULTURE AND LIFE SCIENCES

Pathotype composition and mefenoxam sensitivity of *Plasmopara halstedii* (FARLOW) BERLESE & DE TONI (sunflower downy mildew) in Hungary and the potential use of a botanical pesticide in the management

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1. BACKGROUND AND AIMS

Sunflower (*Helianthus annuus* L.) is one of the important oilseed crop in the world. There are several abiotic and biotic environmental factors that negatively regulate the crop yield of sunflowers and ultimately decline oil production (Rauf 2019). Among these biotic environmental factors, *Plasmopara halstedii* (FARLOW) BERLESE ET DE TONI, the causal agent of sunflower downy mildew, a biotrophic oomycete, infects preferably the sunflowers worldwide (Friskop et al. 2009, Sedlářová et al. 2013).) and leads to crop loss of up to 85% (Ioos et al. 2007). This pathogen not only leads the crop loss but also enhances the cost of protection and resistance breeding in sunflower plants. Therefore, it has become necessary to study this pathogen against the sunflower plants and fulfill the utmost demands of oil to the growing population around the globe.

This disease is mostly initiated by the soil-borne oospores and occasionally from infected seeds. *Plasmopara halstedii* infection in the sunflower usually takes place in the below ground plant parts by direct penetration in the roots (Virányi and Spring 2011). The pathogen mainly infects seedlings via their roots by zoospores leading to systemic infection but sometimes may cause local foliar lesions by airborne sporangia. Moreover, a local infection can turn systemic, which results in the deformation of upper plant parts (Spring, 2009). Root infection leads to seedling damping-off, or severe other symptoms, such as stunted plants (dwarfing), chlorosis of leaves, and white sporulation, which subsequently resulting in yield losses caused by the production of infertile flowers (Gascuel et al. 2015). Yield losses from downy mildew can be substantial, depending on the percentage of diseased plants across the field (Virányi and Spring, 2011).

Plasmopara halstedii rapidly develop races (pathotypes) that can break down the resistance genes in sunflowers (Sedlářová et al. 2016, Bán et al. 2018). *Plasmopara halstedii* is a highly variable and adaptive pathogen, which has about 50 pathotypes in the world nowadays (Spring et al. 2018, Spring 2019, Bán et al. 2021). The high variability of the pathogen significantly makes it difficult the effective disease management in sunflower cultivation. Thus, regular monitoring of the pathotype composition in a region or country is essential.

Downy mildew of sunflower can be controlled by using resistant cultivars carrying dominant *Pl* genes, agrotechnical methods, and chemical treatment (with fungicides) of the seeds with metalaxyl (Albourie et al. 1998). Metalaxyl is a

phenylamide fungicide which provides systemic protection against oomycetes. Mefenoxam (the stereoisomer of metalaxyl) has been widely used for downy mildew control as a seed dressing since 1977 (Melero-Vara et al. 1982, Patil et al. 1991, Schwinn and Margot 1991). This active substance has been extensively applied to control many different oomycetes, including *P. halstedii*, *Phytophthora infestans* (Mont.) de Bary, *Peronospora tabacina* de Bary and *Bremia lactucae* Regel (Schwinn and Staub 1987, Mouzeyar et al. 1995). However, *P. halstedii* has developed resistance against this active ingredient in many countries (Gascuel et al. 2015). To date, there is little or no data available in Hungary on the sensitivity of the pathogen to mefenoxam. In addition, very little is known about plant responses in plants infected with mefenoxam tolerant/resistant *P. halstedii* isolates.

Due to the high variability of the pathogen, traditional control methods need to be complemented by new approaches based on the principles of integrated pest management which are sustainable and economical. As a future alternative to fungicide treatments, efforts were made to control disease via induced resistance and biological antagonism (Sackston et al. 1992). There have been studies to test the effects of a botanical pesticide, neem (*Azadirachta indica* A. Juss), against different pests. Neem-based plant protection products are known to possess antifeedant, antifungal (Schmutterer 1988, Girish and Bhat 2008), nematicidal, insecticidal properties (Girish and Bhat 2008). There is preliminary (positive) data about neem's effect against sunflower downy mildew (Doshi et al. 2020), so more intensive research is needed in this area before its widespread use in the fields.

In view of the above, I have set the following objectives for my work:

- Pathotype identification of *P. halstedii* (sunflower downy mildew) isolates collected from different regions in Hungary in three consecutive years (2017-2019)

- Testing the mefenoxam sensitivity of *P. halstedii* isolates collected in Hungary and characterize host tissue responses to tolerant/resistant isolates with fluorescence microscope

- Investigations on the effectiveness of neem-derived pesticides on *P. halstedii* in sunflower under *in-vitro* and *in-vivo* conditions

2. MATERIALS AND METHODS

2.1 Pathotype identification of *Plasmopara halstedii* isolates collected between 2017 and 2019

2.1.1 Collection of diseased plant materials

Infected leaves of different sunflower hybrids carrying the *Pl6* resistance gene against sunflower downy mildew, were collected from different parts of Hungary between 2017 to 2019. Collected samples of *P. halstedii* isolates were transferred to the lab (Department of Integrated Plant Protection, Institute of Plant Protection, Hungarian University of Agriculture and Life Sciences, Gödöllő, Hungary) and then stored at -70 °C in a deep freezer until use. A total of 22 *P. halstedii* isolates were characterized during the experiment.

2.1.2 Propagation of inoculum using whole seedling immersion (WSI) method

Iregi szürke csíkos (a Hungarian sunflower cultivar susceptible to all the pathotypes of *P. halstedii)* was used for the propagation of pathogen inoculum. Seeds were surface sterilized in 1% NaOCl for 3-5 min, then rinsed in running tap water and germinated between wet filter papers for three days at 20 °C until radicles reached a length of 2 to 5 cm. The white zoosporangia from infected field leaves were washed off into bidistilled water and this suspension was adjusted to a concentration of 35000 sporangia per mL by Burker chamber. The whole seedling immersion (WSI) method (Cohen and Sackston 1973, Körösi et al. 2021) was used for inoculation, i.e., the 3-day old seedlings were incubated in a sporangial suspension at 16 °C in the dark for overnight. The inoculated sunflower seedlings were sown in trays containing horticultural perlite (d = 4 mm). The plants were grown in a growth chamber with a photoperiod of 12 h at 22 °C, light irradiance of 100 µE·m−2 ·s −1. The plants were watered regularly.

