MAGYAR AGRÁR- ÉS ÉLETTUDOMÁNYI EGYETEM

Role of resistance inducers and antioxidant enzymes in the control of sunflower downy mildew caused by (*Plasmopara halstedii* (Farl.) Berl. et de Toni)

DOI: 10.54598/005320

Thesis of PhD Dissertation

Ahmed Ibrahim Alrashid Yousif Mohamed Elamin

A thesis submitted in partial fulfilment of the requirements of Hungarian University of Agricultural and Life Sciences,
for the award of Doctor of Philosophy

2024

The PhD School

Name:	Doctoral School of Plant Sciences
Discipline:	Crop Production and Horticultural Sciences
Head:	Prof. Dr. Lajos Helyes Professor, Head of the PhD School of Plant Sciences, Hungarian University of Agriculture and Life Sciences.
Supervisor:	Dr. Katalin Körösi Associate professor, NVI Hungarian University of Agriculture and Life Sciences, Faculty of Agriculture and Environmental Science, Institute of Plant Protection, Department of Integrated Plant Protection.

.....	.....
Approval of the School Leader	Approval of the Supervisor

Chapter 1: INTRODUCTION

Sunflower (*Helianthus annuus* L.) is one of the most important oilseeds crops worldwide owing to its economic importance, versatility, and high nutritional value. Sunflower oil is widely used for cooking and frying and as an ingredient in many food products such as margarine and salad dressings. In addition, sunflower seeds are used for snacks, animal feed, and in the production of biodiesel (Adeleke & Babalola, 2020). According to the Food and Agriculture Organization of the United Nations (FAO), sunflower is the fourth most important oilseed crop in the world, after soybean, palm, and rapeseed. In 2019, the global production of sunflower seeds was approximately 53 million metric tons, with Russia, Ukraine, and Argentina being the leading producers (FAOSTAT, 2021).

Unfortunately, various diseases, such as *P. halstedii* (Farl.) Berl. et de Toni infection is reducing sunflower production. This pathogen is regarded as one of the most dangerous threats to sunflower crops worldwide (Viranyi and Spring, 2011). For example, Gascuel et al. (2015), *P. halstedii*, the pathogen responsible for sunflower downy mildew, has been documented in many countries where sunflowers grow, indicating the global distribution of the disease. The presence of this pathogen can have a significant effect on sunflower yield, with an estimated 3.5% reduction in commercial seed production when current control measures are used. However, yield losses can reach 100% in fields with high levels of contamination. The stage of host infection determines the symptoms of *P. halstedii* infection.

The widespread use of chemicals has become a significant concern, because they can harm human health and the environment. Owing to their eco-friendly nature, induced resistance can be utilised as an alternative to synthetic chemicals for disease control. Induced systemic resistance occurs when an inducer artificially activates the host plant's natural defensive mechanism (Basavaraj et al., 2019). Systemic acquired resistance (SAR) and induced systemic resistance (ISR) are the two types of induced resistance (Kamle et al. 2020). Chemical inducers and necrotrophy pathogens may activate the SAR, while ISR is triggered due to plant growth-promoting microorganisms colonizing plant roots (Spoel and Dong 2012).

Unlike chemical inducers, botanical pesticides have been applied for thousands of years (Ngegba et al., 2022) and are a new element of the growing

importance of integrated pest management (Ngegba et al. 2022). Among these, the extract of the neem tree (*Azadirachta indica* A. Juss) contains more than 140 biologically active components. Such as azadirone, azadirachtin, flavonoids etc, with azadirachtin as the most efficient active ingredient, are effective against several plant diseases (Kumar et al. 2020; Adusei and Azupio 2022). Preliminary studies have shown that neem leaf extract and azadirachtin perform well against an aggressive isolate of *P. halstedii* pathotype 704 (Doshi et al. 2020).

Several defensive processes are associated with the activation of plant disease resistance and pre-existing chemical and physical barriers to pathogen invasion to avoid or prevent phytopathogenic infections. Other plant resistance mechanisms include inducible defensive responses such as the activation of defense-related enzymes in response to pathogen infection. Integrated pest management (IPM) uses pest biology and ecology to help farmers manage diseases, weeds, insects, and vertebrates in an environmentally and economically sustainable manner (Ehler 2006). IPM is a potential method for controlling sunflower downy mildew (Molinero-Ruiz, 2022).

1.1 Research aims:

1- Testing the efficacy of different biotic and abiotic plant-resistant inducers (BTH and *Trichoderma asperellum*.) and a botanical pesticide, azadirachtin, against seven downy mildew isolates caused by *P.halstedii* under *in vitro* and *in vivo* conditions using the chemical fungicide mefenoxam as a positive control

2- Evaluating the synergistic effects and combination compatibility of (BTH and *T. asperellum*.) and (azadirachtin, BTH, and mefenoxam) to control different *P. halstedii* isolates

3- Evaluate changes in antioxidant enzyme activity levels in susceptible sunflower seedlings pre-treated with BTH, *T. asperellum* and its combinations and infected with the sunflower downy mildew (pathotype 710)

4. Evaluate changes in antioxidant enzyme activity levels in susceptible sunflower seedlings pre-treated with azadirachtin, BTH, mefenoxam and its combinations and infected with the sunflower downy mildew (pathotype 710)

Chapter 2: MATERIAL AND METHODS

2.1 Efficacy of some plant resistance inducers against several sunflower downy mildew (*Plasmopara halstedii* (Farl.) Berl. et de Toni) isolates

Plant and pathogen materials

Seven compatible host-pathogen combinations were examined using with one sunflower genotype (cv. Iregi szürke csíkos) and seven *P. halstedii* isolates. Iregi szürke csíkos is a Hungarian open-pollinated sunflower cultivar with no dominant resistance genes (*Pl* genes) against sunflower downy mildew.

P. halstedii isolates originated from the collection of the Department of Integrated Plant Protection (the Hungarian University of Agriculture and Life Sciences, MATE). Previously, *P. halstedii* isolates were collected from sunflower hybrids with the *Pl*6 resistance gene against sunflower downy mildew between 2014 and 2019. Isolates were stored in a deep freezer at -70°C. The signs and sources of these isolates are listed in Table 1.

Table 1. List and characterization of *Plasmopara halstedii* isolates collected in different regions of Hungary

Isolate code	Location	Collection year	CVF* of the isolate
I1	Abony	2014	704
I2	Körösladány	2014	710
I3	Doboz	2014	704
I4	unknown	unknown	700
I5	Csanytelek	2014	730
I6	Tiszafüred	2014	730
I7	Borsod-Abaúj-Zemplén County	2019	704

The *Trichoderma* spp. isolate (TGAM-G16, Accession number:2538527) used for seed treatment was originally obtained from soil. This isolate, acquired from the Department of Integrated Plant Protection at Hungarian University of Agriculture and Life Sciences MATE in Gödöllő, was selected due to its demonstrated effectiveness against various pathogens

under *in vitro* conditions. These pathogens include *Fusarium oxysporum* f. sp. *lycopersica*, and *Sclerotinia sclerotiorum*, as indicated by unpublished research. Subsequent analysis confirmed the identity of the isolate as *Trichoderma asperellum* based on the ITS sequence region.

