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New data on pathotype distribution and mefenoxam tolerance of *Plasmopara halstedii* in Hungary

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Abstract: Sunflower downy mildew (*Plasmopara halstedii*) is one of the major diseases that can be controlled by using resistant cultivars and seed dressings; however, several isolates have developed tolerance to some fungicides and the resistance has also been overcome by new pathotypes. We aimed to examine the pathotype distribution in Hungary and to test the pathotypes' mefenoxam sensitivity. The isolates, which provided the basis of the research were collected from different regions of Hungary between 2014 and 2017 and, later, their pathotypes were identified. According to our results, pathotype 704 was one of the most widespread in Hungary, but pathotype 730, pathotype 724 and pathotype 700 were also detected. Seven out of ten isolates caused relatively high disease rates on the mefenoxam-treated and inoculated sunflower plants with *P. halstedii*. The pathogen has a high genetic variability which enhances the possibility to develop fungicide resistance. Furthermore, this variability can easily contribute to the breakdown of the resistant genes of the resistant hybrids. Both features can reduce the effectiveness of management; therefore, the continuous monitoring of this oomycete is very important.

Keywords: fungicide tolerance; *Helianthus annuus*; metalaxyl-M; sunflower downy mildew; virulence phenotype

Plasmopara halstedii (Farlow) Berlese et de Toni 1888, the causal agent of sunflower downy mildew, a biotrophic oomycete, is one of the most destructive pathogens of sunflowers worldwide (Sackston et al. 1990; Sedlářová et al. 2013). The importance of the pathogen is also supported by its quarantine status in European seed transport since 1992 (Delmotte et al. 2008). *P. halstedii* mostly causes a primary infection through the roots of the sunflower plants, which results in systemic symptoms, such as stunting and chlorosis. Although the seeds of the systemically infected plants are small and less viable, a significant ratio has been shown to germinate and carry the infection into the next generation (Spring 2001). *P. halstedii* is a highly variable and adaptive pathogen, which has about 50 pathotypes in the world nowadays (Spring et al. 2018; Spring 2019). The high variability of the pathogen significantly threatens the effective disease management in sunflower cultivation. The management of sunflower downy mildew mainly relies on using resistant cultivars carrying dominant *Pl* (polysaccharide lyase) genes, as well as crop rotation and the use of selective fungicides (Vear et al. 1997; Qi et al. 2016). However, new pathotypes easily break down the resistance incorporated into the hybrids after being frequently planted in the rotation system.

Besides the genetic protection and crop rotation, the disease can also be controlled chemically by

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phenylamide fungicides. One such compound is mefenoxam (or metalaxyl-M, the stereoisomer of metalaxyl), which provides the systemic protection against several oomycetes (Schwinn & Staub 1995). Mefenoxam has been widely used for downy mildew control as a seed dressing since 1977 (Melero-Vara et al. 1982; Patil et al. 1991; Schwinn & 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 & Staub 1987; Mouzeyar et al. 1995). Mefenoxam translocates acropetally within the plant and inhibits the ribosomal RNA synthesis – specifically RNA polymerisation – in the pathogen. Resistance against metalaxyl and mefenoxam appeared rather quickly in many pathogens after its introduction, including *Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & De Toni, *Pseudoperonospora cubensis* (Berk. & M.A. Curtis) Rostovzev 1903, *P. tabacina* de Bary; *B. lactucae* Regel 1843, *P. infestans* and *Pythium* spp. (Milgroom 1990; Gisi & Cohen 1996).

Due to the wide application of mefenoxam, resistant isolates of *P. halstedii* were first found in France, then in the USA and Spain (Lafon et al. 1996; Albourie et al. 1998; Gulya 2000; Molinero-Ruiz et al. 2003). It was in the 1980s, when Oros and Virányi (1984) detected the existence of tolerant *P. halstedii* strains in greenhouse experiments in Hungary, but could not prove it in the further tests with field isolates (Virányi & Walcz 2000). Nevertheless, the existence of such resistant isolates was not excluded and presumably was the first weak sign of the fungicide tolerance phenomenon in the Hungarian population of *P. halstedii*. With the increase in the cultivation area of sunflower production in the 2000s in Hungary, downy mildew became more widespread and has recently been considered as one of the most important pathogens of sunflowers. One of the reasons for the increased significance of the disease is the occurrence of new pathotypes in some regions in Europe (and in Hungary, too) after 2010 (Bán et al. 2014a, b, 2018; Trojanová et al. 2018). Another reason may be the decreasing sensitivity of the *P. halstedii* strains to mefenoxam.

