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. INTRODUCTION

Sunflower (*Helianthus annuus* L.) is one of the important oilseed crops in the world. There are several abiotic and biotic environmental factors that negatively influence the yield of sunflowers and ultimately reduce oil production (Rauf 2019). Among these biotic environmental factors, *Plasmopara halstedii* (Farlow) Berlese et de Toni, the causal agent of sunflower downy mildew, 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. 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 develops 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. LITERATURE REVIEW

2.1 Significance of sunflower

Sunflower was introduced to Europe from North and Central America by Spanish explorers. The interest of sunflower for oil extraction was found in Russia. By the end of the 19th century, it had become an agricultural crop and began to be bred. Sunflower is one of the essential seed oil crops in the world. Cultivated sunflower (*Helianthus annuus* L.) is an annual diploid plant (2n=2x=34) and originated from North America. The genus *Helianthus* comprises 53 wild species from which 14 species are annual diploid (2n=2x=34) and 39 species are perennial, including 29 species diploid (2n=2x=34), 4 tetraploids (2n=4x=68) and 6 hexaploids (2n=6x=102) (Moyers and Rieseberg, 2013, Seiler and Jan 2014, Qi et al. 2016). Sunflower is the fourth most cultivated oilseed crop globally, right after oil palm, soybean and rapeseed but second in the European Union. Sunflower produces healthy oil rich in unsaturated fatty acids, and high content of vitamin E. Sunflower plant can cultivate under low water input regimes to compare other oil crops due to their higher adaptability and versatility (Kaya et al. 2012, Gascuel et al. 2015). The most susceptible stages for host plant development are germination and emergence of seeds (Meliala et al. 2000, Virányi and Spring 2011).

Europe is one of the biggest producers of sunflowers and is primarily cultivated in the Southern and Eastern regions. Globally, the most prominent leading producer is Russia, and other producing countries are Ukraine, the USA, Argentina, India, China, Turkey and South Africa (FAO). Among the major sunflower crop producing countries, Russia was the largest cultivator of sunflower crop in terms of harvested area, followed by Ukraine and Argentina and contributed about 56 percent of the total harvested area (FAO). Plant breeders increased the oil content, making it one of the most popular oilseed crops for consumers in the first half of the previous century. However, both (a)biotic stresses are significant constraints for sunflower production worldwide (Rauf 2019).

Sunflower is prone to be attacked by several pests and diseases, resulting in significant yield losses and poor quality of crop production. Diseases are the most significant limiting factor in sunflower production worldwide. Different diseases are dominant in different regions, depending on the prevailing environmental conditions. More than 30 different species of pathogens that attack sunflowers and cause economic loss in production have been identified so far. The most serious ones for the production of oil and confectionery sunflower are downy mildew (*Plasmopara halstedii*), Phomopsis stem cancer (*Diaporthe helianthi*), Sclerotinia stalk and head rot (*Sclerotinia sclerotiorum*), Charcoal rot (*Macrophomina phaseolina*), Verticillium wilt (*Verticillium dahliae*),

Rust (*Puccinia helianthi*), Phoma black stem (*Phoma macdonaldii*), Alternaria (*Alternaria* spp.) and Rhizopus head rot (*Rhizopus* spp.) (Kaya et al. 2012).

2.2 History of *Plasmopara halstedii*

First time, it was reported by Halsted in 1876 from *Eupatorium purpureum* near the Bussay Institution. Later, Farlow (1883) described the pathogen as *Peronospora halstedii* based on samples found on *E. purpureum, Ambrosia artemisiifolia, Bidens frondosa, Rudbeckia laciniata, Silphium terebinthaceum* and the perennial sunflower species *Helianthus strumosus, H. tuberosus* and *H. doronicoides (H. mollis X giganteus).* Schröter (1886) had separated *Plasmopara* from *Peronospora* due to the germination by means of zoospores and instead of forming germ tubes. In 1888, Berlese and co-author de Toni renamed *Peronospora* to the new genus under the name *Plasmopara halstedii* (Farl.) Berl. et de Toni*,* and this name has been generally accepted worldwide (Virányi and Spring 2011). Since then, numerous collections of downy mildew collected on species of Asteraceae and classified as *Plasmopara halstedii* due to morphological similarities of sporangiophores and sporangia. Moreover, Stevens (1913) reported high variability in size and form of *P. halstedii* zoosporangia, which has become one of the most distributed pathogens worldwide. In the middle of the 20th century, this disease caused by *P. halstedii* expanded throughout Europe (Novotelnova 1966). According to Novotelnova's (1966) observation, the pathogen showed geographical dissimilarities varied from Europe to North America. However, Novotelnova renamed *P. halstedii* as *P. helianthi* but could not get acceptance so far (Virányi and Spring 2011).

2.3 Significance of sunflower downy mildew and main characteristics of the pathogen

Sunflower downy mildew is caused by the plant pathogen *P. halstedii* (Farlow) Berlese et de Toni and is one of the most serious diseases affecting sunflower production worldwide. This pathogen had been under quarantine regulation in the European Union since 1992 (Delmotte et al 2008), and more recently it is designated as a regulated non-quarantine pest (RNQP) (EPPO).

Plasmopara halstedii is a *Peronosporaceae*-family obligate biotrophic oomycete that requires a living host to complete its life cycle (Fawke et al. 2015). Haustoria and mycelium help to uptake nutrients from their hosts and release enzymes and effectors into the host's cells. In the absence of resistant cultivars of sunflower and chemical control, it can cause complete loss of sunflower crop and decline yield production. This pathogen is diploid, homothallic and reproduce via both asexually and sexually.

Phytopathogenic oomycetes are different from fungi, including the hemibiotroph genus *Phytophthora*, which causes late blight and obligate biotrophs, which causes downy mildew,

including the genera *Bremia*, *Peronospora*, *Plasmopara*. For downy mildew, the pathosystems *Hyaloperonospora arabidopsidis/Arabidopsis thaliana* and *Bremia lactucae/ Lactuca sativa* are well-studied model systems (Fawke et al. 2015).

Plasmopara halstedii is native to North America, and later reported in Russia and Western Europe around 1960, where it was introduced probably through infected sunflower seeds. This pathogen can be dispersed via wind, infected seeds, but mostly soil-borne (Ioos et al. 2007). The soil-borne pathogen infects seedlings via penetrating underground host tissues, and systemic infection follows. Under favourable conditions, a large number of zoosporangia are produced from oospores, which release motile zoospores which are responsible for leaf infections on neighbouring plants. Sakr et al. (2008), concluded that morphological characteristics of zoosporangia are influenced not only by pathogen genetics but also on growth conditions such as duration of incubation, infected plant parts, and, most importantly, the genotype of the host plant (Sakr et al. 2008).

Mainly primary infections via roots and early secondary infections caused relatively higher yield losses (Allard 1978), resulting in systemic infection of plants by the pathogen (Regnault and Tourvieille 1991, Albourie et al. 1998). Secondary infections on the above-ground parts of sunflower occur via the dispersion of zoosporangia. However, this type of infection slightly affects the sunflower production economically but does not affect the yield significantly; mostly, such infections remain local and temporary (Gulya et al. 1997, Spring 2009). According to Tourvieille et al. (2008), heavy rainfall during the most vulnerable period of sunflower seedlings poses the greatest risk of downy mildew.

It is considered that long-distance spreading of sunflower downy mildew might occur through the exchange of oospore-contaminated seeds (Spring 2001). With the implementation of contamination testing, there are at least possibilities to prevent the introduction of *P. halstedii* and associated phenotypes (Virányi and Spring 2011, Spring 2019). Concerning that *P. halstedii* is characterized by a high level of evolutionary potential (Sakr 2011b, 2012, Virányi and spring 2011), there have been several studies on virulence (Delmotte et al. 2008, Sakr 2011b, 2012, Tourvieille et al. 2000, 2010) and more recently on aggressiveness (Sakr 2011a, b, c, 2012, Sakr et al. 2011, Sakr 2013, Spring 2019, Bán et al. 2021).

Downy mildew has become a major threat to the sunflower crop because of the emergence of new pathotypes and capable of infecting a variable range of sunflower genotypes. Thus, new pathotypes of *P. halstedii* are bypassing sunflower hybrids resistance (Tourvieille 2000, Bán et al. 2021).

Therefore, diversification of resistant sources is a major objective of disease-resistant breeding (Rauf 2019).

2.4 The high variability of *Plasmopara halstedii* – evolution and spread of pathotypes

There are several pathotypes (races or virulence phenotypes) of *P. halstedii*, each with varying degrees of virulence. The widespread cultivation of sunflower hybrids with a rising number of developed resistance genes against *P. halstedii*, which induce genetic changes in the pathogen (Gascuel et al. 2015), is the cause of this high variability. In addition to mutation and sexual recombination, parasexual recombination provides an opportunity for genetic exchange between different pathotypes (Spring and Zipper 2006, Ahmed et al. 2012).

The number of pathotypes is constantly increasing around the world and even accelerated in the past decade. Most recently, 50 different pathotypes of *P. halstedii* had been identified worldwide (Spring 2019, Gilley et al. 2020, Miranda-Fuentes et al. 2021, Bán et al. 2021). Virányi et al. (2015) reviewed the race composition of *P. halstedii* in Europe, as well as in North and South America. Before 1980, there were only two pathotypes of *P. halstedii*: one in Europe (European race) and another in Red River Valley of North America (Red River race). Since 1980, pathologists all around the world have discovered novel *P. halstedii* isolates and identified them into pathotypes (Virányi et al. 2011, Gascuel et al. 2015).

The pathogen has continued to change its virulence character due to the adoption of novel resistance genes in sunflower hybrids (Gulya 2007, Virányi et al. 2015). This pathogen articulated high virulence diversity, especially in the making of pathogenic pathotypes and the spreading of pathotypes that overcome the *Pl6* resistance gene of sunflower is progressing (Bán et al. 2014, Iwebor et al. 2016). Indeed, pathotypes infecting Ha335 containing *Pl6* gene against *P. halstedii* were found in French (304, 307, 314, 334, 704, 707, 714, 717, 774; reviewed by Virányi et al. (2015), Czechian (705, 715; Sedlářová et al. 2016), Hungarian (704, 714; Bán et al. 2014), and Russian (334; Iwebor et al. 2016) sunflower fields over several years. So due to pathogenic variability, the pathogen influences the growth of new sunflower's hybrids and ultimately crop yield loss (Trojanová et al. 2017).

According to Virányi et al. (2015), the highest pathogenic diversity of *P. halstedii* has been recorded in Canada, USA and France, between 2007 and 2013. In France, race 304 was the first to overcome *Pl* resistant genes in 2000. Recently, highly aggressive *P. halstedii* pathotypes have been reported in several areas of Europe, including pathotype 354 in Germany (Spring and Zipper 2018), pathotypes 724 and 734 in Hungary (Bán et al. 2018, Nisha et al. 2021), pathotype 705 in Spain (García-Carneros and Molinero-Ruiz 2017), and pathotypes 705 and 715 in the Czech

Republic (Sedlářová et al. 2016) (for more details review Bán et at. 2021). Pathotype 734 is already widespread in the United States and Russia and is considered very aggressive, having been able to infect hybrids with resistance genes *Pl6* and *Pl7* (Iwebor et al. 2018) and 714 in Italy, which overcome the action of *Pl8* in the line RHA-340. The pathotype 714 has already been described in the Czech Republic, France, Hungary and the United States (Virányi et al. 2015, Bán et al. 2014, Martín-Sanz et al. 2020). Rozynek and Spring (2000) studied pathotypes of sunflower downy mildew in southern Germany. They identified pathotypes 730, 710, 330, 310 and 300 in this region. However, these pathotypes have already been identified in other areas of Europe. In Bulgaria, there are five pathotypes, 300, 330, 700, 721 and 731 identified by Shindrova (2010). Of these, race 700 has the largest distribution area (in northern Bulgaria) and accounts for 46% of the downy mildew population. Alizadeh and Rahmanpour (2005) identified a race as the predominant race of downy mildew on sunflower, *P. halstedii*, for surveyed areas in Iran. Moreover, the identified pathotype was physiologically different from pathotypes identified worldwide determined proposed by Gulya et al. (1991). However, the use of newly introduced differential lines is necessary to ensure the presence of different physiological pathotypes, proposed by Tourvielle et al. (2000).

2.5 International Standardised Nomenclature System for pathotype identification of *P. halstedii*

Gulya et al. (1998) suggested using a triplet code system based on virulence patterns of *P. halstedii* isolates because of the rising number of new pathotypes. Gulya and co-workers described that the pathotype characterization of *P. halstedii* determined by universally accepted international standardised nomenclature system based on sunflower differential lines by using a triplet set of inbred lines containing different major resistance (*R*) genes called *Pl*, and necessitating the identification of further and possibly more durable broad-spectrum resistances (Pecrix et al. 2019). Isolates of *P. halstedii* collected from diseased plants in the field are designated as pathotypes based on virulence profiles in a set of differential lines of sunflower carrying different major *Pl* resistance genes (Gascuel et al. 2015). Susceptible or resistant plants are defined by disease symptoms and mainly via sporulation on the leaves.

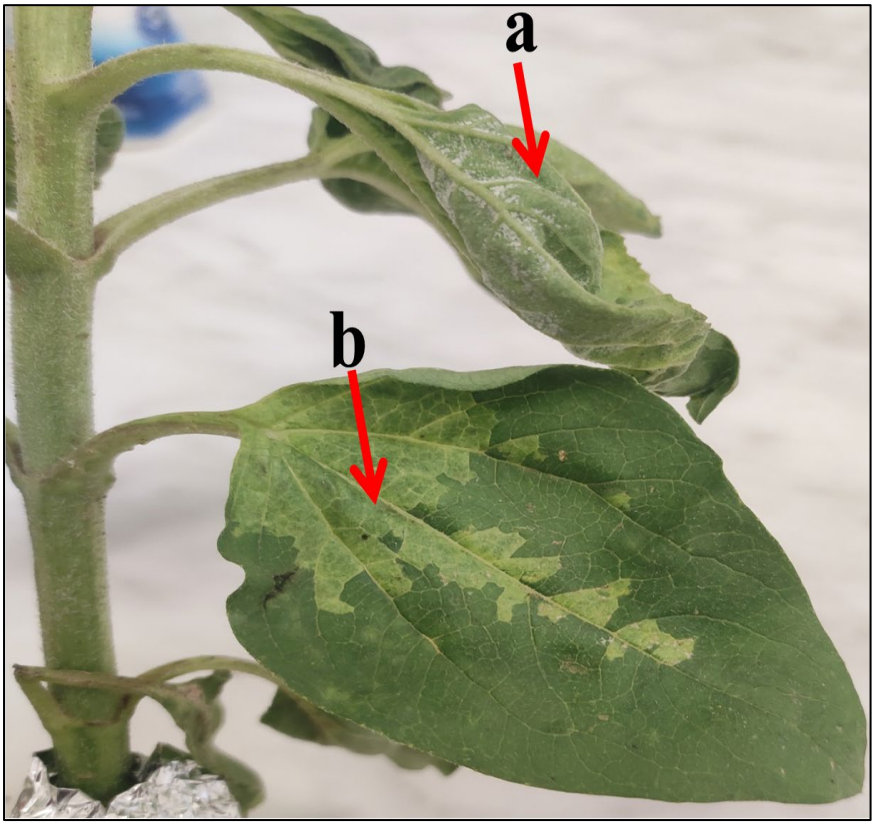
2.6 Symptoms of sunflower downy mildew

The symptoms of downy mildew on sunflower varies according to the age of tissue, the duration of inoculum, cultivars used and the environment that influences the infection process (Spring 2001). In addition to environmental factors, the aggressiveness of the pathogen population also influenced the disease intensity (Göre 2009).

Downy mildew causes white sporulation on the abaxial and adaxial sides of cotyledons, stunted plants to varying degrees, pre- and post-damping-off, chlorosis in the leaves of affected plants,

353 which spreads along the main veins and over the lamella, and eventually leads to plant mortality
354 (Bán et al. 2017) (Figure 1). The cause of dwarfism is unclear; however, it may be due to hormonal
355 changes caused by nutrient-extracted nutrients (Gascuel et al. 2015). Dwarfing of diseased plants,
356 chlorosis along leaf veins, and small heads with sterile seeds are all symptoms of primary infection
357 followed by direct movement of zoospores toward the roots (Jocić et al. 2012, Gascuel et al. 2015).
358 Damping-off can occur as a result of a severe infection. Yield losses from downy mildew can be
359 substantial, depending on the percentage of infected plants and their distribution within the field
360 (Virányi 2008, Markell et al. 2015, Körösi et al. 2020).

361 Secondary infections by zoospores and sporangia that develop beneath the leaves have no impact
362 on disease spread or crop loss. Secondary infections can also become systemic, causing dwarfism
363 of affected plant parts (Spring 2009, Bán et al. 2021). In addition, secondary infection increases
364 the risk of the disease spreading latently through the seeds.



365 **Figure 1.** Signs and symptoms of sunflower downy mildew (a; sporulation, b; chlorosis)
366 (Source: N. Nisha 2022).

367 **2.7 Life cycle of *Plasmopara halstedii***

368 In oomycetes, sexual reproduction can be either homothallic or heterothallic. The pathogen uses
369 the sexual phase (oospores) for overwintering and the asexual phase (zoospores) for secondary
370 infection throughout the sunflower growth season (Gascuel et al. 2015). Overwintering oospores
371 are long-lived and can survive in soil up to 6-8 years (Sakr et al. 2009). Oospores germinate with

zoosporangia which release zoospores that are responsible for secondary infections (Tourvieille et al. 2000). It is considered that the aggressiveness of the pathogen evaluates on the quantity of zoosporangia (Sakr et al. 2008).