Treatment of sunflower seedlings with inducers and inoculation by *P. halstedii*

Sunflower seeds were germinated before treatment and inoculation with *P. halstedii* (except for the mefenoxam treatment and the first *Trichoderma* spp. treatment, see below). For germination, the seeds were soaked in a 1.5% NaOCl solution for 3 min, rinsed in tap water, wrapped in moist filter paper, and kept in the dark at 21°C for three days. Then, the three-day-old sunflower seedlings were treated with the examined inducers, such as BTH and azadirachtin. Seedlings were soaked in an aqueous solution of BTH (20, 40, and 80 ppm) using the chemical inducer Bion 50 WG (Syngenta-Hungary) for 2 hours. NeemAzal T/S (Trifolio-M GmbH, Germany) was used as a botanical pesticide at concentrations of 0.01, 0.1, and 0.2% azadirachtin, with a similar treatment period to BTH. Mefenoxam served as positive control by coating the ungerminated seeds with Apron XL 350 FS (350 g/l mefenoxam, Syngenta AG, Switzerland) according to the EU-registered rate of 3 mg/kg seeds. Mefenoxam-treated seeds were coated homogeneously and kept at 24°C for three days for drying.

For *Trichoderma* spp. treatment, seven-day-old *T. asperellum* TGAM-G16 isolate cultured on PDA was flooded with 10–15 ml of bidistilled water, and conidia were removed by shaking or using a sterile brush under aseptic conditions. The concentration of the conidial suspension was adjusted to $3x10^7$ or $3x10^8$ conidia/ml using a hemocytometer. Gum Arabic (5%) was added to the suspension to aid conidia adherence to the seeds. *Trichoderma* spp.-treated seeds were incubated at 25°C for 3 h before being placed in a growth chamber at 19°C for three days for germination. After germination, the seedlings were re-treated with *T. asperellum* (at one of the above concentrations) before inoculation with various *P. halstedii* isolates.

Inoculation of seedlings by *P. halstedii* was carried out by the whole seedling immersion (WSI) method (Cohen and Sackston, 1973; Sedlářová et al. 2016). Using a hemocytometer, the concentration of the inoculum was

adjusted to $5x10^4$ sporangia/ml. Inoculation was carried out at 16°C overnight.

Experimental setup

The experiment was conducted at the Department of Integrated Plant Protection (Plant Protection Institute, MATE, Gödöllő, Hungary), and the treatments are listed in Table 2.

Table 2: List of Treatments used in single treatment experiment against multiple isolates of *P. halstedii*

Treatment's	**Concentrations**			
	AZA %	BTH-ppm	MX g/kg	*T. asperellum*
BW	-	-	-	
Inoculated (I)	50.000 sporangia /ml			
Mefenoxam (MX)	-	-	3 mg/kg	
MX + I	-	-	3 mg/kg	
BTH	-	20 ppm	-	-
BTH + I	-	20 ppm	-	-
BTH	-	40 ppm	-	-
BTH + I	-	40 ppm	-	-
BTH	-	80 ppm	-	-
BTH + I	-	80 ppm	-	-
AZA	0.01%	-	-	-
AZA + I	0.01%	-	-	-
AZA	0.1%	-	-	-
AZA + I	0.1%	-	-	-
AZA	0.2%	-	-	-
AZA + I	0.2%	-	-	-
T. asperellum	-	-	-	T $3x10^7$
T. asperellum + I	-	-	-	T $3x10^7$
T. asperellum	-	-	-	T $3x10^8$
T. asperellum + I	-	-	-	T $3x10^8$

**(Abbreviations:* bidistilled water (BW), Inoculated (I), Mefenoxam (MX), Benzothiadiazole (BTH), Azadirachtin (AZA), *Trichoderma asperellum (T. asperellum)*)

Combination experiment against multiple isolates: Treatment with inducers and design of the *in-planta* experiments

The combination experiment was conducted at the Department of Integrated Plant Protection (Plant Protection Institute, MATE, Gödöllő, Hungary) following the method described by Cohen et al. (2019), with some modifications. To examine the efficacy of several mixes of mefenoxam, BTH, azadirachtin, and *T. asperellum* at variable doses against infections caused by different pathogen isolates of *P. halstedii* .

Surface-sterilised seeds were germinated in a phytotron for 2-3 days at 19°C. The 3-day-old sunflower seedlings were immersed in aqueous solutions of BTH (Bion 50 WG, Syngenta Hungary), Azadirachtin 1% (Neem Azal® T/S containing 1% azadirachtin A (10 g/L) Trifolio-M GmbH, Germany), for two hours according to different doses and combinations with different ratios, as shown in the list of treatments below.

For *Trichoderma* spp. treatment, seven-day-old *T. asperellum* TGAM-G16 isolate cultured on PDA was flooded with 10–15 ml of bidistilled water, and conidia were removed by shaking or using a sterile brush under aseptic conditions. The concentration of the conidial suspension was adjusted to $3x10^7$ or $3x10^8$ conidia/ml using a hemocytometer. Gum Arabic (5%) was added to the suspension to aid conidia adherence to the seeds. *Trichoderma* spp.-treated seeds were incubated at 25°C for 3 h before being placed in a growth chamber at 19°C for three days for germination. After germination, the seedlings were re-treated with *T. asperellum* and then BTH (at the below concentrations in the table 3) before inoculation with various *P. halstedii* isolates.

Inoculation of seedlings by *P. halstedii* was carried out by the whole seedling immersion (WSI) method (Cohen and Sackston, 1973; Sedlářová et al. 2016). Using a hemocytometer, the concentration of the inoculum was adjusted to $5x10^4$ sporangia/ml. Inoculation was carried out at 16°C overnight.

Experiment setup and assessment

The treated sunflower seeds were grown in 5 cm pots (7 plants per pot) with three repetitions for each treatment and placed in trays (15 pots per tray)

with perlite (d = 0 to 4 mm) at 22°C. Plants were evaluated for plant height, damping-off, sporulation, and chlorosis nine days after inoculation following a spray of bi-distilled water (BW) to stimulate sporulation and then covered with dark bags overnight at 19°C in the phytotron. Macroscopic data were evaluated at 0-4 for sporulation and chlorosis at 10 and 21 days after inoculation (dpi). A randomised block design was used, and the treatments are listed in Table 3.

Table 3: List of Treatments used in combination experiment against multiple isolates of *P. halstedii*

Treatment's	Concentrations			
	AZA %	BTH-ppm	MX g/kg	*T. asperellum*
BW	-	-	-	-
Inoculated (I)	50.000 sporangia/ml			
MX3mg/kg	-	-	3 mg/kg	
MX 3mg/kg+I	-	-	3 mg/kg	
BTH+AZA	0.1%	40 ppm	-	
BTH+AZA+I	0.1%	40 ppm	-	
BTH+MX	-	40 ppm	3 mg/kg	
BTH+MX+I	-	40 ppm	3mg/kg	
MX+AZA	0.1%	-	3mg/kg	
MX+AZA+I	0.1%	-	3 mg/kg	
BTH+MX+AZA	0.01%	20 ppm	3 mg/ kg	
BTH+MX+AZA+I	0.01%	20 ppm	3 mg/kg	
BTH + *T. asperellum*	-	40 ppm	-	T $3x10^7$
BTH+*T. asperellum*+I	-	40 ppm	-	T $3x10^7$

*(*Abbreviations:* bidistilled water (BW), Inoculated (I), Mefenoxam (MX), Benzothiadiazole (BTH), Azadirachtin (AZA), *Trichoderma asperellum (T. asperellum)*)

Statistical analysis

The data were subjected to analysis of variance (one-way and two-way ANOVA), with a p-value of 0.05 for mean separation. Statistical analyses were carried out using the R statistical software

2.2 Interaction of biotic and abiotic inducers benzothiadiazole and *T. asperellum* to activate plant resistance against sunflower Downey mildew caused by *P. halstedii*. pathotype 710

Plant and pathogen materials

The Hungarian sunflower variety cv. Iregi szürke csíkos was selected for this study because of its susceptibility to *P. halstedii* compared with other sunflower genotypes.