Nine days after inoculation, the plants were sprayed with bidistilled water and covered by dark plastic polyethylene bags overnight (at 19 °C) to induce sporulation. Collected sporangia were used as the inoculum for the characterization of pathotypes.

2.1.3 Characterization of *P. halstedii* pathotypes

The preparation of the seeds and inoculum, as well as the method of the inoculation and growing of the plants, were the same as described in the chapter 3.1.3 (Propagation of inoculum using WSI method). However, for the pathotypes characterization, seedlings were inoculated by the concentration of 50 000 sporangia/mL and were sown in trays containing 15 seedlings per each differential line. Plants were grown for 3 weeks.

The disease was evaluated firstly after sporulation, according to the white sporangial coating on cotyledons, and secondly, based on damping-off, as well as according to the chlorosis on true leaves of 21-day old plants. Reaction of plants was determined as susceptible (S) or resistant (R), according to the results of second evaluation.

The pathotype identification of *P. halstedii* isolates was performed by the universally accepted standardized nomenclature method as described by Trojanová et al. (2017) using the nine sunflower differential inbred lines (cv. Iregi szürke csíkos or HA-304 (susceptible lines), RHA–265, RHA-274, PMI-3, PM-17, 803-1, HAR-4, QHP2, and HA-335) all containing different *Pl* resistance genes against *P. halstedii*. A score for each differential line was determined based on the reaction of the plants (S or R) and the location of the differential line inside the triplet: 1, 2, and 4 scores can be given for susceptible lines located in the first, second, and third place inside the triplet, respectively. The pathotype code was determined as the sum of scores by each triplet and results in a three-digit code (coded virulence formula, CVF). The CVF provides information about the virulence pattern of the isolate. The test was repeated twice with two repetitions by each.

2.2 Fungicide sensitivity tests of *Plasmopara halstedii* isolates

2.2.1 Fungicide sensitivity test performed with 10 *P. halstedii* isolates by using WSI method

2.2.1.1 Isolates used for the test

For this experiment we selected 10 *P. halstedii* isolates from the collection of MATE (former SZIU) (isolates from 2014 and 2016) as well as we used some isolates from the 2017 collection.

2.2.1.2 Treatment of seeds with mefenoxam

Seeds were treated with Apron XL 350 FS (350 g/L mefenoxam, Syngenta AG, Switzerland) as per the European registered rate (3 mg/kg seeds) and evenly coated with the fungicide by mixing in a beaker. Treated seeds were kept for drying at room temperature for three days. Non-treated seeds were disinfected by immersion in a 1% Na-hypochlorite solution for 3-5 minutes and then rinsed with running tap water.

2.2.1.3 Preparation of inoculum and set of the 10-isolate experiment

The preparation of inoculum as well as the method of inoculation was same as described in the chapter "2.1.2, i.e., Propagation of inoculum using whole seedling immersion (WSI) method". For non-inoculated plants, seedlings were incubated in a bidistilled water as a control. The seedlings were sown in perlite in pots, containing 5 seedlings per pot.

The *P. halstedii* isolates of the I1–2, I3–6 and I7–10 codes were tested in separate experiments, respectively, under the same conditions. Each experiment was carried out twice with 10 replicates, respectively.

The following treatments and signs were used:

K0 – non-treated with mefenoxam, non-inoculated by *P. halstedii*; M – treated with mefenoxam, non-inoculated by *P. halstedii*; I – non-treated with mefenoxam, inoculated by *P. halstedii*; MI – treated with mefenoxam, inoculated by *P. halstedii*.

2.2.1.4 Disease assessment and measuring plant heights

The disease was evaluated once. Nine days after inoculation, plants were sprayed with bidistilled water and covered with a dark polyethylene bag. Trays were placed in the dark for 24 h at 19 °C to induce sporulation. Plant heights were measured twice. The efficacy of mefenoxam was calculated as the percentage of disease rate of treated and non-treated inoculated plants for all isolates.

Statistical analysis

The data were subjected to ANOVA. Fisher's test at $P < 0.05$ was used for the mean separation. The statistical analyses were performed using the software package Minitab (version 16.1.1.).

2.2.2 Fungicide sensitivity test performed with 8 *P. halstedii* isolates by using soil drench inoculation (SDI) method

For this experiment we selected 8 *P. halstedii* isolates from the collection of MATE. Treatment of seeds was the same as described in the chapters "2.1.2 and 2.2.1.2 i.e., treatment of seeds with mefenoxam".

2.2.2.1 Preparation of inoculum and inoculation using soil drench inoculation (SDI) method

The propagation of inoculum was same as described in the chapter "2.1.2 i.e., Propagation of inoculum and inoculation", except the concentration was adjusted to 50000 sporangia per mL using a Burker counting chamber. Seedlings were sown in perlite in pots (d = 8 cm), containing 5 seeds per pot.

Three days after sowing, seedlings were inoculated by the soil drench method as described by Trojanová et al. (2017) and Goossen and Sackston (1968). The sporangial suspension (2 mL per seedling) was pipetted directly onto the perlite surface of each pot containing the seedlings. For the non-inoculated, bidistilled water was drenched over seedlings as a control.

2.2.2.2 Set of the 8 isolate experiment and evaluation of disease

The plants were kept at 16°C in the dark in a growth chamber for 24 h to ensure infection. After inoculation, plants were grown in a growth chamber at 22°C with a 12 h photoperiod, light irradiance of 100 $\mu E \cdot m^{-2} \cdot s^{-1}$. The plants were watered regularly.