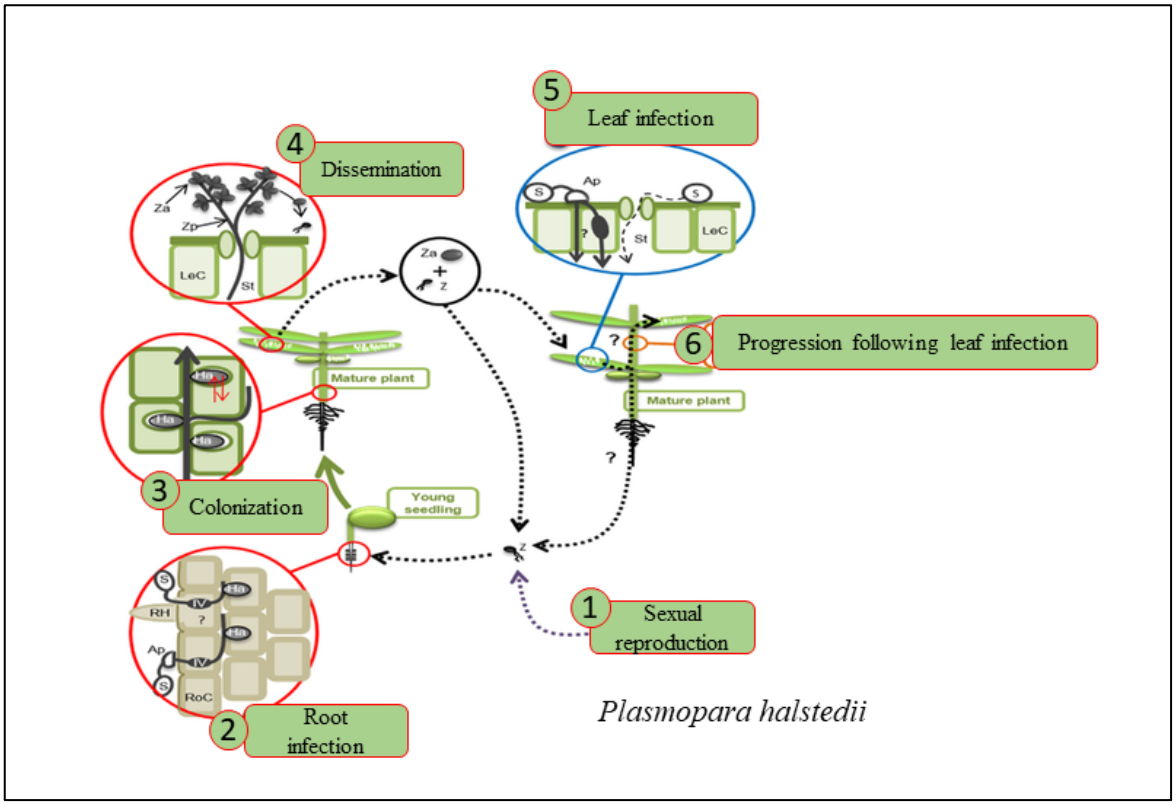


Figure 2. Life cycle of *Plasmopara halstedii.* (Source: Gascuel et al. 2015)

Generally, germination of zoospores (Z) from zoosporangia are produced by overwintering sexual oospores. Zoospores are freely motile asexual spores and flagellated. In the presence of soil free water, zoosporangia rapidly released zoospores and then occurs in contact with a sunflower root (Figure 2 (1)). Zoospores serve as the main source of inoculum after primary infection and germinate in a few hours. Zoospores have two modes of infection, either by direct penetration or indirect penetration. Direct penetration into a roots cell (RoC) with or without formation of an appressorium (Ap), pathogen increases osmotic pressure and enter into root epidermal cells and can be entered through injuries at the base of root hairs (RH) and formation of infection vesicles (IV) occurs (Figure 2 (2)). After penetration of pathogen into susceptible host tissue (compatible), it grows throughout the intercellular and intracellular between cortical cells and starts to colonise towards systematically shoot tissue, formed nutrition elements named haustoria/mycelium (Figure 2 (3)). Under favourable conditions (humidity and temperature), *P. halstedii* shows asexual reproduction structures by releasing zoosporangia (Za) from zoosporangiophores (Zp), and emerge on the lower sides of leaves and cotyledons via stomata (St) and below-ground tissues. Fully developed zoosporangia are the primary means of dissemination and infecting other plant leaves (Figure 2 (4)). Zoospores encyst around leaf trichomes and veins and start to germinate after

penetration into leaf tissues through intercellular spaces of parenchyma cells by making hyphae. Penetration of zoospores via stomata is rarely observed (Figure 2 (5)). Pathogen progression in plant tissues following leaf infections and is poorly characterized (Figure 2 (6)) (Gascuel et al. 2015). Oospores produced during sexual reproduction serve as primary inoculum for the next season (Sakr et al. 2008).

2.8 Management of *Plasmopara halstedii*

The pathogen has both asexual and sexual life cycle, thus making disease management difficult. It is challenging to eradicate this pathogen once it established in an area. The situation is further complicated by the variability of the pathogen, as more and more pathotypes appear year after year and are able to infect the resistant hybrids. In general, therefore, the basic protective measures and effective manner to manage sunflower downy mildew in sunflower is the use of integrated pest management (Barzman et al. 2015).

2.8.1 Integrated pest management (IPM) against *Plasmopara halstedii*

IPM is a sustainable approach for managing pests by combining and integrating all available control measures, including monitoring, crop rotations, crop management and ecology, biological control, mechanical and physical control, pesticide selection, etc., in a way that reduces human health and environmental risks. IPM built on agronomic, mechanical, physical and biological principles and suggested using selective pesticides only when other approaches do not work with other tools (Barzman et al. 2015).

IPM acts in different forms that vary in time and space. It is shaped according to site-specific factors such as regional cropping pattern, field size, type and availability of seminatural habitats, the broader landscape, cultivation practices, pest pressure, R&D efforts, availability of training, farmer attitude, and economics. More sustainable control strategies are needed due to negative impacts of pesticides on human health and the environment, emerging pesticide resistance and stricter regulations on pesticide residues in agricultural products (Spring et al. 2018).

2.8.2 Agricultural methods

Crop rotation is the most effective agronomic measures for pest control and has been used for thousands of years. Crop rotation involves growing a sequence of crop species with the rotation of different species on the same land to break the life cycle of the pathogens (Barzman et al. 2015). Crop rotation practices increase yield and sustainable production. The reduced use of extended rotations largely is due to the introduction of chemical fertilisers and pesticides. Ball et al. (2005) addressed the effects of crop rotation on soil properties such as fertility, organic matter content, water availability, soil structure, aggregation, bulk density and erodibility. Crop rotation is largely

ineffective due to the ability of oospore to survive in the soil for many years until conditions are favourable for germination and infection (Gulya et al. 1997).

In addition to crop rotation, tillage methods, irrigation, weed and biological control have served the purpose of controlling this pathogen. Weed management plays an essential role in disease control because many weeds are host plants for the downy mildew. For example, a common ragweed, *Ambrosia artemisiifolia* was first found to be infected by *P. halstedii* pathogen in Hungary (Vajna 2002, Choi et al. 2009). Volunteer plants eradication is also important because they can act as reservoirs for less and highly virulent pathogen variants (Bán et al. 2021). There are few organism selected as antagonists of Pythium and other phytopathogenic oomycetes that considered to be promising tools against *P. halstedii* due to their taxonomical proximity and similar modes of action (Gulya et al. 1997).

2.8.3 Genetic control, types of resistance to sunflower downy mildew

Genetic resistance is the most effective, economic and environmentally friendly approach for disease management and a sustainable strategy to increase crop yield and reduce fungicides use (Mirzahosein-Tabrizi 2017, Qi et al. 2017).

Disease resistance of sunflowers to *P. halstedii* from plant breeding point of view can be divided into two categories, as it is common for other diseases, as well. The first is qualitative resistance which is mediated by the major *Pl* genes and tends to result in a disease-free plant. The second is quantitative resistance which is controlled by minor genes and tends to affect the rate of disease development (reducing the rate) rather than producing a disease-free plant (Tourvieille et al. 2008). Genetic studies identified 36 major dominant *Pl* resistance genes (Pl_1–Pl_{35}, and Pl_{Arg},) up to 2019 (Ma et al. 2019). Downy mildew resistance genes (*R* genes) have been reported in sunflower and wild species (Pl_1-Pl_{19}, Pl_{21}, Pl_{Arg}) so far, conferring resistance to at least one *P. halstedii* pathotype (Ma et al. 2018). Fifteen of these genes Pl_1, Pl_2, Pl_5–Pl_8, Pl_{13}–Pl_{19}, Pl_{21}, Pl_{Arg} have been introduced into specific linkage groups (LGs) of the cultivated sunflower genome (Kinman 1970, Fick and Zimmer 1974, Miller and Gulya 1984, Miller and Gulya 1991, Seiler 1991, Mouzeyar et al. 1995, Roeckel-Drevet et al. 1996, Vear et al. 1997, Bert et al. 2001, Molinero-Ruiz et al. 2003a, Yu et al. 2003, Mulpuri et al. 2009, Bachlava et al. 2011, Vincourt 2012, Liu et al. 2012, Qi et al. 2015, Qi et al. 2016, Zhang et al. 2017) , and conferring resistance to one or more pathotypes of *P. halstedii* and three quantitative trait loci (QTL) associated with partial resistance of downy mildew were identified on LGs 7, 8, and 10, respectively (Vear et al. 2008a, Vincourt et al. 2012, Qi et al. 2017). *Pl* genes originated mostly from wild *H. annuus* and other *Helianthus* species (*H. argophyllus, H. praecox* and *H. tuberosus*) (Vear et al. 2008b, Gascuel et al. 2015). Introgressive hybridisation with wild species is widely used to broaden the genetic base of cultivated sunflower

(Qi et al. 2016) shown in (Table 1). In addition, Tourvieille et al. (2010) reported that the life expectancy of *Pl* gene seems to be very short (less than 10 years) which is due to the important use of *Pl* gene under conditions of increased infection and various selection pressures (Sakr 2011b). In the last 40 years, several resistance genes against *P. halstedii* pathotypes have become inefficient in sunflower (Ahmed et al. 2012). For instance, it was reported that the downy mildew R genes Pl_6 and Pl_7 were overcome by new pathogen pathotypes in 2009-2010 in the United States (Gulya et al. 2011). In Argentina, the widely used downy mildew R gene Pl_{15} has been overcome since 2013 (Castaño 2018). Therefore, there is further need to research for the characterization of resistances that will be effective against such pathotypes of *P. halstedii*.

Table 2. Resistance genes incorporated in sunflower against downy mildew

Resistance genes	Inbred lines	Linkage groups	References
Pl_1	RHA265, RHA266	LG8	Kinman 1970
Pl_2	RHA274	LG8	Fick and Zimmer,1974
Pl_5	DM-2	LG13	Miller and Gulya 1984
Pl_6	HA335, HA336	LG8	Miller and Gulya 1991
Pl_7	HA337, HA338, HA339	LG8	Miller and Gulya 1991
Pl_8	RHA340	LG13	Miller and Gulya 1991
Pl_{13}	HA-R5	LG1	Mulpuri et al. 2009
Pl_{14}	-	LG1	Bachlava et al. 2011
Pl_{15}	RNID	LG8	de Romano et al. 2010
Pl_{16}	HA-R4	LG1	Roeckel-Drevet et al. 1996, Liu et al. 2012
Pl_{17}	HA 458	LG4	Qi et al. 2015
Pl_{18}	HA-DM1	LG2	Qi et al. 2016
Pl_{19}	-	LG4	Zhang et al. 2017
Pl_{21}	PAZ2	LG13	Vincourt 2012
Pl_{Arg}	Arg1575-2	LG1	Seiler 1991

Two types of sunflower-*P. halstedii* incompatibility responses have previously been found, depending on the host-pathotype combination. Mouzeyar et al. (1994) differentiate between resistance type I and type II. Type I resistance can limit pathogen development to the roots and the hypocotyl basal zone, however type II resistance cannot, allowing the infection to reach throughout

the whole hypocotyl and sporulates on cotyledons. Mouzeyar et al. (1993) proved that *P. halstedii* may infect both susceptible and resistant sunflower lines in a microscopic examination. Hypersensitivity-like reactions, necrosis formation, and cell-division are well known defence reactions in incompatible combinations (Mouzeyar et al. 1993, 1994, Radwan et al. 2011) and fungicide treatments (Mouzeyar et al. 1995). A hypersensitive response (HR) occurs in the hypocotyls of both types I and II resistant plants five days after root infection, but the fate of the infection is determined by both the resistance gene in the host and the avirulence (avr) gene in *P. halstedii.* The pathogen is restricted to the basal part of the hypocotyls in plants with type I resistance, but in plants with type II resistance, the pathogen can penetrate the hypocotyls fully and reach the cotyledons, although this rarely reaches the true leaves (Mouzeyar et al. 1993, 1994). Gulya et al. (1991) and Sackston (1992) were the first to describe this phenomenon, which is known as Cotyledon Limited Infection (CLI), which is a kind of Type II resistance (Radwan 2011). In incompatible plant-pathogen interactions, recognition of a potential pathogen often leads to a hypersensitive reaction (HR), with programmed cell death (PCD) activated at the site of attack to halt the spread of the pathogen. During a HR, a small group of cells in the vicinity of the pathogen undergo rapid PCD, usually within 12–24 hours of inoculation (Hermanns et al. 2003, Radwan et al. 2005). Heller et al. (1997) proposed several mechanisms for latent infection and showed that plants with latent infections have increased cell division activity that prohibits the pathogen in the pith parenchyma and that hypersensitive responses confined the pathogen to the cortical parenchyma.

2.8.4 Chemical control

Seed treatments can be a very effective management tool because they are most active when seedlings germinate, and systemic infection usually occurs within a short time after planting (3 to 15 days) (Gulya et al. 2013, Humann et al. 2016, Humann et al. 2019). Metalaxyl is a systemic phenylalanine fungicide considered a fairly effective measure to control the downy mildew of sunflower as seed dressing (Albourie et al. 1998) and provides systemic protection against oomycete pathogens. The active enantiomer of the racemic fungicide metalaxyl was replaced with mefenoxam. The fungicide is administered at frequencies similar to those used with metalaxyl but at lower rates (Parra and Ristaino 2001). Despite severe resistance problems in the oomycetes, mefenoxam, an active ingredient, has been used widely for control of different oomycete pathogens, including *P. halstedii, Phytophthora infestans, Peronospora tabacina,* and *Bremia lactucae*, because of its excellent preventive, curative and eradicated activities (Morton et al. 1988, Parra and Ristaino 2001, Pintore et al. 2016).

Although reduced sensitivity to metalaxyl (tolerance or resistance to this compound) had already been described in several oomycete fungi soon after the introduction of this chemical into the field, *P. halstedii* retained its sensitivity until recently, except that such tolerant strains could be detected under laboratory conditions (Oros and Virányi 1984).

Oxathiapiproline (OXA) is the first member of the piperidinyl thiazole isoxazoline class of fungicides (FRAC 49) and was recently discovered and developed by DuPont Crop Protection (Pasteris et al. 2016). OXA has been shown to be effective against economically important oomycete pathogens in other crops (Ji et al. 2014, Kness et al. 2016, Patel et al. 2015). This fungicide has a different mode of action than fungicide seed treatments currently available for sunflower and its efficacy and flexibility in application suggest that OXA may be a useful tool for downy mildew control (Humann et al. 2019).

The sensitivity of *P. halstedii* pathotypes to phenylamides needs to be continuously monitored, and fungicides with different modes of action are needed in fields where resistance is observed (Molinero-Ruiz et al. 2005).

2.9 Induced resistance

Active ingredients like metalaxyl or related compounds play a significant role in controlling the disease. However, fungicides are not cost-effective and pose severe environmental hazards (Barzman et al. 2015). Several abiotic and biotic agents have been reported that can induce plant resistance to pathogens. As a future alternative to fungicide treatments, efforts were made to control disease via chemically induced resistance and biological antagonism (Sackston et al. 1992).

Apart from genetic resistance, induced resistance has been considered an effective and long-lasting method for plant disease management. Induced resistance is the activation of plant defence mechanisms triggered by avirulent and virulent pathogens. In broad terms, induced resistance can be divided into two main types: systemic acquired resistance (SAR) and induced systemic resistance (ISR). It is understood that the term induced systemic resistance (ISR) is used to describe resistance caused by non-pathogenic microorganisms, and natural or synthetic elicitors (Kuć 2001, Vallad and Goodman 2004). Induced systemic resistance is a phenomenon that has been extensively studied in many plant–pathogen interactions and is induced by localised infection or by treatments with microbial components or products or by a diverse group of structurally unrelated organic and inorganic compounds (Kuć 2001). In contrast, the term systemic acquired resistance (SAR) is used to describe resistance that is activated after plant exposure to the pathogen and provides protection through a series of induced proteins (Oostendorp et al. 2001, Durrant and Dong 2004, Conrath 2006). The difference between ISR and SAR is that SAR is mediated by

salicylic acid (SA), whereas ISR is mediated by the jasmonic acid (JA) or ethylene pathway (ET). There are many data indicating that plant growth regulators such as salicylates and jasmonates can be used to control fungal diseases (Kepczynska and Kepczynska 2005, Hayat et al. 2010, Kępczyńska and Król 2011). Induced resistance is also triggered by chemical inducers such as salicylic acid (SA), 2,6-dichloroiso-nicotinic acid (INA), Jasmonic acid, Bion 50 WG (benzo (1,2,3)-thiadiazole-7-carbothionic acid-S-methyl ester or acibenzolar-S-methyl, (ASM or BTH)) and DL- β -amino butyric acid (BABA) (Van Loon et al. 1998, Heil and Bostock 2002, Bán et al. 2004, Jayaraj et al. 2004, Vallad and Goodman 2004, Körösi et al. 2009, Körösi et al. 2011, Sillero et al. 2012).

Induced resistance mediated by rhizobacteria has also been studied in various plant species against different pathogens (NandeeshKumar et al. 2009). NandeeshKumar et al. (2008a) reported that plant growth-promoting rhizobacteria (PGPR) strain INR7 induced resistance against *P. halstedii* in sunflower was mediated through enhanced expression of defence mechanisms like catalase, peroxidase, polyphenol oxidase, phenylalanine ammonialyase, and chitinase. Treatment with PGPR strain INR7 effectively reduced the incidence of downy mildew in the sunflower plants in a concentration-dependent manner, and treatment of sunflower seeds with 1×10^{8}cfu/mL of PGPR strain INR7 reduced disease severity and provide 51% protection under greenhouse conditions and 54% in field conditions, respectively. PGPR bacteria could be a beneficial component of integrated disease management (NandeeshKumar et al. 2008a).

