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Investigations on the effect of NeemAzal, a botanical pesticide, against sunflower downy mildew (*Plasmopara halstedii* (Farl.) Berl. et de Toni)

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1. INTRODUCTION

Sunflower (*Helianthus annuus* L.) is the second most significant crop in hybrid breeding, following maize. The main reason for sunflower growth is oil extraction from its seeds, making it an essential oil crop for human food. With up to 9% of the global production of vegetable oils worldwide, sunflower takes position number five after palm oil, soybean, canola oil, and other oils (coconut oil, cottonseed oil, olive oil, palm kernel oil, and peanut oil). In addition to its utilization in human nutrition, sunflower oil possesses various industrial applications, such as serving as a fundamental constituent for polymer synthesis, biofuel, emulsifier, or lubricants.

The prevalence of sunflower diseases is a significant problem for the global cultivation of this crop. This phenomenon is attributable to the frequent and frequently severe assault of various pathogens. Significant yield losses or product quality declines may occur. Even though a large number of pathogens are capable of infecting sunflowers, only a small number have a hazardous impact to yield. The common diseases are downy mildew (*Plasmopara halstedii*) and broomrape (*Orobanche cumana*), followed by white rot (*Sclerotinia sclerotiorum*), Phomopsis stem canker (*Diaporthe helianthi/Phomopsis helianthi*), Alternaria blight (*Alternaria helianthi, A. helianthinficiens*), rust (*Puccinia helianthi*) and black stem (*Phoma macdonaldii*).

Downy mildew caused by *Plasmopara halstedii* is one of the most significant diseases influencing the production of sunflowers. The pathogen has been reported in the majority of sunflower-growing nations. Recent estimates place the global impact on yield at 3.5% of commercial seed production in the presence of current control methods, but yield loss can reach 100% in contaminated fields (Gasuel et al. 2015).

Plasmapora halstedii the pathogen responsible for sunflower downy mildew, exhibits considerable variability with multiple pathotypes or races with varying virulence (Virányi and Spring 2011). This diversity is primarily a result of the widespread cultivation of sunflower hybrids containing resistance genes against *P. halstedii*. These resistance genes induce genetic changes in the pathogen's genome. Factors like mutation, sexual recombination, and parasexual recombination contribute to this variability. The number of pathotypes has steadily increased worldwide, with approximately 50 known pathotypes identified (Bán et al. 2021). Some recent aggressive pathotypes have been discovered in various European regions, challenging cultivating sunflower hybrids with earlier resistance

genes. Due to the pathogen's high variability, the effectiveness of dominant resistance genes in sunflower hybrids diminishes over time. Consequently, assessing the pathotype composition of *P. halstedii* populations is crucial for effective breeding strategies (Bán et al. 2023).

The management of sunflower downy mildew is effectively combated by using integrated pest management (IPM). IPM is a comprehensive strategy emphasizing the interdependence and cooperation of diverse plant protection techniques across various agricultural systems rather than focusing on individual plants and isolated pest species (Barzman et al. 2015). IPM includes resistance breeding and seed coating to prevent sunflower downy mildew. However, resistance breeding and seed coating are vulnerable due to the appearance of new pathotypes and variants of the pathogen. Therefore, the importance of alternative crop protection solutions within IPM, such as botanical pesticides, is increasing. However, the host-parasite relationship must be thoroughly understood before applying a new solution.

This thesis aims to test a botanical pesticide (NeemAzal) as a potential resistance inducer for its effectiveness against sunflower downy mildew. Another goal of this work was to analyze host tissue reactions by light microscopy to understand the background of induced resistance better against *P. halstedii* in sunflowers.

2. LITERATURE REVIEW

2.1 History of sunflower

Sunflower (*Helianthus annuus* L.) is one of the four largest annual edible oil crops, along with soybean, rapeseed, and peanut. American Indians may have cultivated sunflowers before maize (*Zea mays* L.) between 4,500 and 3,000 years ago. When domesticated, American Indians utilized sunflower seeds for sustenance, ceremonial, and ornamental uses (Smith & Bruce, 2014). Spanish explorers introduced the sunflower to Europe 1500 AD, where it flourished in gardens (Putt, 1997). Spanish sunflowers spread fast across France and Italy before migrating north and east into Europe. Sunflower oil was recognized in Russia before 1,800 A.D. Late 19th-century Russian domesticated sunflowers returned to North America. North Dakota and Minnesota began growing sunflowers in the 1950s to produce sunflower oil. By the 1970s, hybrid sunflower farming spread to South Dakota, Kansas, Nebraska, Texas, and California.

Mexican pre-Columbian finds suggest a second domestication hub (Heiser 1998). Blackman et al. (2011) evaluated the sequence diversity of three domestication genes (c4973, HaFT1, and HaGA2ox) and neutral markers in 60 sunflower populations from the U.S., Mexico, and Canada. Their study identified genetic diversity patterns similar to all other domesticated species from an eastern North American domestication location in domesticated and wild Mexican sunflowers. Thus, U.S. and Mexican sunflowers are descended from eastern U.S. sunflowers, likely from Arkansas. According to the USDA, sunflower output reached 51.95 million metric tons in 2022-2023. Russia and Ukraine are also higher than Europe, Argentina, China, Turkey, the U.S., and Kazakhstan. Ukraine and Russia generate 50% of sunflower oil.

2.2 Significance of sunflower

The sunflower production was 57.32 million tons in 2022 (http://www.worldagriculturalproduction.com/crops/sunflower.aspx). Crops produce seeds that are typically 45–53% oil and 15–18 percent protein. Sunflower kernels provide tocopherols, minerals, and vitamins along with oil and protein. Standard sunflower oil is linoleic in nature; however, induced mutations have been exploited to create genotypes with a high-oleic acid content of the oil (> 85%), permitting the production of high-oleic sunflower hybrids. Additionally, genotypes with high concentrations of palmitic, stearic, and linoleic acids have been attained through induced mutations (Skoric 2009).

The two main categories are oilseed and confection, or non-oilseed varieties of sunflower hybrids. Sunflower species produce black seeds that are relatively tiny and have

a thin hull that clings to the achene firmly. Oil from oilseed sunflower is the final product, and while it is suitable for biofuels, it is utilized mainly for human consumption. Large, striped seeds from the non-oilseed kind are used for bird seed blends, human food snacks in the shell or as kernels, and baking components.

2.3 Production of sunflower

2.3.1 Sunflower growing stages

The time necessary for sunflower seeds to mature depends on the plant's variety and growth period. Typically, sunflowers reach maturity 120–150 days into the growing season or 2200–3000 growing degree days after sowing. Schneiter and Miller (1981) categorized sunflower growth stages as vegetative and reproductive (Table 1).

The growth stage classification system comprises two distinct phases, namely the Vegetative (V) and Reproductive (R) stages, which correspond to the developmental stages of plants.The process of vegetative development can be categorized into two distinct phases, namely Vegetative Emergence (VE) and actual leaf development.

The term "Vegetative Emergence" (VE) pertains to the duration that commences from the emergence of the seedling until the initial genuine leaf attains a length of less than 4 cm.

Table 1. The growth stages of sunflower (Schneither and Miller 1981)

Stage	Description
Vegetative Emergence (VE)	The seedling has emerged, and the first leaf beyond the cotyledons is less than four centimeters (cm) in length.
Vegetative Stages (V-number) (For example, V-1,V-2, etc)	These are determined by counting the number of true leaves measuring at least 4 cm in length.
Reproductive Stages (R-1)	The terminal bud forms a miniature floral head rather than a cluster of leaves.
R-2	The embryonic bud extends between 0.5 and 2.0 cm above the closest attached leaf on the stem.
R-3	The embryonic bud extends beyond 2 centimeters above the next leaf.
R-4	The flower begins to unfold.
R-5	This stage represents the beginning of blossoming and is subdivided based on the percentage of the head area that has

	concluded or is in the process of flowering.
R-6	Flowering is complete and the ray flowers are wilting.
R-7	The back of the head has started to turn yellow.
R-8	The back of the head is yellow but the bracts remain green.
R-9	The bracts turn brown and yellow. This is considered physiological maturity.

Plant density significantly impacted the stem circumference, flower diameter, and stem length, while the flowering duration was only marginally affected (Mladenović et al. 2020). According to the data provided by FAO, the optimal plant density for cultivation ranges from 4 to 10 kg/ha. At the same time, the recommended row spacing is approximately 0.9 m, resulting in a total of 60,000 plants per hectare. Both direct seeding and transplanting techniques are employed.

Moisture in the soil is the most significant factor determining density. If the soil has been sufficiently saturated to a depth of 70-90 cm at the time of sowing, it is recommended to maintain a planting density of 20-30 thousand plants per hectare for optimal harvesting outcomes. When the soil moisture content reaches a depth of 130-140 cm, it is recommended to sow 40,000 plants per hectare. When the soil is moistened beyond a depth of 2 meters, it is recommended to maintain a density of 50-60 thousand plants per hectare.

2.3.2 Soil condition and temperature

Achieving high productivity in sunflower production is contingent upon the quality of the soil and seeds utilized. In order to mitigate production risks, it is advisable to select seed that is of high quality, uniform in nature, possesses high germination rates, is characterized by known hybrid varietal purity, and is free from weed seeds and disease. In addition, it is recommended that the optimal soil conditions for the growth of sunflowers are characterized by adequate drainage and a neutral pH range of 6.5 to 7.5. Furthermore, they exhibit tolerance towards clay loam or silty clay loam soils, in addition to their proficiency in sandy loam soils (Berglund 2007).

