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EFFICACY OF BTH AND NEEMAZAL AGAINST AN AGGRESSIVE ISOLATE OF SUNFLOWER DOWNY MILDEW CAUSED BY *PLASMOPARA HALSTEDII*

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Abstract: Sunflower is an important oilseed crop, but its yield is highly affected by devastating diseases such as sunflower downy mildew caused by *Plasmopara halstedii*. The high variability of this pathogen compromises the effective management of sunflowers; therefore, IPM including alternative methods is a promising tool against downy mildew. The goal of our study was to assess the effectiveness of BTH (benzothiadiazole in Bion 50 WG) and NeemAzal as inducers of resistance against sunflower downy mildew. Sunflower seedlings were treated with BTH and NeemAzal before inoculation with varying concentrations of *Plasmopara halstedii* sporangia and incubated overnight at 16°C. Disease severity was assessed using a 0-4 scale, and plant height was measured twice. Histological analysis of sunflower hypocotyls was conducted to examine pathogen structures and host reactions. Both BTH and NeemAzal treatments reduced disease development, with BTH showing greater efficacy in inhibiting pathogen growth and reducing plant height compared to NeemAzal. Histological examination revealed decreased presence of pathogen structures and increased necrosis in treated plants. Further experiments with inducers are recommended. Preliminary results indicate that NeemAzal and BTH reduce the progression of disease symptoms. Plant inducers offer an eco-friendly approach to disease management, including the control of sunflower downy mildew.

Keywords: sunflower downy mildew, *Plasmopara halstedii*, inducers, BTH, NeemAzal, IPM

1. Introduction

Sunflower is considered the fourth most significant oil crop globally, after soybean, oil palm, and canola (Adeleke & Babalola 2020). In 2023, worldwide sunflower oil production reached 21.63 million tons (http1). Diseases have a significant impact on sunflower production. *Plasmopara halstedii* (Farl.) Berl. & de Toni, a biotrophic oomycete, is the causal agent of downy mildew, a significant disease that leads to economic losses in sunflower cultivation (*Helianthus annuus* L.). Severe infections have the potential to result in a complete loss of yield, making sunflower cultivation unfeasible within affected areas (Gascuel et al. 2015).

P. halstedii exhibits remarkable variability and adaptability, with an estimated 50 pathotypes documented worldwide to date (Spring et al. 2018, Spring 2019, Bán et al. 2023). Additionally, new races of *P. halstedii* are frequently observed in fields across various regions worldwide (Tourvieille de Labrouhe 2004, Spring 2019, Körösi et al. 2020, Bán et al. 2021). The wide range of diversity we observe is mainly due to the extensive cultivation of sunflower hybrids that possess genes resistant to *P. halstedii*. Variability is influenced by factors like as mutation, sexual recombination, and parasexual recombination (Spring & Zipper 2006, Ahmed et al. 2012). To develop efficient breeding techniques, it is necessary to evaluate the pathotype composition of *P. halstedii* populations (Bán et al. 2023).

However, the vulnerability of resistance breeding and seed coating arises with the appearance of new pathotypes and variants of the pathogen. Additionally, the distribution of tolerant races of *P. halstedii* to mefenoxam, previously the only effective active ingredient in seed coating, may extend globally (Körösi et al. 2020, Nisha et al. 2023). As a result, the protection of sunflowers against downy mildew presents numerous challenges, emphasizing the necessity for new management methods (Gulya et al. 1997, Bán et al. 2023). Integrated pest management has a crucial role in the management of sunflower downy mildew. The presence of uncertainty in disease

management has led to the development of novel strategies, such as enhancing the plant's defense system via the use of resistance inducers (Oostendorp et al. 2001).

Therefore, the objective of this study was to evaluate the efficacy of some inducers such as benzothiadiazole (BTH) and NeemAzal, a botanical pesticide containing azadirachtin, as a potential inducer of resistance against *Plasmopara halstedii*. BTH, also known as benzo (1,2,3) thiadiazole-7-carbothermic acid S-methyl ester or benzothiadiazole, is a chemical inducer that has been extensively studied for its ability to generate systemic acquired resistance (SAR). The efficacy of the late commercial product Bion 50 WG had been confirmed by its successful use before. *Azadirachta indica* A. Juss, commonly referred to as Neem is a botanical species renowned for its diverse protective properties against pests and diseases, encompassing antifeedant, antifungal, nematicidal, and insecticidal effects. The global recognition of the effectiveness of neem tree seed, leaf, and bark extracts in combating insect infestations and supporting agriculture is well-established. Doshi et al. (2020) have documented the protective efficacy of neem.