The *P. halstedii* isolates 1, 4, 5, 6, 7, 8, 9 and 11 were used during the experiment. Each experiment was carried out twice with 10 replicates, respectively.

2.2.2.3 Disease assessment

Nine days after inoculation, plants were sprayed with bidistilled water and covered with a dark polyethylene bag. Pots were placed in the dark for 24 h at

19°C to induce sporulation. The first evaluation was based on white coating (sporangia) on cotyledons and pre-emergence damping-off, referring to Disease 1. Twenty-one days after inoculation, a second evaluation was made according to chlorosis along the veins of the true leaves and post-emergence damping-off, referring to Disease 2. Plant heights were measured twice (Height 1 and 2) during each disease assessment.

2.2.2.4 Microscopic observations

Histological examinations of cross-sections of sunflower hypocotyls were performed using a fluorescence microscope (Olympus, Japan; filter block BX 50, transmission > 515 nm). Twenty-one days after inoculation, five sunflower hypocotyls were selected and fixed in FAA solution (formalin-acetic acid-ethanol, 10:5:50 by volume) from each treatment. Thin cross-sections (15-20 pieces) were cut with a razor blade from both upper and lower parts of the hypocotyl, and examined for pathogen structures (hyphae, haustoria) and host tissue responses (hypersensitive reaction, cell necrosis).

For the microscopic disease assessment, evaluation was conducted according to Bán et al. (2004) i.e., a 0-4 scale was used for the appearance of pathogen structures and host tissue responses in one, two, three, and four quarters of the cross-sections both in the cortical and pith parenchyma, respectively.

Statistical analysis

Fisher's test at $P < 0.05$ was used for the mean separation. Differences in disease rates, host characteristics (plant height) and host tissue responses (HR and cell necrosis) were assessed by analyses of variance. (ANOVA) followed by the Tukey HSD (Honestly Significant Difference) multiple comparison post-hoc test. Two-way ANOVA was used to examine the interaction between treatment (non-treated, treated) and isolates. Using Ward's method hierarchical cluster analysis was performed to group *P. halstedii* isolates based on their sensitivity to mefenoxam. To examine the correlation between variables, Pearson's correlation coefficient was used for scale variables (disease rates, heights) and Spearman's correlation coefficient was used for ordinal variables (microscopic variables). The IBM SPSS Statistics 27 software was used to conduct the statistical analysis.

2.2.3 Assessing the effect of different concentrations of mefenoxam on 5 *P. halstedii* isolates

The method of inoculation and fungicide treatment with different concentrations was the same as described previously, in the chapters "2.1.3 and 2.2.1.2". The plants were grown in a growth chamber with a photoperiod of 12 h at 22 °C, light irradiance of 100 μE·m−2 ·s −1.

Plasmopara halstedii isolates used in this experiment were Mád1, Mád2, Kömlő, Rákóczifalva and Csanytelek. Mád1, Kömlő, Rákóczifalva and Csanytelek isolates were increased on untreated, while Mád2 isolate on mefenoxam-treated sunflowers.

The whole seedling immersion (WSI) method by Cohen and Sackston (1973) was used for this experiment. The *P. halstedii* isolates were tested in two subsequent experiments with two replicates for each test.

The following treatments were used in the experiment:

- Zero control: Seedlings treated with bidistilled water.
- Infected control: Seedlings inoculated with *P. halstedii* sporangial suspension.
- Mefenoxam (1 mg/kg) treated seeds treated with bidistilled water.
- Mefenoxam (1 mg/kg) treated seeds inoculated with *P. halstedii* sporangial suspension.
- Mefenoxam (3 mg/kg) treated seeds treated with bidistilled water.
- Mefenoxam (3 mg/kg) treated seeds inoculated with *P. halstedii* sporangial suspension.
- Mefenoxam (9 mg/kg) treated seeds treated with bidistilled water.
- Mefenoxam (9 mg/kg) treated seeds inoculated with *P. halstedii* sporangial suspension.
- Mefenoxam (18 mg/kg) treated seeds treated with bidistilled water.
- Mefenoxam (18 mg/kg) treated seeds inoculated with *P. halstedii* sporangial suspension.
- Mefenoxam (30 mg/kg) treated seeds treated with bidistilled water.
- Mefenoxam (30 mg/kg) treated seeds inoculated with *P. halstedii* sporangial suspension.

The evaluation of the disease is the same as described in the chapter, "2.2.2.3".

Statistical analysis

ANOVA followed by a post-hoc Tukey test was performed to compare the different treatments in R software v 3.4.0 R Core Team, while graphs were made in Excel.

2.3 Efficacy of neem-derived pesticides to restrict sunflower downy mildew

2.3.1 *In-vitro* experiment: Examination of the effect of neem-derived pesticides on *P. halstedii* sporangial germination

Sunflower leaves infected by two *P. halstedii* isolates (Mád and Rákóczifalva) stored in deep freezer were soaked in 20 mL bidistilled water to release the sporangia. One milliliter(mL) of sporangia suspension was mixed with 0.5 mL of each tested concentrations of neem leaf extract or azadirachtin solutions, or with 0.5 mL of mefenoxam in an Eppendorf tube. It was agitated gently to mix uniformly and avoid bursting of sporangia, and was incubated at 16 °C for 24 h in the dark in a thermostat. After a 24 h incubation period, samples were observed with a microscope at 200× magnification, to check the effect of neem derived pesticides on the sporangia morphology and release of zoosporangia. Microscopic examination was done for each tested treatment by counting first 50 sporangia/treatment. The experiment was replicated five times with each treatment. Microscopic examination of sporangia in bidistilled water (BW) served as a negative control.