Our goals, therefore, were (*i*) to deliver new data on the pathotype distribution and dominance of sunflower downy mildew and (*ii*) to test the mefenoxam sensitivity of certain *P. halstedii* isolates collected in Hungary and assess their role in the increasing disease severity.

MATERIAL AND METHODS

Collection of diseased plant materials. The *Plasmopara halstedii* isolates, which are used in this study were collected from sunflower hybrids carrying the *Pl6* resistance gene against sunflower downy mildew, from different part of Hungary between 2014 and 2017. The sign and origin of the isolates are shown in Figure 1. The samples were kept at –70 °C in a deep freezer until use.

Propagation of inoculum. The inoculum of *P. halstedii* isolates was propagated on the sunflower *Helianthus annuus* cv. Iregi szürke csíkos, which is susceptible to all known pathotypes of sunflower downy mildew. The sterilisation of the surface of the seeds was undertaken in 1% Na-hypochlorite for 3–5 min then they were washed thoroughly with running tap water. During the germination process, the seeds were placed on wet filter paper, the temperature was kept at 20 °C for 2 to 3 days. The zoosporangia were washed off into double distilled water and this suspension was adjusted to a concentration of 35 000 sporangia/mL by haemocytometer. The whole seedling immersion (WSI) method (Cohen & Sackston 1973) was used for inoculation, i.e., the seedlings were incubated in a sporangial suspension at 16 °C in the dark overnight. The inoculated seedlings were sown in trays containing pure perlite (d = 4 mm). The plants were grown in a growth chamber with a photoperiod of 12 h at 22 °C. Nine days after inoculation, the plants were sprayed with double distilled water and covered by dark plastic bags overnight (at 19 °C) to induce sporulation. The sporangia were collected and the inoculum was kept at –70 °C until use.

Figure 1. Code and location of the origin of the *Plasmopara halstedii* isolates (2014–2017, Hungary)

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Pathotype identification of *P. halstedii* isolates. The pathotype identification of *P. halstedii* isolates was performed as described by Trojanová et al. (2017) using nine sunflower differential lines (*H. annuus* cv. Iregi szürke csíkos/A Hungarian cultivar susceptible to all pathotypes of *P. halstedii*/RHA–265, RHA-274, PMI-3, PM-17, 803-1, HAR-4, QHP-2, HA-335). The preparation of the seeds and inoculum, as well as the method of the inoculation and growing the plants, were the same as described in the chapter "Propagation of inoculum". The disease was evaluated twice; first, after sporulation according to the white coating of the cotyledons and, the second time, according to the symptoms (chlorosis) on the true leaves of the 21-day old plants. The *P. halstedii* isolates were tested in two subsequent experiments with two replicates for each test.

Fungicide sensitivity test. The *P. halstedii* isolates of the I1–2, I3–6 and I7–10 codes were tested in separate experiments, respectively, under the same conditions. For the fungicide sensitivity test, the seeds (except for the controls) were treated with Apron XL 350 FS (350 g/L mefenoxam, Syngenta AG, Switzerland) with registered rate in Europe (3 mg/kg seeds) by mixing them homogeneously with the fungicide and dried at room temperature for 3 days. 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*

The preparation of the seeds and inoculum, as well as the method of inoculation, were the same as described in the chapter "Propagation of inoculum". The seedlings were sewn in perlite in pots (d = 8 cm), containing 5 seeds/pot. Assessment of the disease was undertaken according to symptoms and signs (white coating) on the cotyledons and the number of damped-off plants 9 days after inoculation. The plant heights (for sunflowers inoculated with isolates I1–2 and I3–6) were measured twice, at 9 and 21 days after inoculation, respectively. Isolates I1–2, I3–6 and I7–10 were examined in separate experiments with the same conditions. Each experiment was carried out twice with 10 replicates, respectively. The efficacy of mefenoxam for each *P. halstedii* isolate was calculated as the percentage of the reduction in the disease rate compared to the non-treated inoculated control plants.

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.).