In this study, we used the most virulent pathogen (710). Sunflower leaves infected with this pathogen were stored in a deep freezer at -70°C and later soaked in 100 mL of double-distilled water to release the sporangia. The concentration of the sporangia used in this experiment was 50,000 sporangia /ml.

Evaluation of the efficacy of BTH and *T. asperellum* in controlling sunflower downy mildew pathotype 710: *In vivo* experiments

Experiments were conducted at the Plant Protection Institute of MATE in Gödöllő, Hungary. Surface-sterilised seeds (1% hypochlorite) were grown in a growth chamber using the blotter method at 19°C for 2-3 days. The sunflower seedlings were pre-soaked for 2 h in an aqueous solution of BTH 80 ppm (Bion 50 WG, Syngenta Hungary), *T. asperellum* 3×10^8 conidia/ml, and a combination of *T. asperellum* 3×10^7 conidia/ml + BTH 40 ppm. Sunflower seedlings were inoculated with 50,000 sporangia/ml using the whole seedling immersion procedure (WSI) and incubated overnight at 16°C. Treated sunflower seeds were then planted in 5 cm diameter pots (10 plants per pot) containing perlite (d = 0 to 4 mm) at a temperature of 22°C in the laboratory. The plants were kept in a growth chamber for 21 days and assessed 10 days after infection for plant height, damping off, sporulation, and chlorosis. Disease severity was measured twice, at 10- and 21-days post-inoculation (dpi), using a 0-4 scale. The experiment followed a randomised block design, with the following treatments:

- (0) non-treated, non-inoculated by *P. halstedii*
- (B) BTH 80 ppm-treated, non-inoculated by *P. halstedii*
- (T) *T. asperellum* $3x10^8$ conidia/ml non-inoculated by *P. halstedii*
- (BTH 40 ppm + T $3x10^7$ conidia/ml) non-inoculated
- (I) inoculated with *P. halstedii* and not treated.
- (BTH 80 ppm + I) BTH 80 ppm treatment and inoculation with *P. halstedii*.
- (T + I) *T. asperellum* 3×10^7 conidia/ml + inoculated with *P. halstedii*.
- (B + T + I) BTH 40 ppm + *T. asperellum.* 3×10^7 conidia/ml inoculated with *P. halstedii*.

Measurement of antioxidant activity

Enzyme extraction

Plant samples were collected at four different time points: 0-, 3-, 9-, and 15-days after inoculation (dpi), and all treated plants were included in the analysis. To extract the enzymes, hypocotyl tissue (0.5 g) was mixed with 3 ml of Tris-HCl buffer (50 mM, pH 7.8) containing 1 mM EDTA-Na2 and 7.5% (w/v) soluble polyvinylpyrrolidone, and the mixture was kept at 0–4°C. The suspension was centrifuged at 10,000 rpm for 20 min at 5°C, and the supernatant was collected and stored on ice until use in the enzyme assays. The enzyme activity was measured at a room-temperature Bio-Rad Smart Spec Plus spectrophotometer.

Guaiacol-dependent peroxidase activity measurement (POX)

Guaiacol-dependent peroxidase (POX) activity was measured using the method described by Rathmell and Sequeira (1974) with minor modifications. A 0.1 µL of hypocotyl extract was mixed with 1.950 mL of 50 mM sodium phosphate buffer (pH 6.5) containing 0.1 µL of 50 mM guaiacol to test peroxidase activity. The reaction was initiated by adding 0.1 µL of 32.5 mM H_2O_2, and the actual absorbance rate at 470 nm was measured using a spectrophotometer. At 25°C, the absorbance of 1 mg of enzyme increases by 1.0 per minute.

Polyphenol activity Measurement (PPO)

PPO activity was assessed based on the initial rate of quinone production. To perform the PPO test, 0.2 µl of plant extract was mixed with 1.6 ml of 50 mM sodium phosphate buffer (pH 7.8) and 0.2 µl of 0.2 M catechol. The method modified the approach described by Fehrmann and Dimond in 1967 and involved measuring the increase in absorbance at 400 nm. The enzyme activity was measured at the room-temperature using Bio-Rad Smart Spec Plus spectrophotometer.

Catalase activity measurement (CAT)

CAT activity was determined by measuring the decrease in absorbance at 240 nm and 25°C using a reaction mixture containing 2 ml of 50 mM sodium phosphate buffer (pH 7.0), 0.5 ml of 40 mM H_2O_2, and 0.5 ml of enzyme extract. CAT activity was expressed as U/g FW, where U = 0.1*DA240 nm per minute (Beauchamp & Fridovich, 1971).

Statistical analysis

After normalisation (mean subtraction and division by standard deviation), analysis of variance was performed on the data. Mean separation was calculated using Fisher's test with a significance threshold of 0.05. Statistical analysis was conducted using Minitab-18 software produced by Minitab, LLC, Pennsylvania.

2.3 Effect of azadirachtin (AZA) on the sunflower - *P. halstedii* interaction

Plant and pathogen materials

The Hungarian sunflower variety cv. Iregi szürke csíkos was selected for this study because of its susceptibility to *P. halstedii* compared with other sunflower genotypes.

In accordance with the preliminary experiments, the most virulent isolate, pathotype (710), was selected for this study. Infected sunflower leaves were stored at -70°C and soaked in 100 mL of double-distilled water to extract sporangia (Sudisha et al., 2010). The inoculum for the *in vivo* experiment was prepared by adjusting the sporangial concentration to 4×10^4 sporangi/ml using a haemocytometer following the method of Cohen and Sackston (1973).

Preparation of treatments

Seed coating with mefenoxam

To obtain mefenoxam as a positive control, Apron XL 350 FS (350 g/L mefenoxam, Syngenta AG, Switzerland) was used and applied to the seeds at a rate of 3 mg/kg per the registered EU guidelines. Seeds were homogeneously coated with the product and dried at room temperature.

BTH (Bion: 50 WG)

Pre-germinated seedlings were submerged in aqueous solutions containing the chemical inducer BTH (Bion 50WG, Syngenta, Hungary) at different concentrations for two hours. The BTH concentrations used were 20 ppm, 40 ppm, and 80 ppm.

Preparation of different concentrations of azadirachtin

The commercial product, azadirachtin® T/S, which contains 1% azadirachtin A (10 g/L), has similar efficacy to higher concentrations of up to 4% azadirachtin A. This product is derived from natural neem kernels and has been registered in the European Union by Trifolio-M GmbH, Germany. For this study, 3-day-old seedlings were pretreated with 0.01%, 0.1%, or 0.2% azadirachtin solution for 2 h. One, ten, and 20 mL) were dissolved in 100 mL of distilled water to obtain the desired concentrations.

Effect of botanical and chemical inducers and their combination in *in planta* experiments

Combination treatment against an aggressive *P. halstedii* pathotype 710 isolate was conducted based on Cohen et al. (2019) with some modifications. To assess the efficacy of different ratios and concentrations of mefenoxam, BTH, and azadirachtin against the pathogen strain 710. The experiment was performed in the Integrated Plant Protection Department, MATE (Gödöllő, Hungary).

The seeds were surface-sterilised (1% hypochlorite) and germinated in a phytotron at 19°C for 2-3 days. In the context of this study, the synergistic effects of mefenoxam (MX), azadirachtin 1% (AZA), and benzothiadiazole (BTH) were investigated. Apron XL 350 FS, a fungicidal formulation containing 350 g/L of mefenoxam, manufactured by Syngenta AG, Switzerland, was utilized for seed treatment. The application of mefenoxam to the seeds adhered to the prescribed guidelines set forth by the European Union, with an application rate of 3 mg/kg. To ensure uniform coating, the seeds were meticulously treated with mefenoxam and subsequently allowed to dry at ambient room temperature.