Moreover, seed treatment with plant growth-promoting fungi (PGPF) resulted in improved disease protection against the downy mildew in sunflower (Nagaraju et al. 2012a). Similarly, seed treatment with PGPF, especially *Trichoderma harzianum*, was reported to improve seed and plant growth parameters and induce systemic resistance in sunflower plants against the downy mildew caused by *P. halstedii* (Nagaraju et al. 2012b). β-aminobutyric acid (BABA), a non-protein amino acid, has been shown to induce resistance in plants against a range of microbial pathogens which includes fungi, bacteria, oomycetes, nematodes, viruses and abiotic stresses (Jakab et al. 2001, Conrath et al. 2002, Cohen 2002, Ton and Mauch-Mani 2004, Justyna and Ewa 2013). In addition, it not only induces resistance to stress factors, but can also stimulate plant growth and development (Justyna and Ewa 2013). BTH is a non-toxic synthetic chemical that has been identified as a potent inducer of SAR in several crops (Serrano et al. 2007). Tosi et al. (1998) showed that BTH protected susceptible sunflower plants from *P. halstedii* infection. Bán et al. (2004) also reported that treatment with Bion 50 WG significantly reduced fungal sporulation and plant damped-off in compatible host-pathogen interactions and induced resistance in sunflower. It has been found that BABA and chitosan induced resistance against *P. halstedii* in sunflower was mediated via the

enhanced activation of genes for defence related proteins in susceptible sunflower seedlings (NandeeshKumar et al. 2008a, 2009). Körösi et al. (2011) also reported that BTH, BABA and INA induced systemic resistance against *P. halstedii* in sunflower. BABA also provided significant control of the late blight pathogen *Phytophthora infestans* on tomato (Sharma et al. 2012), and reduced severity of *P. viticola* infestation in grapevines by 62% in field experiments (Tamm et al. 2011). Interestingly, BABA-induced the protection of *Brassica napus* from the fungal pathogen *Leptosphaeria maculans* was also associated with a combination of modes of action, as it induced synthesis of SA and the expression of PR-1, but also exerted a direct fungitoxic effect against the pathogen (Šašek et al. 2012). Neem-derived pesticides could be an alternative to chemical pesticides. In addition to microorganisms, it has also been reported that plant extracts can induce resistance in plants to a number of pathogens. For example, Bhuvaneswari et al. (2012) reported the induction of systemic acquired resistance (SAR) in *Hordeum vulgare* to *Drechslera graminea* by fruit extracts of *Azadirachta indica* Juss. (Neem) by increasing in the activities of phenylalanine ammonia lyase (PAL) and tyrosine ammonia lyase (TAL). Induction of resistance by seed treatment with acibenzolar-S-methyl and methyl jasmonate against *Didymella bryoniae* and *Sclerotinia sclerotiorum* in melon with rapid increase in the activity of chitinase and peroxidase proteins associated with pathogenesis (Buzi et al. 2004). Furthermore, essentials oils were examined with different concentrations against *P. halstedii* and found to be effective to decrease the sporangium quantity (Er et al. 2021). More recently, it was reported that neem-derived pesticides, namely neem leaf extracts (NLE) and azadirachtin (NeemAzal T/S) protect against downy mildew in sunflower's susceptible cultivars (Doshi et al. 2020). Neem is the most studied plant because of its wide range of effects against various plant pests and pathogens (Biswas et al. 2002). For example, Hasan et al. (2005) studied the antifungal effects of neem along with other plant extracts against seed-borne fungi of wheat seeds and reported that the alcoholic extracts of neem completely controlled the growth of *Bipolaris sorokiniana* (Sacc.), *Fusarium* spp., *Aspergillus* spp., *Penicillium* spp. and *Rhizopus* spp. after the treatment on wheat seeds. There have been only few studies of neem against different oomycetes. For instance, Rashid et al. (2004) investigated neem leaf diffusate, neem leaf powder and neem seed cake against *Phytophthora infestans* (Mont.) De Bary and found that neem was effective in controlling the infection. Similarly, different neem products such as crude neem seed oil, crude neem seed oil terpenoid extract, nimbokil and neem leaf decoction has been tested against *P. infestans* by Mirza et al. (2000). The only study examining the effect of neem against *Plasmopara viticola* was conducted by Achimu and Schlösser (1992) where they successfully controlled the pathogen *in vitro* conditions. BTH (acibenzolar-S-methyl) was originally marketed to control powdery mildew in wheat and barley in Europe (Görlach et al. 1996). ASM and INA are considered the best chemical

elicitors available for inducing resistance. They are considered functional analogues of SA and cause a systemic form of induced resistance across a broad range of plant pathogens (Friedrich et al. 1996, Maleck et al. 2000). These chemicals did not exhibit direct antimicrobial activity; however, some cases of antimicrobial activity associated with high elicitor concentrations have been reported (Tosi and Zazzerini 2000, Rohilla et al. 2002, Ghazanfar et al. 2011). For example, ASM was shown to induce SAR in rust (*Uromyces viciaefabae*) and *ascochyta* blight (*Ascochyta fabae*) on faba bean both in the glasshouse and under field conditions (Sillero et al. 2012). ASM has also been reported to control rust infection, caused by *Uromyces pisi* on pea plants, although again control was not complete (Barilli et al. 2010). In this case, ASM induced resistance was associated with increased activity of defence-related enzymes and phenolic content, and indeed there was evidence of activation of defence enzymes by ASM treatment of both susceptible and resistant genotypes (Barilli et al. 2010, Walters et al. 2013).

2.10 Fungicide resistance

Fungicide resistance is a selection mode that describes a fungus's ability to survive and reproduce in the presence of a fungicide and causes poor disease control. However, several key factors influence an organism's susceptibility to fungicides: (i) the pathogen's biology, (ii) the fungicide's mechanism(s) of action, and iii. the rate and frequency of fungicide treatment.

Fungicides are essential tools for preventing and managing plant disease in modern crop production (Vincelli 2014). However, due to the repeated use of fungicides (especially mefenoxam), some novel pathotypes have developed fungicide resistance and have overcome plant genetic resistance. The frequent use of resistant host cultivars is a significant selective driver of pathogen evolution in agro-ecosystems. Typically, a single resistant crop cultivar is widely utilised until the pathogen overcomes its resistance, at which point it is replaced by another. In the presence of significant host selection, this cycle of pathogen evolution is sometimes referred to as the 'boom and bust cycle' (Thompson and Burdon 1992), and it has been described for a variety of powdery mildews and cereal rusts (McDonald and Linde 2002).

The generation of novel virulence in crop pathogen systems is influenced by a number of factors (Brasier 1995, Kaltz and Shykoff 1998). Because pathogens have a shorter generation time than their hosts, they can evolve quickly and improve their local adaptation (Ahmed et al. 2012). However, the evolution of novel pathogenicity is not entirely dependent on recombination, and there are numerous striking examples of evolution through mutation accumulation. For example, in highly clonal populations of wheat rusts, evolution viably mutation was sufficient to allow rapid quick adaptation to resistant host cultivars in highly clonal populations of wheat rusts (Enjalbert et al. 2005).

In the 1960s, a new generation of fungicides was developed, beginning with benzimidazoles. They are highly active and exhibit low phytotoxicity due to their specific mode of action against a target protein in fungal pathogens. The majority of these site-specific fungicides are systemic, which means they can penetrate the cuticle and spread throughout the plant, increasing their activity. Resistance development in pathogen populations and loss of fungicide activity were noticed within a few years following the introduction of site-specific fungicides, with *Botrytis cinerea* being one of the first fungi to develop resistance. Since then, the problem of resistance has gained more attention, and it has become a major focus of fungicide research.

Resistance development is influenced by a number of factors, including the fungicide's chemistry and mode of action, the biology and of the target fungus' biology and reproductive capabilities, and the frequency with which the fungicide is applied (Brent and Hollomon 1998, Hahn 2014). It is a concern for all pesticides, including fungicides, insecticides, and herbicides (Vincelli 2014).

2.10.1 Fungicide resistance of sunflower downy mildew

Field isolates tolerant to metalaxyl were found first in France (Lafon et al. 1996, Delos et al. 1997, Albourie et al. 1998), then in the USA (Gulya et al. 1999), and something similar happened in Spain (Molinero-Ruiz et al. 2003b) and Italy (Covarelli and Tosi 2006). Resistance of *P. halstedii* to mefenoxam has also been reported in Russia (Iwebor et al. 2019, Iwebor et al. 2021).

However, no reduced sensitivity was found in Hungary, although the number of samples examined so far does not allow saying with certainty that this phenomenon is lacking in our country. In the 1980s, Oros and Virányi (1984) demonstrated the presence of tolerant *P. halstedii* strains in greenhouse experiments in Hungary, but could not prove this in further tests with field isolates (Virányi and Walcz 2000). More recently, Körösi et al. (2020) reported the mefenoxam tolerance of *P. halstedii* pathotypes in Hungary.

Sunflower downy mildew is almost worldwide (Spring 2019) and the rapid development of new aggressive pathotypes makes chemical disease control inevitable (Virányi and Spring 2011, Spring et al. 2018). Although variability/diversity of pathogen makes the disease control difficult and develop tolerance to fungicides.

It is believed that the mode of action of metalaxyl is by the selective inhibition of ribosomal RNA synthesis (Davidse et al. 1983, Fisher and Hayes 1984, Davidse 1995). RNA polymerase is the target site for metalaxyl, and an alteration of this target site can lead to resistance in some oomycete pathogens (Davidse et al. 1983, Parra and Ristaino 2001).

3. MATERIALS AND METHODS

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

3.1.1 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 (Table 2). 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 experiments (Table 2) (Appendix 1).

Table 2. List of *Plasmopara halstedii* isolates collected from Hungary during 2017-2019

Isolate number	**Isolate ID**	**Geographic origin**	**Year of collection**	**Sunflower genotype (*Pl* gene)**
1	Ph-20170613-23/1-Hu	Karácsond (HU)	2017	*Pl*6
2	Ph-20170523-2/1-Hu	Martfű (HU)	2017	unknown
3	Ph-20170609-18/1-Hu	Galgahévíz (HU)	2017	*Pl*6
4	Ph-20170621-28/1-Hu	Csongrád (HU)	2017	*Pl*6
5	Ph-20170529-4/1-Hu	Hatvan (HU)	2017	volunteer
6	Ph-20170529-4/2-Hu	Hatvan (HU)	2017	volunteer
7	Ph-20170703-40/1-Hu	Pély (HU)	2017	unknown
8	Ph-20170613-22/1-Hu	Túrkeve (HU)	2017	*Pl*6

9	Ph-20170622-29/C1-Hu	Bonyhád (HU)	2017	*Pl*6
10	Ph-20170622-29/B-Hu	Bonyhád (HU)	2017	*Pl*6
11	Ph-20170606-15/B-Hu	Vésztő (HU)	2017	*Pl*6
12	Ph-20170628-31/1-Hu	Szeged (HU)	2017	Experimental line
13	Ph-20170601-12/1-Hu	Abony (HU)	2017	*Pl*6
14	Ph-20170530-7/1-Hu	Tápé (HU)	2017	*Pl*6
15	Ph-20170630-34/A-Hu	Szamoskér (HU)	2017	*Pl*6
16	Ph-20180601-4/1-Hu	unknown (HU)	2018	*Pl*6
17	Ph-20190522-7/3-Hu	Békésszentandrás (HU)	2019	*Pl*6
18	Ph-20190627-21/1-Hu	Léh (HU)	2019	*Pl*6
19	Ph-20190606-14/1-Hu	Bucsa (HU)	2019	*Pl*6
20	Ph-20190606-14/3-Hu	Kertészsziget (HU)	2019	*Pl*6
21	Ph-20190606-14/4-Hu	Kötegyán (HU)	2019	*Pl*6

22	Ph-20190618-18/2-Hu	Vanyarc (HU)	2019	*Pl*6

3.1.2 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 (Figure 3). 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 $\mu E \cdot m^{-2} \cdot s^{-1}$ (Figure 3). The plants were watered regularly.

Figure 3: Plants growing in the growth chamber

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 (Figure 4). Collected sporangia were used as the inoculum for the characterization of pathotypes.

Figure 4: Plants covered with polyethylene bag to induce sporulation in dark at 19 °C

3.1.3 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 3.1.2 (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* (Table 3). 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) (Table 3). The CVF

provides information about the virulence pattern of the isolate. The test was repeated twice with two repetitions by each.

Table 3. Sunflower differential lines used for pathotype identification for *P. halstedii* in the experiment and resistance genes incorporated (based on Gascuel et al. 2015).

Nomenclature			
Triplet	**Score**	**Sunflower differential lines**	**Resistance gene to *P. halstedii***
1st	1	Iregi Szürke Csíkos	No *Pl* gene
	2	RHA-265	*Pl1*
	4	RHA-274	*Pl2/Pl21*
2nd	1	PMI-3	Pl_{PMI3}
	2	PM-17	*Pl5*
	4	803-1	*Pl5*+[b]
3rd	1	HAR-4	Pl_{15}
	2	QHP-2	$Pl1/Pl_{15}$
	4	HA-335	*Pl6*

3.2 Fungicide sensitivity tests of *Plasmopara halstedii* isolates

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

3.2.1.1 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 (Table 4).

Table 4. *Plasmopara halstedii* isolates used in the 10-isolate experiment during the fungicide resistance tests

Isolate(code)	Locality (county)	Collection (year)	Pathotype (CVF)
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

*CVF (coded virulence formula) was determined during the pathotype identification of the thesis (new results)

3.2.1.2 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.

3.2.1.3 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 "3.1.2, 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 (Table 4). 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*.

3.2.1.4 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 (Figure 4). 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.

3.2.1.5 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.).

3.2.2 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 ((Table 5). Treatment of seeds was the same as described in the chapters "3.1.2 and 3.2.1.2 i.e., treatment of seeds with mefenoxam".

Table 5. *Plasmopara halstedii* isolates used in the 8-isolate experiment during the fungicide resistance tests

Isolate code	Locality (county)	Year of collection	Pathotype (CVF)
1	Mezőkovácsháza (Békés)	2017	724
4	Kömlő (Heves)	2014	704
5	Doboz (Békés)	2014	704
6	Körösladány (Békés)	2014	714
7	Szeghalom (Békés)	2017	724
8	Pély (Heves)	2017	704*
9	Bonyhád (Tolna)	2017	724*
11	Rákóczifalva (Jász-Nagykun-Szolnok)	2012	704

*CVF (coded virulence formula) was determined during the pathotype identification of the thesis (new results)

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

The propagation of inoculum was same as described in the chapter "3.1.2 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) (Table 4). 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.

3.2.2.2 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 µE·m−2 ·s −1 (Figure 3). 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.

3.2.2.3 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.

3.2.2.4 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.

3.2.2.5 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.

3.2.3 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 "3.1.2 and 3.2.1.2". The plants were grown in a growth chamber with a photoperiod of 12 h at 22 °C, light irradiance of 100 $\mu E \cdot m^{-2} s^{-1}$.

Plasmopara halstedii isolates used in this experiment are listed in Table 6. Mád1, Kömlő, Rákóczifalva and Csanytelek isolates were increased on untreated, while Mád2 isolate on mefenoxam-treated sunflowers.

Table 6. Origin of *P. halstedii* isolates used in the experiment with different mefenoxam concentration (pathotypes were identified previously as described in Bán et al. 2021)

Isolate code	**Collection region (County)**	**Collection (year)**	**Pathotype**
Mád1	Borsod-Abaúj-Zemplén	2014	700
Mád2	Borsod-Abaúj-Zemplén	2014	700
Kömlő	Heves	2014	704
Rákóczifalva	Jász-Nagykun-Szolnok	2012	704
Csanytelek	Csongrád-Csanád	2014	730

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, "3.2.2.3".

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.

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

3.3.1 *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

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

3.3.2.1 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.

3.3.2.2 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).

3.3.2.3 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".

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.

4. RESULTS

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

The results of the sunflower downy mildew isolates collected in Hungary between 2017 and 2019 for their pathotype are shown in Table 7.

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 (Table 7). Among all pathotypes, pathotype 704 was the most widespread in collected samples. Differential lines Iregi szürke csíkos, RHA-265, and RHA-274 were completely infected by *P. halstedii* isolates for all samples. Most of these infected plants showed damping-off by the time of the second evaluation, so these lines were highly susceptible to the examined pathotype of sunflower downy mildew. During the study, 734 was identified as a new pathotype in Hungary (Nisha et al. 2021, see details below).

Table 7. Virulence character of *P. halstedii* isolates collected from Hungary in 2017-2019.

Isolate number	**Sunflower genotype (*Pl* gene)**	**Locality**	**CVF of isolate (pathotype)**
1	*Pl*6	Karácsond (HU)	704
2	unknown	Martfű (HU)	704
3	*Pl*6	Galgahévíz (HU)	704
4	*Pl*6	Csongrád (HU)	704
5	volunteer	Hatvan (HU)	700
6	volunteer	Hatvan (HU)	704
7	unknown	Pély (HU)	704
8	*Pl*6	Túrkeve (HU)	700
9	*Pl*6	Bonyhád (HU)	724
10	*Pl*6	Bonyhád (HU)	704
11	*Pl*6	Vésztő (HU)	724
12	Experimental line	Szeged (HU)	714
13	*Pl*6	Abony (HU)	704
14	*Pl*6	Tápé (HU)	704
15	*Pl*6	Szamoskér (HU)	700
16	*Pl*6	unknown (HU)	700
17	*Pl*6	Békésszentandrás (HU)	724
18	*Pl*6	Léh (HU)	**734***

19	*Pl*6	Bucsa (HU)	**734***
20	*Pl*6	Kertészsziget (HU)	**734***
21	*Pl*6	Kötegyán (HU)	730
22	*Pl*6	Vanyarc (HU)	**734***

955 * 734 pathotype has been reported newly in Hungary and published in Nisha et al. (2021)

956 **4.1.1 Identification of a new pathotype, 734, in Hungary**

957 All four isolates examined caused disease on differential lines HA-304, RHA265, RHA-274, PMI-
958 3, PM-17, and HA-335, whereas the other lines showed no symptoms and signs of sunflower
959 downy mildew. Summing the scores given according to the reactions of the differential lines by
960 each triplet, the examined *P. halstedii* isolates were identified as pathotype 734 (Table 8). This
961 pathotype is likely widespread in Hungary because it was detected from three different regions.

962 **Table 8.** Pathotype characterization of *P. halstedii* isolate 734 (S = Susceptible, R = Resistant).