RESULTS

Pathotype identification of *P. halstedii* isolates. The sunflower downy mildew isolates collected in Hungary between 2014 and 2017 were characterised for their pathotype (Table 1). Isolate I2 from Mezőkovácsháza had already been identified by Bán et al. (2018) as pathotype 724 but was re-tested in this study for its virulence phenotype. Out of the nine *P. halstedii* isolates, one (I3) from 2017 was identified as pathotype 700 and two (I8, I10) were identified as pathotype 730. Five isolates were identified as pathotype 704 (I1, I4, I6, I7, I9). A globally new *P. halstedii* pathotype, 724, was detected in two samples (I2, I5) collected in 2017. For isolate I2, we confirmed the result of our previous identification as 724.

Fungicide sensitivity tests. The disease rates of the different *P. halstedii* isolates on the mefenoxam-treated and non-treated sunflowers are shown in Figure 2. 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.* Among these, the highest infection rates were found with the I5 (pathotype 724 from Bonyhád), I9 (pathotype 704 from Körösladány) and I10 (pathotype 730 from Csanytelek) isolates. The downy mildew isolates showing sensitivity to mefenoxam were I1 (pathotype 704 from Tiszaföldvár), I3 (pathotype 700 from Túrkeve)

Table 1. Origin and determined pathotypes of the *Plasmopara halstedii* isolates collected in Hungary in 2014, 2016 and 2017

Isolate (code)	Locality	Collection (year)	Pathotype
I1	Tiszaföldvár	2017	704
I2	Mezőkovácsháza	2017	724
I3	Túrkeve	2017	700
I4	Karácsond	2017	704
I5	Bonyhád	2017	724
I6	Pély	2017	704
I7	Csongrád	2016	704
I8	Tiszafüred	2014	730
I9	Körösladány	2014	704
I10	Csanytelek	2014	730

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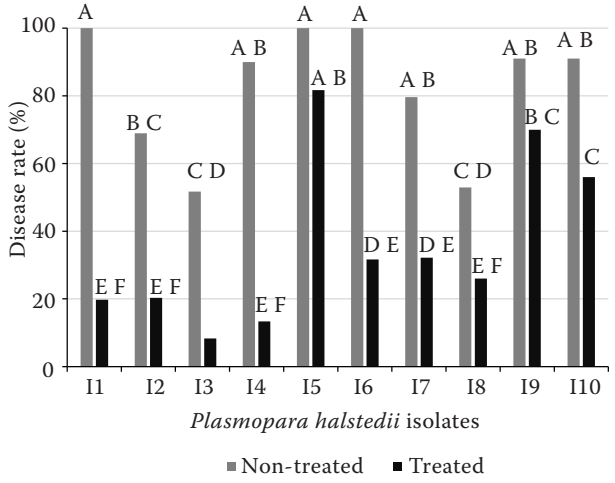

Figure 2. Disease rates (%) on the sunflowers (treated and non-treated with mefenoxam) inoculated by the different isolates of *Plasmopara halstedii* 9 days after inoculation

Non-treated – non-treated with mefenoxam and inoculated with *P. halstedii*; treated – treated with mefenoxam (3 mg/kg seed) and inoculated with *P. halstedii*; ANOVA was performed with Fisher's test; the bars sharing the same letter are not significantly different

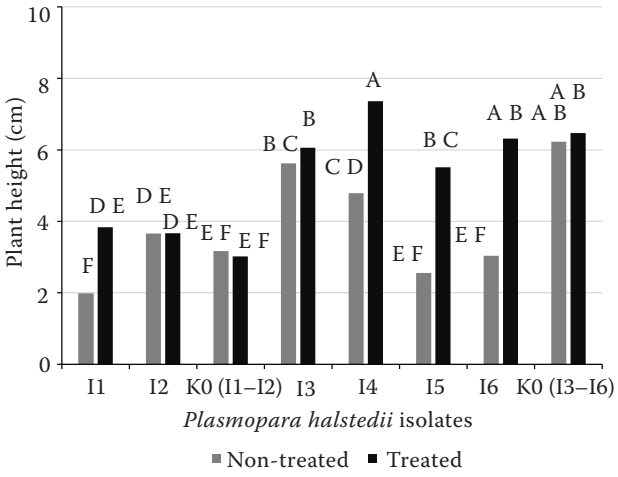

Figure 3. Plant heights of the mefenoxam-treated and non-treated sunflowers 9 days after inoculation with *P. halstedii*

Non-treated – non-treated with mefenoxam and inoculated with *P. halstedii*; treated – treated with mefenoxam (3 mg/kg seed) and inoculated with *P. halstedii*; K0 (I1 – I2) and K0 (I3 – I6) – non-treated and non-inoculated with *P. halstedii* for I1–I2 and I3–I6, respectively; ANOVA was performed with Fisher's test; the bars sharing the same letter are not significantly different

and I4 (pathotype 704 from Karácsond). All of the non-treated and inoculated plants with isolates I1, I5 and I6 showed a damping-off by the end of the experiment (data not shown).