Following the seed treatment with mefenoxam, the seedlings were subjected to a soaking process in solutions containing specified concentrations of BTH (Bion 50 WG, Syngenta Hungary) and AZA (Azadirachtin 1% (Neem Azal® T/S containing 1% azadirachtin A (10 g/L) Trifolio-M GmbH, Germany)) for two hours. The specific details of the concentrations and treatment combinations are elucidated in the comprehensive treatment regimen listed below. Treated seedlings were inoculated with 50.000 sporangia /ml overnight at 16°C using the most aggressive pathotype 710 using the whole seedlings immersion method (WSI).

Experiment setup and assessment

The treated sunflower seedlings were grown in 5 cm pots (10 plants per pot) with five replicates for each treatment in trays (10 pots per tray) in perlite (d = 0 to 4 mm) at 22°C. The plants were kept in a phytotron for 21 days. Nine days post-inoculation, the plants were sprayed with bi-distilled water (BW) to

stimulate sporulation and covered with dark bags overnight at 19°C in a phytotron. Plant height, damping-off, sporulation, and chlorosis were evaluated at 10- and 21-day post-inoculation. Macroscopic observations were scored on a scale of 0-4 for sporulation and 0-5 for chlorosis. The experiment was conducted using a randomised block design and included the treatments listed in Table 4.

Table 4: List of Treatments used in combination experiment against *P. halstedii* pathotype 710

Treatment's	**Concentrations**		
	AZA %	**BTH - PPM**	**MX g/kg**
BW	-	-	-
Inoculated (I)		50.000 sporangia/ml	
BTH	-	80 ppm	-
BTH +I	-	80 ppm	-
AZA	0.2%	-	-
AZA +I	0.2%	-	-
MX3mg/kg	-	-	3 mg/kg
MX3mg/kg+I	-	-	3 mg/kg
BTH+AZA	0.1%	40 ppm	-
BTH+AZA+I	0.1%	40 ppm	-
BTH+MX	-	40 ppm	3 mg/kg
BTH+MX+I	-	40 ppm	3mg/kg
MX+AZA	0.1%	-	3mg/kg
MX+AZA+I	0.1%	-	3 mg/kg
BTH+MX+AZA	0.01%	20 ppm	3 mg/ kg
BTH+MX+AZA+I	0.01%	20 ppm	3 mg/kg

*(*Abbreviations:* bidistilled water (BW), Inoculated (I), Mefenoxam (MX), Benzothiadiazole (BTH), Azadirachtin (AZA))

Measurement of enzyme Activity

Enzyme extraction

Following pathogen inoculation, samples were collected from all treated plants at designated time intervals, including 0-, 3-, 9-, and 15-days post-inoculation (dpi). To extract the enzymes, hypocotyl tissue (0.5 g) was

homogenised in 3 ml of Tris–HCl buffer (50 mM, pH 7.8) containing 1 mM $EDTANa_2$ and 7.5% (w/v) soluble polyvinylpyrrolidone at 0–4°C. Subsequently, the suspension was centrifuged at 10,000 rpm for 20 min at 5°C, and the resulting supernatant was stored on ice and used for enzyme assays.

PPO, POX and CAT activity measurements

Following the enzyme extraction process, the supernatant was stored at low temperature and used for enzyme assays. The enzymatic activity was quantified by spectrophotometry. The enzyme activity was measured at room temperature using a Bio-Rad Smart Spec Plus spectrophotometer (Budapest, Hungary). Guaiacol-dependent POX activity was determined using the modified approach reported by Rathmell and Sequeira (1974). PPO activity was assessed using a modified version of the method described by Fehrmann and Dimond (1967). The reaction mixture for catalase (CAT) activity measurement contained 2 ml of sodium phosphate buffer (50 mM, pH 7.0), 0.5 ml of H_2O_2 (40 mM), and 0.5 ml of enzyme extract. The decrease in absorbance at 240 nm and 25°C indicated H_2O_2 breakdown and CAT activity was calculated as U/g FW, where U = 0.1* DA240 nm per minute (Beauchamp & Fridovich, 1971).

Statistical analysis

The data were submitted for one-way ANOVA and factorial analysis with a confidence interval of 0.05 utilised for mean separation. Statistical analyses were performed using Minitab-18 statistical software (Minitab, LLC, Pennsylvania). All experiments were repeated twice.

Chapter 3: RESULTS

3.1 Efficacy of some plant resistance inducers against several sunflower downy mildew (*Plasmopara halstedii* (Farl.) Berl. et de Toni) isolates

***In vivo* experiment:**

The average disease rate was 1(percentage) (sporulation and damping off) for several *P. halstedii* isolates. This study evaluated the disease rates of several *P. halstedii* isolates on mefenoxam-treated and untreated sunflowers. Mefenoxam was ineffective against some isolates, whereas others showed reduced infection rates. BTH and azadirachtin provided varying levels of protection against different isolates, with the highest dose being the most effective. The biotic inducer, *T. asperellum*, offered increased protection against all isolates.

In the case of the average disease rate 2 (percentage) (chlorosis and damping off) of several *P.halstedii* isolates, The study analysed the disease rates of several *P. halstedii* isolates on mefenoxam-treated and untreated sunflowers, with high disease rates observed in mefenoxam-treated plants. Some isolates were mefenoxam-sensitive. BTH and azadirachtin provided varying levels of protection against different isolates, with the highest dose being the most effective. The biotic inducer *T. asperellum* offered increased protection against all isolates, and moderate protection against isolate I2.

In this study, we assessed the plant height of *P. halstedii*-infected sunflowers under different treatments. No significant difference in size was observed between the non-inoculated, mefenoxam-treated and non-treated plants. Mefenoxam-treated and infected sunflowers developed similarly to the uninfected sunflowers. Certain isolates showed significantly shorter heights than non-treated and infected plants among treated and inoculated plants. The lowest dose of BTH (20 ppm) resulted in shorter or equivalent heights for certain isolates, whereas higher BTH doses (40 and 80 ppm) effectively reduced stunting signs. The smallest dose of azadirachtin (0.01%) did not affect plant stunting, whereas higher doses (0.1% and 0.2%) improved the plant height for specific isolates. Both concentrations of *T. asperellum* reduced the stunting signs, with higher concentrations being more effective.

Combination experiment against multiple isolates: Treatment with inducers and design of the *in-planta* experiments

The study found no significant differences in height between non-inoculated, mefenoxam-treated, and non-treated plants. However, sunflowers inoculated with *P. halstedii* isolates were significantly shorter than non-treated and infected plants. Combinations of different treatments, such as MX+AZA and BTH+MX, resulted in improved plant height for certain isolates. The BTH+AZA+MX mixture reduced stunting symptoms, except in plants infected with isolate I3. Additionally, azadirachtin showed synergistic effects with mefenoxam, Bion, and both, thereby providing greater disease control. Different pathogen isolates responded differently to *T. asperellum* and BTH, with the combination of reduced rates being as effective as the full rate of *T. asperellum* alone.

3.2 Interaction of biotic and abiotic inducers benzothiadiazole and *T. asperellum* to activate plant resistance against sunflower Downey mildew caused by (*P. halstedii*) pathotype 710

Evaluation of the efficacy of BTH and *T. asperellum* in controlling sunflower downy mildew pathotype 710: In vivo experiments

Macroscopic evaluation of in vivo assay

Two experiments were conducted to assess the effects of BTH 80 PPM and the bioagents *T. asperellum* on sunflower downy mildew symptoms. Untreated and infected sensitive (Iregi) plants exhibited signs of downy mildew; however, *Trichoderma* spp. treatment reduced sporulation and chlorosis in susceptible seedlings and improved plant development. BTH at doses of 80 ppm or higher showed similar inhibition of disease symptoms. Inoculated sunflowers treated with biotic or chemical inducers showed significantly lower sporulation signs than the untreated seedlings.