2. Materials and methods

2.1. Sunflower genotype and *Plasmopara halstedii* isolate

The experiment was carried out in the laboratory of the Department of Integrated Plant Protection at the Hungarian University of Agriculture and Life Sciences (MATE) located in Gödöllő, Hungary. A Hungarian open-pollinated cultivar (cv. Iregi Szürke Csíkos) was used in the experiment which is susceptible to all known pathotypes of *P. halstedii* due to the absence of dominant resistance genes against this pathogen but shows tolerance towards other significant diseases. Isolate 3 was used for inoculation, collected in Hungary in 2021, and stored at -70°C in the Department of Integrated Plant Protection (MATE).

2.2 Experimental set-up

The following treatments were used in this experiment:

- Control: non-treated with BTH or Neem and non-inoculated with *P. halstedii*
- BTH: treated with BTH and non-inoculated with *P.halstedii*
- Neem: treated with Neem and non-inoculated with *P.halstedii*
- IC: non-treated and inoculated with *P. halstedii*
- BTH + I: treated with BTH and inoculated with *P.hasltedii*
- Neem + I: treated with Neem and inoculated with *P.halstedii*

A total of 360 seeds of Iregi szürke csíkos were placed in a beaker and immersed in a solution of 1,5 % Na-hypochlorite (NaOCl) solution for 3 to 5 minutes. The seeds were then thoroughly rinsed with running tap water and germinated on moist filter paper at 20 °C until root initials 3 to 5 mm developed. Pre-germinated seedlings were soaked for two hours in 320 ppm BTH solution by using Bion 50WG (Syngenta). The same for Neem pre-germinated seedlings were soaked for two hours in NeemAzal solution (0,1%). The treated seedlings were inoculated with *P.halstedii* by immersing them into the sporangial suspension, which was adjusted first to 50000 sporangia/ml (first experiment) and then to 35000 sporangia/ml (second experiment), using a hemocytometer. Seedlings were then inoculated at 16°C overnight in darkness. The next day, inoculated seedlings were placed into pots containing pure perlite and kept under a 12-hour light/12-hour dark photoperiod with daily irrigation in the phytotron.

Three days after sowing nutrients were given to plants (Volldünger Linz, 0,2 g/l, 0,5 l/tray). The sporulation of the plants was necessary to promote the appearance of the pathogen, which was done 9 days after inoculation. The process was performed by placing the trays in dark-colored bags. Then, before sealing, the plants were sprayed with bi-distilled water, which resulted in high humidity favorable for the appearance of the pathogen on the leaf surface. Following, trays covered with bags were placed in a dark place and stored at 19 °C overnight. The next morning, the bags were removed from the trays from sporulating plants.

2.3 Disease assessment

Disease assessment was conducted by recording the occurrence and the intensity of sporulation, utilizing a scale ranging from 0 to 4. This scale assessed the extent of leaf area covered by sporangia as follows: 0 –no visible sporangiophores (white coating), 1: 25% > of the leaf surface is covered with sporangiophores, 2: 25 –50% of the leaf surface is covered with sporangiophores 3: 50 –75% of the leaf surface is covered with sporangiophores, 4: >75% of the leaf surface is covered with sporangiophores (Oros and Virányi, 1987). The second evaluation was done on 21-day-old plants. In this evaluation, usual symptoms such as leaf chlorosis and/or damping-off were seen. Downy mildew produces significant stunting in affected plants; hence the height of the plants was measured twice, first on 9-day and secondly on 21-day-old plants.

2.4 Examining plant defense reactions by microscopy

Histological examination of cross sections of sunflower hypocotyls was undertaken by light microscopy to check pathogen structures such as hypha and haustoria and host reactions such as necrosis of invaded cells. Plant tissues were fixed in FAA solution (formaldehyde: glacial acetic acid: ethyl alcohol: distilled H_2O 2:1:10:7). All samples of sunflower hypocotyl were divided into the upper and lower parts and then cut into thin slices with a razor blade for examination. Sections were examined unstained. The appearance of infection and necrosis in the cortical and pith parenchyma was evaluated on a scale of 0-4 depending on their occurrence in one, two, three, and four-quarters of the cross-sections (Bán et al. 2004).