The following treatments were used for *in-vitro* experiment:

- Control – bidistilled water + *P. halstedii* sporangial suspension
- 3 mg/kg mefenoxam + *P. halstedii* sporangial suspension
- 10% Neem leaf extract solution + *P. halstedii* sporangial suspension
- 20% Neem leaf extract solution + *P. halstedii* sporangial suspension
- 0.01% NeemAzal solution + *P. halstedii* sporangial suspension
- 0.1% NeemAzal solution + *P. halstedii* sporangial suspension

2.3.2 *In-vivo* experiment: Effect of neem-derived pesticides on *P. halstedii* isolates (Mád and Rákóczifalva) in sunflower

2.3.2.1 Preparation of neem leaf extract (NLE)

The methodology for preparing neem leaf extract was followed according to Doshi et al. (2018) with slight modifications. The air-dried neem leaves were ground into powder using an electric blender. Two concentrations of 10% and

20% (w/v) were prepared by soaking 10 g and 20 g of neem leaf powder, respectively, in 100 mL of distilled water overnight, and then followed by filtration through a non-sterile cheesecloth to remove the coarse leaf materials. The filtered extract was centrifuged at 5000 rpm for 5 min to remove the remaining particles and obtain a clear extract.

2.3.2.2 Preparation of azadirachtin (NeemAzal T/S) (AZA)

A working concentration of 0.01% and 0.1% were prepared of NeemAzal T/S obtained from Trifolio Gmbh, Germany, containing (1% azadirachtin), a registered plant protection commercial product in the European Union, by dissolving 1 mL and 10 mL NeemAzal T/S in 100 mL of distilled water, respectively (Doshi et al. 2020).

2.3.2.3 Germination process and treatments

The sterilization method was the same as discussed previously. After sterilization, seeds were pre-soaked in different concentrations of Neem leaf extract (10 and 20%) and Neem Azal (0.01 and 0.1%) for 4 hours. After 4 hours, seeds were placed on wet filter paper, and germinated at 20 °C for 2 to 3 days (Doshi et al. 2020).

The whole seedling immersion (WSI) method by Cohen and Sackston (1973) was used for the experiment, as described previously. The *P. halstedii* isolates were tested in two subsequent experiments with two replicates for each test.

The following treatments were used in the experiment:

- Non-treated seedlings inoculated with *P. halstedii* sporangial suspension.
- Non-treated seedlings treated with bidistilled water (BW).
- Treated seeds with mefenoxam (3 mg/kg) inoculated with *P. halstedii* sporangial suspension.
- Treated seeds with mefenoxam (3 mg/kg) treated with bidistilled water (BW).
- Seedlings pre-treated with AZA 0.01% and inoculated with *P. halstedii* sporangial suspension.
- Seedlings pre-treated with AZA 0.01% and treated with bidistilled water (BW).
- Seedlings pre-treated with AZA 0.1% and inoculated with *P. halstedii* sporangial suspension.

- Seedlings pre-treated with AZA 0.1% and treated with bidistilled water (BW).
- Seedlings pre-treated with NLE 10% and inoculated with *P. halstedii* sporangial suspension.
- Seedlings pre-treated with NLE 10% and treated with bidistilled water (BW).
- Seeds pre-treated with NLE 20% and inoculated with *P. halstedii* sporangial suspension.
- Seedlings pre-treated with NLE 20% and treated with bidistilled water (BW).

For neem-derived pesticide experiment, thin cross-sections of both upper and lower parts of the hypocotyls were made. The followed was the same as discussed in chapter "3.2.2.4".

The disease was evaluated twice. The evaluation of the disease is the same as described in the chapter "3.2.2.3".

Statistical analysis

For both, *in vitro* and *in vivo* experiments, ANOVA followed by a post-hoc Tukey test was performed to compare the different treatments in R software v 3.4.0 R Core Team.

3. RESULTS

3.1 Pathotype identification of *P. halstedii* isolates collected in Hungary (2017-2019)

The results of the pathotype identification of sunflower downy mildew isolates collected in Hungary between 2017 and 2019 were the followings: out of the 22 *P. halstedii* isolates, nine isolates were characterized as pathotype 704, four as pathotype 700, three as pathotype 724, one as pathotype 714, one as pathotype 730 and four as pathotype 734. Among all pathotypes, pathotype 704 was the most widespread in collected samples. During the study, 734 was identified as a new pathotype in Hungary.

3.1.1 Identification of a new pathotype, 734, in Hungary

All four isolates examined caused disease on differential lines HA-304, RHA265, RHA-274, PMI-3, PM-17, and HA-335, whereas the other lines showed no

symptoms and signs of sunflower downy mildew. Summing the scores given according to the reactions of the differential lines by each triplet, the examined *P. halstedii* isolates were identified as pathotype 734.

3.2 Fungicide sensitivity tests

3.2.1 Fungicide sensitivity tests performed on 10 *P. halstedii* isolates by using the whole immersion method

Seven out of the ten isolates caused relatively high disease rates (ranging from 20 to 80%) on the mefenoxam-treated and inoculated sunflower plants with *P. halstedii*. The efficacy (%) of mefenoxam on the different *P. halstedii* isolates was calculated as the percentage reduction in the disease rate relative to the non-treated infected control. Mefenoxam performed poorly (18–40%) on three *P. halstedii* isolates (I5, I9, I10) and gave moderate (41–60%) protection against two isolates (I8, I7). The protection was good (61–80%) to excellent (> 81%) on five isolates (I1, I2, I3, I4, I6).

As the stunting of the infected plant is a significant symptom of *P. halstedii*; hence, the plant height was measured twice for some isolates during the experiments. There was no significant difference between the heights of the non-inoculated, mefenoxam treated and non-inoculated, non-treated plants in any of the experiments at any time of recording the information.