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 (Figures 3 and 4). 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. Furthermore, the mefenoxam-treated and inoculated sunflowers grew similarly to the non-inoculated ones at the first evaluation (Figure 3). The plant heights were significantly lower for the non-treated sunflowers inoculated with the *P. halstedii* isolates, I1, I4, I5 and I6, than that of treated plants at the first evaluation. The non-treated plants inoculated with I1, I5 and I6 isolates showed a damping-off by the time of the second evaluation (Figure 4). The non-treated, inoculated plants with the I2 and I3 isolates

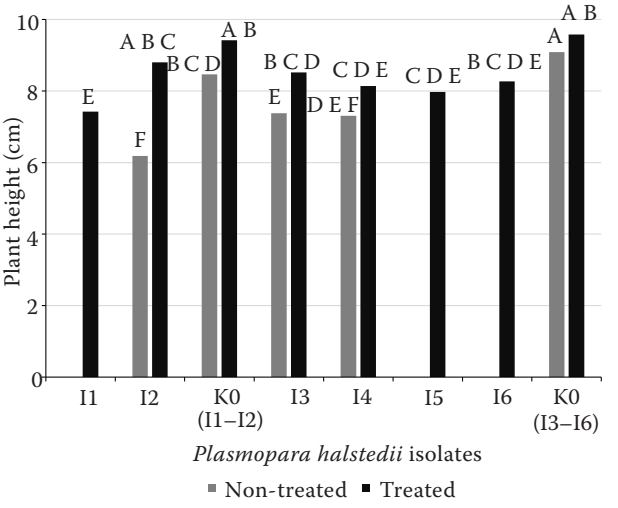

Figure 4. Plant heights of the mefenoxam-treated and non-treated sunflowers 21 days after inoculation with *P. halstedii*

Non-treated – non-treated with mefenoxam and inoculated with *P. halstedii*; treated – treated with mefenoxam (3 mg/kg seed) and inoculated with *P. halstedii*; K0 (I1 – I2) and K0 (I3 – I6) – non-treated and non-inoculated with *P. halstedii* for I1–I2 and I3–I6, respectively; ANOVA was performed with Fisher's test; the bars sharing the same letter are not significantly different

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showed significantly lower heights than the treated ones at the second evaluation.

DISCUSSION

Plasmopara halstedii, the causal agent of sunflower downy mildew, may severely threaten the sustainable production of sunflowers all around the world. Beside common plant production practices, resistance breeding against new pathotypes and the fungicidal treatment of sunflower seeds with mefenoxam are the main components to control this pathogen. Testing the efficacy of these control methods is an important task in sustainable agriculture; therefore, surveys in the populations of different pathogens with respect to their virulence phenotype and fungicide tolerance are essential elements of integrated plant protection.

The pathotype characterisation of *P. halstedii* is based on an internationally accepted methodology (Trojanová et al. 2017). In our study, we used field isolates, which were increased on a susceptible sunflower variety. Though the field isolates are probably genetically heterogeneous and may represent a mixture of fungicide tolerant and sensitive isolates, as well as different pathotypes, the single spore method with *P. halstedii* works little efficiency and is very complicated. In order to reduce the heterogeneity, isolates were collected from single, infected leaves, from a single lesion.