The combination of *T. asperellum* and BTH exhibited the highest efficacy in reducing symptoms, followed by *T. asperellum* and BTH alone. Overall, the treatments significantly reduced the severity of downy mildew infection compared to the controls, with biological inducer being particularly effective. The activator treatment also reduced stunting in susceptible plants. *T. asperellum* and the combination of *T. asperellum* and BTH 40 PPM showed the highest reduction in stunting symptoms. BTH 80 PPM resulted in shorter

plant height compared to *that of T. asperellum*-treated plants. However, the combination of *T. asperellum* and BTH did not differ significantly from other treatments in terms of dwarfing symptoms. The experiments demonstrated that *T. asperellum* acts as a biocontrol agent and improves plant growth and resistance to infection.

Measurement of antioxidant activity

This study examined the formation of oxidative enzymes in sunflowers and found that the lowest enzyme values were observed in the non-inoculated non-treated control group. The chemical inducer BTH and biological inducer *T. asperellum*, individually or in combination, increased enzyme activity. The combination of *T. asperellum* with BTH 40 PPM resulted in the highest induction of all enzymes compared with the other treatments. When BTH and *T. asperellum* were applied alone, BTH was more effective in increasing oxidative enzyme activity; however, the combination showed the greatest efficacy.

BTH was particularly effective in enhancing peroxidase (POX) activity, whereas combining BTH and *T. asperellum* was most effective for polyphenol oxidase (PPO). One-way ANOVA revealed a significant difference in the decrease in H_2O_2 between the different days and treatments. Catalase (CAT) activity increased from 0 to 15 d. p. i., with BTH being more effective than *T. asperellum* alone. The combination of *T. asperellum* and BTH showed enzyme activities equivalent to those of the full dose of BTH throughout the growing season. In conclusion, the combination of BTH and *T. asperellum* can induce systemic resistance to downy mildew in sunflowers with an impact comparable to that of BTH alone. The increase in POX, PPO, and CAT enzyme activity was associated with resistance reactions, but not necessarily correlated with the level of resistance, but rather with the severity of necrotic symptoms in resistant tissues.

3.3 Effect of azadirachtin (AZA) on the sunflower - *P. halstedii* interaction.

Effect of botanical and chemical inducers and their combination in *in planta* experiments

Macroscopic evaluation of in vivo assay

This study focused on reducing sunflower dwarfism caused by *P. halstedii* using multiple inducers and their combinations. The treated plants showed significantly reduced stunting symptoms compared with the untreated inoculated plants. Non-inoculated plants treated with various combinations also exhibited enhanced growth and development, without signs of phytotoxicity. Additionally, the treatments significantly decreased sporulation and chlorosis symptoms induced by *P. halstedii*, demonstrating their efficacy compared to the chemical control. Mefenoxam treatment did not show significant differences compared to the untreated inoculated plants. Different combinations of AZA, BTH, and mefenoxam were applied to the seedlings, and the resulting plants were infected with the *P. halstedii* isolates. All combinations effectively reduced disease symptoms without causing phytotoxicity. Overall, this study highlights the potential of these inducers and their combinations in mitigating sunflower dwarfism and reducing disease severity.

Measurement of antioxidant activity

The study examined the enzyme activity of sunflower seedlings treated with an inducer was compared to that of untreated controls. The treated seedlings exhibited significantly higher levels of peroxidase (POX), polyphenol oxidase (PPO), and catalase (CAT) enzyme activities than the untreated uninoculated controls. In infected seedlings, the enzyme activity increased notably from 0 to 15 days post-inoculation (d.p.i.). The maximum enzyme activity was observed on day 15 post-inoculation. Treated and inoculated seedlings displayed the highest enzyme activity, followed by treated seedlings without inoculation, and control seedlings with inoculation. azadirachtin and its combinations induced systemic resistance against downy mildew in sunflowers, similar to the effect of BTH. The activation of these defense-related enzymes was not directly correlated with the level of resistance, but rather with the severity of necrotic symptoms in resistant tissues.

The peroxidase activity was significantly higher on day 15 in the treated and inoculated seedlings than in the control seedlings. Treated and infected seedlings and untreated seedlings exhibited enhanced enzyme activity from day 0 to day 15 post-infection compared to untreated, non-inoculated plants. The combinations of inducers showed similar or non-significantly different peroxidase enzyme activities compared with the single treatments.

On day 15, the seedlings treated with inducers and then inoculated showed the highest levels of polyphenol oxidase (PPO) activity, whereas untreated seedlings had the lowest activity. Treated and inoculated seedlings exhibited the highest PPO enzyme activity. The combinations of inducers (BTH+MX+AZA+I, BTH+AZA+I, BTH+I, and AZA+I) in treated and inoculated seedlings displayed higher PPO activity than the susceptible inoculated and uninoculated untreated controls. There was no significant difference in PPO activity or duration between the single full treatment and combinations with half doses of different inducers.

On day 15, the seedlings treated with inducers and then inoculated had the highest levels of catalase (CAT) activity, while treated seedlings without inoculation showed the lowest activity. Treated and inoculated seedlings exhibited the highest catalase enzyme activity. The combinations of inducers (BTH+MX+AZA+I, BTH+AZA+I, BTH+I, and AZA+I) in treated and inoculated seedlings displayed higher CAT activity than the susceptible inoculated and uninoculated untreated controls. There was no noticeable difference in CAT activity or duration between the single full treatment and combinations with half doses of different inducers.

Chapter 4: DISCUSSION

Fungicides have been widely used to control plant diseases, but they negatively affect human health and the environment. Therefore, the disease management trend is shifting towards the use of biocides and resistance inducers. Metalaxyl is a systemic fungicide extensively used to protect delicate cultivars against downy mildew caused by species which grouped to Peronosporales order and for commercial purposes, such as seed production (Singh and Shetty, 1990). This fungicide has multiple known mechanisms of action, including inhibition of sporangial germination and inhibition of asexual sporulation in infected plants sprayed with 250 ppm of metalaxyl. Metalaxyl and metalaxyl-M have been investigated for their efficacy in controlling downy mildew on sunflowers; however, their potency against *P. halstedii* has been found to decrease over time (Albourie et al. 1998; Gulya, 2000; Körösi et al. 2020). This study found that mefenoxam used as a seed coating showed a moderate protection rate when applied to different isolates of *P. halstedii*, but some isolates were resistant to mefenoxam when applied at a rate of 3 mg/kg.

Plant resistance can be developed using various factors such as microorganisms (bacteria, fungi, viruses, and mycorrhiza), chemicals (ASM, BABA), plant extracts, and cell wall fragments. These factors can contribute to the activation of the plant defense system in response to pathogen invasion either locally or systemically (Walters & Fountaine, 2009; Walters et al. 2013). Several studies have shown that certain neem components inhibit numerous fungal diseases (Schmutterer, 1988). Aqueous leaf extracts of *Azadirachta indica* and *Reynoutria sachalinensis* have also been found to effectively induce resistance against cucumber powdery mildew and leaf stripe disease in barley (Daayf et al. 1995; Paul & Sharma, 2002).

This study found that the novel biological *T. asperellum* is highly efficient against numerous pathotypes of *P. halstedii*, and is beneficial for both the prevention and enhancement of plant growth. A similar study showed that treatment with *Trichoderma harzianum* (TRIC8) significantly increased hypocotyl length, outperforming both the untreated control and fungicide (ai: Metalaxyl-m) treatments (Özer et al. 2021). *T. harzianum*, isolated from the plant rhizosphere, has also been shown to induce resistance to *P. halstedii* and stimulate plant growth (Nagaraju et al. 2012).

