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)

PhD Dissertation

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Declaration

I hereby declare that the work presented in this thesis has not been submitted for any other degree or professional qualification, and is the result of my independent work.

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

BABA	Beta aminobutyric acid
BCA	Biocontrol agents
BGPF	Plant growth-promoting fungi
BTH	benzo[1,2,3]thiadiazole-7-carbothioic acid-S-methyl ester
BW	Bidistelled water
℃	Celsius
CAT	Catalase
DPI	Days post infection
DS	Disease severity
EDTA-Na_2	Ethylenedinitrilotetraacetic acid disodium salt dihydrate
HCL	Hydrochloric acid
IPM	Integrated pest management
ISR	Induced systemic resistance
JA	Jasmonic acid
MIC	Minimal inhibitory concentration
MX	Mefenoxam
AZA	Azadirachtin
PDA	Potato dextrose Agar
PH	*Plasmopara halstedii*
POX	Guaiacol Peroxidase
PPO	Polyphenol oxidase
PAL	Phenylalanine ammonia-lyase
PR	Pathogenesis-Related Protein
PVP	polyvinylpyrrolidone
rpm	Rotary per mint
SA	Salicylic acid
SAR	Systemic acquired resistance
SDM	Sunflower downy mildew
SOD	Superoxidase
T	*Trichoderma asperellum*
w/v	Weight per volume
WSI	Whole seedlings immersion method

Chapter 1: INTRODUCTION

1.1 Background

Sunflower (*Helianthus annuus* L.) is one of the most important oilseed 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). Sunflower plants can adapt to a variety of environmental variables. It is primarily a cross-pollinated crop with excellent production potential and is suitable for sawing throughout the year because of its photosensitivity (Qi et al. 2016). This plant has been discovered in several parts of the world, including Tabasco, Mexico, at the San Andres dig site (Murphree, 2012). In 1510, some early Spanish explorers brought sunflower seeds from the Americas to Europe, according to another legend (Putt, 1997). Therefore, the sunflower has become one of the four plants known to have been domesticated in eastern North America (Smith, 2006).

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). Sunflower is a fast-growing oilseed crop that is often ready for harvest within 90–120 days (Joksimovic et al. 2006; Yousef, 2021). In terms of economic value, the sunflower industry generates significant revenue for farmers, seed companies, and the food-processing industry. For example, in the United States, sunflower production contributes more than $1.5 billion to the economy each year (National Sunflower Association, n.d.). Similarly, sunflower production in the European Union (EU) generates an estimated €6 billion annually (European Commission 2021).

Unfortunately, various diseases, such as *P. halstedii* (Farl.) Berl. et de Toni infection is reducing sunflower production. The pathogen is regarded as one of the most dangerous threats to sunflower crops worldwide (Viranyi and Spring, 2011). To acquire successful disease management, it is essential to be acquainted with the physiological capacity, living needs, and biology of the pathogen. Certain diseases exhibit tremendous phenotypic variations, particularly in terms of pesticide sensitivity and virulence (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 a global distribution of the disease. The presence of this pathogen can have a significant impact on sunflower yield, with an estimated 3.5% reduction in commercial seed production when current control measures are used.

However, in fields with high levels of contamination, yield losses can reach up to 100%. The host stage of infection determines the symptoms of *P. halstedii* infection (Meliala *et al.* 2000). Primary infection, followed by the direct movement of zoospores into the roots, results in the dwarfing of infected plants, chlorosis along leaf veins, and small heads with sterile seeds (Jocic et al. 2012). Damping-off may develop from a severe infection. Zoospores and sporangia that form under leaves do not transmit the disease or cause crop damage. Secondary infections can become systemic and cause plant dwarfism (Spring 2009). Secondary infections can spread latently with seeds, and the widespread use of chemicals has become a significant concern because they harm human health and the environment.

Owing to its 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). One of the eco-friendly ways of controlling plant disease is by using biotic agents that stimulate the host's defensive mechanisms. Biological agents, such as microbes and plant extracts, have been used in various host-pathogen systems to elicit systemic resistance in multiple crops. At the cellular level, the accumulation of lignin, callose, phenols, and other antimicrobial compounds is the primary resistance mechanism supplied by chemical inducers (Basavaraj et al. 2019).

Several defensive processes are associated with the activation of plant disease resistance and pre-existing chemical and physical barriers against pathogen invasion to avoid or prevent phytopathogenic infection. Other plant resistance mechanisms include inducible defensive responses, such as the activation of defense-related enzymes in response to pathogen infection. Peroxidases are defense-related enzymes involved in several plant defense systems that play crucial roles in the interaction between plants and pathogens (Shivakumar et al. 2003). These effects include activation of the pathogen hypersensitivity response, generation and suberisation of ethylene, and biosynthesis of lignin (Shivakumar et al. 2003).

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 (Kogan, 1998; Ehler, 2006). IPM uses less toxic pesticides or natural biocontrols only when an economic threshold of insect damage is achieved (Kogan, 1998; Ehler, 2006). Long-term pest control requires IPM as part of a crop management system redesigned to reduce insect pressure, pesticide use, and farmer costs, while protecting the environment and human health (Barzman et al. 2015; Colbach & Cordeau, 2018; Pretty, 2018). IPM is a potential method for controlling sunflower downy mildew (Ruiz 2022).

1.2 Problem statement

The widespread use of chemicals has raised significant concerns owing to their negative impacts on human health and the environment. To address this issue, induced resistance can be utilised as an eco-friendly alternative to synthetic drugs for disease control. Induced systemic resistance occurs when an inducer activates the host plant's natural defensive mechanism. Biotic agents such as microbes and plant extracts can also be used to stimulate the host's defensive mechanisms as a means of plant disease control. Various host-pathogen systems utilise biological agents to elicit systemic resistance in multiple crops.

At the cellular level, the primary mechanism of resistance against pathogens involves drugs that cause the accumulation of lignin, callose, phenols, and other antimicrobial compounds. The combination of different chemicals or active ingredients can help prevent pesticide resistance. The integrated components must be homogenised and work synergistically to affect the target organism. Thus, it is essential to evaluate the synergistic effects of different ingredients to enhance the plant's defense against pathogens and assess how this synergistic effect affects pathogens growing inside the plant.

P. halstedii is a biotrophic oomycete pathogen that causes downy mildew disease in sunflower plants. This pathogen causes significant yield losses and poses a serious threat to sunflower production worldwide. However, *P. halstedii* can overcome both the genetic and chemical resistance mechanisms. The pathogen can evolve quickly, and new races that can overcome previously resistant sunflower varieties can emerge. In addition, the use of chemical compounds can lead to the development of resistant pathogen populations. Therefore, a multifaceted approach, including the utilisation of genetic and chemical resistance mechanisms, as well as cultural practices, is necessary to effectively manage the downy mildew disease caused by *P. halstedii* in sunflower crops.

1.3 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 differing in virulence and aggressiveness 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 *Trichoderma asperellum*.) and (azadirachtin, BTH, and mefenoxam) to control different *P. halstedii* isolates

3- Evaluate the 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 the 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: LITERATURE REVIEW

2.1 Classification, history, and phylogenetic distribution of *P. halstedii*

There are approximately 500 species belong to the Oomycetes order recognised as pathogens for animals, plants, fungi, bacteria, and other species. Most oomycete infections worldwide cause substantial reductions in crop yields (Walker & van West, 2007). Oomycetes (lso known as water moulds) are often considered to be saprophytic and harmful obligatory parasites (Vallance et al. 2009). Grapevine downy mildew, sunflower downy mildew, late blight in potatoes and tomatoes, seedling blights, damping-off, root rot, and foliar blights are the most prominent diseases caused by the genus Oomycetes (Vallance et al. 2009).

Downy mildew in sunflowers is caused by the biotrophic pathogen *P. halstedii*, which exists in soil, seeds, and as an airborne organism (Wehtje & Zimmer, 1978). *P. halstedii* belongs to the Peronosporales group of oomycete pathogens, one of the largest groups of oomycetes. Other groups of downy mildew pathogens include hemi-biotrophs fungi, such as the *Phytophthora* genus, and other obligate pathogens (Gascuel et al. 2015). All downy mildew pathogens are obligate and require specific environmental and nutritional conditions to complete their life cycles in live plants. The genus Plasmopara includes several other plant pathogenic species, including *Plasmopara viticola*, the causal agent of grapevine downy mildew (Kamoun et al. 2015).