The germination period of sunflower seedlings is contingent upon several factors, including the specific cultivar, geographical location, and climatic conditions experienced throughout the growth cycle. The most favorable period for seedling growth is between May 15th and June 15th, following the conclusion of the freezing period. The optimal temperature for seedling growth is recommended to be 45°F (7°C), while a temperature of 50°F (10°C) is suggested for the germination process.

2.3.3 Nutrients supply during the vegetation period of sunflower

The growth of sunflowers necessitates a minimum of 16 components. Certain elements, such as oxygen, hydrogen, and carbon, are sourced from the surrounding atmosphere and aqueous environments. Additional constituents were acquired from the soil. Nitrogen, phosphorus, and sulfur are commonly found to be deficient in soils across various climatic regions. Furthermore, regions with significant precipitation typically exhibit deficiencies in potassium, calcium, and magnesium. Deficiencies in iron, manganese, zinc, copper, molybdenum, boron, and chlorine are infrequent yet possible in diverse climatic zones (Franzen 2010).

Nutrients greatly influence the development and growth of crops. One of the most important elements influencing sunflower seed output is nutrient management. Nitrogen is one of the key elements among the several nutrients that improve the metabolic processes that, in turn, promote the crop's vegetative, reproductive, and yield development (Koutroubas et al. 2008). Since nitrogen is the most scarce nutrient, plants should be able to get substantial amounts of it from the soil (Nasim et al. 2012). The forms of nitrate (NO_3), urea ($CO(NH_2)_2$), and ammonium (NH_4+) are easily absorbed by plants. Research has shown that nitrogen sources and rates considerably impacted sunflower production and agronomic traits (Malik et al. 1999; Yassen et al. 2011). According to Hasan and Mukhtar (2000), sunflowers reacted more favorably to a greater rate of nitrogen in the form of urea. However, Malik et al. (1999), Osama et al. (2010), Yassen et al. (2011), and Soleymani et al. (2013) have all observed increases in plant height, head diameter, 1000 seed weight, and seed production when utilizing ammonium nitrate.

The diameter of the head (HD) is a crucial factor in determining the yield of sunflower plants. Nitrogen fertilizer has been observed to exert a significant influence on HD. This phenomenon may be linked to the overall promotion of vegetative growth due to nitrogen supplementation. According to Wajid et al. (2012), the response of nitrogen fertilizer on HD varied with different N levels, and it was observed that the response increased with an increase in nitrogen fertilizer.

According to Franzen (2010), the application of fertilizers ought to be based on a soil test. Nitrogen applications can be implemented pre-planting, during seeding, post-seeding, or through a combination of these methods, contingent upon the guidance provided by the soil test. Potassium and phosphorus can be applied pre-tillage during either the spring or fall season. The optimal fertility requirements for a 2,000-pound sunflower crop are contingent upon the outcomes of soil sample analysis, with a typical recommendation

consisting of 100 pounds of nitrogen as well as 50 pounds each of phosphate and potassium.

2.3.4 Water supply during the growth period

Sunflowers exhibit drought tolerance due to their extensive root system, which enables them to extract water from depths beyond the reach of other plant species. The observed root length of the plant is 4 meters. However, it has been noted that the plant can extract nutrients and water from depths beyond the measured root length. Consequently, farmers retain stubble on their fields during winter to increase soil moisture retention (Berglund 2007).

Proper water management is not only to reduce water deficit levels but also to obtain higher yields within existing water resources. The application of traditional flood irrigation methods are responsible for considerable loss of water, reduction in crop yields, and disturbance of eco-system, widespread salinity, and waterlogging problems. This has reduced the overall irrigation efficiency hardly up 30% (Ishfaq 2002).

2.3.5 Significance of crop rotation

Crop rotation is a highly effective method for mitigating the incidence of diseases and pests that may have affected prior crops. Implementing a suitable crop rotational sequence for sunflowers is recommended to effectively manage overwintering pathogens, insect populations, weeds, water use, and fertility management. Additionally, this practice can aid in minimizing pathogen inoculum levels in the soil. In regular crop rotations, the failure to alternate fields and planting sunflowers nearby can escalate disease pressure. The occurrence of Sclerotinia stalk and head rot, commonly known as white mold, in the Northern Great Plains region, has been observed to cause a substantial reduction in crop yield. Additionally, it has been noted that this particular pathogen can infect various other broadleaf crops as well (Bolton et al. 2006). Crop rotations of a minimum of four years, incorporating cereals, are frequently recommended to manage ailments such as Sclerotinia stalk and head rot as well as sunflower downy mildew.

2.3.6 Harvesting

Typically, sunflowers are among the final crops to undergo harvesting during autumn, as the occurrence of fall frosts aids in the desiccation of the crop. The harvesting of sunflowers typically occurs when the moisture content of the seeds is less than 16 percent. According to Berglund (2007), harvesting sunflowers at a seed moisture level exceeding 16 percent may lead to scuffing during the harvesting process, shrinkage during drying, and molding in storage.

2.4 Integrated Pest Management (IPM) in sunflower

2.4.1 General aspects of Integrated Pest Management

Integrated Pest Management is the recommended approach to mitigate yield loss caused by disease and pests. IPM is a comprehensive approach to managing pests and diseases that integrates biological, cultural, and chemical methods. This approach aims to mitigate the economic, health, and environmental risks associated with pest and disease (Barzman et al, 2015).

IPM comprises eight fundamental principles: prevention and suppression, monitoring, decision-making, seven control options, and evaluation. The initial approach involves implementing preventive and suppressive measures, such as crop rotation, utilization of resistant cultivars, and expansion of biodiversity within the field, as part of the overall management strategies. The second principle pertains to implementing pest monitoring strategies that rely on the farmer's routine surveillance practices and using predictive models. The third principle pertains to decision-making processes that consider prior strategies and the possible implementation of thresholds to assess the level of insect and disease infestation. In situations where intervention becomes necessary, it is recommended to prioritize non-chemical approaches, specifically the utilization of biological control, as outlined in Principles 4-7. The assessment of agricultural safeguarding techniques (Principle 8) through a multi-seasonal lens is imperative for the implementation of comprehensive plant protection strategies (Barzman et al. 2015).

2.4.2 IPM against sunflower diseases with special respect to *Plasmopara halstedii*

According to Leite (2014), disease impacts an estimated annual loss of 12% of global sunflower production, thereby emerging as the primary constraint for crop production in most regions. The most common diseases are downy mildew caused by *Plasmopara halstedii*, white rot caused by *Sclerotinia sclerotiorum*, Phomopsis stem canker caused by *Phomosis healiathi,* Alternaria blight caused by *Altermaria helianthi* and *A. helianthinficiens,* rust caused by *Puccinia helianthi*, and black stem caused by *Phoma macdonaldii* (Gulya et al. 2019).

One of the most important ways to control diseases is to choose resistant varieties and hybrids against the main diseases of the given region. Several resistant hybrids are available against the different pathotypes of sunflower downy mildew, broomrape, and phomopsis stem canker (Leite 2014). In those areas where the agressive pathotypes of *P. halstedii* have been identified (e.g. the eastern part of Hungary, in some regions in France and Spain, and most of the sunflower growing area in the United States), cultivating

resistant hybrids against all pathotypes is essential (Spring 2019, Bán et al. 2021). Broomrape resistance against the different races of this pest is also an essential trait in sunflower hybrids. In addition, considering tolerance against white rot (*Sclerotinia sclerotiorum*) is an important task upon hybrid choice. Many sunflower hybrids are tolerant against either stalk rot or head rot caused by *S. sclerotiorum*. Finally, resistance against phomopsis stem canker is a vital charachteristic of a hybrid at present (Bán et al. 2023).

In addition to agrotechnical practices such as crop rotation and proper hybrid selection, harmonious nutrient supply and chemical protection play a vital role in sunflower protection. In particular, seed treatment is effective against sunflower downy mildew and sclerotinia, while spraying is mainly targeted at stem and head diseases, most notably sclerotinia (Gulya et al. 1997, Bán et al. 2023).

2.5 Sunflower downy mildew

2.5.1 Significance and history of *Plasmopara halstedii*

Plasmopora halstedii (Farl.) Berl. et de Toni, a biotrophic oomycete, is responsible for causing downy mildew, a significant disease that affects sunflower production. The pathogen has been documented in most countries where sunflower seed cultivation occurs. It has been estimated that the global impact on yield in commercial seed production is expected to decrease by 3.5%. However, in a contaminated field, the yield losses may reach 100% (Gascuel et al. 2015).

Plasmopara halstedii forms different variants called pathotypes. There are approximately 50 pathotypes of this pathogen worldwide (Bán et al. 2021). Newly emerged aggressive pathotypes may break the resistance of sunflower hybrids, as was the case many times in sunflower production. Moreover, tolerant strains of *P. halstedii* to mefenoxam, previously the only effective active ingredient in seed coating, could also be distributed worldwide (Körösi et al. 2021; Nisha et al. 2023). Consequently, the protection of sunflowers against downy mildew has many difficulties, and new methods in its management are essential (Gulya et al. 1997; Bán et al. 2023).