3.2.2 Fungicide sensitivity tests performed on 8 *P. halstedii* isolates by using the soil drench inoculation method

3.2.2.1 Disease rates and plant heights

Disease rates (%) and heights of mefenoxam treated and non-treated sunflower plants inoculated with different *P. halstedii* isolates were measured. According to the sporulation of the pathogen on the cotyledons and pre-emergence damped-off plants (Disease 1), mefenoxam-treated sunflowers inoculated with the isolates 1, 4, 5, 6, 7, and 9 showed significantly lower infection rates compared to non-treated ones. However, there were no significant differences in disease rates between treated and non-treated plants inoculated with isolates 8 and 11. The situation was similar with Disease 2 (ratio of chlorotic, post-emergence damped-off plants and healthy sunflowers), but there was no difference in the disease rate of treated and non-treated plants inoculated with isolates 7 in addition to isolates 8 and 11.

Plants of mefenoxam treated sunflowers inoculated with *P. halstedii* isolates 1, 4, 5, 6 were significantly higher than that of the non-treated inoculated plants nine days after inoculation. On the contrary, there was no significant difference in plant heights between treated and non-treated sunflowers inoculated by isolates 7, 8, 9, and 11. However, by the second recording date, the height of the treated plants was significantly higher than the non-treated plants for all isolates except 11.

3.2.2.2 Microscopic observations of host tissue responses

Host tissue responses of sunflowers to infection by *P. halstedii* in hypocotyl cross-sections were examined. Similar tissue responses were observed in most treated and non-treated plants infected with different isolates, but the intensity of the pathogenic spread and plant responses were variable. In general, intercellular hyphae and intracellular haustoria were detected in the hypocotyl of non-treated plants both in the cortical and the pith parenchyma 21 days after inoculation. By contrast, cell browning under normal light and an intense fluorescence of cells showing a hypersensitive-like reaction could be detected in cross-sections of several mefenoxam-treated sunflowers. Moreover, the development of cellular necrosis by vigorous cell division and the strong fluorescent response of surrounding cells was also frequently observed in treated and inoculated plants.

Hyphae were able to spread to a significantly greater extent in the cortical and pith parenchyma of non-treated plants inoculated with isolates 1, 4, 5, and 7 compared to mefenoxam-treated plants. In contrast, more hyphae were found in the cortical and pith parts of mefenoxam-treated sunflowers inoculated with *P. halstedii* isolate 8 than in non-treated ones. The situation was similar for the appearance of hyphae of isolate 11 in the pith. In addition, hyphae were significantly more abundant in the cortical part of non-treated than treated sunflowers inoculated with isolate 9, whereas there was no significant difference in hyphal distribution between treated and non-treated sunflowers for isolate 6.

Generally, fluorescence microscopy of cross-sections of sunflower hypocotyls revealed a relatively higher rate of hypersensitive-like reaction and necrosis (cell death) in the cortical than in the pith parenchyma in this experiment. The hypersensitive reaction was prominent in non-treated plants inoculated with isolate 5 and to a smaller extent in non-treated sunflowers inoculated with isolates 1, 4, 6, and 11 in the cortical parenchyma. Necrosis in the pith parenchyma cells was minimal in each sample.

3.2.2.3 Assessing the sensitivity of *Plasmopara halstedii* isolates to mefenoxam

Cluster analyses of sunflowers based on disease rates and plant heights inoculated by different *P. halstedii* isolates was performed. Four distinct clusters could be identified determined by macroscopic parameters. Cluster 1 includes non-treated plant samples inoculated with isolates 5, 6, 9, 11, and mefenoxam-treated plants from 11, which were found to have high infection levels in both sampling periods. Therefore, the pathogen was able to penetrate the upper parts of these sunflowers. Plant heights were the lowest in this group. In Cluster 2 are samples of the other part of non-treated and inoculated plants, where the first infection value (Disease 1) was relatively high, like Cluster 1. However, unlike the first cluster, the second time point for disease assessment (Disease 2) resulted in much lower infection values and less plant dwarfing in Cluster 2 members. In this case, the pathogen could only penetrate to a lesser extent above the hypocotyl.

Clusters 3 and 4 mainly include samples of inoculated plants treated with mefenoxam. In contrast to the initial infection rates, there was no significant difference between the two clusters in the second survey. However, the plant height values were significantly higher for Cluster 3 members.

Cluster analyses of sunflowers based on the examined microscopic variables inoculated by different *P. halstedii* isolates was presented. Three distinct clusters could be identified by microscopic parameters, such as hyphal spread, the occurrence of hypersensitive-like reaction, and the development of necrosis in the cortical and pith parenchyma. Samples of non-treated inoculated plants are in the first two clusters, while mefenoxam-treated plants can be found in all three clusters. Moreover, treated plants inoculated with isolates 4 and 5 are equally represented in the first two clusters.

For Cluster 1 samples, the pathogen could invade both the cortical and pith parenchyma. Not only the spread of hyphae but also the HR and necrosis in different tissue sections were significant in Cluster 1 samples compared to the other two clusters. Besides non-treated ones, treated sunflowers inoculated with the *P. halstedii* isolates 8 and 11 are included in the first cluster. Unlike the sunflowers in the first cluster, the distribution of hypha of samples in Cluster 2 was accompanied by HR and necrosis only in the cortical parenchyma but not in the pith. Most of the treated sunflower samples, except for isolates 6, 8, and 11, are in Cluster 3, with little hyphae detected in the cortical tissues. No plant response was detected in these sunflowers.

3.2.2.4 Correlations among macroscopic and microscopic parameters

Pearson correlation based on the examined macroscopic variables (disease rates, plant heights) was performed. During the second evaluation, a strong negative correlation was found between the disease rate and plant height values of both non-treated and treated plants. Similarly, there was a strong negative correlation between the initial disease rates and the final plant height values of treated plants in the experiment. In contrast, a high positive correlation could be detected between the initial and final plant height data of both treated and non-treated plants. In addition, a strong positive correlation was found between the initial and final disease values of mefenoxam-treated sunflowers.