After World War II, Eastern European scientists reexamined and corrected the taxonomic classification of *P. halstedii*, a finding that has been referenced in other studies (Zimmer & Hoes, 1978; W. Sackston (1981), Viranyi and Oros (1991), Gulya et al. 1997). In 1883, Farlow attempted to describe the downy mildew pathogen *Peronospora halstedii* based on pathogen samples collected from various perennial sunflower species (*Helianthus strumosus*, *H. tuberosus*, and *H. doronicoides* (*Helianthus mollis X giganteus*)) as well as from several flowering weeds belonging to the Asteraceae family, including *Aster dactyloides*, *Rudbeckia laciniata*, *Ambrosia artemisiifolia*, *Silphium terebinthaceum*, *Bidens frondosa*, and *Eupatorium purpureum*.

In 1876, Halsted-type downy mildew was first discovered in the *Eupatorium purpureum* weed near the Bussey faculty. Schröter (1886) distinguished between *Plasmopara* and *Peronospora* based on the germination of *Peronospora* through zoospores instead of germ tubes (Thines & Choi, 2016). Later, Berlese and de Toni (1888) reclassified the taxon as *P. halstedii* (Farl) Berl and de Toni. The host range used in this study consisted only of perennial *Helianthus* species, not annuals, from seven different families (Viranyi & Spring, 2011). During the field experiment, no symptoms of downy mildew were observed in any other host plants, except for *H. annuus* and a few other annual species of the *Helianthus* genus. As a result, Novotelnova (1962) decided to rename the species complex using Farlow's nomenclature of *P. halstedii* for the species

identified on Eupatorieae and separating the downy mildew on sunflowers as *Plasmopara helianthi* Novot. The genetic material of the two pathotypes, Farlow's *P. halstedii* and *P. helianthi* Novot, has been re-evaluated using modern molecular technology following the oomycete phylogeny. This re-categorisation of the two pathotypes after almost 50 years has altered all taxonomic levels (Viranyi and Spring, 2011).

2.2 *P. halstedii* host range and pathotype distribution

P. halstedii was originally detected in North America, which is the native home of *H. annuus*, the *Helianthus* species with the widest global spread (Sackston 1981). Among the 49 *Helianthus* species, only 11 are susceptible to *P. halstedii* according to the US National Fungus Collection, BPI storage, and infection in wild sunflowers is exceedingly rare (Viranyi & Spring, 2011). The pathogen *P. halstedii* has been identified worldwide, with the exception of Australia (Gulya, 2007; Virányi, 2008). However, the origins of *P. halstedii* remain obscure and complex (Viranyi & Spring, 2011). Most of the pathotypes have been discovered in Europe, Spain, Germany, and France. Nevertheless, this pathogen has also been found in Canada, South Africa, and the United States (Virányi, 2008). In addition, five pathotypes of *P. halstedii* (770, 330, 300, 730, and 710) were distributed across North and South America, Europe, and Africa. France accounted for the greatest number of newly identified pathotypes in the last few years (Vear et al. 2007).

P. halstedii is believed to have a high potential for developing new pathotypes owing to genetic changes, with over 35 pathotypes reported in 2006 (Gulya, 2007), which increased to 41 pathotypes in 2014 (Viranyi et al., 2015), and 50 pathotypes were recorded in 2018 (Spring, 2019). In Hungary, six of the 14 pathotypes of *P. halstedii* in Europe were discovered before 2010 (Bán et al. 2014), with the novel pathotype 704 identified in mid-June 2012 (Bán et al. 2014) and pathotype 724 discovered in 2018 (Bán et al. 2018). Pathotype 734 was recently reported by Nisha et al. (2021). New pathotypes of the pathogen emerge as a direct result of breeding and selection processes for susceptible pathogen genes (Viranyi & Spring, 2011). Gulya (1998) proposed the use of the Limpert and Muller naming method to identify *P. halstedii* pathogens, and a new nomenclature for *P. halstedii* pathotypes was approved in 2000 at the ISA Conference in Toulouse (Gulya, 1998).

P. hastedii has been found in various wild plants, including ragweed (*Ambrosia artemisiifolia*) (Walcz et al. 2000). According to Komjáti et al. (2007), the pathogen has been observed to transfer to *H. annuus* from other related species like *Xanthium strumarium*. Several downy mildew symptoms were identified in commercial farms of the Florida plant *Ageratum houstonianum* (floss flower) in 2016 and 2018. The infection was identified as *P. halstedii* after

laboratory testing and microscopic analysis of the symptoms. Pisani et al. (2019). Artificial cross-infection of sunflowers with zoosporangia isolated from various wild species under laboratory conditions has proven to be effective (Cohen & Sackston, 1973). However, under field circumstances, the natural infection of cultivated sunflowers with sporangia of downy mildew from wild Asteraceae host species has yet to be demonstrated (Viranyi & Spring, 2011).

2.3 Common diseases affecting sunflowers and their economic impacts

Sunflower cultivation is prone to approximately 40 different plant diseases; however, only a few of these infections are considered the most detrimental, causing significant decreases in seed yield. Among the fungal diseases that affect sunflowers are downy mildew, rust, Sclerotinia stalk, phoma black stem, Verticillium wilt, head rot, and leaf spot (Sackston, 1981; Mukhtar, 2009; Markell et al.2015).

Downy mildew caused by *P. halstedii* has a considerable economic impact on production regions (Jocic et al. 2010). For example, Gascuel et al. (2015) reported that the damage caused by *P. halstedii* infection could potentially reduce commercial sunflower seed output by 3.5%, and that the infection may progress up to 100% in contaminated fields. Limiting sunflower cultivation in severely infected areas is crucial to prevent the spread of the disease (Gascuel et al. 2015). *P. halstedii* has a broad host range and can potentially promote disease in various plants, including *P. umbelliferarum* on *Umbelliferae*, *Plasmopara viticola* on grapevine, *Plasmopara geranii*, and *Plasmopara pusilla* on geranium. The pathogen has a significant impact on flower production of *Helianthus argophyllus*, *Helianthus debilis*, *Helianthus petiolaris*, and *Helianthus annuus*, as well as many other Helianthus species (Gascuel et al. 2015).

Table 1: List of the most common sunflower diseases (Mukhtar, 2009).

Number	Name disease	Pathogen	Reference
1	Charcoal rot	*Macrophomina phaseolina*	(Jalaluddin, 2008).
2	Sclerotinia stalk wilt and head rot	*Sclerotinia sclerotiorum*	(Mirza & Yasmin, 1984).
3	Alternaria blight	*Alternaria* spp.	(Sackston, 1978)
4	Septoria leaf spots	*Septoria helianthi*	Henry & gilbert (1924) a
5	Rhizopus head rot	*Rhizopus* spp.	(Mirza & beg, 1983)
6	Verticillium wilt	*Verticillium dahliae*	
7	Rust	*Puccinia helianthi schwein*	(Qureshi & Jan 1993).
8	Downy mildew	*P. halstedii* (farl.) Berl and de Toni	(Viranyi, 1990)
9	Seed borne diseases	Alternaria spp, *Aspergillus* spp, *Cladosporium* spp, *Curvularia* spp, *Drechslera* spp, *Fusarium* spp and *Penicillium* spp. *Phomopsis macdonaldii*	(Mukhtar,2009).
10	Phomopsis stem canker	*Phomopsis helianthi, Diaporthe gulyae*	Berglund (2007), Gulya et al. (1997), Thompson et al. (2011)

2.4 Biology, life cycle, and infection mechanisms of *P. halstedii*

Oomycetes were previously classified as fungi because of their similar appearance and ecological role; however, molecular studies have revealed that they are actually a distinct group of organisms more closely related to algae than fungi (Agrios, 2005). Oomycetes, including *P. halstedii*, possess filamentous structures and absorb nutrients from their surroundings. They can reproduce both sexually and asexually, with asexual reproduction involving the production of motile zoospores that can move towards the plant host (Gascuel et al. 2015). In laboratory studies, *P. halstedii* displays both sexual and asexual reproduction (Spring 2000a). Although asexual reproduction is crucial during the growing season for disease development, sexual reproduction is necessary to produce overwintering oospores (Gascuel et al. 2015).

In the context of downy mildew infection in sunflowers, certain climatic and environmental conditions are known to promote growth. The extent of damage to sunflower plants may range from 1 to 100 percent, depending on the severity of the infection (Jocic et al. 2010). Sunflower seedlings are susceptible to primary infections during seed germination, although this vulnerability is limited to a short period (Zimmer, 1971). Oospores or airborne sporangia produce motile zoospores that infect seedlings, either before or after emergence. Subsequently, the infection spreads to intracellular and intercellular hyphae, which then branch into the leaf and stem tissues (Wehtje & Zimmer, 1978).

In the context of sunflower infections, systemic infection is initiated once hyphae reach the apical meristem, although the precise penetration site responsible for the systemic illness has not been conclusively identified (Wehtje & Zimmer, 1978). Previous research has indicated that root hairs and epidermal cells represent the primary points of entry for the initial infection (Novotelnova, 1962), whereas hypocotyls have been shown to be the main entry site for systemic infections originating from the hypocotyls (Cohen & Sackston, 1973). Secondary infections are responsible for the development of systemic symptoms that mainly affect apical meristems, although such symptoms are infrequent in field plants.