Furthermore, in agreement with the findings of Sharma et al. (2010) and Abd El-Rahman and Mohamed (2014), the application of biotic inducers (*T. harzianum*) and abiotic inducers (BTH), either individually or in combination, led to a reduction in the severity of chocolate spot disease compared to untreated infected controls. This study also assessed the effectiveness of *T. asperellum* in enhancing resistance to downy mildew in sunflower. In conclusion, various factors, such as microorganisms, chemicals, and plant extracts, can contribute to the activation of plant defense systems and help develop plant resistance. *T. asperellum*, BTH, and azadirachtin were found to be highly efficient in preventing sunflower downy mildew infection and enhancing plant growth. The use of fungicides, such as mefenoxam, as a seed coating has also been found to be less effective in managing sunflower downy mildew.

Use of combinations of different chemical ingredients is a common strategy for controlling plant diseases. This approach can provide several advantages over the use of a single chemical, such as increased efficacy, reduced risk of resistance development, and lower environmental impact. However, it is important to note that chemical combinations should be used with caution, as some combinations can lead to unwanted interactions that may affect their efficacy or cause harm to non-target organisms. Therefore, it is crucial to carefully evaluate the compatibility and effectiveness of the different chemical combinations before their use in the field.

For example, the findings of this study were consistent with those of previous research conducted by Cohen et al. (2019), which indicated that the use of Plenaris (oxathiapiprolin, Syngenta) on sunflower seedlings can decrease *P. halstedii-induced* downy mildew. Plenaries were found to have a synergistic effect when used in conjunction with Bion (BTH, acibenzolar-S-methyl) and apron (mefenoxam). Similarly, Jhala et al. (2017) investigated the efficacy of six fungicides and the neem component azadirachtin against *Alternaria porri* in onions. This study found that difenoconazole completely inhibited mycelial growth at various concentrations, followed by tebuconazole. However, the combination of difenoconazole and azadirachtin as a foliar spray was most effective in controlling the disease.

According to a recent study, the plant defence activator benzothiadiazole (BTH) suppresses the formation of downy mildew on sunflowers. Following BTH treatment, the concentration of three gene

transcripts in the sensitive sunflower genotype increased: glutathione S-transferase (GST), defensin (PDF), and catalase (CAT) (Körösi & Virányi, 2008). Basavaraj et al. (2019) reported that seed priming with *P. fluorescens*, *T. virens*, and neem leaf extract can enhance the growth of pearl millet plants and offer protection against blast disease. The researchers observed an increase in PAL, POX, LOX, and β-1,3-glucanase activities, which supported the findings of studies on disease prevention. The aqueous extract of neem leaves effectively controlled the leaf stripe pathogen (*Drechslera graminea*) on barley as well as the fungicide Bavistin (carbendazim, BASF India Limited). Treated leaves display increased activity of phenylalanine ammonia lyase (PAL) and tyrosine ammonia lyase (TAL), accompanied by rapid accumulation of fungitoxic phenolic compounds (Paul and Sharma, 2002). Thus, it is important to establish a valid system for the cultivation and distribution of seed samples for the pathotyping of new isolates. Additionally, new sources of resistance to downy mildew, including non-race-specific or durable resistance, must be identified. Attention should also be given to alternative types of resistance, such as the use of abiotic and biotic resistance inducers. Chemical control will continue to be necessary; however, the efficacy of fungicides should be improved by discovering new molecules and/or mixtures of various compounds to combat fungicide tolerance (Virany & Spring, 2011).

Ban et al. (2023) suggested implementing integrated management practices to minimize the risk of developing resistance to fungicides and to ensure effective and sustainable management of sunflower downy mildew. However, alternative control methods may only provide partial effectiveness if not integrated into Integrated Pest Management (IPM). For these methods to be fully effective, it is necessary to provide broader advisory and training services to farmers in order to understand new research findings and their practical implementation. Dar et al. (2020) pointed out that the use of fungicides to control the disease may have drawbacks, such as accumulating toxic compounds in the crop and ecosystem, being expensive, time-consuming, and partially effective. Therefore, various biocontrol agents have been tested to control this disease. Overall, using a combination of different chemical ingredients can be an effective strategy for controlling plant diseases, but careful consideration and evaluation of their compatibility and effectiveness are required.

Chapter 5: CONCLUSIONS AND RECOMMENDATIONS

- Frequent evaluation of commercial active ingredients is necessary to combat fungicide resistance and pathotype development in *P. halstedii*. Natural materials such as plant extracts and biocontrol agents are promising elements in IPM for sunflower downy mildew.
- *In vitro* studies have confirmed that azadirachtin (AZA) effectively inhibits sporangial germination, and its combination with BTH and mefenoxam (MX) demonstrates significant efficiency. The response to chemical inducers and number of empty sporangia varied among the isolates.
- *T. asperellum* is highly effective against various pathotypes of *P. halstedii* causing sunflower downy mildew. It suppresses zoospore release and induces antioxidant enzyme activity, making it suitable for seed treatment. The combination of *T. asperellum* and BTH functioned synergistically.
- Mixing azadirachtin with BTH and/or mefenoxam in different doses significantly reduces disease severity in sunflower seeds infected with *P. halstedii*, with no observed phytotoxicity.
- We observed that *T. asperellum* exhibited compatibility with BTH, as demonstrated under *in vitro* conditions. Furthermore, our results indicate that BTH did not have any antimicrobial effect against *T. asperellum*. This suggests that the combination of *T. asperellum* and BTH can be effectively utilised without negatively impacting the growth of *T. asperellum*.
- Reduced rates of azadirachtin or BTH alone are less effective, but their combination is as effective as the full rate of mefenoxam or BTH, indicating synergy.
- Different isolates of the pathogen responded differently to treatments with *T. asperellum*, BTH, and their combination. *T. asperellum* and BTH had synergistic effects when used together.
- *T. asperellum* is effective in inducing resistance against downy mildew in sunflowers by seedlings treatment. It improves seed quality; promotes plant growth; and activates catalase, peroxidase, and polyphenol oxidase as resistance measures.
- BTH and *T. asperellum* are both effective in reducing disease severity and inducing antioxidant activity in sunflowers infected with downy mildew. Their combination creates a more significant effect owing to their distinct mechanisms of action.

- Combination of AZA and BTH establish systemic resistance against downy mildew in sunflower, akin to the impact observed with BTH alone. The activities of POX, PPO, and CAT enzymes increase during resistance reactions, but do not correspond to the level of resistance, but rather to the severity of necrotic symptoms in resistant tissues.