Spearman correlation of the examined microscopic variables was performed. There was a strong positive correlation in the occurrence of hyphae in different parenchymatic plant parts (cortical and pith) of both non-treated and treated inoculated sunflowers. Moreover, strong positive correlations were found among the presence of hyphae in the cortical parenchyma tissues and the appearance of hypersensitive reaction and necrosis, respectively, in treated plants. In addition, a strong positive correlation could be confirmed for the establishment of necrosis in the cortical part and the occurrence of hyphae in the pith of mefenoxam-treated and inoculated sunflowers.

3.2.3 Effect of different concentrations of mefenoxam on 5 *P. halstedii* isolates in sunflower

3.2.3.1 Disease rates

The values of non-treated, inoculated plants with Mád1 and Mád2 isolates were significantly higher than those of other *P. halstedii* isolates. Disease values gradually decreased for all plants inoculated with different isolates by increasing mefenoxam concentration. Disease values of plants inoculated with Kömlő, Rákóczifalva, and Csanytelek isolates were halved already at 3 mg/kg mefenoxam concentration compared to control plants. In contrast, in the Mád isolates, the halving occurred at 18 mg/kg concentration.

Interestingly, in sunflowers inoculated with Mád isolates, there was no difference in Disease 1 and 2 values between 18 and 30 mg/kg treatments with mefenoxam. In addition, no or minimal infection was found on plants inoculated with Kömlő, Rákóczifalva, and Csanytelek isolates at 9, 18, and 30 mg/kg mefenoxam concentrations during the assessment period.

3.2.3.2 Plant heights

In the case of the inoculated plants with Mád 1 isolate, there was no significant difference in Height 1 values between control (I) and treated plants in the first assessment. However, during the second assessment, only plants treated with 30 mg/kg mefenoxam could grow higher than t In the case of the inoculated plants with Mád 2 isolate, there was no significant difference in Height 1 values between control (I) and treated plants in the first assessment. However, similarly to Mád 1 isolate, during the second assessment, only plants treated with 18 and 30 mg/kg mefenoxam could grow higher than the control.

In the case of the inoculated plants with Kömlő isolate, there was no significant difference in Height 1 values between control (I) and treated plants in the first assessment. Twenty-one days after inoculation, it could be detected that inoculated plants treated with 18 mg mefenoxam were significantly higher than the control.

During the first evaluation of plants inoculated by the Rákóczifalva isolate, no significant difference was observed among all the plant heights of inoculated sunflowers except plants treated with 1 mg mefenoxam, which were significantly higher. However, all treated and inoculated plants were significantly higher than the control 21 days after inoculation.

In the case of the inoculated plants with Csanytelek isolate, there was no significant difference in Height 1 values between control (I) and treated plants in the first assessment. However, twenty-one days after inoculation, it could be detected that all treated and inoculated plants were significantly higher than the control.

3.3 Effects of neem-derived pesticides on sunflower downy mildew

3.3.1 *In-vitro* experiment: Examination of the effect of neem-derived pesticides on *P. halstedii* sporangial germination

The microscopical examination of sporangia was done 24 h after treatment with neem leaf extract and NeemAzal T/S (1% azadirachtin). For the Rákócifalva isolate, the statistical analysis showed that all the neem-derived pesticide treatments significantly decreased the number of empty sporangia, thus inhibiting germination. For the Mád isolate, all the treatments, except AZA 0.1%, were found to be significantly better than the control (no treatment) at reducing the

number of empty sporangia (which includes completely or partially empty sporangia.

3.3.2 Pre-treatment effect of neem-derived pesticides on *Plasmopara halstedii* isolates in *in-vivo* conditions

3.3.2.1 Neem effects on disease rates and plant heights

On the two assessment dates, both concentrations of neem leaf extract and NeemAzal T/S and mefenoxam treatment were found to reduce the sporulation of *P. halstedii* isolates Mád and Rákóczifalva significantly. Thus, the pre-treatments significantly affected the sporulation, pre-damping-off, chlorosis, and post-damping-off caused by *P. halstedii*.

The plant heights for first and second evaluation were measured. Plant heights of non-inoculated sunflowers were similar all over this experiment. The plant heights of inoculated sunflowers with Mád isolate were significantly higher than that of the control for the mefenoxam treated and lower for the NLE10% treated plants in the first evaluation. The plant heights of inoculated sunflowers with the Rákócifalva isolate were significantly higher than that of the control for the AZA0.01 treated plants in the first evaluation. During the second evaluation, the heights of inoculated and treated plants with different neem-products were significantly higher than the control (except NLE10% Mád and AZA0.01 Rákóczifalva treatments).

3.3.2.2 Microscopic observations

Intercellular hyphae and haustoria were able to spread in the cortical and pith parenchyma of most treated and non-treated plants inoculated with different *P. halstedii* isolates. In addition, in plants treated with mefenoxam and inoculated with the Mád isolate, hyphae of the pathogen were significantly more abundant both in the cortical and pith parenchyma than that of the non-treated plants. The opposite was true for the mefenoxam-treated plants inoculated by the Rákóczifalva isolate.

In sunflowers treated with neem-derived pesticides and inoculated by Mád isolate significantly more hyphae could be observed compared to non-treated ones in the cortical than in the pith parenchyma. No hypersensitive reaction and necrosis could be detected in the pith parenchyma for all treatments in both isolates. More necrosis in the cortical parenchyma cells was observed in AZA 0.01% treated

sunflowers than in inoculated controls for Rákóczifalva isolate. Similarly, we could detect more necrosis in the cortical part of sunflowers treated with both concentrations of AZA and mefenoxam inoculated by the Mád isolate.

4. DISCUSSION

4.1 Pathotype composition of sunflower downy mildew (*P. halstedii*) in Hungary

Downy mildew of sunflower is one of the most widespread diseases caused by *P. halstedii* and affects the crop worldwide. Moreover, *P. halstedii* has several pathotypes with varying degrees of virulence. Thus, the background knowledge of the distribution of *P. halstedii* pathotypes is of utmost importance for effective pest management (Virányi et al. 2015, Spring 2019, Bán et al. 2021, Miranda-Fuentes et al. 2021).