In 1883, Farlow described the *Peronospora halstedii* pathogen, utilizing pathogen samples obtained from various plant species, including *Eupatorium purpureum, Ambrosia artemisifolia, Bidens frondosa, Rudbeckia laciniata, Silphium terephthalaceum,* and the perennial sunflower species *Helianthus strumosus, H. tuberosus,* and *H. doronicoides*. Viranyi and Spring. (2011) highlight that the initial reference to a sample of *Eupatorium purpureum* collected by Halsted near the Bussey Institution dates back to 1876.

The *Plasmopara* genus was distinguished from Peronospora based on its zoospore germination mechanism instead of germ tube formation. Subsequently, Berlese and de Toni (1888) classified the organism as *Plasmopara halstedii* (Farlow) Berlese & de Toni and placed it in a newly established genus. As per the provided description, the range of hosts encompassed seven genera, comprising four perennial Helianthus species, while lacking any annuals. *Plasmopara halstedii* was identified in North America by examining various downy mildew collections found on the Asteraceae species. The classification was based on the physical resemblance of sporangiophores and sporangia, although initial observations noted the presence of size and shape heterogeneity (Viranyi and Spring 2011).

The earliest documented instances of downy mildew on Helianthus annuus can be traced back to the 1890s. Subsequently, by the 1920s, this disease emerged as a significant threat to sunflower production in multiple states across the United States, as reported by Henry and Gilbert in 1924. According to Virányi et al. (2015), the spread of the phenomenon occurred in the 1960s, first reaching Russia and the Balkan Peninsula, specifically Bulgaria, Greece, and Turkey, and later extending to various other European countries, such as Hungary, Italy, France, Spain, Germany, and the former Czechoslovakia. During the latter half of the 20th century, sporadic occurrences of the disease, as mentioned earlier, disseminated throughout Europe, which were attributed to the implementation of intensive farming practices and favorable weather patterns. In 1977, *P. halstedii* was categorized as a dangerous pathogen, posing a significant risk to sunflower cultivators in Europe. From 1992, *P. halstedii* had been subjected to quarantine restrictions in the European Union (Delmotte et al. 2008). Now it is a regulated, non-quarantine pest (RNQP) in many countries (Bán et al. 2023).

2.5.2 Symptoms and signs of sunflower downy mildew

The systemic, obligatory parasite downy mildew produces intercellular mycelium with spherical haustoria inside the plant cells and sporangia that arise from stomata. The slender sporangia exhibit monopodial branching, which produces zoosporangia at the terminal ends of the branches. According to Zimmer and Hoes (1978), the zoosporangia fragment and discharge biflagellate zoospores.

The manifestation of symptoms in immature sunflower plants due to root infections is of the utmost severity. It has a considerable impact on the yield in the agricultural field. Studies have demonstrated that infections exhibit maximum efficacy within five days after seed germination. However, infections may still transpire until the sunflower plants attain

the four-leaf stage, typically taking 2 to 4 weeks.

The pathogen is characterized by a white layer on the underside of leaves, composed of asexual reproductive structures, including sporangiophores and sporangia that contain zoospores. Secondary infection can occur through sporangia (zoospores) during vegetation, leading to local symptoms such as angular leaf lesions. While the secondary infection is less prevalent than the primary infection, it can substantially reduce crop yield (Meliala et al. 2000). The symptomscomprise stunted growth of affected plants, chlorosis of leaves along the veins upright positioning of heads, and either sterile or nonviable seeds. Plant damping-off may occur due to severe infestation during the initial phases of sunflower growth (Gascuel et al. in 2015 and Ban et al. in 2023).

According to Spring (2009), local symptoms can progress into a systemic infection in the upper plant portions, which can ultimately lead to latent seed contamination by the pathogen.

2.5.3 Life cycle of downy mildew

According to Ioos et al. (2007), *P. halstedii* is predominantly a pathogen transmitted through soil and seed. The life cycle of *P. halstedii* commences with the sexually produced oospores, which are thin-walled and resistant structures that usually overwinter (Gulya et al., 1997) (Figure 1.). Oospores have been observed to occur in plant residue contaminated from the previous sunflower harvest and in the seeds of plants that have been infected systemically. After winter, the oospores undergo germination, especially under moist conditions during the spring season. According to Zimmer and Hoes (1978), certain oospores can remain dormant for up to fourteen years. The genus Plasmopara predominantly employs indirect zoospore germination, as Grenville-Briggs and Van West (2005) noted.

The zoospores of *P. halstedii* can infect young sunflower plants (Gasuel et al.2015).The entry of pathogen germ tube into root tissues can occur via three distinct mechanisms: primarily through the formation of an appressorium, as well as through lesions, particularly at the bases of root hairs, or by direct penetration through the wall of epidermal cells without an appressorium (Gasuel et al.2015). Upon attachment to the roots, the zoospore undergoes encystment through the secretion of a wall, leading to the loss of its flagella. According to Spring et al. (2018), the produced cystoscope subsequently infiltrates the plant tissues through a germ tube. The mycelium of the pathogen traverses the intercellular spaces to reach the plant's aerial portions, releasing altered hyphae (haustoria) into the cells to obtain nutrients. Next, sporangia with zoospores occur on the

underside of the leaves as a white coating.

The pathogen's biotrophic nature results in many diseased plants surviving until harvest, serving as a reservoir for the pathogen's dissemination. At the culmination of the developmental period, oospores are produced on the stem through sexual reproduction (oogamy). According to Viranyi et al. (2011) and Spanu et al. (2017), the oospores of *P. halstedii* are considered to be quiescent structures that can persist in the soil for a maximum of ten years. *Plasmopara halstedii* is primarily a pathogen that is found in soil. However, it can endure in various other sources such as seeds (known as seed-borne), host vegetation, and sunflower crop residues, which include oospores and mycelium.

The infection is contingent upon a potent pathogen, a susceptible host plant, and favorable environmental circumstances. The favorable environmental conditions for the growth of downy mildew during the sowing of sunflowers are characterized by cool temperatures ranging from 10 to 15 °C and high moisture levels. According to Gulya et al. (2019) and Baldini et al. (2008), the temperature mentioned serves as a barrier to disseminating the pathogen's mycelium throughout the plant. Furthermore, it has been observed that an increase in soil temperature (exceeding 20°C) after the sowing of sunflowers significantly restrains the germination of oospores and subsequent infection, as reported by Gulya et al. (2019) and Humann et al. (2019), even in instances of intense precipitation and irrigation.

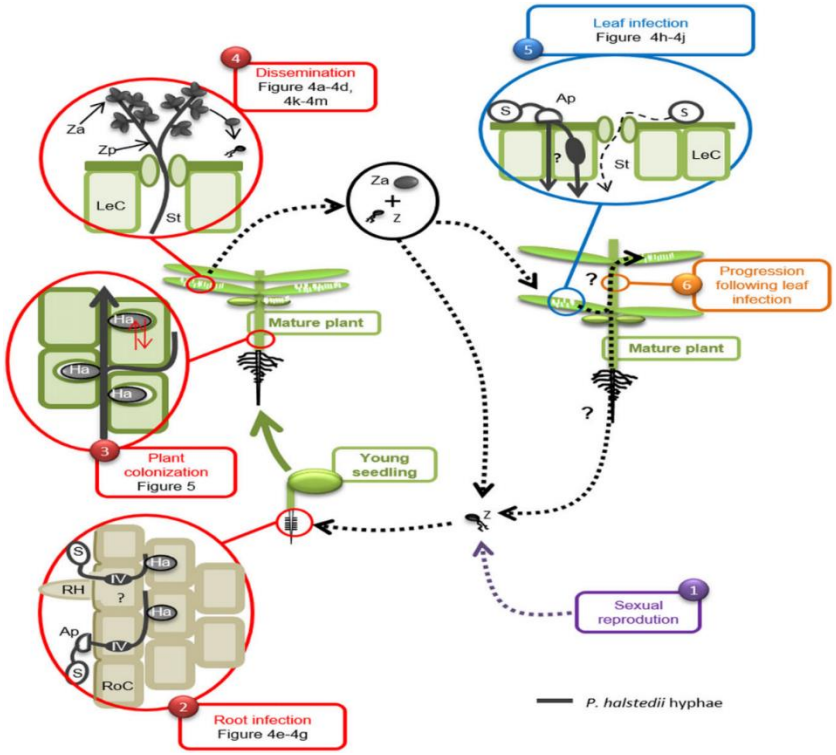

Figure 1. Life cycle of *P. halstedii.*

2.5.4 Downy mildew management

The primary means of managing sunflowers against downy mildew include agrotechnical techniques, fungicide seed treatments, and genetic resistance.

Utilizing non-host cultivars in crop rotation is a vital strategy that disrupts the pathogen's life cycle. It is essential to adhere to a crop rotation period of at least four years because the oospores can persist in the soil for an extended period (Debaeke et al. 2014). Nevertheless, the viability of these dormant structures notably diminishes after the four-year mark (Virányi and Spring 2011).

Tillage plays a vital role in sunflower cultivation as it is necessary for the healthy growth of sunflower roots. It also assists in handling any contaminated crop remains. However, the extensive adoption of modern weed control methods in various farming systems significantly lowers the risk of *P. halstedii* remaining and proliferating (Bán et al. 2023).