Differential lines	**Reaction of plants**	**Score**	**Pathotype Code (CVF)**
Iregi szürke csíkos	S	1	**7**
RHA-265	S	2	
RHA-274	S	4	
PMI-3	S	1	**3**
PM-17	S	2	
803-1	R	0	
HAR-4	R	0	**4**
QHP-2	R	0	
HA-335	S	4	

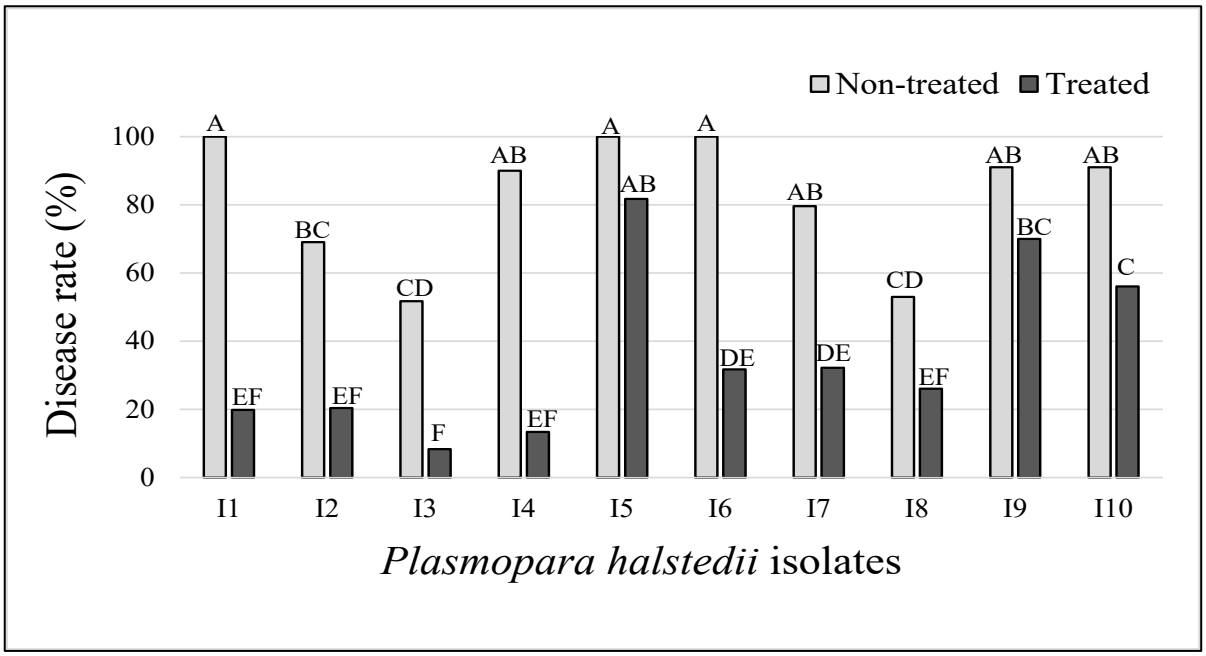
963

4.2 Fungicide sensitivity tests

4.2.1 Fungicide sensitivity tests performed on 10 *P. halstedii* isolates by using the whole seedling immersion (WSI) method

The disease rates of the different *P. halstedii* isolates on the mefenoxam-treated and non-treated sunflowers are shown in Figure 5. 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) and I4 (pathotype 704 from Karácsond). All the non-treated and inoculated plants with isolates I1, I5 and I6 showed a damping-off by the end of the experiment. 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).

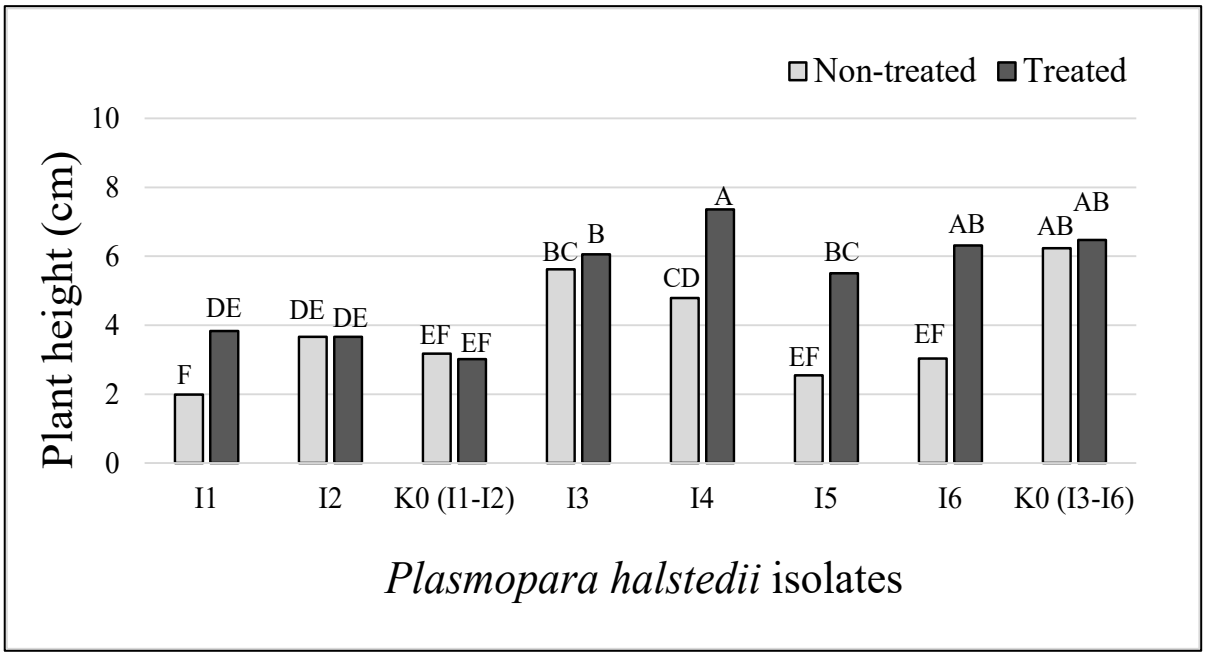
As the stunting of the infected plant is a significant symptom of *P. halstedii*; hence, the plant height was measured twice for some isolates during the experiments (Figures 6 and 7). There was no significant difference between the heights of the non-inoculated, mefenoxam treated and non-inoculated, 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 6). 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 7). The non-treated, inoculated plants with the I2 and I3 isolates showed significantly lower heights than the treated ones at the second evaluation.



991
992 **Figure 5.** Disease rates (%) on the sunflowers (treated and non-treated with mefenoxam)
993 inoculated by the different isolates of *Plasmopara halstedii* 9 days after inoculation.

994 Non-treated – non-treated with mefenoxam and inoculated with *P. halstedii*; treated – treated with
995 mefenoxam (3 mg/kg seed) and inoculated with *P. halstedii*; ANOVA was performed with Fisher's test; the
996 bars sharing the same letter are not significantly different. Isolate codes are in Table 4.

997



998
999 **Figure 6.** Plant heights of the mefenoxam-treated and non-treated sunflowers 9 days after
1000 inoculation.

1001 Non-treated – non-treated with mefenoxam and inoculated with *P. halstedii*; treated – treated with
1002 mefenoxam (3 mg/kg seed) and inoculated with *P. halstedii*; K0 (I1 – I2) and K0 (I3 – I6) – non-treated
1003 and non-inoculated with *P. halstedii* for I1–I2 and I3–I6, respectively; ANOVA was performed with
1004 Fisher's test; the bars sharing the same letter are not significantly different. Isolate codes are in Table 4.

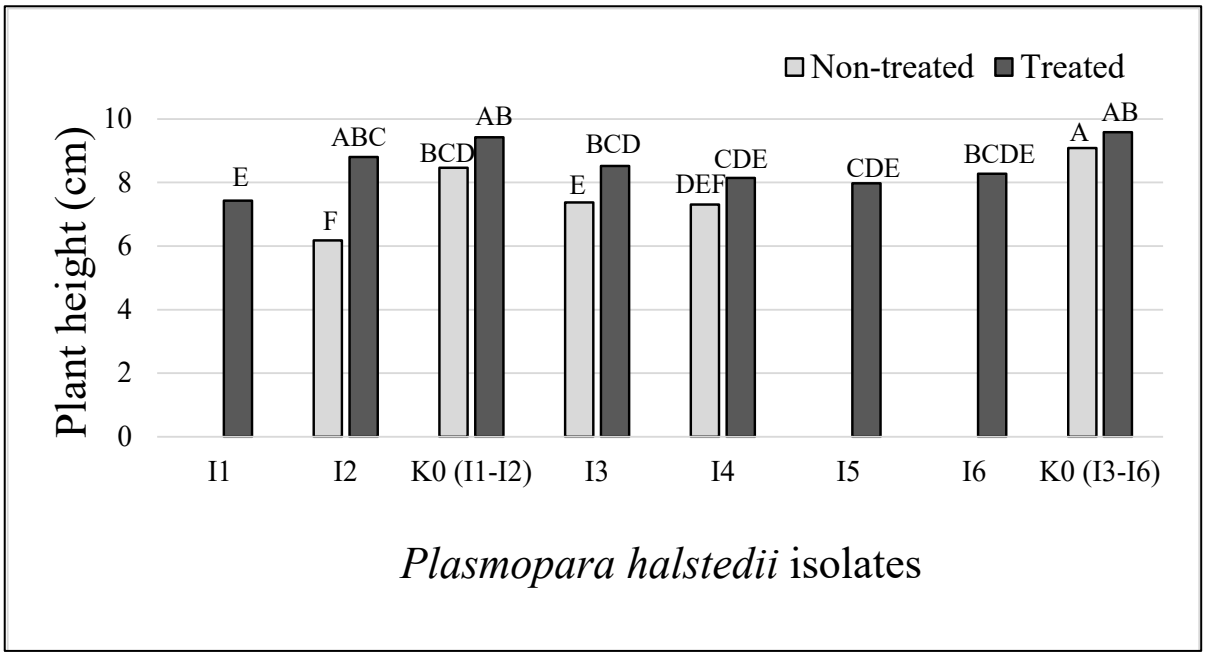


Figure 7. Plant heights of the mefenoxam-treated and non-treated sunflowers 21 days after inoculation with *P. halstedii.*

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. Isolate codes are in Table 4.

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

4.2.2.1 Disease rates and plant heights

Disease rates (%) and heights of mefenoxam treated and non-treated sunflower plants inoculated with different *Plasmopara halstedii* isolates are shown in Figure 8. According to the sporulation of the pathogen on the cotyledons and pre-emergence damped-off plants (Disease 1, Figure 8A), 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, Figure 8B), 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 (Figure 8C). 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 (Figure 8D).

For all parameters tested (Disease 1-2, Height 1-2), the interaction between isolate and treatment was significant (for Disease 1: $F=12.06$, $p<0.001$, for Disease 2: $F=5.36$, $p<0.001$, for Height 1: $F=6.61$, $p<0.001$, for Height 2: $F=7.37$, $p<0.001$), i.e., the impact of treatment varied between isolates.

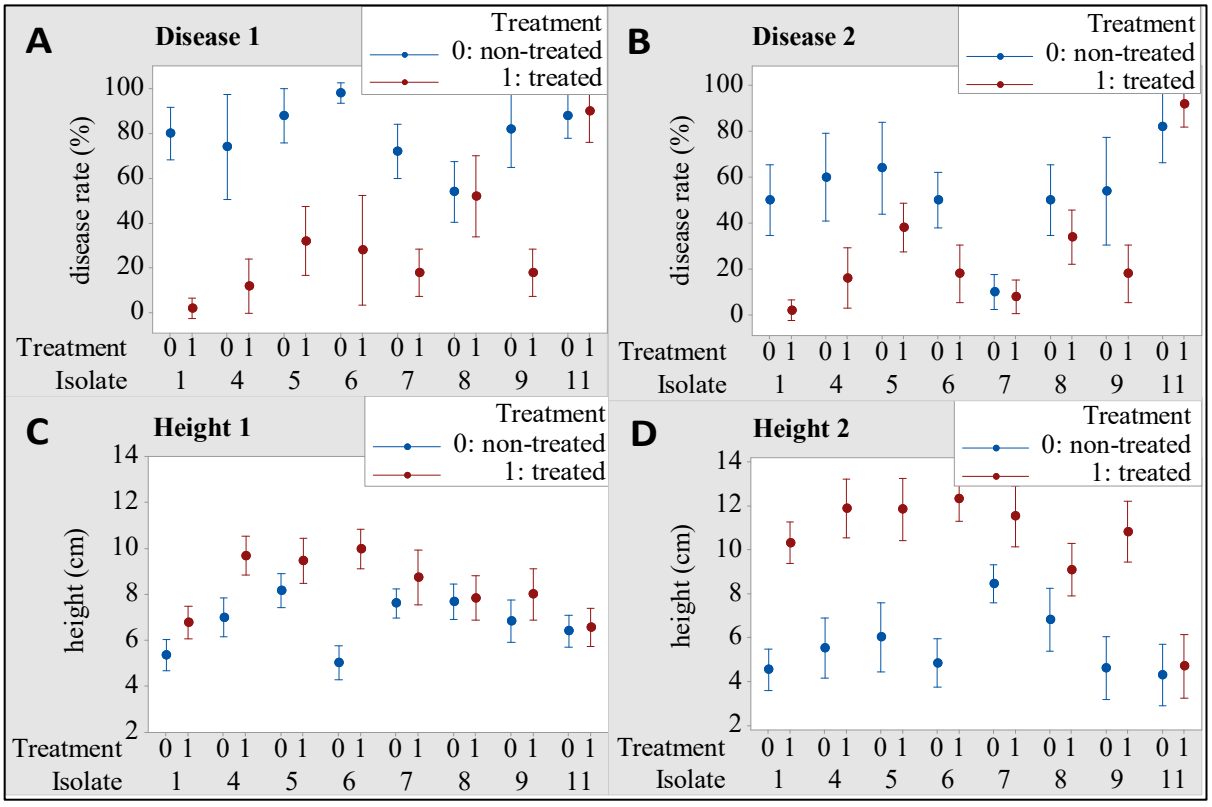


Figure 8. Disease rates (A, B) and heights (C, D) of mefenoxam treated and non-treated sunflower plants inoculated with different *Plasmopara halstedii* isolates.

Disease 1: ratio of sporulating, pre-emergence damped-off plants and healthy sunflowers nine days after inoculation.
Disease 2: ratio of chlorotic, post-emergence damped-off plants and healthy sunflowers 21 days after inoculation.
Height 1: height of sunflowers nine days after inoculation (heights of damped-off plants were taken as zero).
Height 2: height of sunflowers 21 days after inoculation (heights of damped-off plants were taken as zero).
Treatment: non-treated (0) and treated (1) with mefenoxam (3 mg/kg seed).
Isolate: code of *Plasmopara halstedii* isolates used in the experiment (1, 4, 5, 6, 7, 8, 9, 11) (for more details, see Table 5)
Bars represent the 95 % confidence intervals ($p \geq 0,5$). Individual standard deviations were used to calculate the intervals.

4.2.2.2 Microscopic observations of host tissue responses

Host tissue responses of sunflowers to infection by *P. halstedii* in hypocotyl cross-sections are shown in Figure 9. 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 (Figure 9A). Under UV light, fluorescence appeared in the intercellular spaces around hyphae, giving the image a dotted look (Figure 9B). By contrast, cell browning under normal light (Figure 9C) and an intense fluorescence of cells showing a hypersensitive-like reaction (Fig 9D) could be detected in cross-sections of several mefenoxam-treated sunflowers. Moreover, the development of cellular necrosis by vigorous cell division (Figure 9E) and the strong fluorescent response of surrounding cells (Figure 9F) was also frequently observed in treated and inoculated plants.

The rate of pathogen hyphal spread and host tissue responses are shown in Figure 10. 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 (Figure 10A and B). 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 (Figure 10A and B).

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 (Figure 10C-F). 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 (Figure 7C and D). However, it was not significant for the two latter compared to mefenoxam-treated plants. The occurrence of cell necrosis in the cortical part was intensive in non-treated plants inoculated with isolates 4, 5, and 6. For the latter, it was not significant compared to mefenoxam-treated sunflowers (Figure 10E). Necrosis in the pith parenchyma cells was minimal in each sample (Figure 10F).