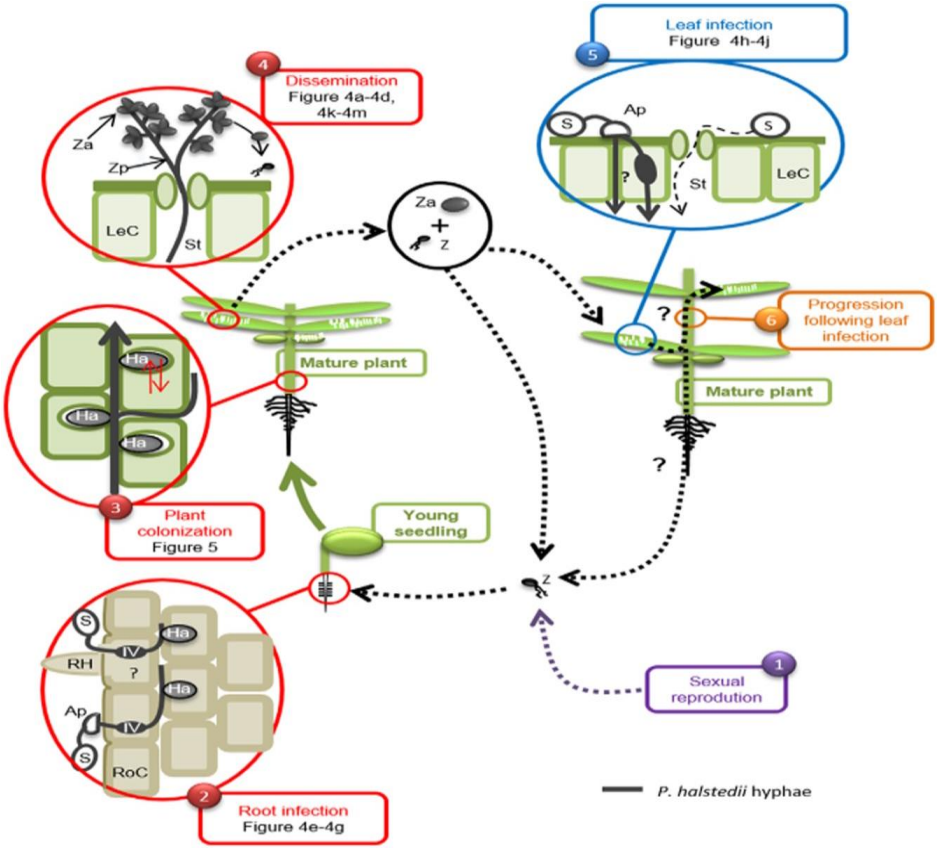


Figure 1: Life cycle of sunflower downy mildew (*P. halstedii).* (Gascuel, et al. 2015).

The life cycle of *P. halstedii* comprises several stages. First, during spring, biflagellate zoospores (Z) are discharged into soil-free water from zoosporangia formed by overwintering sexual oospores. Upon contact with a sunflower root, spore (S) encystment occurs, and there are two possible entry points for zoospores: (i) directly into a root cell through an appressorium, a swelling where the pathogen raises osmotic pressure and drills root epidermal cells, or (ii) via lesions typically found at the base of the root hair (RH). Plant epidermal cells infected with appressoria form infected vesicles (IV). To reach and colonise shoot tissues, pathogen hyphae (thick gray line) must travel further through the intercellular gaps between the cortical root cells. *P. halstedii* forms many infection-and-nutrition-supporting structures called haustoria during this stage of infection (Ha). Ideal circumstances for *P. halstedii* to emerge on the abaxial surface of leaves and cotyledons via stomata (St) occur during moderate temperatures in late spring and summer. Dissemination structures, called zoosporangiophores (Zp), are generated and convey zoosporangia (Za), which can spread to other plant leaves and contaminate them. They released up to 20 zoospores (Z). Appressoria form when spores encyst in leaf trichomes or near veins encyst and germinate within a few hours (thick gray line). Stomatal penetration (thin dotted line) is observed in some cases. Pathogen development in plant tissues following leaf infection remains poorly understood.

2.5 Symptoms of *P. halstedii* infection

According to Musetti et al. (2005), the primary infection of sunflower downy mildew is caused by zoospores in the root system that germinate in early spring. In contrast, *P. viticola* infection in grapevines begins in leaves and fruits. The initial manifestation of sunflower downy mildew induced by zoospores occurs in the roots, leading to crop loss due to sterile blooms (Figure 1), as reported by Sackston (1981). The infection causes severe symptoms, including dwarfism, resulting from hormonal imbalance in the plant and nutrient uptake by the pathogen. Additionally, spores present on leaf surfaces may serve as secondary sources of infection for surrounding plants (Sackston, 1981). Pathogen sporulation in flower bodies causes sunflower loss, which leads to the development of sterile flowers. Consequently, various pathological symptoms have evolved, including damping-off from root damage (Gascuel et al. 2015). Mouzeyar et al. (1993) observed that infected plants exhibited dwarfism, chlorosis, root browning, and alterations in secondary metabolism (Figure 2).

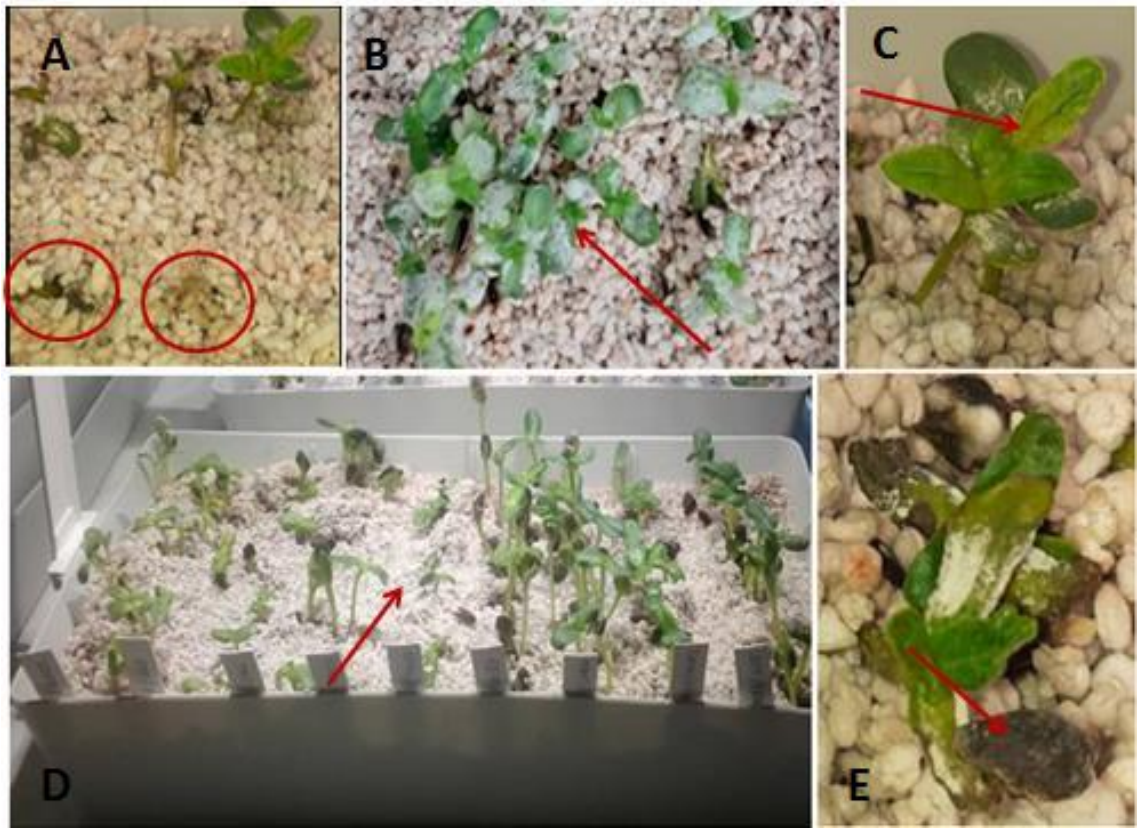


Figure 2: *P. halstedii* disease symptoms and signs under laboratory conditions, in case of artificially infected sunflower seedlings. Damping-off (A), sporulation on the leaf surface (B), leaf chlorosis (C), plant stunting, represented by the arrow (D) (all cases is in the susceptible variety (Iregi)) (Source: Ahmed Yousif)

2.6 Conventional control methods for the sunflower downy mildew

2.6.1 Metalaxyl as a fungicide for controlling sunflower downy mildew

Metalaxyl is a systemic fungicide derived from amino acids and alanine, with the chemical formula Methyl DL-(2,6-dimethyl phenyl)-N(2methoxyacetyl)-N alaninate (Singh & Shetty, 1990). It has been extensively used against various Peronosporales species, including downy mildew, to protect vulnerable cultivars during commercial seed production (Singh and Shetty 1990). Metalaxyl prevents sporangial germination and asexual sporulation in infected plants when sprayed at a concentration of 250 ppm, and the delivery route influences the infectiousness of sporangia (Singh & Shetty, 1990). Upon application, the fungicide is absorbed by plant parts and disseminated through water into the surface-tread seeds (Singh & Shetty, 1990). Fungicide has been detected in all plant components for up to 45 d, with leaves accumulating more than stems and roots for 60 d (Singh & Shetty, 1990).Sunflower downy mildew can be effectively controlled using resistant sunflower cultivars and seed-coating fungicides such as metalaxyl-M (mefenoxam) (Molinero-Ruiz et al. 2008). Molinero-Ruiz et al. (2008) evaluated the effectiveness of two active

components, Metalaxyl and Metalaxyl-M, in controlling downy mildew on sunflowers. However, numerous studies have reported that these compounds are less effective against *P. halstedii* (Gulya, 2000; Albourie et al. 1998).