Another way to manage sunflower downy mildew by using fungicide is seed coating (Körösi et al. 2021). Furthermore, today, farmers have a range of options to choose from when it comes to seed coating methods, including film coating with a polymer, pelleting, or encrusting, allowing them to select the most suitable technique for their specific growing conditions. Seed pellets and polymers serve the dual purpose of binding seed-applied treatments like pesticides and indirectly protecting the plants by ensuring uniform and larger sunflower seed sizes, which enhances the seeding process (Bán et al. 2023).

According to Molinero-Ruiz (2019) and Molinero-Ruiz and Melero-Vara (2003), genetic resistance is the primary means of controlling this particular pathogen. The discovery of additional *P. halstedii* populations exhibiting heightened virulence capable of overcoming resistance alleles in sunflower crops (Molinero-Ruiz et al. 2003),(Garcia-Carneros et al. 2017) poses a significant threat to the efficacy of resistant sunflower hybrids. The limited duration of protection against downy mildew provided by dominant resistance genes incorporated into sunflower hybrids can be attributed to the high variability of the pathogen (Virányi et al. 2010).

In sunflowers, two types of resistance to downy mildew are caused by Pl genes. Type I resistance prevents symptoms on shoots and keeps the pathogen from appearing above the base of hypocotyls. Type II resistance results in limited sporulation on cotyledons, with no symptoms on the upper part of the plant, preventing the pathogen from reaching true leaves. Different sunflower genotypes exhibit either type I or type II resistance based on their Pl resistance genes, such as Pl5 and Pl6. These resistances have been effective against

Plasmopara halstedii pathotypes, but in the last 15 years, most of them, except for $Pl_{Arg,}$ have been overcome. For instance, eight pathotypes have overcome the Pl2 and Pl6 genes (Gasuel et al.2015).

2.5.5 Chemical resistance inducers

BTH, also known as benzo (1,2,3) thiadiazole-7-carbothermic acid S-methyl ester or benzothiadiazole, is a widely researched chemical inducer that activates systemic acquired resistance (SAR). Its effectiveness has been demonstrated through the successful use of the commercial product Bion 50 WG. The activation of resistance in various cultivars against various diseases, such as powdery mildew in barley and wheat, pea rust, and Sclerotinia diseases in cantaloupe and soybean, has been demonstrated using BTH. Sunflower's efficacy was comparable in its ability to combat downy mildew and rust disease (Bán et al. 2017).

The botanical species Azadirachta indica A. Juss, known as Neem, exhibits various protective properties against pests and diseases, including antifeedant, antifungal, nematicidal, and insecticidal effects. The global recognition of the efficacy of neem tree seed, leaf, and bark extracts in managing insect infestations and agriculture is widely acknowledged. Doshi et al. (2020) reported the protective efficacy of neem seed extract and a commercial neem product against *Plasmopara viticola* (Berk. and M.A. Curtis) Berl. And de Toni in grapevine, with a demonstrated efficacy of over 90%.

According to Doshi et al. (2020), the results of the in vivo experiment for sunflower against downy mildew indicate that pre-treated seedlings, aged three days, exhibited a significant reduction in infection when exposed to both concentrations of neem leaf extracts (containing more compounds in addition to azadirachtin) and a commercial neem-based pesticide product NeemAzal T/S (denoted as AZA)(Doshi et al. 2020). This experiment was first for sunflower downy mildew.

Examining the host tissue reactions of BTH-treated and Neem-treated susceptible sunflower plants, cell necrosis and secondary cell division were observed, similar to genetically resistant sunflowers that have been inoculated. Mouzeyar et al.(1993) demonstrated that P. halstedii could infect susceptible and resistant sunflower lines, although a susceptible plant can react to the pathogen's growth to a diminished extent. According to his experiment, the pathogen starts systemic colonization once it invades the plant. In response to contact with the pathogen, the cytoplasm of infected cells fills with fluorescent compounds before degenerating. These cells disintegrate, and their walls combine into a solid mass surrounding the pathogen structures. The walls of neighboring

cells closest to the pathogen become lignified as they divide. These effects inhibit pathogen growth without first destroying it, which explains why the pathogen may continue to grow in the plant. The intensity of a plant's reaction may differ according to genotype; the lower the parasite obstructs the hypocotyl, the more intense and earlier the reaction.

The lignification of the cell wall and cell division consistently occurs in a circular pattern surrounding the center of the lesion, whereby the newly generated walls are positioned perpendicular to the circle's radius.

3. MATERIALS AND METHODS

3.1 Set up of the experiments

The experiment conducted in the laboratory of the Plant protection at the Hungarian University of Agriculture and Life Sciences (MATE) in Gödöllő, Hungary. Iregi Szürke Csíkos seeds was used for this experiment. This is an old Hungarian sunflower cultivar susceptible to all known pathotypes of *P. halstedii* because it does not include resistance genes against this pathogen but shows tolerance to other critical diseases. Isolate 3 was used for inoculation originated from the collection of the Department of Integrated Plant Protection.

3.1.1 Preparation of seeds and inoculum

The Iregi szürke csíkos 360 seeds were measured to set up our experiment, then placed them in a beaker and soaked them for disinfecting in 1,5% Na-hypochlorite (NaOCl) solution for 3–5 min, rinsed thoroughly with running tap water, and germinated on moist filter paper at 20 °C until 3-5 mm root initials had been developed (Figure 1).

Figure 2. Germinated sunflower seeds (photo: B. Arbnora, 2022)

For the tests, isolate 3 of *P. halstedii* was used from the Department of Integrated Plant Protection (MATE) collection, which was stored at -70 °C. This isolate originated from Hungary and was collected in 2021. The sporangia from the infected leaves were brushed into 100 ml of bi-distilled water, and the suspension concentration was adjusted to 50,000 sporangia/ml using a hemocytometer.

3.2 Treatments

The following treatments were used in this experiment:

- negative control: non-treated with BTH or NeemAzal and non-inoculated with *P. halstedii;*
- chemical control: treated with BTH or NeemAzal and non-inoculated with *P. halstedii;*
- infected control: non-treated but inoculated with P. halstedii ;
- BTH- treated and Neem-treated: treated with BTH or NeemAzal inducer and inoculated with *P.hasltedii.*

Figure 3. Preparing BTH solution for the experiment (photo: B. Arbnora 2022)

Pre-germinated seedlings were soaked for two hours in 320 ppm BTH solution prepared by measuring 0,096 g Bion 50WG (containing 50 % BTH) and pouring it into 149,9 g water. The same for Neem pre-germinated seedlings were soaked for two hours in NeemAzal solution (0,1%) prepared with 0.15 ml NeemAzal poured in 149,85 ml water. The treated seedlings were inoculated with *P.halstedii* by immersing them into the sporangial suspension, which was adjusted to 50 000 sporangia/ml in the first experiment and to 35 000 sporangia/ml in the second experiment, using a hemocytometer. Seedlings were then inoculated at 16° C overnight in darkness.

Figure 4. Treatment of sunflower seeds with BTH (photo: D.Galymzhankyzy)

Figure 5. Treatment of sunflower seeds with NeemAzal (photo: D.Galymzhankyzy)

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 phytotron (Figure 6-7.).

Figure 6. Sown sunflower seedlings in perlite before emergence (photo: D. Galymzhankyzy)

Figure 7. Sunflower plants placed into phytotron (photo: D. Galymzhankyzy)

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 planting.

The process was performed by placing the trays in dark-colored bags (Figure 8). Then, before sealing, the plants were sprayed with bi-distilled water, which promoted the formation of 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 (Figure 9).

Figure 8. Inoculated sunflower plants sprayed for sporulation (photo: D.Galymzhankyzy)

Figure 9. Sporangium coating on sunflower plants after sporulation (photo: D.Galymzhankyzy)

3.3 Disease assessment

Disease assessment was made by calculating the ratio of diseased (sporulated and damped-off plants) as well as healthy plants on 9-day-old plants. Plant height was measured twice, first on 9-day-old plants, and secondly on 21-day-old plants.

The first evaluation was done 9 – day-old plants; the bags were removed from the

trays and we could see a white coating (sporangiophores and sporangia) on the leaves. The disease rate was evaluated by using a 0-4 scale, where:

0 – no visible sporangiophores (white coating).
1 - < 25% of leaf disc surface is covered with sporangiophores
2 – 25 –50% of leaf disc surface is covered with sporangiophores
3 – 50 –75% of leaf disc surface is covered with sporangiophores
4 – >75% of leaf disc surface is covered with sporangiophores

The second evaluation was done on 21-day-old plants. In this evaluation, height was measured, too. In addition, leaves were checked for *P. halstedii* symptoms such as curling, different forms of leaves, necrosis, chlorosis, stunting, and damping-off.

3.4 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 H2O = 2:1:10:7) (Figure 10A). All samples of sunflower hypocotyl were divided into the upper and lower parts and then were cut into thin slices with a razor blade for examination (Figure 10B). Sections were examined unstained.

As per the methodology outlined by Bán et al. (2004), a scale ranging from 0 to 4 was used to observe both pathogen structures and host responses. Briefly, the sections were theoretically partitioned into four quarters, encompassing the cortical and pith parenchyma. The investigation involved analyzing the occurrence of the pathogen and the plant's reactions in each of these quarters.