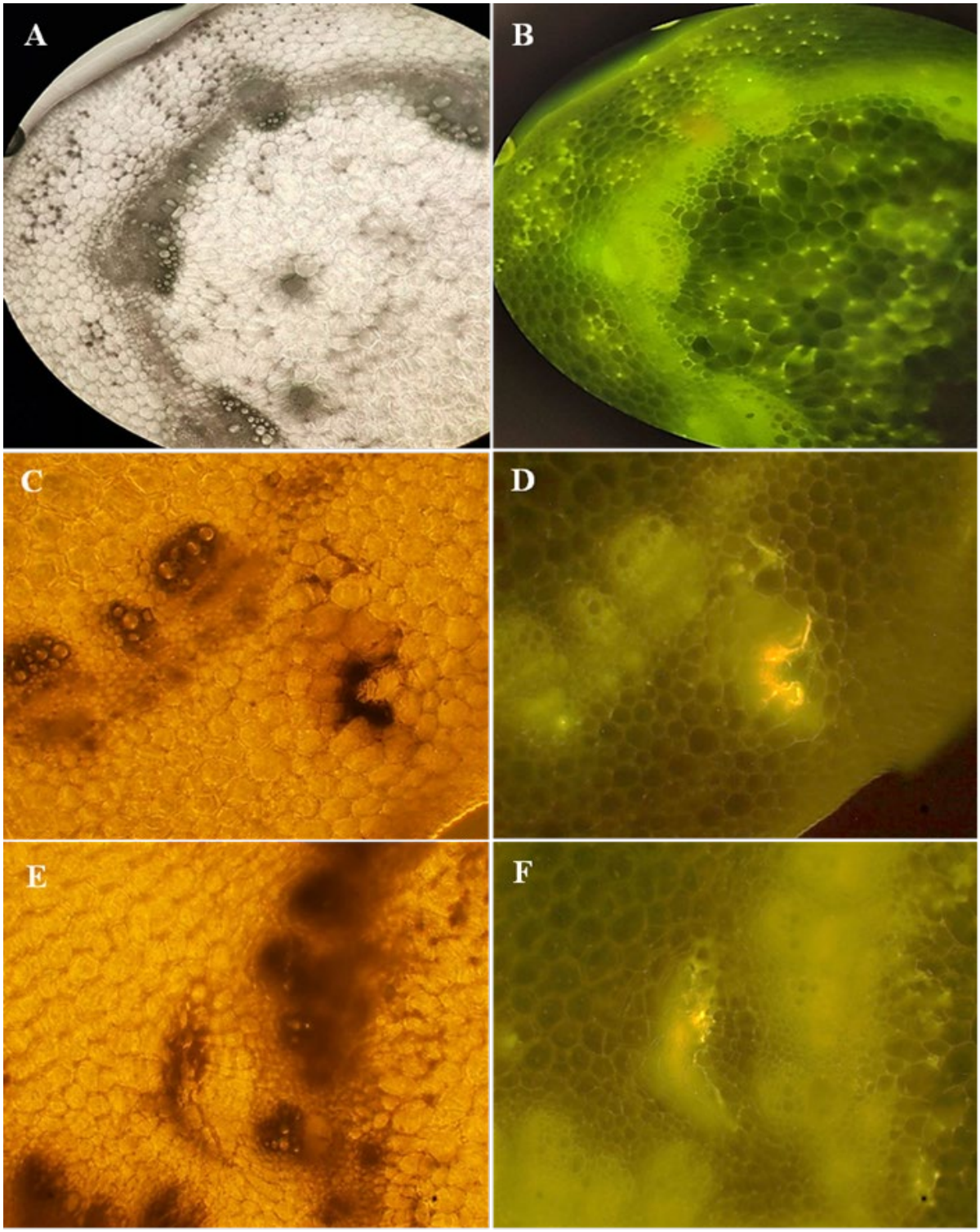


Figure 9. Light micrographs of mefenoxam-activated resistance responses in hypocotyl cross-sections of sunflower. Hyphae of *Plasmopara halstedii* invade cells of non-treated, inoculated susceptible plants (cv. Iregi szürke csíkos) without any host tissue responses in normal (A) and in UV light (B) (λ = 485 nm), at 21 dpi. Browning (C), autofluorescence (hypersensitive reaction) (D), and necrosis (E: normal light, F: UV light) of cortical parenchyma cells neighboring invaded cells as a host tissue response to the pathogenic attack of mefenoxam-treated, inoculated plants, at 21 dpi. Scale bar = 100 µm

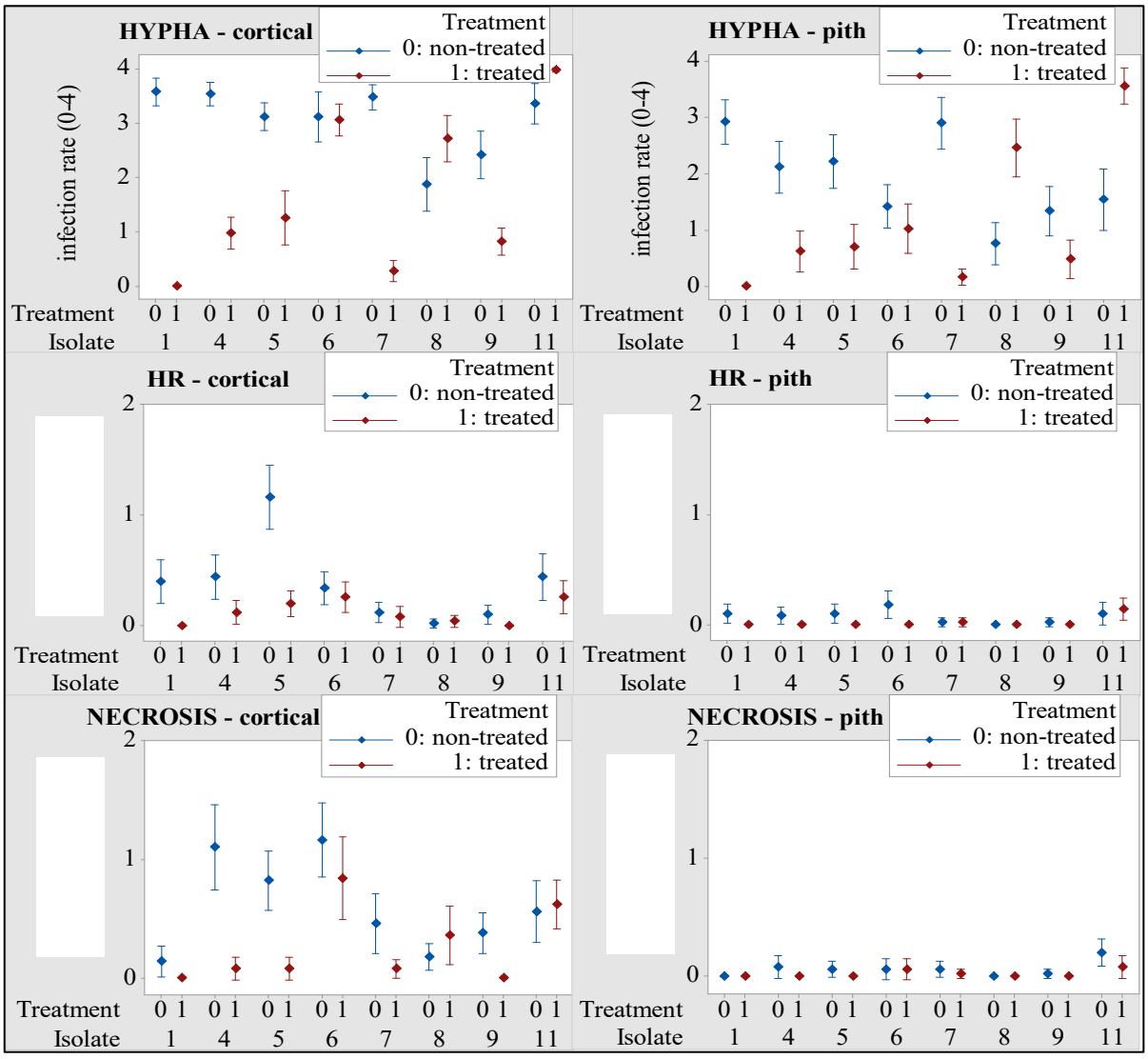


Figure 10. Occurrence of the pathogen hypha (A, B) and host tissue responses such as hypersensitive reaction (C, D) and necrosis (E, F) in the cortical and pith parenchyma of mefenoxam treated and non-treated sunflower plants inoculated with *Plasmopara halstedii*.

Treatment: non-treated (0) and treated (1) with mefenoxam (3 mg/kg seed).
Isolate: code of *Plasmopara halstedii* isolates used in the experiment (1, 4, 5, 6, 7, 8, 9, 11)
The infection rate and the intensity of the host reaction were measured on a 0-4 scale.
Vertical lines represent 95% confidence intervals (95% CI) of the mean values of disease rates and heights

4.2.2.3 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 are shown in Table 9. 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 (Table 9). 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 (Table 9).

Table 9. Cluster analyses of sunflowers based on disease rates and plant heights inoculated by different *P. halstedii* isolates.

Variables	Cluster 1	Cluster 2	Cluster 3	Cluster 4
Disease 1 (%)	90.2 ± 6.9 **d**	72.2 ± 12 **c**	20.4 ± 12.3 **a**	38.2 ± 13.3 **b**
Disease 2 (%)	74.5 ± 10.8 **C**	29.6 ± 10.4 **B**	15.9 ± 8.6 **A**	27.3 ± 10.2 **AB**
Height 1 (cm)	6.0 ± 0.8 **a**	7.1 ± 0.4 **b**	9.7 ± 0.8 **c**	7.4 ± 0.6 **b**
Height 2 (cm)	4.0 ± 1.0 **A**	7.5 ± 0.6 **B**	11.7 ± 1.1 **D**	9.3 ± 0.9 **C**

Data represent the means of variables for each cluster. Values followed by means represent standard deviation. Different letters (e.g., A, a) indicate significant differences based on the Tukey HSD post-hoc test ($p<0.05$).
Cluster 1: isolates 1, **5, 6, 9, 11** non-treated, **11** treated.
Cluster 2: isolates **1**, **4**, **7**, **8** non-treated.
Cluster 3: isolates **1, 4, 5, 6**, 7, 9 treated.
Cluster 4: isolates 1, 4, 5, 6, **7, 8, 9** treated, 8, 9 non-treated.
Bold isolate numbers indicate dominance of that isolate in that cluster compared to other clusters.

Cluster analyses of sunflowers based on the examined microscopic variables inoculated by different *P. halstedii* isolates is presented in Table 10. 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 (Table 10). 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.

Table 10. Cluster analyses of sunflowers based on the examined microscopic variables inoculated by different *P. halstedii* isolates.

Variables	Cluster 1	Cluster 2	Cluster 3
H_Cort	3.7 ± 0.3 **C**	3.0 ± 0.5 **B**	0.2 ± 0.2 **A**
HR_Cort	0.4 ± 0.4 **c**	0.2 ± 0.2 **b**	0 **a**
NEC_Cort	0.7 ± 0.5 **C**	0.5 ± 0.4 **B**	0 **A**
H_Pith	3.6 ± 0.4 **c**	0.5 ± 0.3 **b**	0 **a**
HR_Pith	0.1 ± 0.2 **B**	0 **A**	0 **A**
NEC_Pith	0.1 ± 0.2 **b**	0 **a**	0 **a**

Data represent the means of variables for each cluster. Values followed by means represent standard deviation. Different letters (e.g., A, a) indicate significant differences based on the Tukey HSD post-hoc test ($p<0.05$).
Cluster 1: isolates **1, 4, 5, 7** non-treated, **8, 11** treated
Cluster 2: isolates **4, 5, 6, 8, 9, 11** non-treated, **6** treated
Cluster 3: isolates **1, 4, 5, 7, 9** treated.
Bold isolate numbers indicate dominance of that isolate in that cluster compared to other clusters. The underlined isolates were equally represented in the clusters concerned.

4.2.2.4 Correlations among macroscopic and microscopic parameters

The results of Pearson correlation based on the examined macroscopic variables (disease rates, plant heights) are shown in Table 11. 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.

Table 11. Pearson correlation among the examined variables (disease rates, plant heights).

Variable	Disease 1	Disease 2	Height 1	Height 2
Panel A: Non-treated (n=80)				
Disease 1	1	0..346**	-0.465**	-0.550**
Disease 2		1	-0.439**	**-0.713****
Height 1			1	**0.737****
Height 2				1

Panel B: Treated (n=80)				
Disease 1	1	**0.701****	-0.368**	**-0.700****
Disease 2		1	-0.329**	**-0.722****
Height 1			1	**0.741****
Height 2				1

Disease 1: ratio of sporulating, damped-off plants and healthy sunflowers nine days after inoculation.
Disease 2: ratio of chlorotic, damped-off plants and healthy sunflowers 21 days after inoculation.
Height 1: height of sunflowers nine days after inoculation (heights of damped-off plants were taken as zero).
Height 2: height of sunflowers 21 days after inoculation (heights of damped-off plants were taken as zero).
Treatment: non-treated and treated with mefenoxam (3 mg/kg seed).
**Correlation is significant at the 0.01 level (2-tailed).
Values in bold indicate a strong correlation between variables.

The Spearman correlation of the examined microscopic variables is presented in Table 12. 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.

Table 12. Spearman correlation among the examined microscopic variables

Variable	**H_Cort**	**HR_Cort**	**NEC_Cort**	**H_Pith**	**HR_Pith**	**NEC_Pith**
Panel A: Non-treated (n=200)						
H_Cort	1	0,211**	0,291**	**0,508****	0,158**	0,150**
HR_Cort		1	0,240**	0,193**	**0,375****	0,080
Nec_Cort			1	0,223**	0,155**	0,172**
H_Pith				1	0,156**	0,248**
HR_Pith					1	0,106*
Nec_Pith						1
Panel B: Treated (n=200)						
H_Cort	1	**0,327****	**0,488****	**0,759****	0,174**	0,153**
HR_Cort		1	0,072	0,213**	0,241**	0,029
Nec_Cort			1	**0,547****	0,079	0,180**
H_Pith				1	0,204**	0,169**

HR_Pith					1	0,129*
Nec_Pith						1

H: hyphae of *Plasmopara halstedii*
HR: hypersensitive reaction of invaded cells
Nec: necrosis
Cort: cortical parenchyma
Pith: pith parenchyma
**Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).
Values in bold indicate a strong correlation between variables.

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

4.2.3.1 Disease rates

Disease 1 and 2 values are shown in Figures 11 and 12. 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 (Figures 11 and 12).

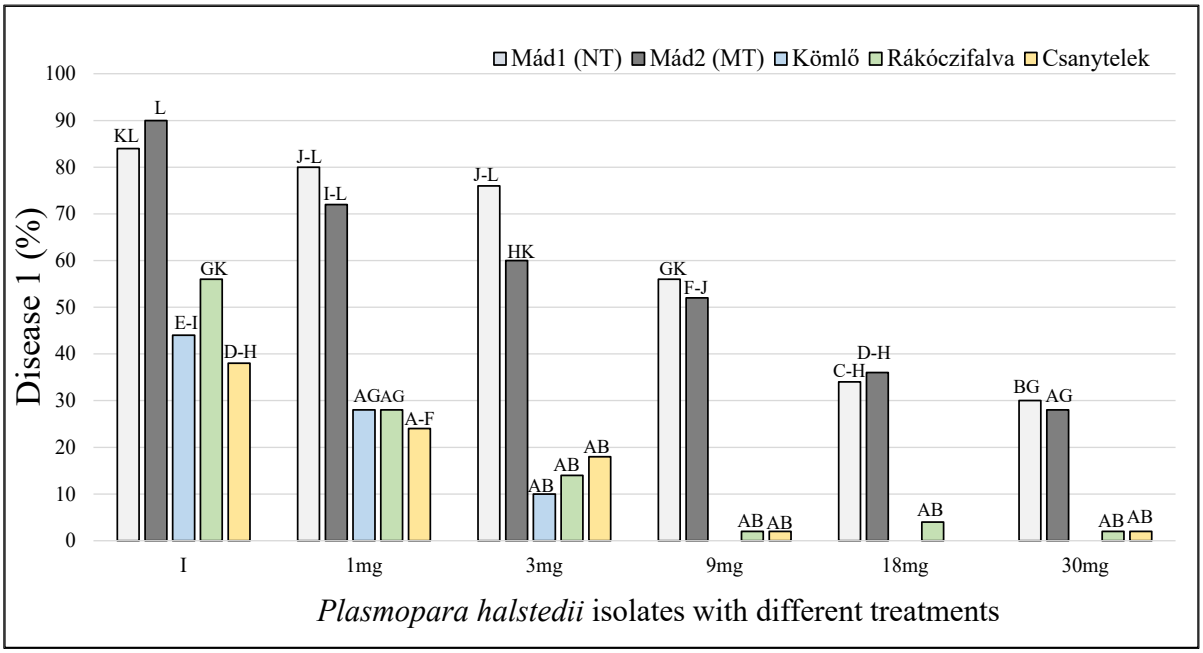


Figure 11. Disease 1 (%) values of sunflowers treated with different concentrations of mefenoxam and inoculated by 5 isolates of *Plasmopara halstedii* 9 days after inoculation.
The different letters displayed above the columns in the figure indicate a significant difference between treatments ($p < 0.05$).

Mád1,2, Kömlő, Rákóczifalva, Csanytelek: codes of different *P. halstedii* isolates referring to their place of origin. Mád1 (NT) - the isolate from Mád was propagated (increased) on untreated plants with mefenoxam, Mád2 (MT) - the isolate from Mád was propagated (increased) on plants treated with mefenoxam at 3 mg/kg.
I: control which was inoculated with *P. halstedii* sporangial suspension,
1, 3, 9, 18, and 30 mg: treatment with different concentrations of mefenoxam (1, 3, 9, 18, and 30 mg/kg seeds) and inoculated with *P. halstedii* sporangial suspension

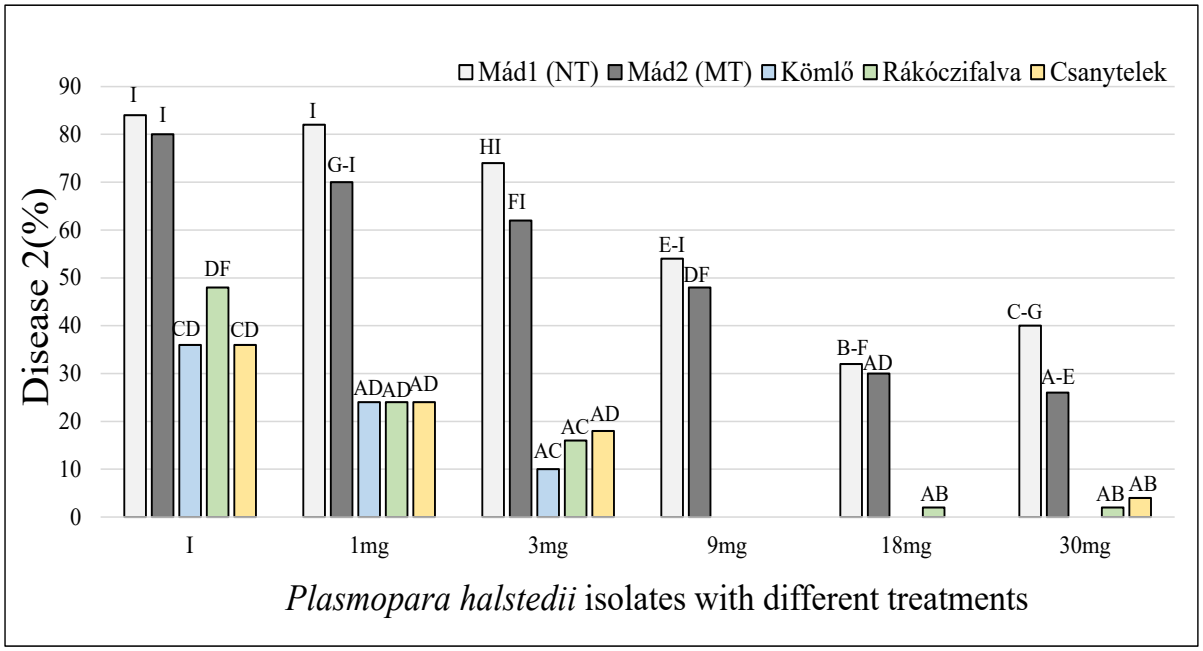


Figure 12. Disease rates 2(%) on the sunflowers treated with different concentration of mefenoxam and inoculated by the different isolates of *Plasmopara halstedii* 21 days after inoculation.
The different letters displayed above the columns in the figure indicate a significant difference between treatments ($p < 0.05$).
Mád1,2, Kömlő, Rákóczifalva, Csanytelek: codes of different *P. halstedii* isolates referring to their place of origin. Mád1 (NT) - the isolate from Mád was propagated (increased) on untreated plants with mefenoxam, Mád2 (MT) - the isolate from Mád was propagated (increased) on plants treated with mefenoxam at 3 mg/kg.
I: control which was inoculated with *P. halstedii* sporangial suspension,
1, 3, 9, 18, and 30 mg: treatment with different concentrations of mefenoxam (1, 3, 9, 18, and 30 mg/kg seeds) and inoculated with *P. halstedii* sporangial suspension.