2.6.2 Genetic resistance to *P. halstedii*

Sedláová et al. (2016) reported that *P. halstedii* is capable of rapidly evolving and potentially generating a race that can overcome sunflower resistance genes. PI genes found in sunflowers, which have been traced back to the wild species *Helianthus annuus* and other *Helianthus* species, are considered to be the primary source of resistance (Vear et al. 2008). These genes encompass approximately 20 genes that confer resistance to various *P. halstedii* pathotypes (Gascuel et al. 2015). Currently, more than 40 major resistance genes for downy mildew have been reported, including Plv-Plz, Pl1-Pl35, and PlArg. These genes have been identified in several studies. Wild sunflowers possess desirable traits and exhibit resistance to downy mildew and other diseases (Molinero-Ruiz 2022). PI genes utilise two different mechanisms to confer resistance against *P. halstedii*: type (A) resistance, which does not present visible symptoms on shoots and exhibits no pathogen growth on the upper part of the hypocotyls; and type (B) resistance, where sporulation occurs only on the cotyledons, whereas the upper sections and true leaves of the plant remain asymptomatic (Mouzeyar et al. 1993).

The PI gene group is known to confer resistance to all wild sunflower variants of *P. halstedii*. Although plants may harbour multiple resistance genes, they are only resistant to a single pathotype (Seiler, 1998) and novel pathotypes of *P. halstedii* can easily overcome resistance. Tourvieille et al. (2005) suggested a novel breeding strategy that combined significant genes to reduce the severity of downy mildew infection. The inbred line RHA340 is a resistant hybrid carrying the PI8 gene, and is one of the nine differential lines used to characterise pathogenic *P. halstedii* pathotypes (Martin-Sanz et al. 2019). In the spring of 2018, *P. halstedii* overcame resistance in commercial varieties with the PI8 gene (Martin-Sanz et al. 2019). However, Vear et al. (2007) utilised a different type of resistance that does not depend on the pathogen-host interaction by utilising partial resistance in sunflower varieties that do not possess the PI gene, which is sufficient to reduce the disease.

2.7 Induced resistance in plants

In the mid-1980s, induced resistance in plants became a novel phenomenon investigated by scientists as a potential approach for managing plant diseases. The development of ecologically friendly, non-toxic, and safe techniques for plant disease prevention prompted the study of the genes involved in plant defense and how signals influence the function of these genes, which has

become a new field of research (Hammerschmidt & Kuc, 2013). Biotic and abiotic stimuli, including microbes (bacteria, fungi, viruses, and mycorrhiza), chemicals (e.g. benzothiadiazole and acibenzolar-S-methyl), plant extracts, and cell wall fragments, can trigger plant resistance. These conditions may activate the plant defense system locally or systemically to prevent further pathogen invasion (Walters & Fountaine, 2009). SAR can be induced by chemical inducers and necrotrophic infections, whereas ISR is triggered by plant growth-promoting rhizobacteria (PGPR) that invade plant roots (Spoel & Dong, 2012).

2.7.1 Types of induced systemic resistance in the plants

The phenomenon of induced systemic resistance (ISR) has been extensively studied to better understand how plants can manage plant diseases. When infected by necrotrophic pathogens, plants accumulate chemical compounds and create physical barriers to defend themselves against infection. However, there is no direct method to eradicate or inhibit pathogens (Conrath et al. 2001). This resistance is mediated by a transactional signalling pathway involving ethylene and jasmonic acid (Hammerschmidt, 1999). In contrast, systemic acquired resistance (SAR) is initiated by the accumulation of pathogen-related protein groups or salicylic acid in the host plant following disease or parasite infestation (Pieterse et al. 1998). SAR is triggered by a pathogenic infection that causes necrotic lesions, either due to a successful infection or the host's hypersensitivity response to the pathogen (Walters et al. 2013). This resistance is more common and effective in suppressing many phytopathogens.

Chemical inducers such as salicylic acid or benzothiazole (BTH) and 2,6-dichloro isonicotinic acid (INA), which operate like salicylic acid, may activate SAR (Hammerschmidt and Ku, 1982; Kessmann et al. 1994). Research suggests that induced resistance may arise from direct induction and cell priming (Ahmad et al. 2010). Activation of SAR in the host plant results in the systemic expression of pathogenesis-related (PR) proteins, which are typically located in the apoplast and can have direct contact with the pathogen during the infection process (Gulya et al. 1997). Among these PR proteins, chitinases and glucanases are particularly important for resistance and other antimicrobial functions (Gulya et al. 1997). Plants transformed with PR genes have been shown to resist various pathogens, which supports the role of PR gene expression in SAR (Alexander et al. 1993; Liu et al. 1994).

However, plants genetically modified with PR genes may not exhibit high levels of resistance to all phytopathogens (Linthorst et al. 1989). Furthermore, studies have shown that PR proteins are responsible for the systemic resistance phenotype in plants (Walters et al. 2013). In SAR, PR proteins utilise two distinct strategies to suppress pathogens. The first strategy involves hydrolytic activity on the pathogen cell wall, inhibiting the development of diseases caused by

pathogens such as fungi, oomycetes, and bacteria (Van Loon, 1997; Van Loon et al. 1998). However, determining the precise mechanisms of action of PR proteins in plants remains a primary challenge. Specific approaches are necessary to demonstrate the direct antibacterial effects, pathogen lysis, and increased elicitor release in plants with acquired resistance.