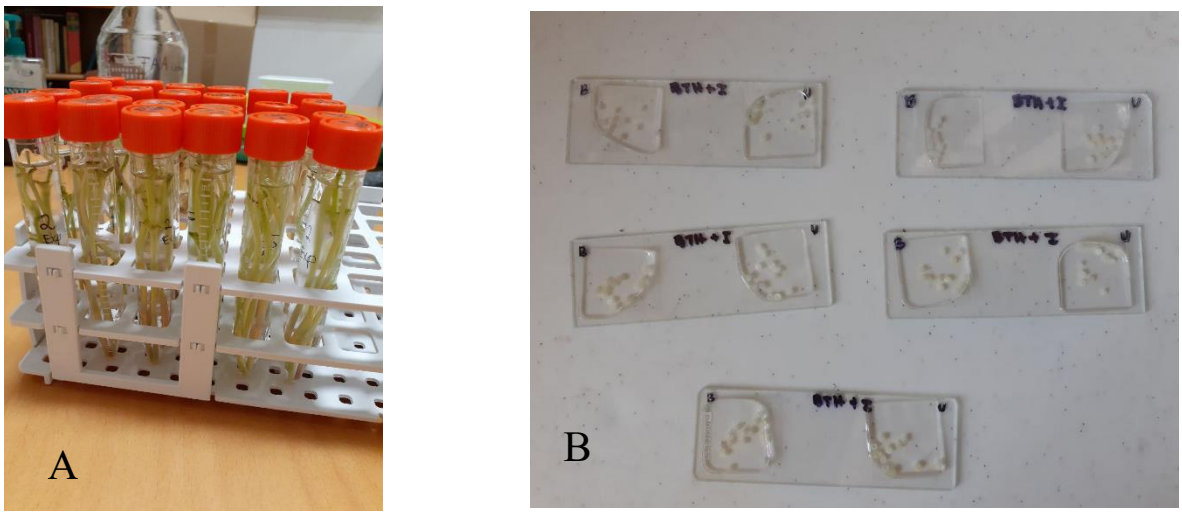

Figure 10. Preparation of hypocotyls

(A) Sunflower hypocotyl fixed in FAA; (B) Preparation of cross sections of sunflower hypocotyl (photo: D.Galymzhankyzy)

3.5 Data Analysis

After the collection of the data for disease rates, host characteristics (plant height), and host responses (cell necrosis) as well as pathogen structures (hyphae) were assessed by the one-way analysis of variance (ANOVA). We used Fisher's HSD post-hoc test ($p < 0,05$) to determine which treatments are statistically different.

4. RESULTS

4.1 Disease rates during experiments

Figure 11 demonstrates the disease rates of *P. halstedii* during the evaluation of the first experiment. The highest infection rate was measured on the non-treated and inoculated plants with *P. halstedii* (IC) and Neem-treated inoculated plants (Neem+I), while the lowest one on BTH-treated and inoculated (BTH+I) plants during both evaluations. There was no significant difference between the disease rates of Neem-treated and control plants in this experiment. However, the disease rates of BTH-treated inoculated sunflowers were significantly lower than either non-treated or Neem-treated inoculated plants.

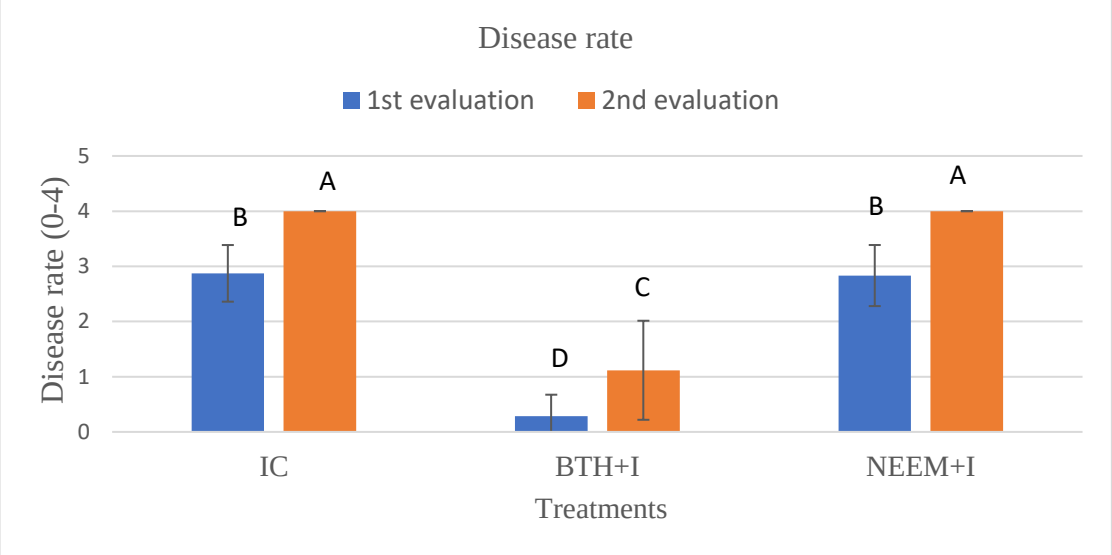

Figure 11. **First experiment:** Disease rates of non-treated and treated sunflowers inoculated with *Plasmopara halstedii* (inoculum concentration: 50 000 sporangia/ml)

Legend: IC: inoculated control without any treatments, BTH+I: BTH-treated and inoculated with *P. halstedii*, NEEM+I: Neem-treated and inoculated with *P. halstedii*. Vertical lines represent 95% confidence intervals (95% CI) of the mean values of disease rates. Different letters (A, B, etc.) indicate significant differences based on the Fisher's HSD post-hoc test ($p < 0,05$) among variables.

Figure 12 shows the result of the second experiment with the reduced concentration of sporangial suspension (35 000 sporangia/ml) of *P. halstedii*. As in the previous experiment, there was no significant difference between the disease rates of inoculated non-treated and Neem-treated plants in any of the recordings. However, the difference

between them was greater than in the first experiment. So, the disease rates of Neem-treated inoculated plants was lower (though not significantly) than that of the control sunflowers during the measurements. Similar to the first experiment, BTH-treated plants were significantly less diseased than control (inoculated non-treated) plants during both evaluations. In contrast, we could not detect a significant difference in disease between BTH-treated and Neem-treated inoculated plants in the second evaluation.

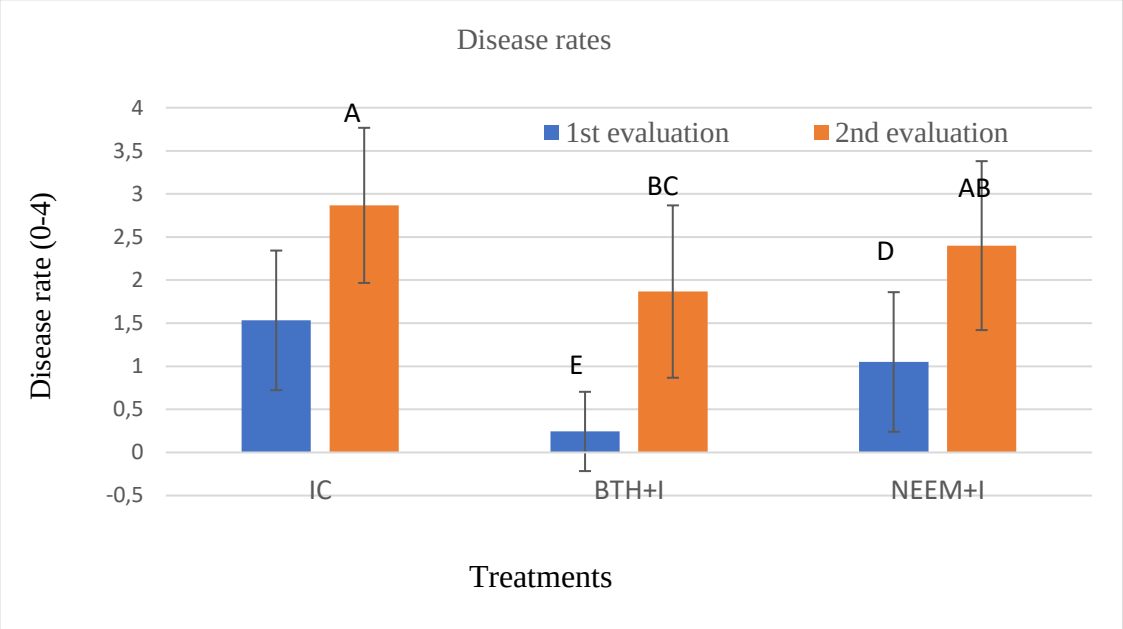

Figure 12. **Second experiment:** Disease rates of non-treated and treated sunflowers inoculated with *Plasmopara halstedii* (inoculum concentration: 35 000 sporangia/ml)

Legend: IC: inoculated control without any treatments, BTH+I: BTH-treated and inoculated with *P. halstedii*, NEEM+I: Neem-treated and inoculated with *P. halstedii*. Vertical lines represent 95% confidence intervals (95% CI) of the mean values of disease rates. Different letters (A, B, etc.) indicate significant differences based on the Fisher's HSD post-hoc test ($p < 0,05$) among variables.

4.2 Plant Heights

Figure 13 shows the plant heights during the first experiment. We found that non-treated and inoculated as well as neem-treated and inoculated plants (Neem+I) were significantly shorter than 0 control and neem-treated (non-inoculated) plants both in the first and second evaluations. Furthermore, there was no significant difference among the heights of BTH- treated plants and BTH-treated and inoculated (BTH+I) sunflowers in both evaluation. However, BTH-treated non-inoculated plants were significantly lower than Neem-treated non-inoculated and 0 control sunflowers during the experiment.