2.5 Data Analysis

Data from duplicate experiments were analyzed using one-way ANOVA. Fisher's HSD post-hoc test ($p < 0.05$) was used to discern significant differences between treatments.

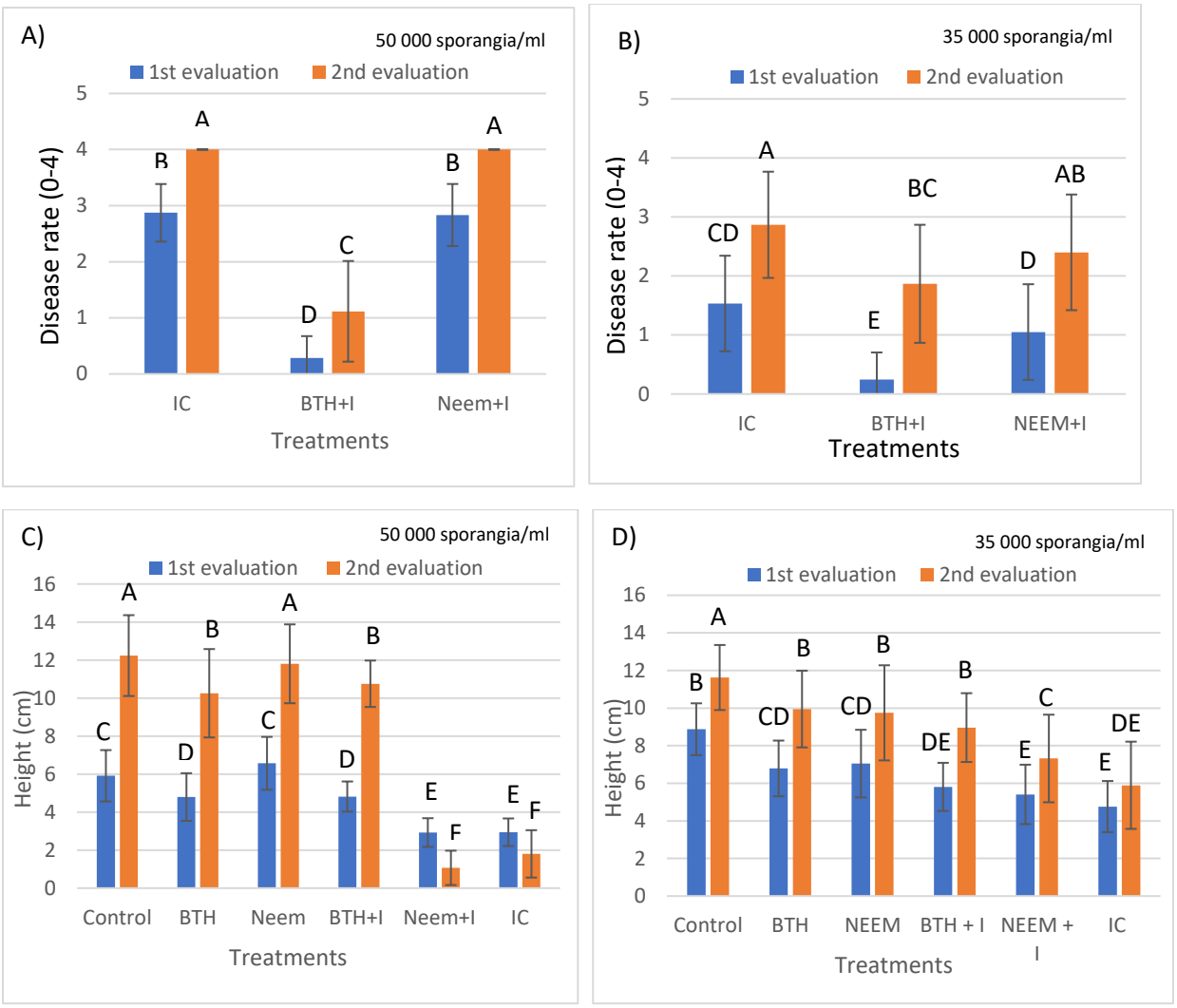
3. Results

3.1 Disease rates and heights

Figure 1A-B shows that disease rates of *P. halstedii* significantly increased by the second evaluation, regardless of treatment applying either concentration of the inoculum. However, BTH-treated and inoculated plants were significantly less diseased than Neem-treated inoculated sunflowers in both experiments (irrespective of inoculum concentration) during the examination period. In addition, there were no significant differences between the disease rates of non-treated and Neem-treated inoculated plants when using either inoculum concentration (Figure 1A-B). Treatment with inducers (BTH or Neem) significantly reduced plant heights of non-inoculated sunflowers comparing to non-treated ones except for Neem-treatment in the first experiment (with the concentration of 50000 sporangia/ml) (Figure 1C-D). Heights of BTH-treated and inoculated sunflowers were significantly greater than non-treated inoculated ones and compared to Neem-

treated inoculated plants irrespective of inoculum concentration. In contrast, there were no significant difference in the plant heights of Neem-treated and non-treated inoculated plants.