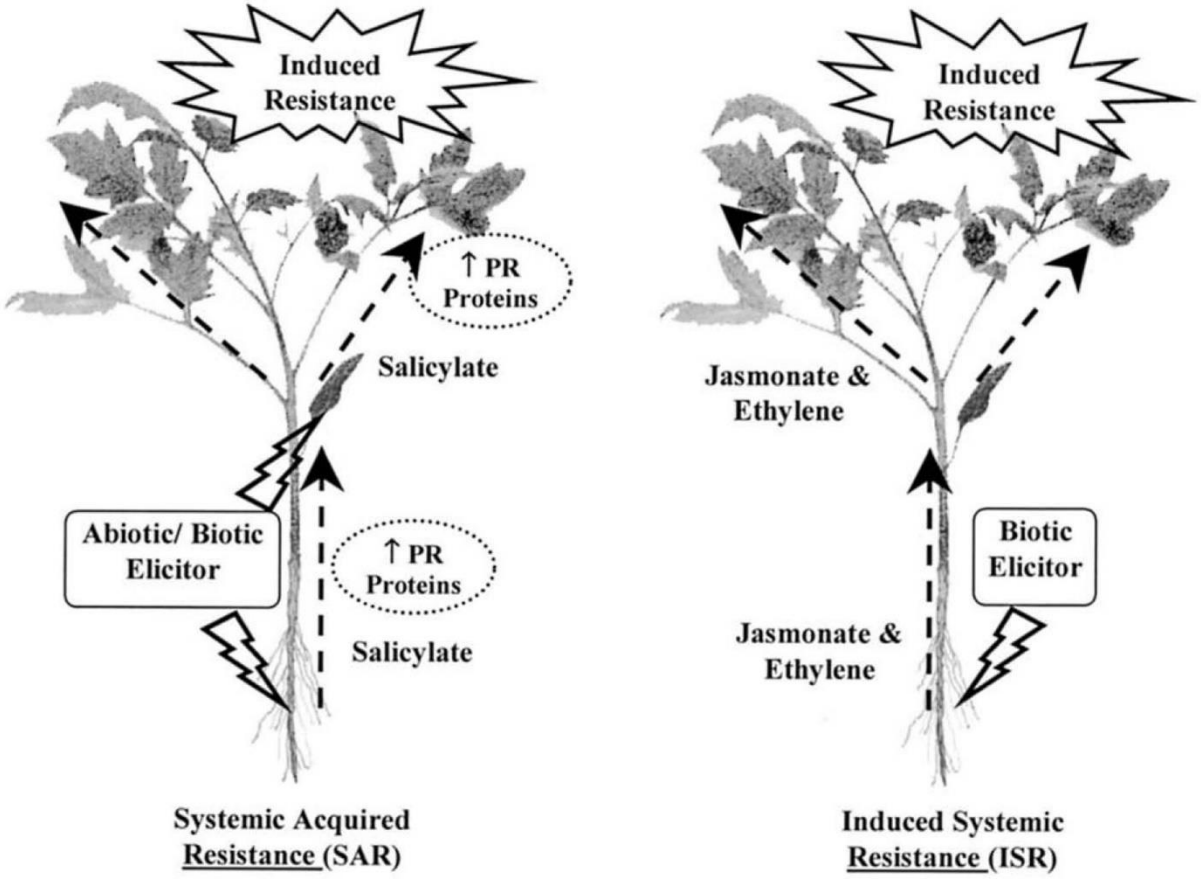


Figure 3 Comparison of Induced Resistances: SAR vs ISR (Vallad & Goodman, 2004).

2.7.2 Factors affecting induced resistance in plants

The efficacy of induced resistance agents is influenced by several factors, including host genotype (Tucci et al. 2011). Studies have shown that the application of BABA and ASM to foliar pathogens across different barley varieties results in diverse induced responses, with some varieties showing no response (Walters et al. 2011). When BABA was used as a resistance inducer in tomato genotypes, a strong response was observed against *Phytophthora infestans*, which causes late blight disease. However, the variability of pathogen isolates significantly affects the resistance response and infection incidence in this case (Sharma et al. 2010). Resistance also increases with leaf age, suggesting a systemic action of γ-aminobutyric acid in tomato leaves (Cohen & Gisi, 1994). In addition to obligate pathogens, mycorrhizal infection may induce plant resistance only to insects and necrotrophic diseases (Pozo & Azcón-Aguilar, 2007).

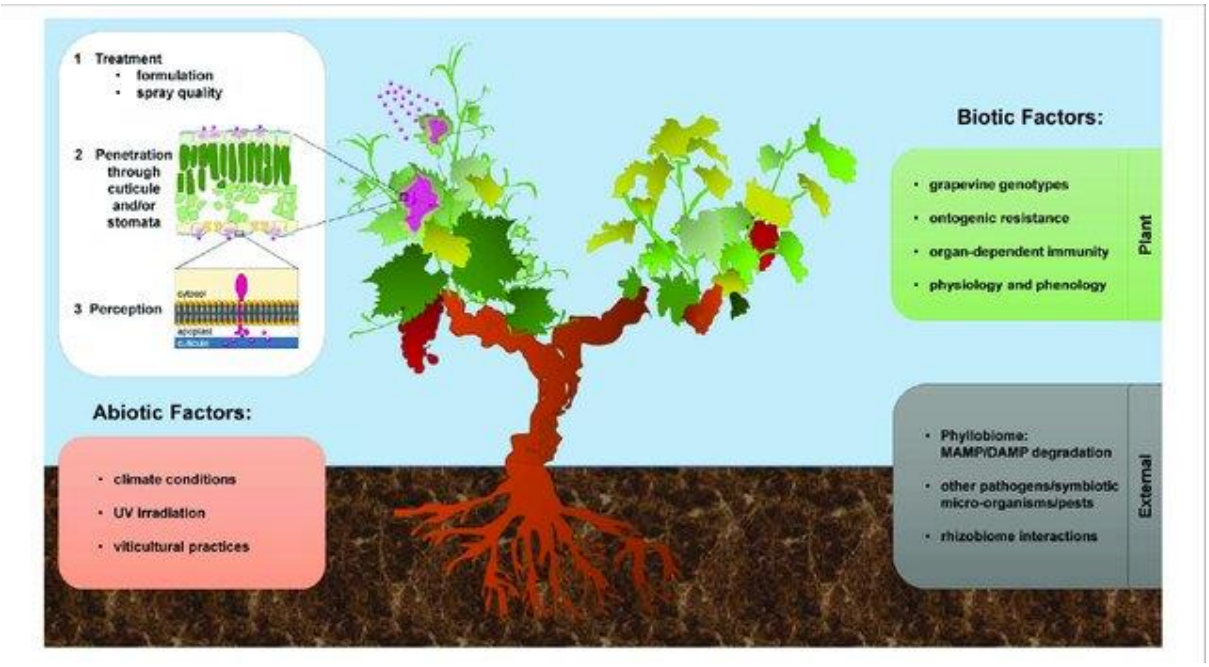


Figure 4: Factors influencing Induced Systemic Resistance Efficiency (Héloir et al., 2019).

Insects, fungi, endophytic bacteria, and nematodes are crucial contributors to plant resistance development (Kosaka et al. 2001; Walters et al. 2005; Bostock, 2005; Kang et al. 2007). Thus, induced plant resistance and gene expression may be influenced by biotic and abiotic factors. For instance, the application of ASM in the field did not result in significant changes in gene expression in treated and untreated plants (Herman et al. 2007). A plant's resistance system is modulated by various factors, including age or life history. Early flowering cultivars show less resistance, whereas perennial varieties with later flowering can induce plant resistance upon treatment with ASM (Heil & Ploss, 2006).

2.7.3 Chemical plant resistance inducer agent (BTH)

Incorporating elicitors into plants can activate their defense systems without physical or chemical invasion. Physical elicitors, such as high and low temperatures, ultraviolet and gamma radiation, and other forms of radiation, are examples of such elicitors. In addition, certain compounds can mimic the actions of signalling molecules like SA or JA, their derivatives, or simulate pathogen attack, such as chitosan, benzothiadiazole (BTH), and 1-methyl cyclopropane (Ruiz & Gómez, 2013). These compounds may interact with plant receptors, inducing defensive reactions and, in some cases, hypersensitivity. Peaches treated with BTH exhibited a lower incidence of *Penicillium expansum* infection than untreated peaches. Hyphal wall components extracted from *Phytophthora* species have been shown to activate the metabolic cascade involved in active defence in tomatoes, producing an internal emergency signal to combat infections. Elicitors have been developed to improve plant resistance to infections. Although they do not kill

pathogens, they stimulate plant defense mechanisms, such as increased synthesis of phenolic compounds (Ruiz & Gómez, 2013).

Various biotic and abiotic stimuli can trigger plant defence mechanisms against pathogen invasion (Lyon, 2007). Probenazole (Oryzemate), the first chemical plant disease inducer, was developed by a Japanese company in 1975. Since then, numerous types of resistance inducers, including biological and chemical elicitors such as acibenzolar-S-methyl (ASM, also known as Bion from Syngenta) and chitosan extract (Elexa), have been developed (Walters et al. 2013). For many years, benzothiadiazole (BTH, trade named Bion 50 WP), a chemical plant resistance activator, has been extensively studied for its potential to provide adequate resistance against wheat powdery mildew (Sticher et al. 1997).

Studies in Italy and Hungary have shown that BTH has a positive impact on the development of resistance to sunflower Downy mildew in both field and greenhouse settings (Tosi et al. 1998; Bán et al. 2004). The application of various elicitors to plants has been shown to be an effective strategy for increasing the phenolic content. Once BTH was proven to be capable of activating systemic acquired resistance (SAR), it was widely accepted. Post-harvest banana and mango treatments triggered increases in polyphenol oxidase (PPO), peroxidases, and total phenolic content (TPC). In addition to the aforementioned studies on enzyme activity, researchers have investigated the beneficial effects of BTH on polyphenolic compounds in various plant species. For instance, grapevines treated in the field with BTH have greater resistance to Botrytis cinerea and produce more resveratrol and anthocyanins (Ruiz & Gómez, 2013).

2.7.4 Plant extracts as resistance inducers against plant diseases

As conventional agricultural chemicals are often harmful to the environment, eco-friendly options, such as botanical products, have been explored as safe alternatives for plant protection. Therefore, there is a need for other strategies to enhance a plant's natural resistance to infections. Using natural-based plant protection solutions can boost crop yields and provide effective control against phytopathogens, while being more environmentally friendly than chemical pesticides, as they are biodegradable. *Frangula alnus* bark extract (FABE) and *Rheum palmatum* root extract (RPRE) have been found to provide significant resistance to *P. viticola* in Vitis vinifera leaves through various mechanisms, including the activation of HR, induction of phytoalexin accumulation, and promotion of peroxidase activity (Godard et al. 2009). Devaiah et al. (2009) discovered that *D. metel* extract increased salicylic acid (SA) concentration and the levels of defence-related enzymes, peroxidase, -1,3-glucanase, and chitinase. *Azadirachta indica* and

Reynoutria sachalineusis water-leaf extracts have been found to enhance plant resistance to powdery mildew and leaf stripe disease in barley (Daayf et al. 1995; Paul & Sharma, 2002).