Chapter 6: NEW SCIENTIFIC RESULTS

1. All concentrations of biopesticides AZA (0.01, 0.1% and 0.2%) as well as both concentrations of biological agent activators *T. asperellum* ($3x10^7$ and $3x10^8$ conidia/ml) demonstrated effective inhibition of empty sporangial *in vitro.*
2. Both concentrations of chemical inducer BTH (40 and 80ppm) were significantly more effective than botanical inducer and fungicide in reducing disease symptoms (disease 1, disease 2, and plant stunting) against multiple isolates of *P.halstedii* isolates I1,I3,I4 and I6.
3. *T. asperellum* exhibits high efficacy against P. *halstedii* pathotypes I1, I3, I4, I5, I6 and I7 under *in vivo* conditions reducing the disease severity at low concentrations ($3x10^7$ and $3x10^8$ conidia/ml) under *in vivo* conditions.
4. Azadirachtin demonstrated high efficiency against multiple pathotypes of *P. halstedii*, I1, I2, I3, I4 and I6 reducing the disease severity at highest concentration AZA 0.2% under *in vivo* conditions.
5. The effectiveness of combining reduced concentrations of *T. asperellum* ($3x10^7$ conidia/ml) with BTH at 40 ppm proved to be more efficient than using the full dosage of the chemical fungicide mefenoxam when used alone against multiple isolates I1 to I7 of *P. halstedii*
6. Different *P. halstedii* isolates exhibit disparate responses to the treatments, particularly notable among the isolates I2, I4, I6, and I7, which display heightened aggressiveness and induce elevated infection rates.
7. We proofed that the combination of *T. asperellum* $3x10^7$ conidia/ml with the resistance inducer BTH 40 ppm decreased disease severity and elicited superior antioxidant activity, particularly in terms of CAT, POX, and PPO. Moreover, the efficacy of this combination treatment was comparable to that of plants treated solely with the chemical inducer BTH 80 ppm.
8. We proofed that azadirachtin has the potential to induce systemic resistance (SAR) against downy mildew in sunflowers. Alternatively, the combination of AZA 0.1% and BTH 40 ppm can confer systemic resistance akin to that observed with BTH 80ppm alone. However, the enzymatic activities of POX, PPO, and CAT increase during resistance reactions, though they do not correlate with the level of resistance but rather with the severity of necrotic symptoms in resistant tissues. The ability of both chemical, botanical inducers and fungicide to substantially reduce disease severity and induce antioxidant activity, with evidence of synergistic effects when combined.

Publications associated with this research

Articles related to the topic

Published, peer-reviewed articles

Yousif, A. I. A., Almuslimawi, A., Turóczi, G., Kiss, J., Kovács, A., & Körösi, K. (2023). Efficacy of some plant resistance inducers against several sunflower downy mildew (*Plasmopara halstedii* (Farl.) Berl. et de Toni) isolates. *Acta Biologica Szegediensis*, *67*(1), 75-86.

Doshi, P., Nisha, N., **Yousif, A. I. A**., Körösi, K., Bán, R., & Turóczi, G. (2020). Preliminary investigation of the effect of neem-derived pesticides on *Plasmopara halstedii* pathotype 704 in sunflower under in vitro and in vivo conditions. Plants, 9(4), 535.

Nisha, N., Körösi, K., Perczel, M., **Yousif, A. I. A**., & Bán, R. (2021). The first report of the occurrence of an aggressive pathotype, 734, of *Plasmopara halstedii* causing sunflower downy mildew in Hungary. Plant Disease, 105(3), 711-711.

Bán, R., Kovács, A., Nisha, N., Pálinkás, Z., Zalai, M., **Yousif, A. I. A**., & Körösi, K. (2021). New and highly virulent pathotypes of sunflower downy mildew (*Plasmopara halstedii*) in seven countries in Europe. Journal of Fungi, 7(7), 549.

Körösi, K., Kovács, A., Nisha, N., Bóta, I., Perczel, M., **Yousif, A. I. A**., ... & Bán, R. (2020). New data on the pathotype distribution and mefenoxam tolerance of *Plasmopara halstedii* in Hungary. Plant Protection Science, 57(1), 31-37.

Articles published during the PhD study

Wiratno, M. S., Ankardiansyah, P. P., & **Yousif, A. I. A**. (2018). Biological control of root-knot nematodes (*Meloidogyne* spp.) in pepper plants utilising endophytic bacteria *Pseudomonas* sp. and *Micrococcus* sp. J. Pepper Ind, 9, 11-22.

Munif, A., Butarbutar, E., Pradana, A. P., **Yousif, A. I. A**. (2022). Plant-parasitic nematodes associated with common horticultural weeds. Pakistan Journal of Phytopathology, 34(1), 01-11.

Pradana, A. P., Adiwena, M., **Yousif, A. I. A**. (2022). Formula Bakteri Endofit Untuk Meningkatkan Pertumbuhan Bibit Jagung Pada Tanah Masam Podsolik Merah-Kuning. Jurnal Ilmiah Inovasi, 22(1), 30-41.

Pradana, A. P., Hoesain, M., Asyiah, I. N., Adiwena, M., Budiman, A., & **Yousif, A. I. A.** (2022). Biocontrol of root-knot nematode *Meloidogyne incognita* in arabica coffee seedling by using fortified bacterial consortium. Coffee Science-ISSN 1984-3909, 17, e172063-e172063.

Adiwena, M., Murtilaksono, A., Ngau, M., Rachmadaniar, R., Egra, S., Pradana, A. P., & **Yousif, A. I. A.** (2024). The Application of Botanical Pesticides to Control Fusarium Wilt on Asparagus Beans. Journal of Applied Agricultural Science and Technology, 8(1), 1-11.

Conferences, Posters and Presentations during the PhD study:

Yousif, A. I. A, Pratik Doshi, György Turóczi, Katalin Körösi, Nisha Nisha, Rita Bán: Preliminary study on the effect of different plant resistance inducers against sunflower downy mildew (*Plasmopara halstedii*). International Sunflower Conference, Novi Sad, Serbia, 21-24 June 2021. Poster, an abstract and a short presentation by the first author.

Kevein Ruas Oliveira, Katalin körösi, Pratik doshi, Nisha nisha**, Yousif, A. I. A**., György turóczi Priscila lupino gratão & Rita bán. Changes in the antioxidant enzyme activity level of sunflower (Helianthus annuus l.) Inoculated by *Plasmopara halstedii* (sunflower downy mildew) and treated with neem. International Sunflower Conference, Novi Sad, Serbia, 21-24 June 2021. Poster Sessions.

Rita Bán, Attila Kovács, Nisha Mihaly, Katalin Körösi, Zoltán Pálinkás, Mihály Zalai, **Yousif, A. I. A**, Mihály Perczel, József Kiss: Occurrence of *Plasmopara halstedii* (sunflower downy mildew) pathotypes in Hungary. International Sunflower Conference, Novi Sad, Serbia, 21-24 June 2021. Poster and a short presentation by the first author.

Nisha February, Attila Kovács, Katalin Körösi, Rita Bán**, Yousif, A. I. A**, Arbnora Berisha, Mihály Perczel. Fungicide tolerance of *Plasmopara halstedii* (sunflower downy mildew) to mefenoxam in Hungary. International Sunflower Conference, Novi Sad, Serbia, 21-24 June 2021. Poster Sessions.

Conferences abstracts:

Bán R., Körösi K., Nisha N., **Yousif A.I.A,** Preczel M.: Hazai napraforgó-peronoszpóra (Plasmopara halstedii) izolátumok változékonysága és fungicid érzékenysége [Pathotype distribution and fungicide sensitivity of *Plasmopara halstedii* isolates from Hungary]. Növényvédelmi Tudományos Napok, 2020. február 18-19., Budapest. Összefoglalók, p.45.

Nisha, N., Kovács, A., Körösi, K., Bán, R**., Yousif, A. I. A**., Perczel, M., Kiss, J.: Investigations on the fungicide sensitivity of *Plasmopara halstedii* (sunflower downy mildew) isolates to mefenoxam in Hungary. Növényvédelmi Tudományos Napok 2019. február 19-20., Budapest. Összefoglalók, p.99.

Chapter 7: REFERENCES

Abd El-Rahman, S. S., & Mohamed, H. I. (2014). Application of benzothiadiazole and Trichoderma harzianum to control *Faba bean* chocolate spot disease and their effect on some physiological and biochemical traits. Acta physiologiae plantarum, 36, 343-354.