4.2.3.2 Plant heights

The stunting of the plants is a significant symptom of *P. halstedii*; hence, the plant height was measured twice for all isolates during the experiments.

The heights of sunflowers connected to experiments with Mád 1 *P. halstedii* isolate are shown in Figure 13. After 9 days (Height 1), compared to control plants (BW), a significant height reduction was observed for the non-inoculated plants treated with different concentrations of mefenoxam. The situation was similar during the second evaluation (Height 2) except for non-inoculated plants treated with 3 and 9 mg/kg mefenoxam. 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 the control.

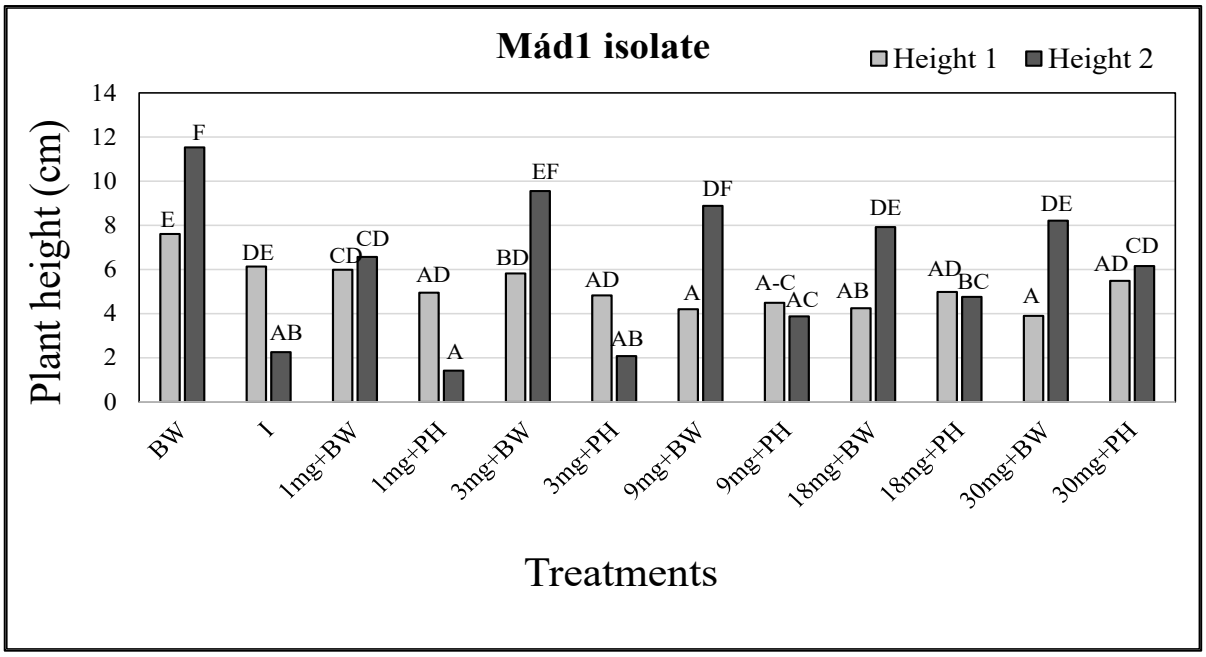


Figure 13. Plant heights of sunflowers treated with different concentrations of mefenoxam inoculated with Mád1 isolate of *Plasmopara halstedii.*

The different letters displayed above the columns in the figure indicate a significant difference between treatments ($p < 0.05$). BW: treated with bidistilled water and non-inoculated with *P. halstedii*, I: inoculated with *P. halstedii* sporangial suspension, 1, 3, 9, 18, and 30 mg: concentrations of mefenoxam, Height 1: height of sunflowers nine days after inoculation, Height 2: height of sunflowers 21 days after inoculation. The two height values (Height 1 and 2) are not statistically comparable in the figure.

The heights of sunflowers connected to experiments with Mád 2 *P. halstedii* isolate are shown in Figure 14. After 9 days (Height 1), compared to control plants (BW), a significant height reduction was observed for the non-inoculated plants treated with higher concentrations of mefenoxam (9, 18, 30 mg). During the second evaluation of non-inoculated sunflowers (Height 2), plants treated with 1, 18, and 30 mg mefenoxam were significantly lower than the control plants. 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.

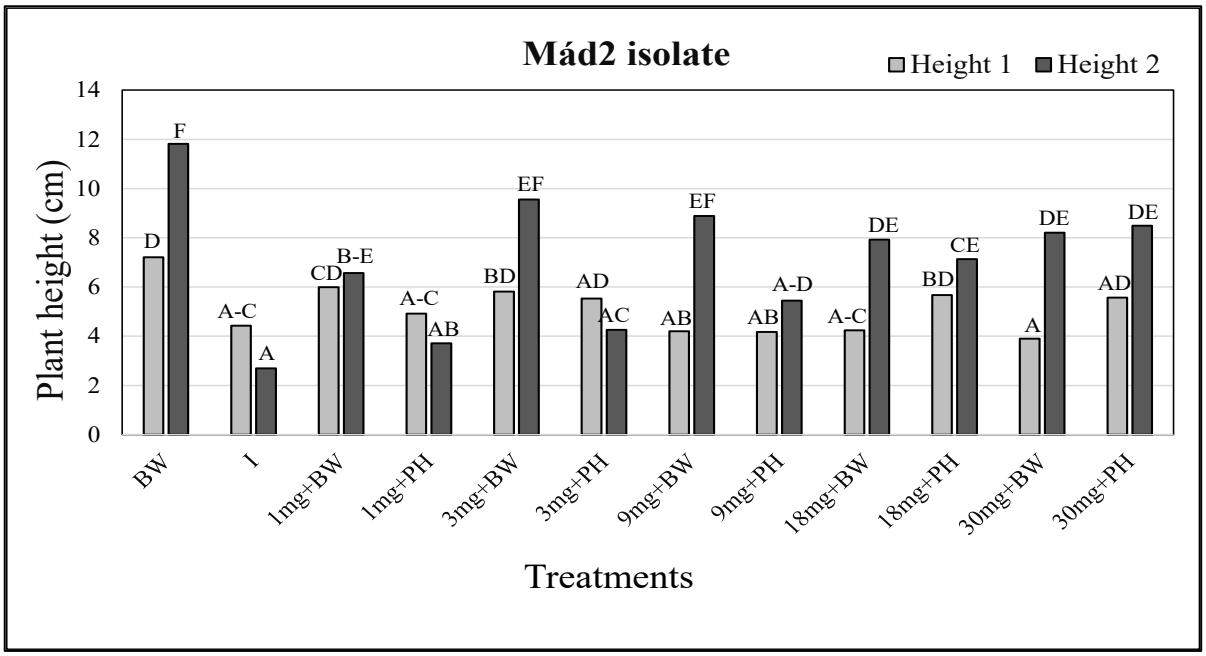


Figure 14. Plant heights of sunflower treated with different concentrations of mefenoxam inoculated with Mád2 isolate.

The different letters displayed above the columns in the figure indicate a significant difference between treatments ($p < 0.05$). BW: treated with bidistilled water and non-inoculated with *P. halstedii*, I: inoculated with *P. halstedii* sporangial suspension, 1, 3, 9, 18, and 30 mg: concentrations of mefenoxam, Height 1: height of sunflowers nine days after inoculation, Height 2: height of sunflowers 21 days after inoculation. The two height values (Height 1 and 2) are not statistically comparable in the figure.

The heights of sunflowers connected to experiments with Kömlő *P. halstedii* isolate are shown in Figure 15. There was no significant difference among the plant heights of non-inoculated sunflowers 9 days after inoculation (Height 1). During the second evaluation, only non-inoculated plants treated with 30 mg mefenoxam were significantly lower than non-treated ones. 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.

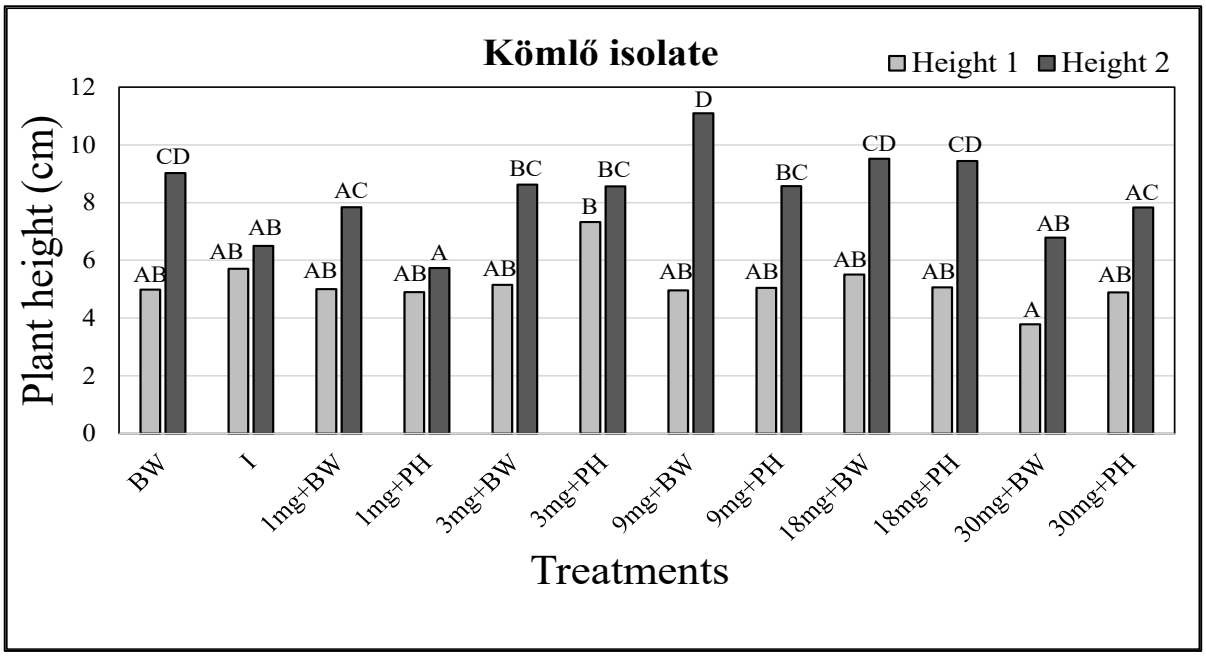


Figure 15. Plant heights of sunflower treated with different concentrations of mefenoxam inoculated with Kömlő isolate.

The different letters displayed above the columns in the figure indicate a significant difference between treatments ($p < 0.05$). BW: treated with bidistilled water and non-inoculated with *P. halstedii*, I: inoculated with *P. halstedii* sporangial suspension, 1, 3, 9, 18, and 30 mg: concentrations of mefenoxam, Height 1: height of sunflowers nine days after inoculation, Height 2: height of sunflowers 21 days after inoculation. The two height values (Height 1 and 2) are not statistically comparable in the figure.

The heights of sunflowers connected to experiments with Rákóczifalva *P. halstedii* isolate are shown in Figure 16. There was no significant difference among the plant heights of non-inoculated sunflowers 9 and 21 days after inoculation (Height 1 and 2). During the first evaluation, 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.

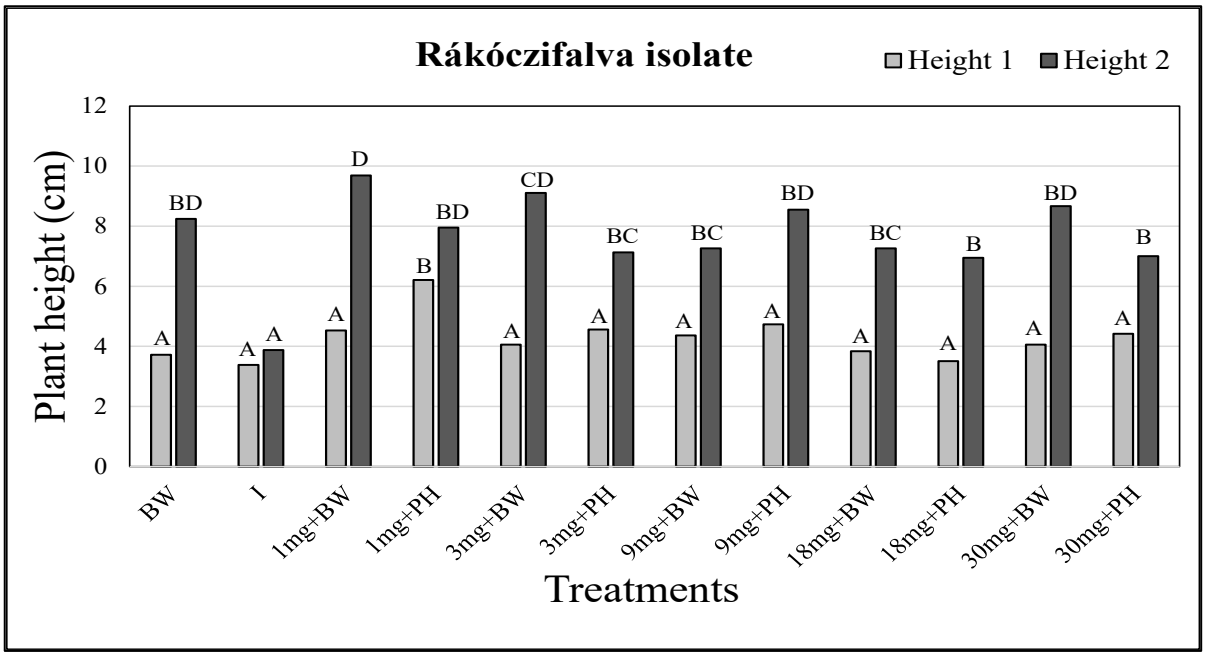


Figure 16. Plant heights of sunflower treated with different concentrations of mefenoxam inoculated with Rákóczifalva isolate.

The different letters displayed above the columns in the figure indicate a significant difference between treatments ($p < 0.05$). BW: treated with bidistilled water and non-inoculated with *P. halstedii*, I: inoculated with *P. halstedii* sporangial suspension, 1, 3, 9, 18, and 30 mg: concentrations of mefenoxam, Height 1: height of sunflowers nine days after inoculation, Height 2: height of sunflowers 21 days after inoculation. The two height values (Height 1 and 2) are not statistically comparable in the figure.

The heights of sunflowers connected to experiments with Csanytelek *P. halstedii* isolate are shown in Figure 17. There was no significant difference among the plant heights of non-inoculated sunflowers 9 and 21 days after inoculation (Height 1 and 2) except for plants treated with 1 and 9 mg of mefenoxam. 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.

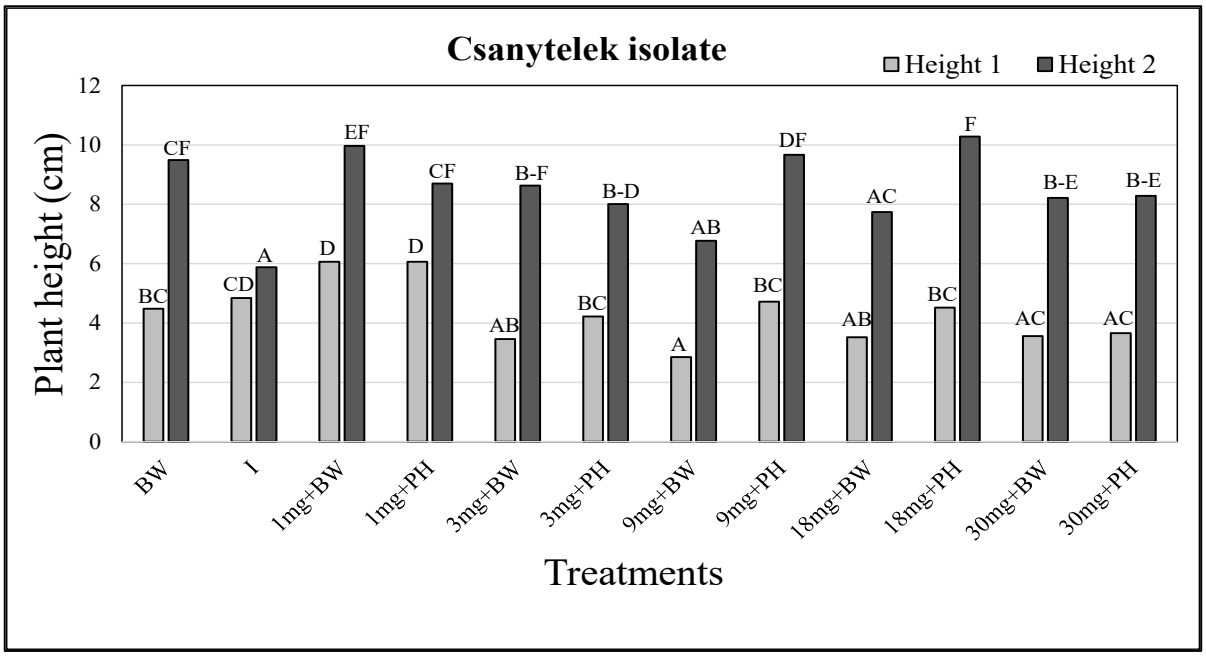


Figure 17. Plant heights of sunflower treated with different concentrations of mefenoxam inoculated with Csanytelek isolate.

The different letters displayed above the columns in the figure indicate a significant difference between treatments ($p < 0.05$). BW: treated with bidistilled water and non-inoculated with *P. halstedii*, I: inoculated with *P. halstedii* sporangial suspension, 1, 3, 9, 18, and 30 mg: concentrations of mefenoxam, Height 1: height of sunflowers nine days after inoculation, Height 2: height of sunflowers 21 days after inoculation.