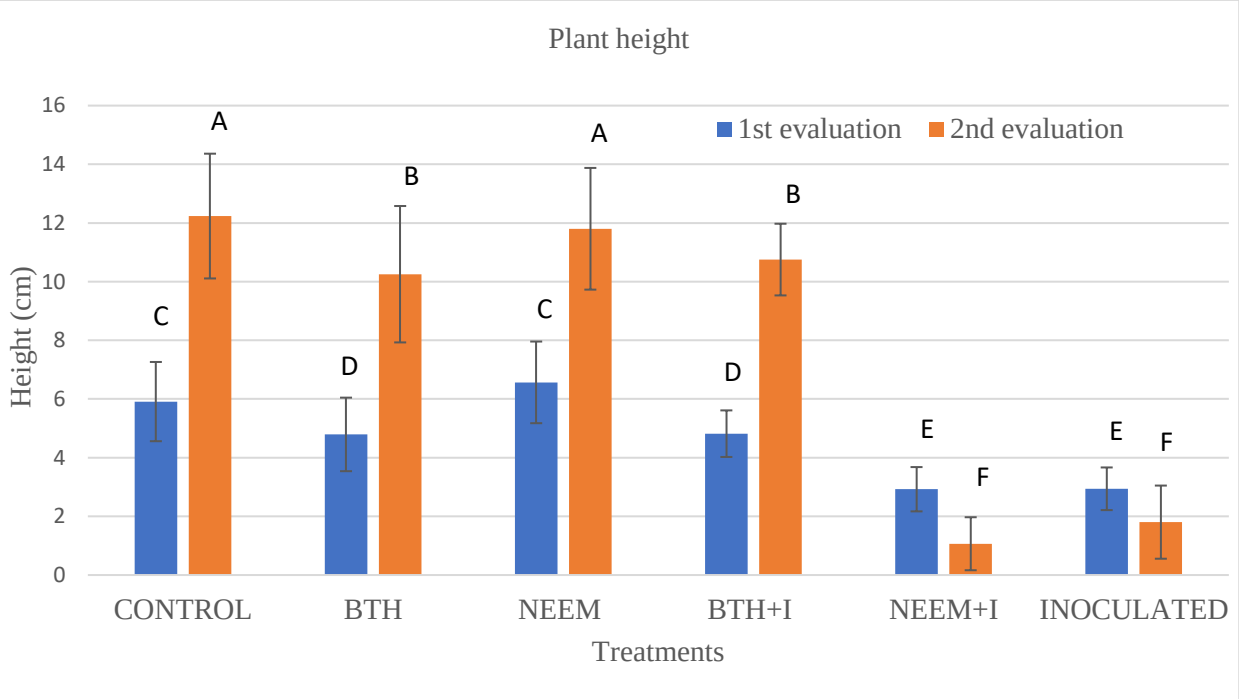

Figure 13. **First experiment:** Heights of 9-day-old plants at the first evaluation and 21-day-old sunflowers at the second evaluation

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, INOCULATED: 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.

Figure 14 illustrates the heights of 9-day-old plants at the first evaluation and 21-day-old sunflowers at the second evaluation. As a result, non-treated inoculated plants and Neem-treated inoculated plants were significantly lower than their controls during the two

evaluations. In contrast, the height of the BTH-treated and non-treated inoculated plants did not differ significantly. Moreover, there was no significant difference in heights among the BTH- treated (non-inoculated) and Neem-treated (non-inoculated) and BTH-treated and inoculated with *P. halstedii* in any time of evaluation.

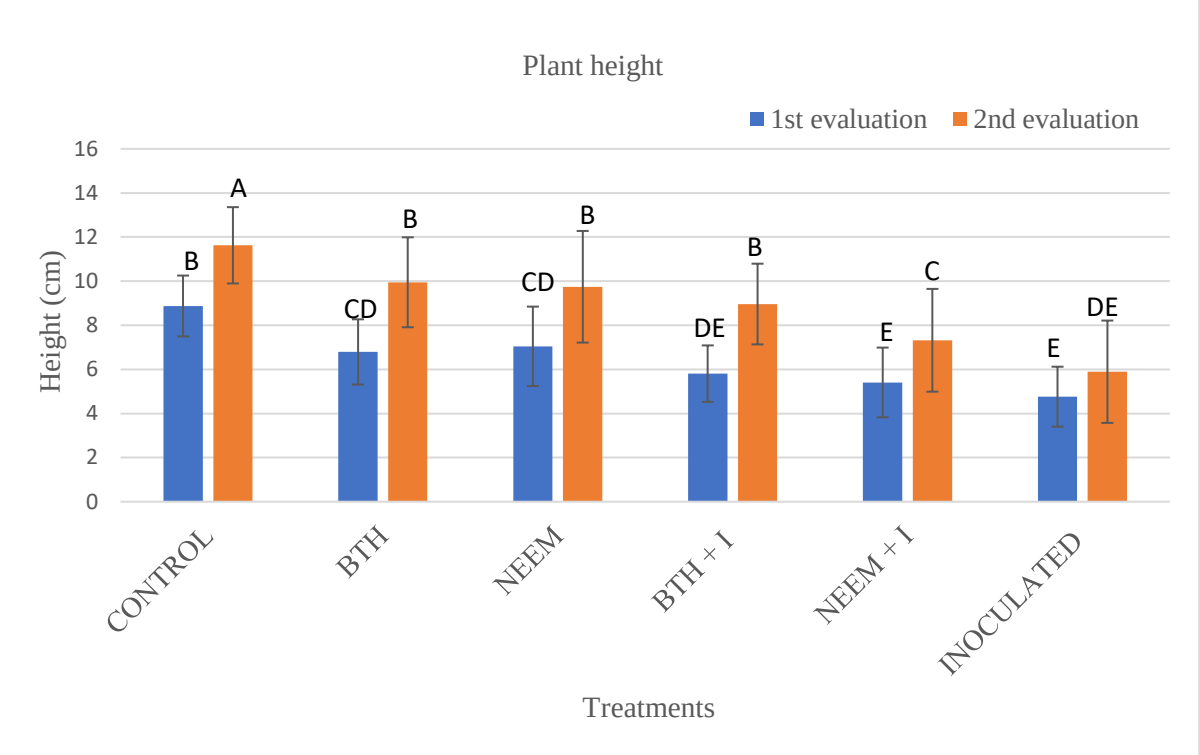

Figure 14. **Second experiment:** Heights of 9-day-old plants at the first evaluation and 21-day-old sunflowers at the second evaluation

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, INOCULATED: 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.

4.3 Microscopic observations

Figure 15 shows the pathogen hyphae in the intercellular spaces of a non-treated inoculated sunflower hypocotyl. The intercellular hyphae and haustoria were detected either in the cortical or the pith parenchyma, and could be seen as dots under light microscope. No host responses such as hipersensitive reaction and necrosis could be identified.

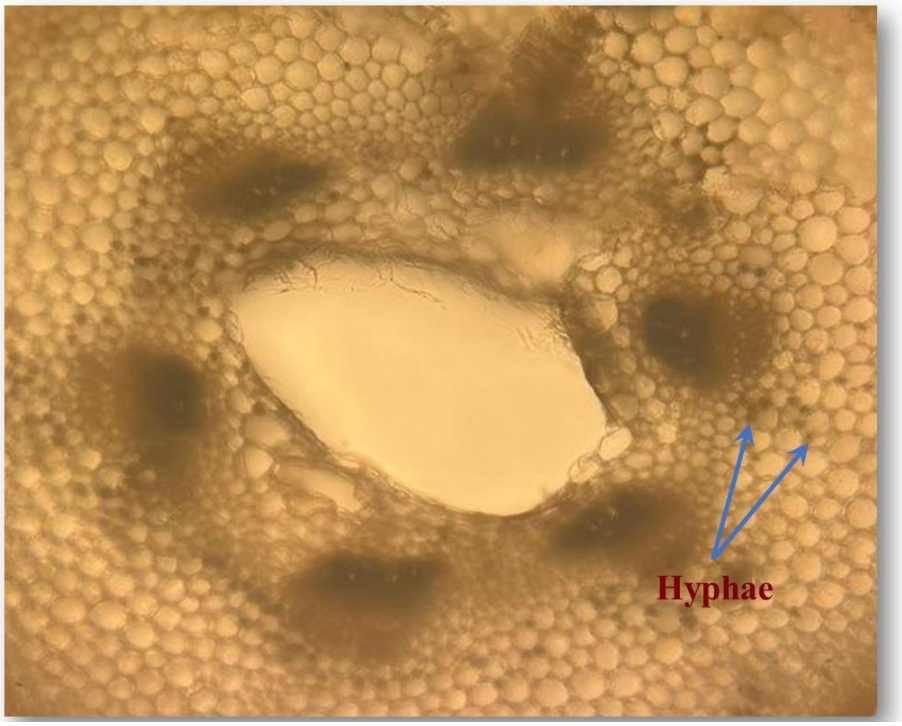

Figure 15. Light micrograph of a cross section of a non-treated and inoculated sunflower: hyphae (arrows) and haustoria of *Plasmopara halstedii* (sunflower downy mildew) in the cortical and pith parenchyma (photo: D. Galymzhankyzy)

Figure 16 presents the development of a cellular browning and necrosis (arrow) surrounded by cell elongation (arrows) in a Neem-treated sunflower hypocotyl section.