Adeleke, B. S., & Babalola, O. O. (2020). Oilseed crop sunflower (Helianthus annuus) as a source of food: Nutritional and health benefits. Food Science & Nutrition, 8(9), 4666-4684.

Bán, R., Kiss, J., Pálinkás, Z., & Körösi, K. (2023). Placing Management of Sunflower Downy Mildew (Plasmopara halstedii (Farl.) Berl. et de Toni) under an Integrated Pest Management (IPM) System Approach: Challenges and New Perspectives. Agronomy, 13(4), 1029.

Basavaraj, G., Murali, M., Lavanya, S., & Amruthesh, K. (2019). Seed priming with biotic agents invokes defense response and enhances plant growth in pearl millet upon infection with Magnaporthe grisea. *Biocatalysis and Agricultural Biotechnology*, *21*, 101279.

Beauchamp, C., & Fridovich, I. (1971). Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Analytical biochemistry, 44(1), 276-287.

Cohen, Y., Rubin, A. E., & Galperin, M. (2019). Novel synergistic fungicidal mixtures of oxathiapiprolin protect sunflower seeds from downy mildew caused by *Plasmopara halstedii*. PLoS One, 14(9), e0222827.

Cohen, Y., Sackston, W., 1973. Factors affecting infection of sunflowers by Plasmopara halstedii. Can. J. Bot. 51, 15–22.

Dar, A. A., Sharma, S., Mahajan, R., Mushtaq, M., Salathia, A., Ahamad, S., & Sharma, J. P. (2020). Overview of purple blotch disease and understanding its management through chemical, biological and genetic approaches. Journal of Integrative Agriculture, 19(12), 3013-3024.

Doshi, P., Nisha, N., Yousif, A. I. A., Körösi, K., Bán, R., & Turóczi, G. (2020). Preliminary investigation of effect of neem-derived pesticides on Plasmopara halstedii pathotype 704 in sunflower under in vitro and in vivo conditions. *Plants*, *9*(4), 535.

Ehler, L.E., 2006. Integrated pest management (IPM): definition, historical development and implementation, and the other IPM. Pest Manag. Sci. 62, 787–789.

FAOSTAT, 2020. https://www.fao.org/faostat/es/#data/QCL. Last accessed October 19, 2021

Fehrmann, H., Dimond, A. E. (1967): Peroxidase activity and phytophthora resistance in different organs of the potato plant. Phytopathology 57: 69-72.

Gascuel, Q., Martinez, Y., Boniface, M. C., Vear, F., Pichon, M., & Godiard, L. (2015). The sunflower downy mildew pathogen P lasmopara halstedii. Molecular Plant Pathology, 16(2), 109-122.

Gulya, T., 2000. Metalaxyl resistance in sunflower downy mildew and control through genetics and alternative fungicides. Presented at the Proceedings of the 15th International Sunflower Conference, Toulouse, France.

Jhala, P., Mali, B. L., & Meena, M. K. (2017). Effective management of purple blotch of onion caused by Alternaria porri (Ellis) through host resistance, fungicides and botanicals. International Journal of Current Microbiology and Applied Sciences, 6(5), 1737-1745.

Kamle, M., Borah, R., Bora, H., Jaiswal, A. K., Singh, R. K., & Kumar, P. (2020). Systemic acquired resistance (SAR) and induced systemic resistance (ISR): Role and mechanism of action against phytopathogens. *Fungal Biotechnology and Bioengineering*, 457–470.

Körösi, K., & Virányi, F. (2008). Molecular changes in downy mildew-infected sunflower triggered by resistance inducers. In Proceedings of the 17th International Sunflower Conference (Vol. 1, pp. 157-161).

Körösi, K., Kovács, A., Nisha, N., Bóta, I., Perczel, M., Yousif, A. I. A., & Bán, R. (2020). New data on the pathotype distribution and mefenoxam tolerance of Plasmopara halstedii in Hungary. *Plant Protection Science*, *57*(1), 31-37.

Molinero-Ruiz, L. (2022). Sustainable and efficient control of sunflower downy mildew by means of genetic resistance: a review. *Theoretical and Applied Genetics*, 1-15.

Nagaraju, A., Sudisha, J., Murthy, S.M., Ito, S., 2012. Seed priming with Trichoderma harzianum isolates enhances plant growth and induces resistance against *Plasmopara halstedii*, an incitant of sunflower downy mildew disease. Australas. Plant Pathol. 41, 609–620.

Ngegba, P. M., Cui, G., Khalid, M. Z., & Zhong, G. (2022). Use of botanical pesticides in agriculture as an alternative to synthetic pesticides. *Agriculture*, *12*(5), 600.

Özer, N., Coşkuntuna, A., & Şabudak, T. (2021). Trichoderma harzianum-induced defense in sunflower (Helianthus annuus L.) against Plasmopara halstedii with changes in metabolite profiling of roots. Biocontrol Science and Technology, 31(12), 1403-1418.

Paul, P. K., & Sharma, P. D. (2002). Azadirachta indica leaf extract induces resistance in barley against leaf stripe disease. *Physiological and molecular plant pathology*, *61*(1), 3-13.

Rathmell, W. G., Siqueira, L. (1974): Soluble peroxidase in fluid from intercellular spaces of tobacco leaves. Plant Physiology, 53: 317-318.

Schmutterer, H., 1988. Potential of azadirachtin-containing pesticides for integrated pest control in developing and industrialized countries. J. Insect Physiol. 34, 713–719.

Sedlářová, M., PoSPíchalová, R., Trojanová, Z.D., Bartůšek, T., Slobodianová, L., Lebeda, A., 2016. First report of *Plasmopara halstedii* new pathotypes 705 and 715 on sunflower from the Czech Republic–short communication. Plant Prot. Sci. 52, 182–187

Sharma, K., Butz, A., Finckh, M., 2010. Effects of host and pathogen genotypes on the inducibility of resistance in tomato (*Solanum lycopersicum*) to *Phytophthora infestans*. Plant Pathol. 59, 1062–1071.

Sharma, K., Butz, A., Finckh, M., 2010. Effects of host and pathogen genotypes on the inducibility of resistance in tomato (*Solanum lycopersicum*) to *Phytophthora infestans*. Plant Pathol. 59, 1062–1071.

Shukla, V., Khurshid, M. D., & Kumar, B. (2020). A review on phytochemistry and pharmacological activity of Azadirachta indica (Neem). Int. J. Pharm. Biol. Sci, 10, 172-180.

Singh, S., Shetty, H., 1990. Efficacy of systemic fungicide metalaxyl for the control of downy mildew (Sclerospora graminicola) of pearl millet (Pennisetum glaucum). Indian J. Agric. Sci. 60, 575–581.

Spoel, S.H., Dong, X., 2012. How do plants achieve immunity? Defense without specialized immune cells. Nat. Rev. Immunol. 12, 89–100.

Sudisha, J., Niranjana, S. R., Sukanya, S. L., Girijamba, R., Lakshmi Devi, N., & Shekar Shetty, H. (2010). Relative efficacy of strobilurin formulations in the control of downy mildew of sunflower. *Journal of Pest Science*, *83*, 461-470.

Viranyi, F., & Spring, O. (2011). Advances in sunflower downy mildew research. European Journal of Plant Pathology, 129, 207-220.

Viranyi, F., Spring, O., 2011. Advances in sunflower downy mildew research. Eur. J. Plant Pathol. 129, 207–220.

Walters, D., Fountaine, J., 2009. Practical application of induced resistance to plant diseases: an appraisal of effectiveness under field conditions. J. Agric. Sci. 147, 523–535.

Walters, D.R., Ratsep, J., Havis, N.D., 2013. Controlling crop diseases using induced resistance: challenges for the future. J. Exp. Bot. 64, 1263–1280.