The two height values (Height 1 and 2) are not statistically comparable in the figure.

4.3 Effects of neem-derived pesticides on sunflower downy mildew

4.3.1 *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, Figure 18).

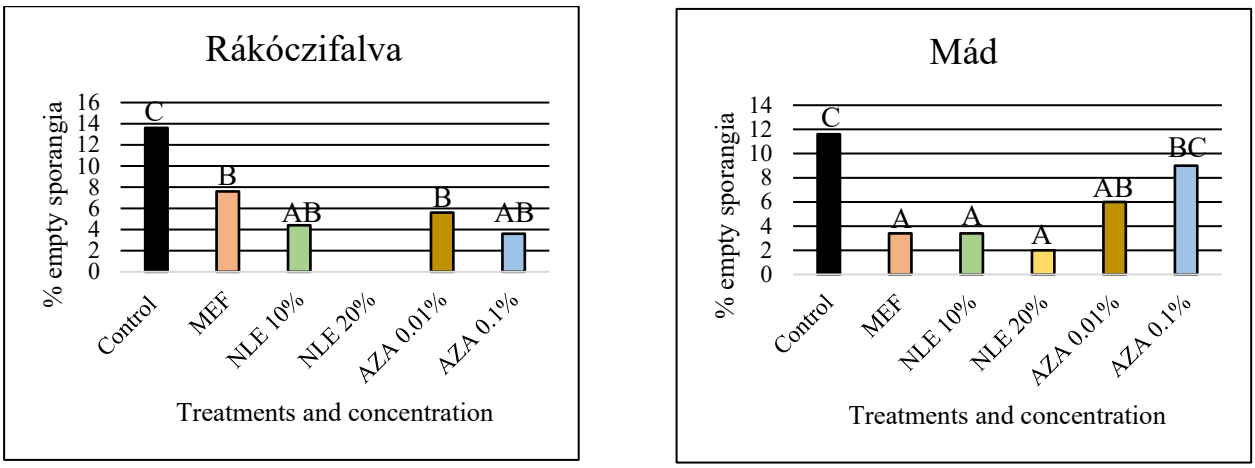


Figure 18. Effect of two different concentrations of neem leaf extract (NLE) and NeemAzal T/S (AZA), respectively, on the germination of *P. halstedii* sporangia of two isolates (Rákóczifalva and Mád). Mefenoxam (MEF) was used as a positive control.

Different letters according to Tukey's test indicate significant difference at 95% confidence level.

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

4.3.2.1 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 (Figures 19 and 20). 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 are shown in Figures 21 and 22. 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).

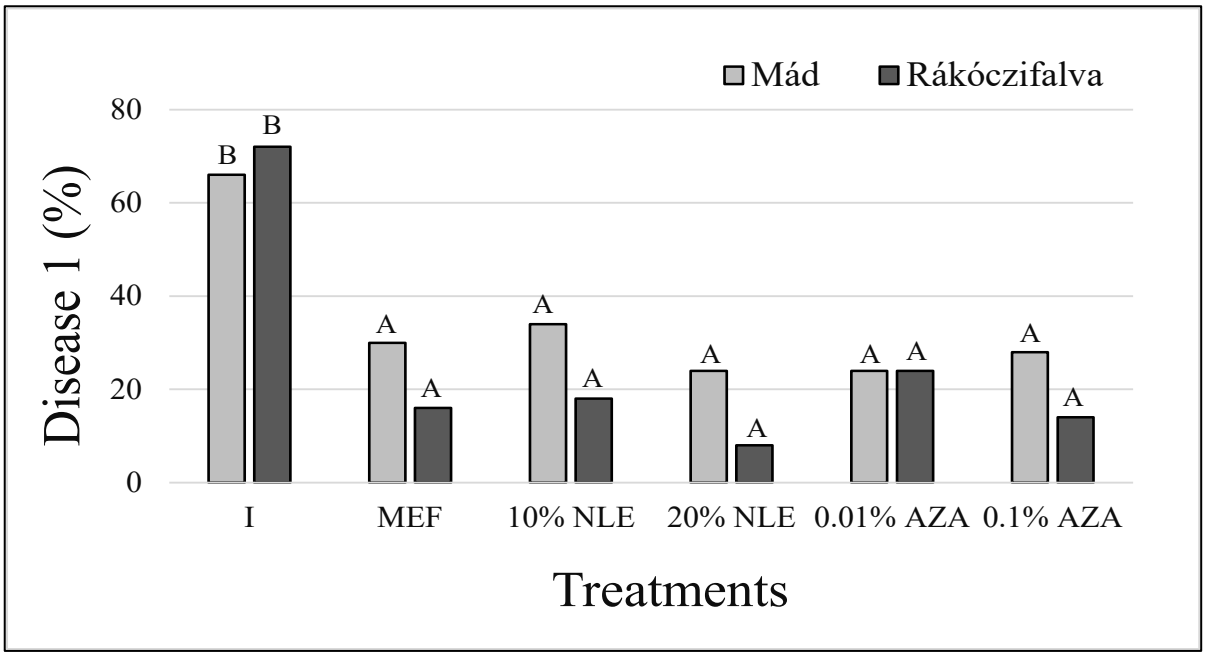


Figure 19. Disease 1 (%) of the sunflowers treated with neem leaf extract (NLE) and NeemAzal T/S (AZA) after 9 days inoculation.

I; non-treated with mefenoxam and inoculated with *P. halstedii*, MEF; treated – treated with mefenoxam (3 mg/kg seed) and inoculated with *P. halstedii*, 10% NLE; 20% NLE; 0.01% AZA; 0.1% AZA; inoculated with *P. halstedii*. Bars sharing the same letter are not significantly different.

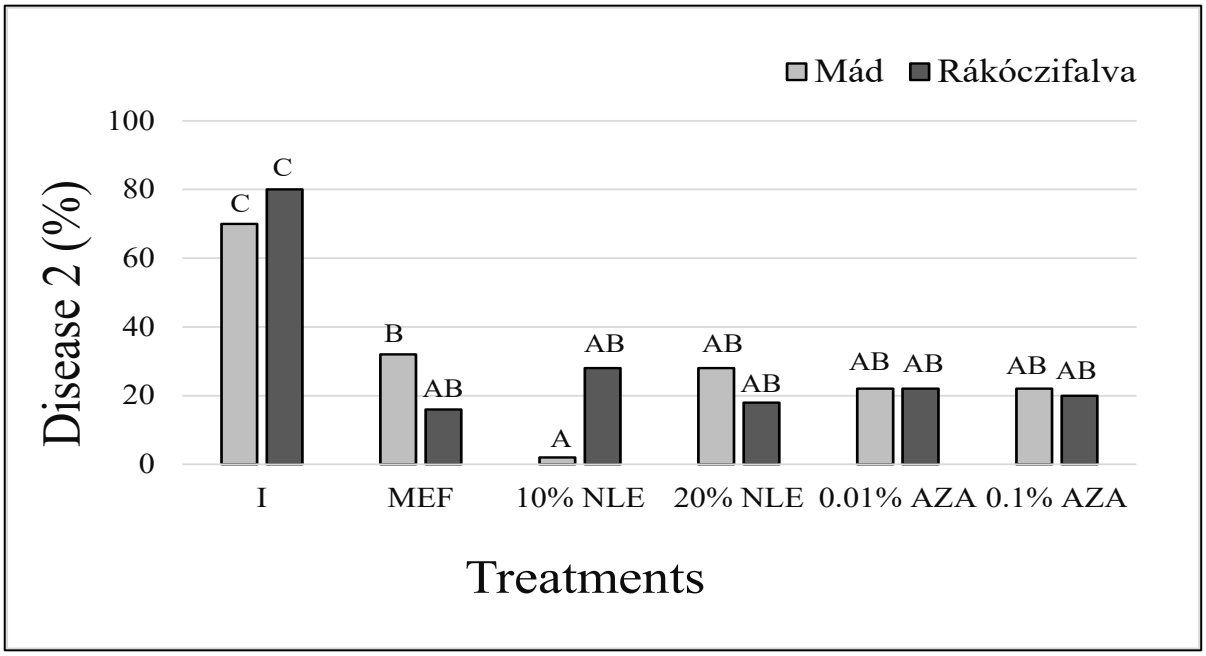
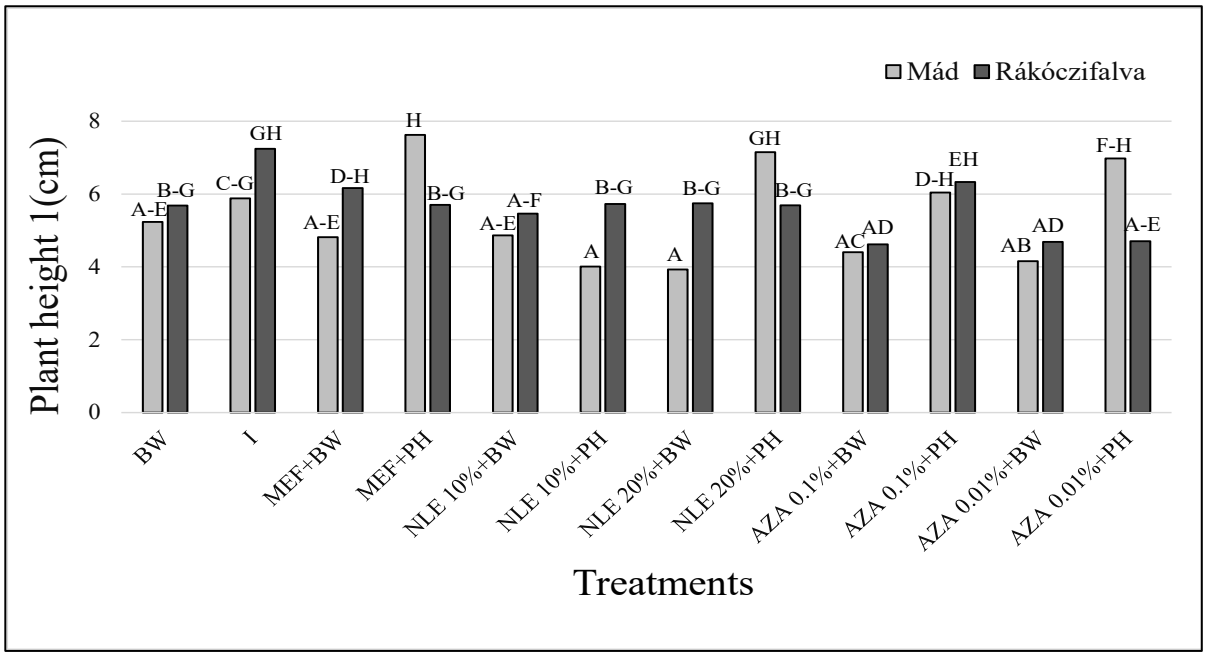
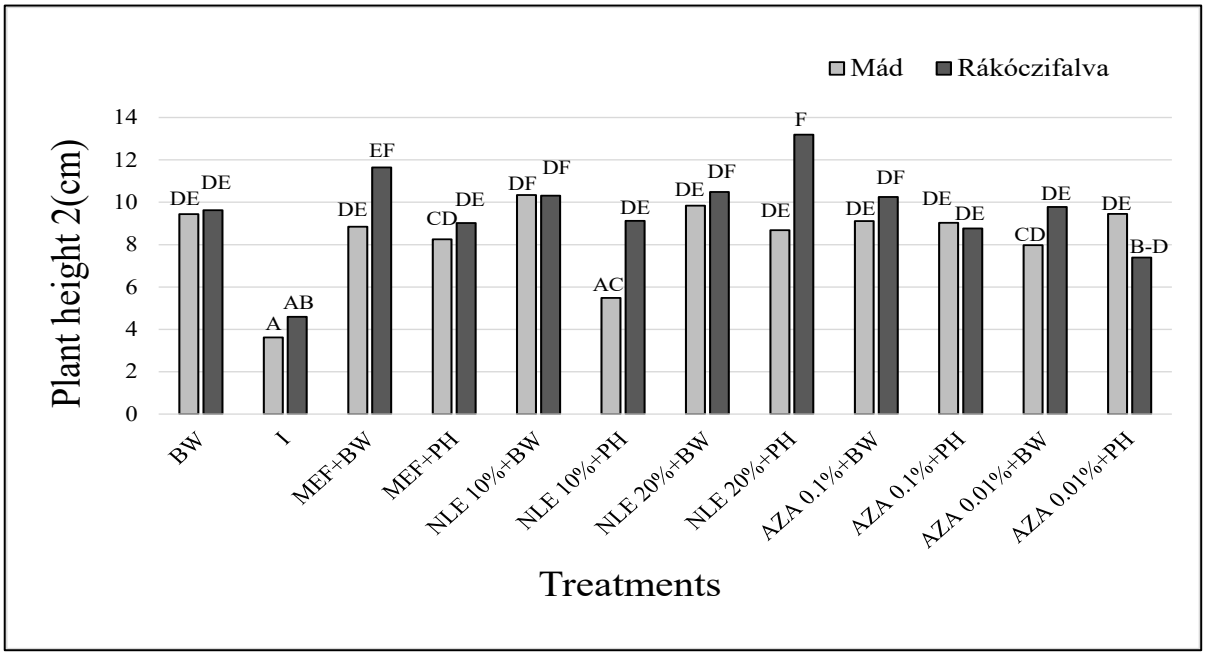


Figure 20. Disease 2 (%) on the sunflowers treated with the concentration of neem leaf extract and NeemAzal T/S (AZA) after 21 days inoculation.

I; non-treated with mefenoxam and inoculated with *P. halstedii*, MEF; treated – treated with mefenoxam (3 mg/kg seed) and inoculated with *P. halstedii*, 10% NLE; 20% NLE; 0.01% AZA; 0.1% AZA; inoculated with *P. halstedii*. Bars sharing the same letter are not significantly different.



1361 **Figure 21.** Plant heights of the neem leaf extract and NeemAzal T/S (AZA) treated sunflowers 9
1362 days after inoculation with *P. halstedii.*
1363 BW: treated with bidistilled water, I: non-treated with mefenoxam and inoculated with *P. halstedii*,
1364 MEF+BW: treated with mefenoxam (3 mg/kg seed) and bidistilled water, MEF+PH: treated with
1365 mefenoxam and inoculated with *P. halstedii,* 10% NLE; 20% NLE; 0.01% AZA; 0.1% AZA: treated with
1366 different concentrations of neem leaf extract or azadirachtin and inoculated with *P. halstedii*. Mád,
1367 Rákóczifalva: isolates of *P. halstedii*. Bars sharing the same letter are not significantly different.



1368
1369 **Figure 22.** Plant heights of the neem leaf extract and NeemAzal T/S (AZA) treated sunflowers
1370 21 days after inoculation with *P. halstedii.*

1371 BW: treated with bidistilled water, I: non-treated with mefenoxam and inoculated with *P. halstedii*,
1372 MEF+BW: treated with mefenoxam (3 mg/kg seed) and bidistilled water, MEF+PH: treated with
1373 mefenoxam and inoculated with *P. halstedii,* 10% NLE; 20% NLE; 0.01% AZA; 0.1% AZA: treated with

different concentrations of neem leaf extract or azadirachtin and inoculated with *P. halstedii*. Mád, Rákóczifalva: isolates of *P. halstedii*. Bars sharing the same letter are not significantly different.

4.3.2.2 Microscopic observations

The rate of pathogen hyphae and host reactions, such as hypersensitive reaction and necrosis are shown in Figure 23. 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 hypha 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.

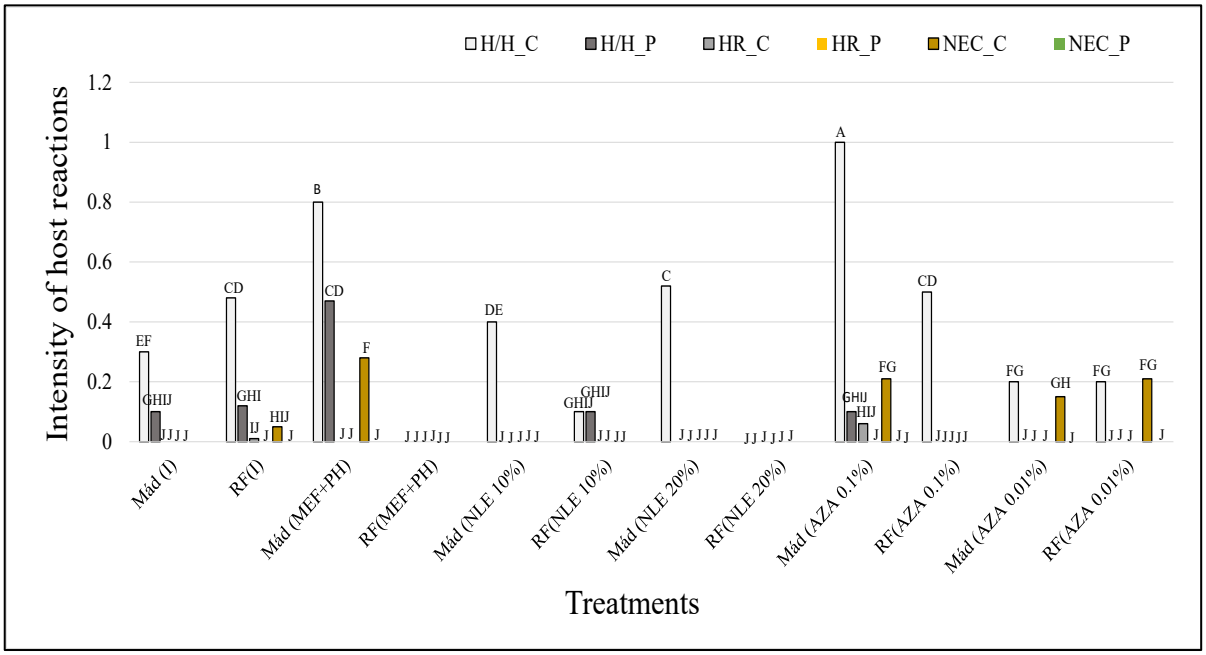


Figure 23. Effects of neem leaf extract (NLE), NeemAzal T/S (AZA) and mefenoxam (MEF) on the occurrence of the pathogen hypha and haustoria (H/H) and host tissue responses such as hypersensitive reaction (HR) and necrosis (NEC) in the cortical (C) and pith (P) parenchyma inoculated with *Plasmopara halstedii* isolates (Mád and RF: Rákóczifalva).