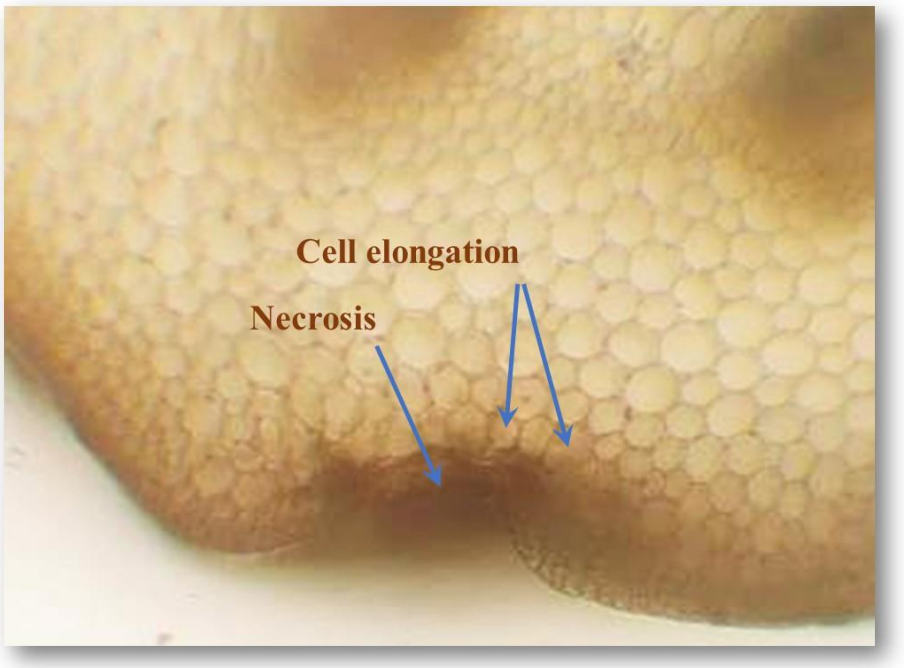

Figure 16. Light micrograph of a cross section of a Neem-treated and inoculated sunflower: host response such as cell necrosis and elongation of cells (arrows) next necrotic ones to pathogen invasion in the cortical parenchyma (photo: D. Galymzhankyzy)

Figure 17 demonstrates the development of hyphae in the host tissues and host reaction (necrosis) of treated and non-treated sunflower to pathogen invasion. Significantly less hyphae were found in the BTH-, and Neem-treated than that of the non-treated inoculated sunflowers. Although more necrosis could be detected in the treated than in non-treated inoculated plants in the cortical parenchyma, it was not significant statistically.

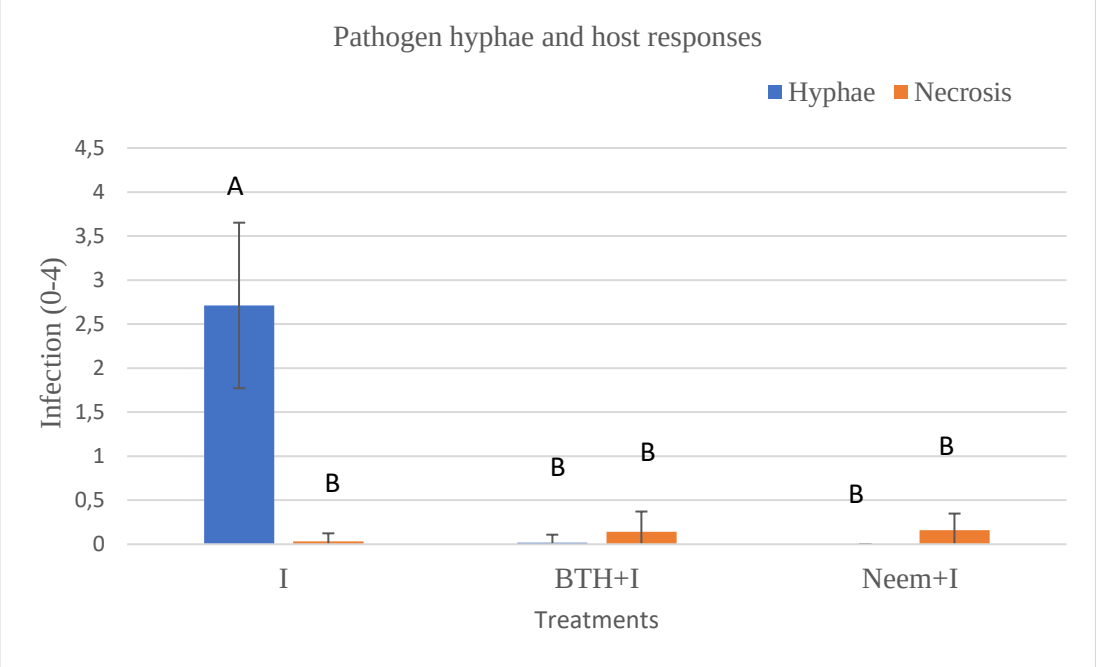

Figure 17. Effect of BTH and Neem treatment on host response of sunflowers inoculated by sunflower downy mildew (*Plasmopara halstedii*)

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.

5. CONCLUSION AND RECOMMENDATIONS

In my research work, I tested a botanical pesticide (NeemAzal) as a potential resistance inducer for its effectiveness against sunflower downy mildew. Another goal of this work was to analyze host tissue responses by light microscopy to understand the background of induced resistance better against *P. halstedii* in sunflowers.

Beside NeemAzal, another inducer, BTH (benzothiadiazole) was used and served as a kind of control in my experiments where we applied two different concentrations of the *P. halstedii* inoculum. According to our results, both BTH and NeemAzal restricted disease development of *P.halstedii* in treated and inoculated plants. The BTH treatment reduced pathogen development (infection rate) and also stunting of plants which latter was recorded by height measurements. The same result was observed by Bán et al. (2004) with *P. halstedii* and Bán et al. (2017) when they used BTH as a resistance inducer against white rot *(Sclerotinia sclerotiorum)* in sunflower. Since, many crops have been demonstrated to activate resistance when exposed to BTH to a variety of disease, including powdery mildew in barley and wheat, pea rust (Barilli et al. 2010) as well as *Sclerotinia* disease in melon (Buzi et al. 2004) and soybean (Dann et al. 1998).

The effect of Neem inducer was lower than BTH effect for reducing disease rate and plant height during our experiment. It contrasted with the result obtained by Doshi et al. (2020), where they tested two neem-derived substances for their effectiveness in preventing *P.halstedii* pathotype 704 under *in vivo* and *in vitro*. They examined neem leaf extracts and commercial neem-based pesticide product NeemAzal T/S (1% azadirachtin). *In vivo*, both concentrations of neem leaf extract and NeemAzal (0.1%) significantly reduced all sporulation in pre-treatment sunflowers. While the reason is still unclear why Neem-treatment failed to decrease downy mildew disease in a greater extent in our experiment, one possible explanation can be the high aggressivity of the isolate we used.

The histological examination of host responses revealed that BTH and NeemAzal treatments significantly reduced the presence of hyphae and necrosis in cortical and pith parenchyma of hypocotyl. The most of intercellular hyphae and haustoria were in cortical parenchyma of hypocotyls. The same result was showed by Bán et al. (2017) where they observed effect of BTH and micorrhyza fungi against *Sclerotinia sclerotorium* in three sunflower genotypes with various resistance. Our results are also consistent with Bán et al. (2004), where they evaluate BTH (Bion 50WG) effectiveness to downy mildew pathogen. According to their result, BTH-treated sunflowers showed a relatively higher level of

necrosis than non-treated ones. The same result were taken from another chemical ingredient mefenoxam (metalaxyl-M) which reduce the downy mildew by blocking the rRna biosynthesis of the pathogen (Nisha et al. 2023). The hypocotyl of plants showed similar tissue responses for infected plants without treatment and treated plants with BTH and Neem than in our experiments. Meanwhile, treated sunflowers responded to the pathogen by necrosis surrounded by intensive cell division.

The rate and intensity of plant reactions of treated sunflowers may vary. They can occur in the root or in various portions of the hypocotyl. However, most of these reactions were in the underneath part of the hypocotyl, so the pathogen in induced (treated) plants could not reach the upper part of the plants (Nisha et al. 2023).

Examinig and summarizing the results obtained from the BTH and Neem experiment against *P.halstedii*, the causal agent of sunflower downy mildew, we came to the conclusion that it would be worthwhile to perform several such experiments with inducers. We received preliminary result that both inducers reduce the development of disease symptoms. Plant inducers, such as NeemAzal and BTH, open up a new, environmentally friendly option for disease control, including sunflower downy mildew (Bán et al. 2023).

SUMMARY

The sunflower is the second most crucial crop for hybrid breeding, primarily valued for its seed oil extraction, and ranks fifth globally in vegetable oil production. It is widely used in human nutrition and has various industrial applications, including polymer synthesis, biofuel, emulsifiers, and lubricants. Given its rising popularity, it's crucial to maintain and continually expand our knowledge about it, with a particular focus on disease management.

Pathogens in sunflower can lead to substantial harm, with one noteworthy example being sunflower downy mildew caused by the pathogen *Plasmopora halstedii.* This disease has the potential to result in significant or even complete crop loss. Managing this disease is made even more challenging due to the emergence of new pathogen variants and the development of resistance to fungicides resulting from the use of a single plant protection product.