Pathotype characterization of *P. halstedii* is based on an internationally accepted methodology with 9 sunflower differential lines to serve as a standard method worldwide (Trojanová et al. 2017). Recently, it has been found that several pathotypes have overcome the *PI6* resistance gene incorporated into a wide range of sunflower hybrids and led to the emergence of highly aggressive pathotypes (Sedlářová et al. 2016, García-Carneros and Molinero-Ruiz 2017, Bán et al. 2018, Spring and Zipper 2018) and *PI8* (Martin-Sanz et al. 2020).

More recently, Bán et al. (2021) updated the distribution of pathotypes of sunflower downy mildew in seven European countries and reported 18 new pathotypes in six countries. This dissertation is part of this work, which presents data for Hungary from 2017 to 2019. As a result, besides the dominance of high virulent pathotypes such as 704, the presence of less virulent pathotypes (700 and 730) was also confirmed in our study from 2017 to 2019. In addition, we identified pathotype 734 for the first time in Hungary (and Central Europe) during this period (Nisha et al. 2021). Previously the occurrence of the globally new pathotype, 724, has been reported only in Hungary, from two regions in Békés county (Mezőkovácsháza and Szeghalom) (Bán et al. 2018). Later, pathotype 724 was also detected in Romanian samples in 2019 (Bán et al. 2021). In this work, we confirmed its presence in two more sites in Békés county (Békésszentandrás and Vésztő).

Finally, this work supports the previous considerations by Virányi et al. (2015) that there is a shift in the pathotype composition of sunflower downy mildew in

Hungary. However, many more samples and frequent sampling would be needed to prove this pathogenic shift.

4.2 Mefenoxam-sensitivity of Hungarian *P. halstedii* isolates

The widespread use of mefenoxam has resulted in a decline in efficacy against sunflower downy mildew in some western European countries (Lafon et al. 1996; Albourie et al. 1998, Molinero-Ruiz et al. 2003) and the USA (Gulya 2000).

In our first fungicide resistance study, 10 isolates of *P. halstedii* were tested using the WSI (whole seedling immersion) method. Here, we were interested in how the registered rate of mefenoxam (3mg/kg seed) influences the development of initial symptoms and signs (sporulation, early damping-off, decrease in plant height) of different *P. halstedii* isolates originated mainly from hybrids where mefenoxam was applied as a seed coating. Mefenoxam performed poorly or only moderately in the case of half of the examined *P. halstedii* isolates in our test. Although a limited number of samples have been analysed, these results provide the first evidence of mefenoxam tolerance of sunflower downy mildew in high oleic sunflower hybrids in Hungary.

Continuing the sensitivity studies with additional *P. halstedii* isolates, the SDI (soil drench inoculation) method was used in the next series of 8 isolates, which better models the natural infection of the pathogen. We were also curious to see if there were differences in plant responses such as hypersensitive reaction and cell necrosis in plants infected with isolates of different sensitivities. We performed detailed statistical analyses here to show differences.

Both mefenoxam-treated and non-treated plants formed two relatively distinct groups (clusters) based on the cluster analysis of disease rates and plant heights in the 8 isolate experiment. Treatment with mefenoxam had different effects on different *P. halstedii* isolates, according to disease rates and plant heights.

Pearson correlation, especially during the second evaluation, showed a strong negative correlation between the disease rate and plant height values of both non-treated and treated plants. In the case of treated plants, this negative correlation is presumably related to fungicide tolerance (resistance) since if the pathogen can spread within the plant, the growth-reducing effect is exerted.

Host tissue responses of sunflowers (susceptible, resistant) inoculated with *P. halstedii* have already been examined by several authors (Allard 1978, Wehtje et

al. 1979, Gray and Sackston 1985, Mouzeyar et al. 1993, 1994, Bán et al. 2004, Radwan et al. 2011). We first revealed a clear difference in host tissue responses of mefenoxam-treated susceptible sunflowers inoculated with various *P. halstedii* isolates. Treated sunflowers inoculated by some isolates (6, 8, and 11) showed hyphal growth in the cortical and pith parenchyma. The cortical part could also detect a moderate hypersensitive reaction and necrosis. For other *P. halstedii* isolates, we could detect a limited or no mycelial growth in the mefenoxam-treated plants, which was accompanied by weak or no reactions of treated sunflowers in their hypocotyls.

In our 8 isolate study, cluster analyses of sunflowers based on the microscopic variables showed clear differentiation of three groups of mefenoxam-treated sunflowers inoculated by different *P. halstedii* isolates. However, microscopic studies allowed us to estimate the sensitivity (tolerance) more accurately, showing refined interaction with non-treated plants.

Further studies are needed to explore the reasons for the differences in tissue responses to sensitive and tolerant isolates of *P. halstedii* in sunflower. In addition, how plant defense mechanism contributes to the effectiveness of fungicides also has to be elucidated.

In the third part of the fungicide sensitivity studies, the effects of different mefenoxam concentrations were tested against 5 isolates of *P. halstedii* with the WSI method for inoculation. One isolate (Mád) was propagated on both non-treated (Mád1) and mefenoxam treated plants (Mád2); the others could only be propagated on non-treated plants. Although EC50 values were not established, we estimated the mefenoxam concentration (interval) at which at least half of the plants showed symptoms and signs of *P. halstedii*. These values varied, ranging from 18 to 30 mg for two isolates (Mád1 and 2) and from 1 to 3 mg for the others. On this basis, the Mád isolate is further evidence of the presence of mefenoxam tolerance/resistance in Hungary, as it was only effective against it at several folds of the registered concentration (3 mg/kg seeds).