Methanolic extracts from eight medicinal plants were evaluated for their effectiveness against Plasmopara halstedii, the causative agent of sunflower downy mildew. Among these extracts, Morinda citrifolia, Zingiber officinale, and Tinospora cordifolia showed the most promising results in reducing sporulation of *P. halstedii* in both whole leaf and leaf disc assays. Phytochemical analysis of these extracts revealed the presence of various bioactive compounds including alkaloids, glycosides, steroids, flavonoids, reducing sugars, phenolics, tannins, saponins, and terpenoids (Niranjana et al. 2014). Singh and Shetty (1990) reported that extracts of garlic (Ajoene) and ginger provide effective protection against powdery mildew infection in pea plants. Similarly, *Datura metel* extract has been found to activate plant resistance against *pearl millet* downy mildew (Shivakumar et al. 2003). Various defensive enzymes including peroxidase, phenylalanine ammonia lyase, and polyphenol oxidase have been explored to enhance plant resistance to phytopathogens (Shivakumar et al. 2003). However, limited research has been conducted on the plant extracts, particularly neem extracts, as inducers of plant disease resistance.

2.7.5 Azadirachtin as botanical fungicide and disease resistance stimuli

The Meliaceae family includes *Azadirachta indica*, commonly known as neem, an evergreen Indian tree that holds promise as a source of compounds with pest control, antimicrobial, and medicinal properties (Udoh et al. 2012). For centuries, neem has been used as traditional therapy for various human ailments in India. There are approximately 700 herbal formulations based on the neem in Ayurveda, Siddha, Unani, Amchi, and other regional health practices. Its potential use as an herbal pesticide and in various therapeutic formulations has garnered significant interest in China, the United States, France, Germany, and Italy (Aronson et al. 2016). The US National Academy of Science acknowledged the therapeutic benefits of neem in a report titled "Neem-A Tree for Solving Global Problems" in 1992. The seed, bark, leaves, and roots of the tree contain neem active chemicals, and more than 300 active compounds have been identified in various parts of the tree. Essential limonoids include azadirachtin, salannin, and nimbin (Siddiqui et al. 2004). Neem limonoids are known for their potent antioxidant, anti-inflammatory, and anti-cancer properties. Azadirachtin, gedunin, and nimbolide are the most extensively studied neem limonoids (Nagini 2014). These limonoids have been found to be effective against cancer by halting the growth and division of cancer cells and preventing apoptosis, invasion, and the formation of new blood vessels. They target multiple oncogenic pathways, and can be used for chemoprevention and treatment (Nagini, 2014).

Owing to its insect-repellent properties, neem extract is commonly used in herbal pesticide formulations, and its active components inhibit the growth of Gram-positive and Gram-negative bacteria (Aronson et al. 2016). In addition, various neem components have been reported to inhibit numerous fungal diseases, such as powdery mildew in peas (Schmutterer, 1988). Neem fruits are the principal source of active compounds against insects (Schmutterer, 1988). Neem leaves have been utilised as botanical pesticides in storage and agriculture (Schmutterer, 1988). Different levels of chemical compounds, such as triterpenoids and azadirachtin, have been isolated from neem seeds, and these chemicals are particularly effective against insects at low concentrations (Campos et al. 2016).

In addition to their antibacterial properties, research has shown that plant extracts may be used to induce resistance against plant diseases (Daayf et al. 1995; Paul & Sharma, 2002). The use of azadirachtin extracts reduced a variety of pathogenic developmental features. In this context, azadirachtin has shown several modes of action in pea leaves, which inhibit the advancement of pathogens by lowering the quantity and formation of multiple germ tubs, haustoria, branches, and colonisation. Furthermore, neem azal induces a hypersensitive reaction (HR) that produces proteins in intercellular fluids (Singh & Prithiviraj, 1997). In tomatoes, neem fruit extract (*A. indica*) significantly increased the activities of phenylalanine ammonia-lyase (PAL), tyrosine ammonia-lyase (TAL), polyphenol oxidase (PPO), peroxidase (POX), and isoenzymes of PPO and POX (Bhuvaneshwari & Paul, 2012).

Figure 5: A. Neem Seed Kernels. **B.** NEEM AZAL® TS Organic pesticide extracts from Neem seed kernels. (Source: Google Images).

2.8 Biocontrol agents as resistance inducers

Biological management or the use of non-pathogenic microbes to manage plant diseases may reduce disease incidence in various ways. Competition for space and resources, the ability of antimicrobial chemical synthesis to inhibit pathogen development, and induced resistance heighten the plant's defensive response (Daayf et al. 2003). *Bacillus sp.*, *Pseudomonas sp.*, *Streptomyces sp.*, fungi such as Coniothyrium and Gliocladium, and the well-known biocontrol agent *Trichoderma* spp. are often used in the biocontrol of plant diseases (Nelson, 2004). Several natural plant disease management strategies have been modelled after some of these biocontrol agents (Nelson, 2004).

These microorganisms reduce disease incidence by directly interfering with the pathogen's life cycle, interfering with its pathogenicity as a result of biochemical substances generated by organisms against the targeted pathogen, or indirectly activating the plant's immune system (Nelson, 2004). The significant impact of bioagents is ascribed to *Trichoderma* spp., *which* stimulates the plant's immune defense system (El Assiuty et al. 1986, p.). *Bacillus subtilis* may produce antibiotics that impede the development of pathogens, such as subtillin, bacillin, and abacillomicin (Loeffer et al. 1986). Moreover, the PGPR strain INR7 (*Bacillus* spp.) significantly enhances resistance to sunflower downy mildew in plants, reducing infection by 51% and 54% under greenhouse and field conditions, respectively (Nandeeshkumar et al. 2008). The application of PGPR (*Bacillus* spp.) strain INR7 to sunflower seedlings also resulted in the activation of several enzymes, including polyphenol oxidase (PPO), chitinase (CHI), catalase (CAT), phenylalanine ammonia-lyase (PAL), and peroxidase (POX), which contributed to the increased resistance of seedlings to sunflower downy mildew (Nandeeshkumar et al. 2008).

2.8.1 *Trichoderma* spp. as a promising model for controlling plant diseases

Trichoderma spp. are free-living microorganisms associated with plants that act as opportunistic symbionts, conferring several benefits to their host plants. Among the various rhizobacteria, including *Pseudomonas*, *Bacillus*, *Streptomyces*, *Enterobacter*, and *Trichoderma* spp., the latter have evolved different mechanisms to assist plants in combating diseases and improving their growth (Belimov et al. 2001; Harman et al. 2004). For nearly 70 years, *Trichoderma* spp. have been known to attack other fungi, produce antibiotics that affect bacteria, and serve as biocontrol agents (Weindling and Fawcett, 1936). Recent studies have revealed diverse modes of action and potential for commercial applications. *Trichoderma* spp. are proficient producers of extracellular enzymes, with cellulase being the most prominent example (Mandels 1975).