Our experiment aims to test a botanical pesticide (NeemAzal) as a potential resistance inducer for its effectiveness against sunflower downy mildew. Another goal of this work was to analyze host tissue reactions by light microscopy to understand the background of induced resistance better against *P. halstedii* in sunflowers. Our experiments were performed under laboratory conditions. To set up our experiment, we used cv. Iregi szürke csíkos, which is susceptible to all *P.halstedii* pathotypes. Bion 50WG (with BTH) and a botanical pesticide NeemAzal were used as inducers. Pre-germinated seedlings were soaked in BTH (320ppm) and NeemAzal (0.1%) solutions. Following, we evaluated the degree of sporulation on cotyledons, plant height, and the degree of chlorosis on true leaves of sunflower plants. Based on disease rate and plant height we obtained that BTH and NeemAzal had positive effects against the pathogen. However, NeemAzal treatment reduced pathogen development considerably less than BTH treatment. As a result of the histological examinations of host responses, BTH and NeemAzal treatments significantly reduced the presence of hyphae and necrosis in cortical and pith parenchyma of the hypocotyl.

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APPENDICES

Appendix 1: Analysis of variance for disease susceptibility data (1st experiment)

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0,05

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	6	IC1; IC2; BTH+I(1); BTH+I(2); Neem+I(1); Neem+I(2)

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	5	691,1	138,223	135,59	0,000
Error	354	360,9	1,019		
Total	359	1052,0			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
1,00968	65,70%	65,21%	64,52%

Means

Factor	N	Mean	StDev	95% CI
IC1	60	2,783	1,027	(2,527; 3,040)
IC2	60	4,000	0,000	(3,744; 4,256)
BTH+I(1)	60	0,283	0,783	(0,027; 0,540)
BTH+I(2)	60	1,117	1,795	(0,860; 1,373)
Neem+I(1)	60	2,833	1,107	(2,577; 3,090)
Neem+I(2)	60	4,000	0,000	(3,744; 4,256)

Pooled StDev = 1,00968

Fisher Pairwise Comparisons

Grouping Information Using the Fisher LSD Method and 95% Confidence

Factor	N	Mean	Grouping			
Neem+I(2)	60	4,000	A			
IC2	60	4,000	A			
Neem+I(1)	60	2,833		B		
IC1	60	2,783		B		
BTH+I(2)	60	1,117			C	
BTH+I(1)	60	0,283				D

Means that do not share a letter are significantly different.

Appendix 2 : Analysis of plant height variance (1st experiment)

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0,05

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	12	height control 1; height control 2; height BTH 1; height BTH2; height NEEM 1; height NEEM 2; height INFECTED 1; height INFECTED 2; height BTH+I 1; height BTH+I 2; height NEEM+I 1; height NEEM+I 2

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	11	10555	959,581	114,46	0,000
Error	708	5936	8,384		
Total	719	16491			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
2,89549	64,01%	63,45%	62,78%

Means

Factor	N	Mean	StDev	95% CI
height control 1	60	5,912	2,700	(5,178; 6,646)
height control 2	60	12,235	4,253	(11,501; 12,969)
height BTH 1	60	4,793	2,507	(4,059; 5,527)
height BTH2	60	10,253	4,649	(9,519; 10,987)
height NEEM 1	60	6,567	2,783	(5,833; 7,301)
height NEEM 2	60	11,802	4,148	(11,068; 12,536)
height INFECTED 1	60	2,940	1,454	(2,206; 3,674)
height INFECTED 2	60	1,803	2,493	(1,069; 2,537)
height BTH+I 1	60	4,813	1,588	(4,079; 5,547)
height BTH+I 2	60	10,752	2,443	(10,018; 11,486)
height NEEM+I 1	60	2,925	1,510	(2,191; 3,659)
height NEEM+I 2	60	1,067	1,807	(0,333; 1,801)

Pooled StDev = 2,89549

Fisher Pairwise Comparisons

Grouping Information Using the Fisher LSD Method and 95% Confidence

Factor	N	Mean	Grouping				
height control 2	60	12,235	A				
height NEEM 2	60	11,802	A				
height BTH+I 2	60	10,752		B			
height BTH2	60	10,253		B			
height NEEM 1	60	6,567			C		
height control 1	60	5,912			C		
height BTH+I 1	60	4,813				D	
height BTH 1	60	4,793				D	
height INFECTED 1	60	2,940					E
height NEEM+I 1	60	2,925					E
height INFECTED 2	60	1,803					F
height NEEM+I 2	60	1,067					F

Means that do not share a letter are significantly different.

Appendix 3: Analysis of variance for disease susceptibility data (2nd experiment)

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0,05

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	6	IC1; IC2; BTH+I (1); BTH+I (2); NEEM+I (1); NEEM+I(2)

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	5	234,6	46,919	15,89	0,000
Error	324	956,6	2,953		
Total	329	1191,2			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
1,71830	19,69%	18,45%	16,73%

Means

Factor	N	Mean	StDev	95% CI

IC1	60	1,533	1,631	(1,097; 1,970)
IC2	60	2,867	1,818	(2,430; 3,303)
BTH+I (1)	45	0,244	0,933	(-0,259; 0,748)
BTH+I (2)	45	1,867	2,018	(1,363; 2,371)
NEEM+I (1)	60	1,050	1,630	(0,614; 1,486)
NEEM+I(2)	60	2,400	1,976	(1,964; 2,836)

Pooled StDev = 1,71830

Fisher Pairwise Comparisons

Grouping Information Using the Fisher LSD Method and 95% Confidence

Factor	N	Mean	Grouping			
IC2	60	2,867	A			
NEEM+I(2)	60	2,400	A	B		
BTH+I (2)	45	1,867		B	C	
IC1	60	1,533			C	D
NEEM+I (1)	60	1,050				D
BTH+I (1)	45	0,244				E

Means that do not share a letter are significantly different.

Appendix 4 : Analysis of plant height variance (2nd experiment)

Method

Null hypothesis	All means are equal
Alternative hypothesis	Not all means are equal
Significance level	α = 0,05

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	12	control 1; control 2; BTH 1; BTH 2; NEEM 1; NEEM 2; BTH+I (1); BTH+I (2); NEEM+I (1); NEEM+I(2); INFECTED 1; INFECTED 2

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	11	2908	264,35	19,02	0,000
Error	678	9423	13,90		
Total	689	12330			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
3,72795	23,58%	22,34%	20,89%

Means

Factor	N	Mean	StDev	95% CI
control 1	60	8,873	2,772	(7,928; 9,818)
control 2	60	11,625	3,461	(10,680; 12,570)
BTH 1	60	6,790	2,978	(5,845; 7,735)
BTH 2	60	9,948	4,080	(9,003; 10,893)
NEEM 1	60	7,045	3,602	(6,100; 7,990)
NEEM 2	60	9,745	5,063	(8,800; 10,690)
BTH+I (1)	45	5,807	2,561	(4,716; 6,898)
BTH+I (2)	45	8,962	3,676	(7,871; 10,053)
NEEM+I (1)	60	5,408	3,173	(4,463; 6,353)
NEEM+I(2)	60	7,318	4,673	(6,373; 8,263)
INFECTED 1	60	4,760	2,736	(3,815; 5,705)
INFECTED 2	60	5,893	4,642	(4,948; 6,838)

Pooled StDev = 3,72795

Fisher Pairwise Comparisons

Grouping Information Using the Fisher LSD Method and 95% Confidence

Factor	N	Mean	Grouping			
control 2	60	11,625	A			
BTH 2	60	9,948		B		
NEEM 2	60	9,745		B		
BTH+I (2)	45	8,962		B		
control 1	60	8,873		B		
NEEM+I(2)	60	7,318			C	
NEEM 1	60	7,045			C	D
BTH 1	60	6,790			C	D
INFECTED 2	60	5,893				D E
BTH+I (1)	45	5,807				D E
NEEM+I (1)	60	5,408				E
INFECTED 1	60	4,760				E

Means that do not share a letter are significantly different.

Appendix 5 : Histological examination of host responses

Method

Null hypothesis	All means are equal

Alternative hypothesis	Not all means are equal
Significance level	α = 0,05

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
Factor	6	I H; I N; BTH+I H; BTH + I N; Neem+I H; Neem+I N

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Factor	5	367,2	73,4371	154,30	0,000
Error	526	250,3	0,4759		
Total	531	617,5			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0,689876	59,46%	59,08%	58,16%

Means

Factor	N	Mean	StDev	95% CI
I H	59	2,712	1,885	(2,535; 2,888)
I N	59	0,0339	0,1825	(-0,1425; 0,2103)
BTH+I H	106	0,0189	0,1943	(-0,1128; 0,1505)
BTH + I N	106	0,1415	0,4668	(0,0099; 0,2731)
Neem+I H	101	0,000000	0,000000	(-0,134853; 0,134853)
Neem+I N	101	0,1584	0,3933	(0,0236; 0,2933)

Pooled StDev = 0,689876

Fisher Pairwise Comparisons

Grouping Information Using the Fisher LSD Method and 95% Confidence

Factor	N	Mean	Grouping	
I H	59	2,712	A	
Neem+I N	101	0,1584		B
BTH + I N	106	0,1415		B
I N	59	0,0339		B
BTH+I H	106	0,0189		B
Neem+I H	101	0,000000		B

Means that do not share a letter are significantly different.

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