Figure 1.: Disease rates (A, B) and plant heights (C, D) of non-treated and BTH- or Neem-treated sunflowers inoculated with *Plasmopara halstedii* (inoculum concentration: A, C: 50,000, B, D: 35,000 sporangia/ml)



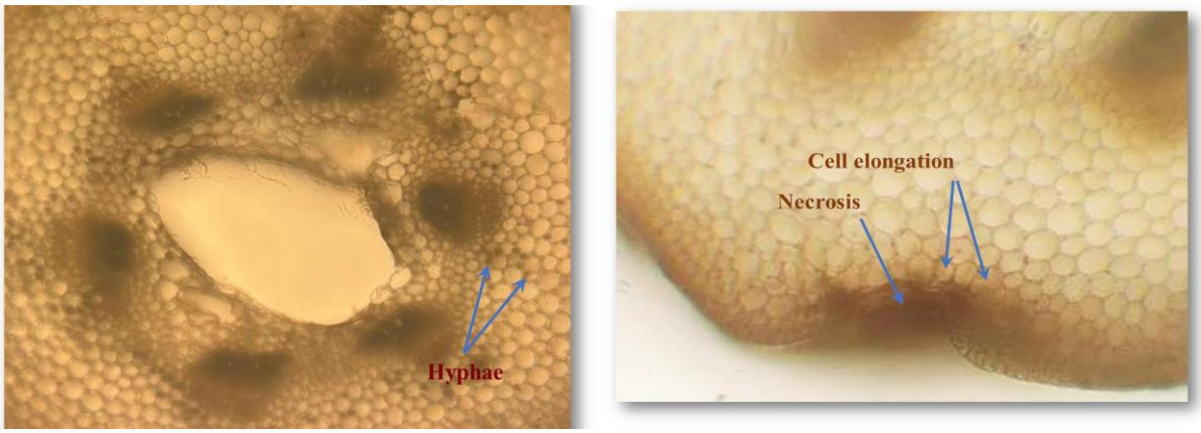
Source: Author's own editing/ A.Berisha (2024)

Legend: **Control**: Non-treated and non-inoculated with *P. halstedii*, **BTH**: BTH-treated and non-inoculated, **Neem**: Neem-treated and non-inoculated, **BTH+I**: BTH-treated and inoculated, **Neem+I**: Neem-treated and inoculated, **IC**: non-treated and inoculated control. Vertical lines represent 95% confidence intervals (95% CI) of the mean values of plant heights. Different letters (A, B, etc.) indicate significant differences based on the Fisher's HSD post-hoc test ($p < 0,05$) among variables.

3.2 Examination of host reactions

Figure 2. A) shows the presence of pathogen hyphae in the intercellular spaces of a non-treated inoculated sunflower hypocotyl. The hyphae could be seen as brown dots either in the cortical or the pith parenchyma under a light microscope. *Figure 2. B)* indicates A neem-treated inoculated sunflower hypocotyl section displaying the progression of cellular browning and necrosis (arrow), accompanied by cell elongation in the surrounding area (arrows).