I: stands for inoculated, non-treated control. Bars sharing the same letter are not significantly different.

5. DISCUSSION

5.1 Pathotype composition of sunflower downy mildew (*Plasmopara 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. Therefore, knowing the virulence diversity within the pathogen population of sunflower downy mildew has become essential for resistance breeding and quarantine measures. 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).

The differentiation of the pathogen started in the 1970s when the first resistance gene was implemented in the sunflower line RHA266 (Vranceanu and Stoenescu 1970, Spring 2019). The acceleration of the process has made it necessary to develop a protocol to standardize the pathotyping process. Today the 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). Although several attempts have been made to develop modern methods for this purpose, they have not yet been widely used in practice due to their many drawbacks (Gascuel et al. 2016). Therefore, we decided to use the traditional and widely accepted method of Trojanová et al. (2017) for pathotype identification.

Gulya (2007), Virányi et al. (2015), and Spring (2019) have previously summarized the pathotype distribution of *P. halstedii* worldwide. Recently, it has been found that several pathotypes have overcome the *Pl6* resistance gene incorporated into a wide range of sunflower hybrids and led to the emergence of highly aggressive pathotypes (Sedlárová et al. 2016, García-Carneros and Molinero-Ruiz 2017, Bán et al. 2018, Spring and Zipper 2018). Moreover, Martin-Sanz et al. (2020) reported a virulent pathotype 714, which has overcome to *Pl8* resistance gene.

Before 2010, Gulya (2007) reported about five pathotypes (100, 330, 700, 710, and 730) considered relevant in Hungary. There was a significant change in the virulence character of *P. halstedii* populations detected between 2007 and 2013 in Hungary and worldwide. However, despite new pathotypes, less virulent pathotypes such as 700 and 730 were still predominant in Hungary from 2007 to 2014 (Virányi et al. 2015).

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). This pathotype is likely widespread in Hungary because it was detected from three different regions of the country: Borsod-Abaúj-Zemplén county (Léh), Békés county (Bucsa, Kertészsziget) and Nógrád county (Vanyarc). The possibility that pathotype 734 is present in Hungary has been raised before (Iwebor et al. 2018), but previous isolates were proved weak for proper identification. This pathotype has already been widespread among hot races in the USA and Russia (Spring 2019) and is considered highly aggressive, which was able to overcome the effect of resistance genes *Pl6* and *Pl7*.

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). In addition, further spread into the western part of the country was proved as we identified the pathotype 724 in Bonyhád (Tolna county). This fact is noteworthy because no highly virulent pathotypes have been reported from the western part of Hungary so far. Moreover, the only data available in this part of the country on sunflower downy mildew pathotypes are from Martonvásár (pathotype 700).

It is remarkable that, to a smaller extent, less virulent pathotypes such as 700 and 730 could be identified from sunflowers with resistance genes against these strains during our survey. The exact reason for this is still unknown, but other authors report similar cases for different pathogens (Kema et al. 2018, Seybold et al. 2020). A highly aggressive pathotype likely represses the host's defense mechanisms, creating favorable conditions for the less virulent (or avirulent) pathotypes. It is even likely that lower virulence in these strains is associated with higher fitness, contributing to their persistence.

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.

Several factors may be responsible for the emergence of new *P. halstedii* pathotypes such as favorable weather conditions, the emergence of mefenoxam-resistant *P. halstedii* isolates (Körösi et al. 2020), and the spread of minimal tillage systems. In Hungary, however, mainly short crop

rotation and, in many cases, inadequate weed management may promote the distribution of new aggressive *P. halstedii* pathotypes (Bán et al. 2016, 2021).

5.2 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). Some data supported by greenhouse experiments were available in Hungary by Oros and Virányi (1984), but they could not prove it in further tests with field isolates of *P. halstedii* (Virányi and Walcz 2000). Similarly, resistance to mefenoxam has already been reported in other populations of different oomycetes (Schwinn and Staub 1987, Lamour and Hausbeck 2000, Parra and Ristaino 2001,).

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 analyzed, these results provide the first evidence of mefenoxam tolerance of sunflower downy mildew in high oleic sunflower hybrids in Hungary. Furthermore, like Gulya (2000), our results did not find any correlation between the virulence phenotype (CVF) and the fungicide resistance characteristic of different *P. halstedii* strains, i.e., there were also sensitive and resistant strains characterized by either the 704 or 724 pathotypes.

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. The development of subsequent symptoms (e.g., leaf chlorosis, late damping-off) caused by the pathogen was monitored in addition to the initial symptoms. 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. The sunflowers in Cluster 1 (non-treated and inoculated by isolates 5, 6, 9, 11) had relatively high initial and subsequent infection rates, indicating that the pathogen could penetrate unhindered into the upper parts of the plant. This was associated with significant growth inhibition of these plants.

On the other hand, the reaction was similar in mefenoxam-treated plants inoculated by isolate 11; therefore, it appears to be mefenoxam resistant. Although this is a typical reaction of susceptible sunflowers to the pathogen, it is interesting that in Cluster 2, non-treated plants (inoculated with isolates 1, 4, 7, 8) were characterized by the decreased spreading of the pathogen to the above plant parts. This difference between the two clusters (mainly non-treated plants) can probably be explained by the different aggressiveness of the *P. halstedii* isolates tested as indicated by other authors (e.g., Sakr 2009). Nevertheless, the two clusters of mefenoxam-treated and inoculated plants (Clusters 3 and 4) also differed, mainly in the degree of initial disease rate and in the development of plant heights. In conclusion, 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. This negative correlation is not surprising, as many authors have reported such effects of the pathogen on plant development in susceptible, non-treated sunflowers (Virányi and Oros 1991, Gascuel et al. 2015). 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 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). Mouzeyar et al. (1993, 1994) pointed out that *P. halstedii* could infect susceptible and resistant sunflower lines in a microscopic investigation. Although to a smaller extent, even a susceptible plant can react to the pathogen's spread. Our results with fluorescent microscopy of non-treated sunflowers also supported this.

Moreover, the speed and intensity of host tissue response to *P. halstedii* in a resistant sunflower may vary, and it can appear in the root or different parts of the hypocotyl (Mouzeyar et al. 1993). Previous authors also described a hypersensitive-like response in the hypocotyl of mefenoxam-treated susceptible sunflowers (Mouzeyar et al. 1995). They found that all metalaxyl concentrations and application modes provided complete protection against *P. halstedii*. However, only one *P. halstedii* isolate was tested in the latter work, which seemed sensitive to the active ingredient.

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. This phenomenon was very similar to what usually occurs in non-treated susceptible plants with the plant response appearing to be a delayed host reaction to a pathogenic attack (Mouzeyar et al. 1993, Gascuel et al. 2015). 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. Because of the lack of massive mycelial growth in the hypocotyl, it is likely, that the pathogen was arrested in the root tissues by the chemical.

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. Those in the first two groups (clusters) showed increased (isolates 8 and 11) or moderate tolerance (isolate 6) to mefenoxam, while isolates in the third group showed sensitivity. Disease rate and plant height values of treated and inoculated sunflowers with these tolerant or resistant isolates also supported this. However, microscopic studies allowed us to estimate the sensitivity (tolerance) more accurately, showing refined interaction with non-treated plants. In addition, only isolate 11 could be defined with more decreased sensitivity with the evaluation of visible symptoms.

In addition to its direct toxic effect on the pathogen, metalaxyl (mefenoxam) activates the host defense system, which might result in increased sunflower resistance, restricting pathogen development (Cahill et al. 1993). In previous research, histological alterations such as haustoria encapsulation by callose deposits (Hickey and Coffey 1980) or the development of limited hypersensitive-like lesions were also reported, followed by metalaxyl treatment in some host-parasite interactions where the pathogen was sensitive to the chemical (Ward et al. 1980, Lazarovits and Ward 1982, Stössel et al. 1982, Mouzeyar et al. 1995). However, the question remains whether the direct (fungistatic) or indirect effect (through the host) of metalaxyl is more significant against the sensitive pathogen in different host-parasite relationships.

Examining metalaxyl-sensitive and tolerant *Phytophthora megasperma* isolates in soybean Cahill and Ward (1989) pointed out that metalaxyl enhanced the release of phytoalexin elicitors (glyceollin) in culture fluids of the sensitive isolate but not in those of the tolerant isolate. Releasing elicitors due to metalaxyl treatment could induce host reactions in compatible interactions with the sensitive isolate. In our study, the effective host tissue responses against the sensitive *P. halstedii* isolates likely occurred at a very early stage of infection in the roots of mefenoxam-treated sunflowers. Despite this, the reaction of mefenoxam-treated plants to tolerant isolates could appear later in the hypocotyl, which the delayed stimulation of elicitor activity can explain by the chemical. Our results with the Spearman correlation also demonstrate this. It

showed that the spread of the tolerant isolates in the cortical parenchyma of treated plants correlated positively with the appearance of HR and necrosis.

Interestingly, more abundant hyphae were found in the pith of treated than non-treated plants inoculated by isolates 8 and 11 (considered as tolerant or resistant). This is in line with the results of Cahill and Ward (1989). They reported a better growth of metalaxyl-tolerant *Phytophthora megasperma* isolates in the presence of the chemical *in vitro* and *in vivo*. Previous authors assumed that metalaxyl could serve as a nutrient and raised the idea of other tolerance mechanisms and different interactions with the host (soybean) for those tolerant isolates. In addition, the more significant presence of the pathogen in the pith of sunflowers has been shown to facilitate the spread of the pathogen to the upper parts of the plant (e.g., epicotyl) (Heller et al. 1997).

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).

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

The effect of two different neem-derived pesticides, such as neem leaf extract (NLE) and NeemAzal T/S, was tested in different concentrations for the first time against *P. halstedii in vitro* and *in vivo* conditions by Doshi et al. (2020). The authors reported that those neem-derived pesticides could be valuable for controlling the downy mildew of sunflower, but only one *P. halstedii* isolate was used in those studies, which was sensitive to mefenoxam. Previously, Mirza et al. (2000) tested the neem products on *Phytophthora infestans 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 *Ph. infestans*. It was shown that all these products could potentially manage potato late blight disease. Similarly, Rashid et al. (2004)

1597 also observed that all the neem products significantly inhibited the different developmental stages
1598 of the above pathogen.

1599 We tested neem leaf extracts (NLE) and azadirachtin (AZA as NeemAzal T/S) in different
1600 concentrations against two different *P. halstedii* isolates by treating plants with the ingredients for
1601 a longer exposure time (4 h) than Doshi et al. (2020). In addition, we studied host tissue responses
1602 (hypersensitive reaction and cell necrosis) of neem-treated, inoculated plants with a fluorescent
1603 microscope to explore the histological background of protection.

1604 Under *in vitro* conditions, all the treatments except the higher concentration of NLE showed
1605 significant inhibition of the sporangial germination of Rákóczifalva isolate. Similarly, except for
1606 AZA 0.1%, all the treatments were significantly better than the inoculated control at reducing the
1607 total number of empty sporangia for Mád isolate.

1608 When tested *in vivo* in our study, both concentrations of neem leaf extracts and NeemAzal T/S
1609 significantly reduced the sporulation and chlorosis of *P. halstedii* isolates as compared to
1610 inoculated control plants. Our results were consistent with the findings of Achimu and Schlösser
1611 (1992), where neem seed extract and commercial neem products were effective
1612 against *Plasmopara viticola* in the grapevine. Similarly, Krzyzaniak et al. (2018) also found that
1613 the plant extract successfully controlled *P. viticola*. The reduction of infection in the pre-treatment
1614 may be due to sunflower sensitizing defense response against *P. halstedii*, reported by Fernandez
1615 et al. (2004), where they tested the essential oil obtained from *Bupleurum gibraltarium* against the
1616 pathogen. They reported that pre-treatment with oil might activate the defense response of the
1617 seedlings against *P. halstedii*.

1618 Host tissue responses of neem-treated sunflowers inoculated by *P. halstedii* were first examined
1619 by fluorescent microscope in our study. We observed a similar tissue response (cell necrosis) in
1620 neem-treated and inoculated plants as previously observed in BTH (benzothiadiazole as Bion
1621 50WG) treatments against sunflower downy mildew (Bán et al. 2004) and sclerotinia (Bán et al.
1622 2017). Similarly, we could detect more necrosis in the cortical part of sunflowers treated with both
1623 concentrations of AZA and mefenoxam inoculated by the Mád isolate as compared to the non-
1624 treated control. This was true for plants treated with 0.01 % AZA and inoculated with the
1625 Rákóczifalva isolate. Therefore, it seems that azadirachtin induces similar host tissue responses in
1626 diseased plants to mefenoxam and benzothiadiazole, which can play a role in restricting *P.*
1627 *halstedii* in susceptible sunflowers.

1628

6. 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.

7. 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.

8. SUMMARY

Downy mildew of sunflower (*Helianthus annuus* L.), caused by the obligate biotrophic oomycete *Plasmopara halstedii* Farl. Berl. et de Toni, is one of the most destructive pathogens of sunflowers worldwide. In the absence of resistant sunflower cultivars and seed treatment, it can cause complete loss or decline in yield production. *Plasmopara halstedii* rapidly develops pathotypes that can break down the resistance genes in sunflowers. Therefore, knowing the virulence diversity within the pathogen population of sunflower downy mildew has become essential for resistance breeding and quarantine measures. Fungicide resistance of the pathogen is another increasing problem worldwide.

In view of the above, I have set the following objectives for my work: (i) Pathotype identification of *P. halstedii* (sunflower downy mildew) isolates collected from different regions in Hungary in three consecutive years (2017-2019), (ii) Testing the mefenoxam sensitivity of *P. halstedii* isolates collected in Hungary and characterize host tissue responses to tolerant/resistant isolates with fluorescence microscope, (iii) Investigations on the effectiveness of neem-derived pesticides on *P. halstedii* in sunflower under *in-vitro* and *in-vivo* conditions

As a result of our survey, pathotype 704 was the most widespread in the collected samples of *P. halstedii*. The presence of less virulent pathotypes (700 and 730) was also confirmed in our study from 2017 to 2019. During the study, 734 was identified as a new pathotype in Hungary. We confirmed the previous statements that there is a shift in Hungary's pathotype composition of sunflower downy mildew towards highly virulent pathotypes.

Mefenoxam performed poorly or only moderately in the case of several *P. halstedii* isolates in our test. Microscopic studies allowed us to estimate the sensitivity (tolerance) more accurately. We first revealed a clear difference in host tissue responses of mefenoxam-treated susceptible sunflowers inoculated with various *P. halstedii* isolates. The effects of different mefenoxam concentrations were also tested against 5 isolates of *P. halstedii*. From these, mefenoxam was only found to be effective at several folds of the registered concentration (3 mg/kg seeds) against one isolate (Mád).

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. 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.

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2411 **Appendix 1.** Data of *Plasmopara halstedii* isolates used in the experiments of the thesis

Isolate ID in MATE collection (year, month, day, number, country)	**Locality**	**Code of isolate for pathotype identification**	**Code of isolate for 10-isolate experiment**	**Code of isolate for 8-isolate experiment**	**Code of isolate for 5-isolate experiment**	**Pathotype (CVF*) of isolate, new result in dissertation (CVF published before, not new in thesis)**
Ph-20170613-23/1-Hu	**Karácsond**	1	I4	-	-	704
Ph-20170523-2/1-Hu	Martfű	2	-	-	-	704
Ph-20170609-18/1-Hu	Galgahévíz	3	-	-	-	704
Ph-20170621-28/1-Hu	Csongrád	4	-	-	-	704
Ph-20160621-5/1B-Hu	Csongrád	-	I7	-	-	(704)
Ph-20170529-4/1-Hu	Hatvan	5	-	-	-	700
Ph-20170529-4/2-Hu	Hatvan	6	-	-	-	704
Ph-20170707	Pély	7	I6	8	-	704

3-40/1-Hu						
Ph-20170613-22/1-Hu	Túrkeve	8	I3	-	-	700
Ph-20170622-29/C1-Hu	Bonyhád	9	I5	9	-	724
Ph-20170622-29/B-Hu	Bonyhád	10	-	-	-	704
Ph-20170606-15/B-Hu	Vésztő	11	-	-	-	724
Ph-20170628-31/1-Hu	**Szeged**	12	-	-	-	714
Ph-20170601-12/1-Hu	**Abony**	13	-	-	-	704
Ph-20170530-7/1-Hu	Tápé	14	-	-	-	704
Ph-20170630-34/A-Hu	Szamoskér	15	-	-	-	700
Ph-20180601-4/1-Hu	unknown (Hungary)	16	-	-	-	700
Ph-20190522-7/3-Hu	**Békés-szentandrás**	17	-	-	-	724

Ph-20190627-21/1-Hu	Léh	18	-	-	-	734
Ph-20190606-14/1-Hu	**Bucsa**	19	-	-	-	734
Ph-20190606-14/3-Hu	Kertészsziget	20	-	-	-	734
Ph-20190606-14/4-Hu	Kötegyán	21	-	-	-	730
Ph-20190618-18/2-Hu	**Vanyarc**	22	-	-	-	734
Ph-20170608-16/1B-Hu	Mezőkovácsháza	-	I2	1	-	(724)
Ph-20140626-23/1-Hu	Kömlő	-	-	4	Kömlő	(704)
Ph-20140527-9/1-Hu	Doboz	-	-	5	-	(704)
Ph-20140527-7/2-Hu	Körösladány	-	-	6	-	(714)
Ph-20140527-7/1-Hu	Körösladány	-	I9	-	-	(704)
Ph-2017060	Szeghalom	-	-	7	-	(724)

8-17/1C-Hu						
Ph-20120626-7/1-Hu	Rákóczifalva	-	-	11	Rákóczi-falva	(704)
Ph-20170523-1/1	Tiszaföldvár	-	I1	-	-	(704)
Ph-20140521-6/1-Hu	Tiszafüred	-	I8	-	-	(730)
Ph-20140611-11/1-Hu	Csanytelek	-	I10	-	Csanyteleк	(730)
Ph-20140521-5/1-Hu	Mád (1,2)	-	-	-	Mád1, Mád2	(700)

2412

2413