According to our results on the pathotype identification of the selected *P. halstedii* isolates between 2014 and 2017, pathotype 704 was one of the most widespread in Hungary during this period. Based on the examinations of thirty-three isolates collected between 2012 and 2014 in different regions of Hungary, pathotypes 704 was dominant, infecting commercial sunflower hybrids with the *Pl6* resistance gene (Bán et al. 2014a, b; 2016). The remaining five isolates were pathotype 700, 710, and 730 in those studies, which were known to be widespread and dominant in Hungary before 2010. The presence of pathotype 704 was proven in all the sampling sites representing the eastern part of the country between 2012 and 2014 (Bán et al. 2014a). In our previous work, we reported on the occurrence of a globally new pathotype, 724, from two locations in Hungary (Bán et al. 2018). In this present study, we re-tested one of these *P. halstedii* isolates (I2) and repeatedly proved that its virulence phenotype is 724. Moreover, besides the two previous locations, we found this pathotype in another site (Bonyhád, I5) as well. Though a limited number of *P. halstedii* isolates were examined in 2017, our present data are in line with the previous findings on the dominance of pathotype 704 in Hungary. Based on our previous and current results, it is likely that pathotype 704 and other virulent pathotypes are continuously spreading throughout Hungary. Pathotype 704 was identified in the USA and in some European countries in the last few decades (Spring 2019). Among other aggressive pathotypes, 704 has overcome the resistance of the *Pl6* gene which is incorporated into many sunflower hybrids. The widespread use of these hybrids has resulted in the emergence and worldwide spread of other new, aggressive pathotypes, such as pathotype 354 in Germany (Spring & Zipper 2018), pathotype 705 in Spain (García-Carneros & Molinero-Ruiz 2017) as well as pathotypes 705 and 715 in the Czech Republic (Sedlářová et al. 2016). Not only the widespread use of the hybrids, but also an insufficient crop rotation system and inadequate weed management system can contribute to the distribution of new *P. halstedii* pathotypes. These insufficient practices in sunflower production coupled with rainy weather around the time of sowing can be a major reason of the accelerated emergence and spread of new pathotypes over the past 20 years in some regions of Hungary and all around the world (Bán et al. 2016).

Although to a lesser extent, we also identified pathotype 700 during our survey in 2017. The existence of the avirulent pathotypes such as 700, 710 and 730 on the sunflower hybrids carrying the *Pl6* resistance gene was proven in our previous study, as well (Bán et al. 2016). This is not unknown, since previous works (e.g., Brown & Sharp 1970; Adhikari & McIntosh 1998) also reported the appearance of a normally avirulent pathotype on a resistant host. It is likely, that the co-infection of sunflower carrying the *Pl*6 gene with compatible (e.g., 704 and 714) and incompatible (e.g., 700) pathotypes of the sunflower downy mildew allows the normally avirulent pathotype (e.g., 700) to infect and reproduce. Currently, many studies highlight the phenomenon of "induced susceptibility", where a virulent pathotype can suppress the resistance mechanisms of the host creating a conducive environment for less virulent (or avirulent) pathotypes or pathogens (Kema et al. 2018, Seybold et al. 2020). Further studies are needed

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to elucidate this phenomenon in the sunflower–*P. halstedii* interactions.

The occurrence of pathogens resistant to pesticides is increasing worldwide, so application of an integrated pest management system is of great importance (Barzman et al. 2015). Being a soilborne disease, sunflower downy mildew can be effectively managed by a seed dressing with the compound mefenoxam. However, as with other phenylamide compounds, the fungicide resistance risk to mefenoxam is high and the descendent sensitivity in *P. halstedii* population could be detected in some Western European countries and the USA. Firstly, Albourie et al. (1998) in France, then Gulya (2000) and Molinero-Ruiz et al. (2003) pointed out that some *P. halstedii* isolates were not controlled by the mefenoxam concentration registered for the seed treatment and found differences in the metalaxyl sensitivity of the sunflower downy mildew isolates.

According to our results, mefenoxam performed poorly or only moderately in the case of half of the examined *P. halstedii* isolates when the registered rate was applied. Although a limited number of samples have been analysed so far, our results have provided first evidence for the existence of mefenoxam tolerance of sunflower downy mildew in high oleic sunflower hybrids in Hungary.

Similar to Gulya (2000), we did not find any correlation between the virulence phenotype and the fungicide resistance characteristic of different *P. halstedii* strains, i.e., there were also sensitive and resistant strains characterised by either the 704 or 724 pathotype. The decrement in plant heights shows that mefenoxam could effectively balance the stunting effect of downy mildew even in the treated plants inoculated by isolate (I5), which was found to be tolerant to the compound.

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 follow up on 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 to get good quality and high yields (Spring & Gómez-Zeledón 2020).

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