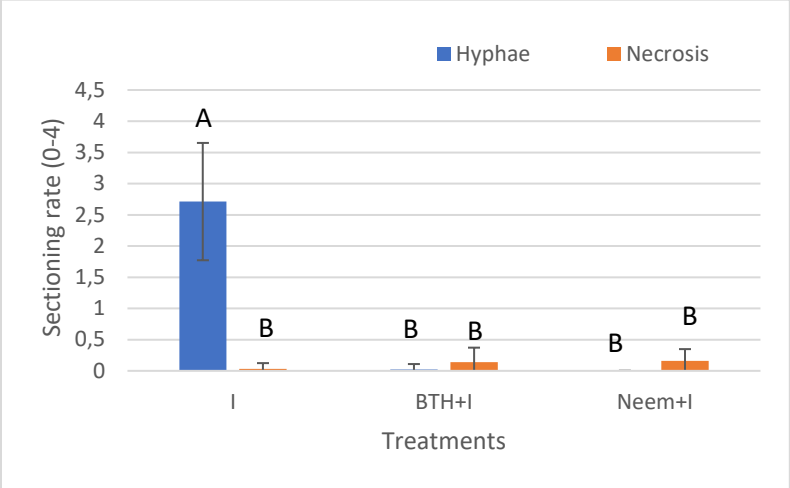
Figure 2. *A) Light micrograph of a cross-section of a non-treated and inoculated sunflower: hyphae and haustoria (arrows) of Plasmopara halstedii (sunflower downy mildew) in the cortical parenchyma. B) Light micrograph of a cross-section of a Neem treated and inoculated sunflower: host response such as cell necrosis and elongation of cells next necrotic ones to pathogen invasion in the cortical parenchyma.*



Source: Author's own editing/ A.Berisha (2024)

Figure 3. illustrates the extent of hyphae within host tissues and the resulting host reaction (necrosis) in both treated and non-treated sunflowers following pathogen invasion. Sunflowers treated with BTH and Neem exhibited significantly fewer hyphae compared to non-treated plants. While there was a higher presence of necrosis in treated plants within the cortical parenchyma compared to non-treated plants, this difference was not significant.

Figure 3. *Effect of BTH and Neem treatment on fungal elements (hyphae) and host response (necrosis) of sunflowers inoculated by sunflower downy mildew (Plasmopara halstedii)*



Legend: **I**: non-treated and inoculated, cort.: cortical parenchyma, pith: pith parenchyma, **BTH+I**: BTH-treated and inoculated, **Neem+I**: Neem-treated and inoculated, Vertical lines represent 95% confidence intervals (95% CI) of the mean values of infection rate. Different letters (A, B, etc.) indicate significant differences based on the Fisher's HSD post-hoc test ($p < 0,05$) among variables.

Source: Author's own editing/ A.Berisha (2024)

4. Discussion

The resistance-inducing potential of a botanical pesticide NeemAzal and an intensively studied chemical inducer, BTH (benzothiadiazole) were examined against an aggressive isolate of sunflower downy mildew in this study. In addition, pathogen structures and host tissue responses were detected by fluorescence microscope to understand better the mechanisms underlying induced resistance in the sunflower-*Plasmopara halstedii* host-parasite interaction.

As in previous studies (Bán et al. 2004, Körösi et al. 2011) BTH limited the development of downy mildew in the treated and inoculated plants. Moreover, it was also efficient in decreasing stunting of treated sunflowers. Previously, many crops have been demonstrated to activate resistance when exposed to BTH to a variety of diseases, including powdery mildew in barley and wheat, rust in pea (Barilli et al. 2010) as well as white rot disease in melon (Buzi et al. 2004), and soybean (Dann et al. 1998). Our findings on histological analysis of host responses also confirmed earlier results where the application of BTH decreased the abundance of hyphae and induced necrosis in the hypocotyl's cortical and pith parenchyma (Bán et al. 2004).

In our experiment, the effect of Neem inducer on reducing disease rate was very low. It was in contrast with the results reported by Doshi et al. (2020), who examined the efficacy of two substances derived from neem in inhibiting *P.halstedii* (an isolate identified as pathotype 704) *in vivo*. An explanation for the limited efficacy of Neem treatment in reducing downy mildew disease in our experiment remains uncertain; however, the high aggressivity of the isolate used may provide some insight.

After examining and summarizing the outcomes of the BTH and Neem experiment conducted against *P. halstedii*, we can conclude that conducting additional experiments with inducers,

especially with Neem would be beneficial. Our findings confirmed that BTH inducer effectively decreases the progression of disease symptoms, on the other hand, Neem didn't have a positive effect in our case. In spite of this lack of positive results with NeemAzal, plant inducers may represent a promising and environmentally friendly strategy for disease control against sunflower downy mildew (Bán et al. 2023).

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