4.3 Efficacy of neem-derived pesticides to restrict sunflower downy mildew

We tested neem leaf extracts (NLE) and azadirachtin (AZA as NeemAzal T/S) in different concentrations against two different *P. halstedii* isolates by treating plants with the ingredients for a longer exposure time (4 h) than Doshi et al. (2020). In addition, we studied host tissue responses (hypersensitive reaction and

cell necrosis) of neem-treated, inoculated plants with a fluorescent microscope to explore the histological background of protection.

Under *in vitro* conditions, all the treatments except the higher concentration of NLE showed significant inhibition of the sporangial germination of Rákóczifalva isolate. Similarly, except for AZA 0.1%, all the treatments were significantly better than the inoculated control at reducing the total number of empty sporangia for Mád isolate.

Previously, Mirza et al. (2000) tested the neem products on *Phytophthora infestans* (Mont.) de Bary *in vitro*. They reported the effectiveness of all the products, namely crude neem seed oil, nimbokil (a commercial formulation of neem oil), crude terpenoid extract of neem seed oil, and neem leaf decoction against mycelial growth, sporangial germination, and sporangium production of *P. infestans*. It was shown that all these products could potentially manage potato late blight disease.

When tested *in vivo* in our study, both concentrations of neem leaf extracts and NeemAzal T/S significantly reduced the sporulation and chlorosis of *P. halstedii* isolates as compared to inoculated control plants. Our results were consistent with the findings of Achimu and Schlösser (1992), where neem seed extract and commercial neem products were effective against *Plasmopara viticola* in the grapevine.

Host tissue responses of neem-treated sunflowers inoculated by *P. halstedii* were first examined by fluorescent microscope in our study. We observed a similar tissue response (cell necrosis) in neem-treated and inoculated plants as previously observed in BTH (benzothiadiazole as Bion 50WG) treatments against sunflower downy mildew (Bán et al. 2004) and sclerotinia (Bán et al. 2017). Similarly, we could detect more necrosis in the cortical part of sunflowers treated with both concentrations of AZA and mefenoxam inoculated by the Mád isolate as compared to the non-treated control. This was true for plants treated with 0.01 % AZA and inoculated with the Rákóczifalva isolate. Therefore, it seems that azadirachtin induces similar host responses in diseased plants to mefenoxam and benzothiadiazole, which can play a role in restricting *P. halstedii* in susceptible sunflowers.

5. CONCLUSIONS AND RECOMMENDATIONS

The high variability of *P. halstedii* is an important trait of the pathogen allowing it to overcome the resistance genes and the effectiveness of the compounds such as mefenoxam. Therefore, the key task and goal of the future research is to monitor the pathotype composition and fungicide resistance of the pathogen. This facilitates the efficient resistance breeding and the development of new active substances against the pathogen in order to get good quality and produce high yields.

Also, the broader use of integrated plant protection could significantly slow down the evolution of new pathotypes of *P. halstedii*.

Plasmopara halstedii has several pathotypes with varying degrees of virulence. Recently, highly aggressive pathotypes have emerged worldwide. Information on the virulence diversity within the population of sunflower downy mildew become essential for resistance breeding and quarantine measures. Moreover, a highly aggressive pathotype might repress the host's defense mechanisms, creating favorable conditions for the less virulent pathotypes. Thus, an even more diverse population of sunflower downy mildew can threaten the effectiveness of control methods against the pathogen. Integrated pest management, therefore, is an essential tool to manage *Plasmopara halstedii*. In addition, the introduction of new methods in pathotyping is urgent because of the uncertainties of previous methods.

The widespread use of mefenoxam has resulted in a decline in efficacy against sunflower downy mildew in Hungary, in Europe and in the USA. Mefenoxam performed poorly or only moderately in the case of several *P. halstedii* isolates in our study. In conclusion, treatment with mefenoxam had different effects on different *P. halstedii* isolates, according to disease rates and plant heights. Further studies are needed to explore the reasons for the differences in tissue responses to sensitive and tolerant isolates of *P. halstedii* in sunflower. In addition, how plant defense mechanism contributes to the effectiveness of fungicides also has to be elucidated.

Fungicidal resistance/tolerance to mefenoxam requires the introduction of newer, effective agents to protect against the pathogen. This also calls for the research and introduction of new alternative control methods and innovative management tools against the disease. For this, the effect of two different neem-derived pesticides, such as neem leaf extract (NLE) and NeemAzal T/S, was tested in different concentrations against *P. halstedii* isolates under *in vitro* and *in vivo* in our study.

Host tissue responses of neem-treated sunflowers inoculated by *P. halstedii* were examined for the first time by fluorescent microscope in our study. This research on neem-derived pesticides efficacy against downy mildew is a first step to control this disease. Further research is needed for alternative methods. Botanical pesticides, such as neem products may play an important and effective method in the future against *P. halstedii* and other pathogens.

6. NEW SCIENTIFIC RESULTS

I. We proved the dominance of high virulent pathotypes such as 704 and the presence of less virulent pathotypes (700 and 730) between 2017 and 2019 in Hungary. We first showed a highly virulent pathotype (724) in the western part of Hungary.

II. We identified pathotype 734 for the first time in Hungary and Central Europe and prooved its occurrence from three different regions of Hungary.

III. We confirmed the previous statements that there is a shift in Hungary's pathotype composition of sunflower downy mildew towards highly virulent pathotypes.

IV. We proved the presence of mefenoxam tolerant/resistant *P. halstedii* isolates in the Hungarian sunflower downy mildew population.

V. We first revealed differences in host tissue responses such as hypersensitive reaction and cell necrosis of mefenoxam-treated susceptible sunflowers inoculated with various *P. halstedii* isolates.

VI. We first found that neem leaf extract and azadirachtin were effective against two *P. halstedii* isolates *in vivo* and *in vitro.*

VII. We first observed a similar host tissue response (cell necrosis) in neem-treated and inoculated plants as previously observed in BTH (benzothiadiazole as Bion 50WG) treatments against sunflower downy mildew.

THE PUBLICATIONS OF THE AUTHOR RELATED TO THE DISSERTATION

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