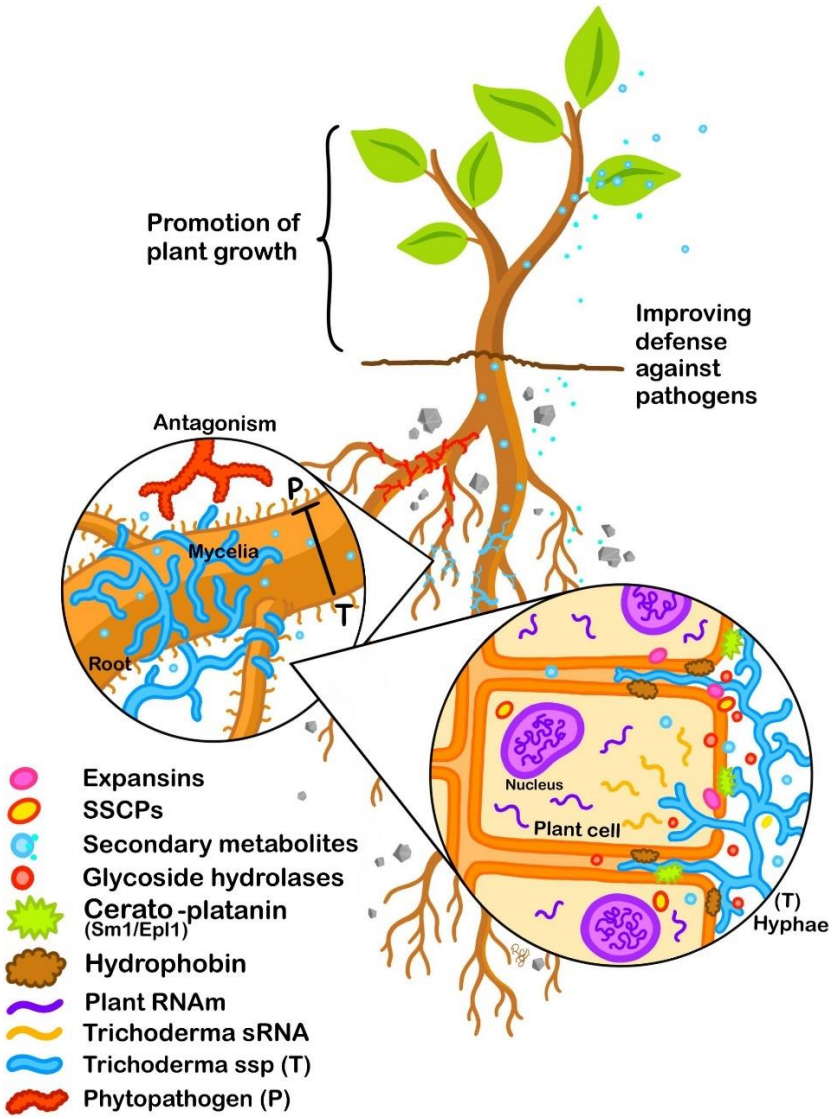


Figure 6: Effectors mediating *Trichoderma* spp. and plant interaction (Ramírez-Valdespino et al 2019).

The antagonistic activity of *Trichoderma* spp. toward phytopathogenic fungi and the release of *Trichoderma* spp. compounds that function as effectors are highlighted. These chemicals alter the hormonal equilibrium and defensive mechanisms of plants, thereby facilitating invasion. This beneficial relationship enhances plant development and increases the resistance to phytopathogens. (Ramírez-Valdespino et al 2019). *Trichoderma* spp. produces various extracellular enzymes that play a crucial role in the control of biological diseases in plants (Elad et al. 1982). In the early 1990s, several studies revealed a complex mixture of biocontrol enzymes and several of these genes were isolated and sequenced (Benitez et al. 1998; Lorito, 1998). Studies have demonstrated that combining multiple enzymes from *Trichoderma* spp. and other species can be more effective than using a single enzyme to prevent fungal growth, and synergistic effects have been observed (Lorito et al. 1996). The efficacy of these enzymes in plants has been tested by inserting their genes into plants, making them resistant to various plant pathogens (Bolar et al. 2001). This section addresses the importance of biocontrol agents in reducing or inhibiting

Oomycetes- and *P. halstedii-induced* infections. The application of several biocontrol agents (spraying, soaking, spraying, and soaking combined) isolated from the maize rhizosphere (*T. viride, T. harzianum, G. virens,* and *B. subtilis*) proved effective in preventing downy mildew in maize (Sadoma et al. 2011).

2.9 Role of antioxidant enzymes in plant disease control

The harmful effects of reactive oxygen species (ROS) on plants can be counteracted by antioxidants, which can have either enzymatic or non-enzymatic structures. Non-enzymatic antioxidants include vitamins C and E, selenium, zinc, beta-carotene, carotenoids, taurine, hypotaurine, and glutathione. Enzymatic antioxidants include superoxide dismutase (SOD), catalase, glutaredoxin, and glutathione reductase (Sağol et al. 1999). Antioxidants can be classified as natural or synthetic, based on their solubility in water or fat. Antioxidants can be categorised into enzymatic and non-enzymatic types, with only plants producing enzyme-based antioxidants. SOD and CAT are the most common antioxidants in vivo, with glutathione peroxidase (GPx) being another important enzyme (Agarwal et al. 2015). Numerous defense mechanisms are involved in avoiding or preventing phytopathogenic infections, including activating plant disease resistance and enhancing chemical and physical barriers against pathogen invasion. Inducible defense responses, such as activating defense-related enzymes in response to pathogen infection, contribute to additional resistance mechanisms in plants. Among these enzymes, peroxidases are crucial for plant-pathogen interactions and in several plant defense mechanisms (Shivakumar et al. 2003). These include activating the hypersensitive reaction to the pathogen, promoting ethylene production and suberisation, and facilitating lignin biosynthesis (Shivakumar et al. 2003).

In addition, peroxidase is often involved in the production of H_2O_2 during oxidative bursts (Lamb and Dixon, 1997). Applying copper sulphate in combination with CHN1.5/20 to grapevine leaves resulted in the accumulation of phytoalexins in plant tissues, thereby increasing plant resistance. When CuSO4 was applied alone, phytoalexin production was effectively enhanced in the grapevine leaves (Aziz et al. 2006). *Bacillus subtilis* KS1, isolated from grape berry skin, showed efficient activity against the fungus causing grapevine downy mildew (Furuya et al. 2011). The antagonistic activity of *B. subtilis* KS1 against *P. viticola* was examined based on iturin A production (Furuya et al. 2011).Heil and Ploss (2006) discovered that certain wild plants possess enzymes related to their immune system, and their capacity to produce these enzymes is a result of prior natural infection. Although wild plants naturally exhibit resistance and constitutive enzyme activity, ASM can induce resistance and activate additional defence enzymes (Heil &

Ploss, 2006). Chitosan has been found to enhance the early response of sunflower plant defences against downy mildew (Nandeeshkumar et al. 2008).

2.9.1 Peroxidase Enzyme

Enzymes called peroxidases, which are haeme-containing enzymes, oxidise multiple substrates at the cost of H_2O_2 at the rate of one electron. These enzymes (EC 1.11.1.7; H_2O_2 oxidoreductase), which are haeme-containing, perform one-electron oxidation of several different substrates at the cost of H_2O_2 and are known as peroxidases.

$$2RH + 2H_2O = 2RH + H_2O_2 \quad (1)$$

This has been observed in *Chlorophyta, Euglenophyta, Rhodophyta*, and other plant kingdoms. Plant peroxidases, fungal peroxidases, and bacterial peroxidases belong to the same superfamily as eukaryotic and prokaryotic haeme-containing peroxidases (Barceló 2003). All *Byophyta, Pteridophyta*, and *Spermatophyta* have been previously investigated (Barceló, 2003). Three structural types of this superfamily have been discovered that are distantly related (Barceló, 2003). Plants produce various multifunctional peroxidases (Gill and Tuteja 2010). Different isoforms of plant peroxidases and sophisticated expression regulations are involved in many physiological processes throughout the plant life cycle (Passardi *et al.* 2005). Peroxidases transform substrates into electron donors to reduce H_2O_2 to water (phenols, amines, organic acids, and glutathione). They aid xenobiotic detoxification, phytoalexin production, lignin and suberin biosynthesis, respiration, nitrogen exchange, mycorrhizal formation, and plant growth and development (Maksimov et al. 2015). Peroxidases generate superoxide anions, hydrogen peroxide, and hydroperoxide (Minibayeva et al. 2009). A boost in peroxidase activity may suggest plant tolerance to stress, given its physiological relevance and function in defensive responses to abiotic and biotic stresses (Maksimov et al. 2015; Minaeva et al. 2018).

2.9.2 Catalase enzyme

Catalases are antioxidant enzymes that catalyse the oxidation of hydrogen peroxide to produce water and atomic oxygen. Catalases can be split into three classes based on their structure and sequence: traditional catalase, catalase-peroxidase, and pseudo-catalase, also known as Mn-catalase (Zhang et al. 2010). At least eight species, Penicillium, *Aspergillus niger, Saccharomyces cerevisiae, Staphylococcus, Micrococcus lysodeiktious, Thermoascus aurantiacus, Bacillus subtilis, and Rhizobium radiobacte,* can produce catalases (Zhang et al. 2010). Catalases are used in several industries, including the food and textile industries, to remove hydrogen peroxide used in bleaching or sterilisation (Liu & Kokare. 2017).

Catalase (CAT) is another type of dismutase. It contains an active site haeme group that transforms two hydrogen peroxide molecules into oxygen and water (Kehrer et al. 2010). A small quantity of hydrogen peroxide attaches to the active site and interacts with a second hydrogen peroxide molecule to generate component I of catalase. The Km of catalase for H_2O_2 is in the m mol l range despite its extraordinarily high Vmax. Owing to its high Km, catalase degrades hydrogen peroxide in peroxisomes, the largest subcellular organelles containing catalase (Kehrer et al. 2010).

(Kehrer et al. 2010) $H_2O_2+H_2O_2 \rightarrow O_2+2H_2O$

Catalase is a widely distributed antioxidant enzyme that converts hydrogen peroxide to water and oxygen. Several pathogens produce catalase to defend themselves against oxidative stress and hydrogen peroxide assaults (Iwase et al. 2013). A recent study found that a catalase-deficient mutant pathogen is more vulnerable to oxidative stress caused by hydrogen peroxide and immune cell assaults (which also entail hydrogen peroxide) than its wild-type counterpart. Measuring pathogen catalase activity can help us to understand the underlying processes of their pathogenicity, particularly their resistance to oxidative stress (Day et al. 2000).

2.9.3 Polyphenol oxidases (PPOs) in plants

PPO is found in various plants. Depending on the type of tissue studied, PPO activity may differ significantly across different organs and even between other parts of the same organ. PPOs are found in both the soluble component of the cell and membrane-bound organelles of the cell (mitochondria, peroxisomes, and chloroplasts). There are differences according to the ontogenic state of the tissue and the degree of membrane binding. As a result, the overall PPO activity is higher and is primarily found in bound forms in early green fruits than in ripe fruits, when it typically decreases. A greater proportion of soluble forms was present (J. Nicolas et al. 2003).

More polyphenol oxidase activity is present in infected resistant plants than in susceptible or healthy plants. This is crucial for disease resistance because polyphenol oxidase activity converts phenolic compounds to quinones, which are more toxic to pathogens than phenols. Polyphenol oxidases increase infection resistance and production of oxidation products. During fruit ripening, lipoxygenases build up and degrade diene, a fungi-toxic compound, in young, immature fruits. Usually, ripening fruits are affected by such incidents. Non-pathogenic fungi stimulate the production of epicatechin, which prevents lipoxygenases, in various fruits. By reducing diene breakdown, epicatechin prevents the anthracnose fungus from decomposing and ripening fruits. The peroxidase enzyme converts phenolics into quinones and hydrogen peroxide. The latter is antimicrobial and accelerates the polymerisation of phenolic compounds into

compounds resembling lignin, by generating highly reactive free radicals. These substances accumulate in cell walls and papillae and stop pathogen development (J. Nicolas et al. 2003).

Furthermore, the optimum pH of some enzymatic preparations varied depending on the phenolic substrate used. Little research has been conducted on the impact of temperature, rather than pH, on PPO activity. The optimal temperature ranges between 15°C and 40°C, depending on the same variables that affect the pH. PPO have been found in a wide range of plants. PPO activity varies from one organ to another and within an organ depending on the tissue under consideration.

2.10 Integrated pest management (IPM) and its importance in crop protection

According to integrated pest management (IPM), pests, plants, and the environment are all interdependent parts of a system. To favour the desired plants and control pest issues, IPM comprises selecting plant species and cultivars and managing the surrounding environment (Flint 2012). Detecting and quantifying pest and disease incidence, as well as making informed decisions about when to deploy interventions, monitoring, or the regular, systematic observation of crops and plants, is crucial. Pests can be controlled in places with pest issues using methods designed for them. IPM prefers non-chemical pest control methods, such as cultural, physical, and biological methods, but it does not rule out chemical methods. If pesticides are necessary, the least disruptive environmental treatments, pest parasites, and predators are chosen (Flint, 2012).

IPM maximises the synergy between a wide collection of pest management strategies (biological, chemical, cultural, and mechanical) coherently coordinated at the scale of the cropping system, rotations, and technical operations of each crop, to decrease pesticide use and preserve crop production (Barzman et al. 2015; Swanton & Stephan, 1991). IPM systems require an in-depth understanding of pest biology and the interaction effects among pest control strategies to produce long-term synergies that disrupt the habitats of pest species and prevent outbreaks of highly adapted pests (e.g. chemical resistance/tolerance) (Barzman et al. 2015).

A sustainable IPM approach should incorporate all viable measures, including prudent and targeted application of pesticides, to reduce pest load. However, IPM applications face several challenges. IPM is more time-consuming and complex than chemical pesticides, because it utilizes many strategies against all insects. To ensure that the IPM method is administered at the best time and location, the insect populations must be monitored. Finding agricultural professionals with the knowledge required to provide farmers unbiased advice on the most efficient IPM techniques can be challenging in various parts of the world (Ehler 2006; Kleijn et al. 2019). Therefore, considerable agricultural system reform, knowledge sharing, and sociocultural change are required

to effectively deploy IPM to support sustainable agriculture. IPM is a potential method for controlling sunflower downy mildew (Molinero-Ruiz, 2022).

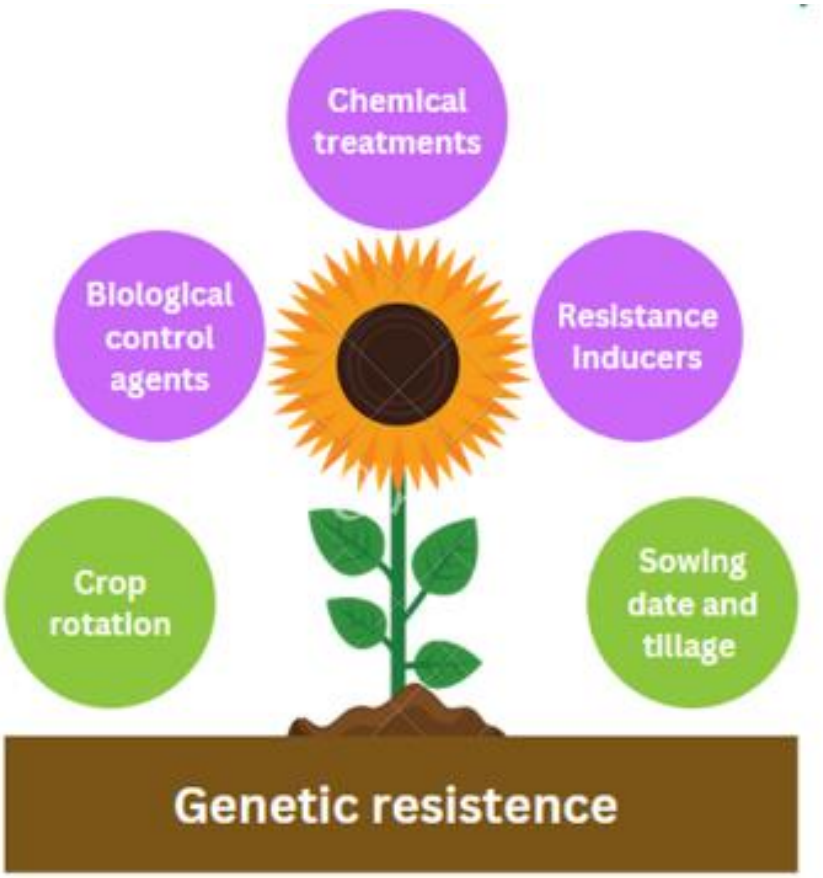


Figure 7: Integrated pest management strategies for sunflower downy mildew (own editing)

Chapter 3: MATERIALS AND METHODS

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

3.1.1 Plant and pathogen materials

Seven compatible host-pathogen combinations were examined using 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 2.

Table 2. The isolates of *P. halstedii* used in this study (location, collection yaer and pathotype)

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

3.1.2 *T. asperellum* as biocontrol agents

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.

3.1.3 The Effects of BTH, azadirachtin, and *T. asprellum* on sporangia morphology and release of *P. halstedii* : A microscopic examination

Sunflower leaves infested with *P. halstedii* were used to release sporangia by immersing them in 100 mL of bi-distilled water. The resulting sporangia suspension was then mixed with different concentrations of BTH (20, 40, and 80 ppm azadirachtin (0.01, 0.1%, and 0.2%), and *T. asprellum* (3×10^7 and 3×10^8 conidia/ml), as well as a positive control of mefenoxam. The samples were incubated overnight in the dark at 16°C, and after 24 h, sporangial morphology and zoosporangial release were observed under a microscope at 200x magnification. The first 50 sporangia in each treatment were counted microscopically, and a germination scale was used to identify sporangia morphology based on microscopic examination. The study was repeated five times for each treatment with distilled water as the control. The germination scored 0 -filled sporangia, and 1 - completely or partly empty sporangia and was based on the assumption that every discharged zoospore has the potential to infect the host plant.

3.1.4 Effects of BTH, azadirachtin, and *T. asprellum* on sunflower downy mildew disease severity and plant growth

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 *T. asperellum* treatment seven-day-old *T. asperellum* (TGAM-G16 isolate) cultured on PDA were 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 haemocytometer. 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* (with 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.

Figure8 indicates that the Pre-treated and/or inoculated sunflower seedlings (25 plants/treatment) were sown in pots (d=5 cm, five plants per pot) filled with horticultural perlite (d = 0-4 mm) and kept at 22°C in a growth chamber (12 h photoperiod, light irradiance of 100 µE·m−2 ·s −1). The plants were grown for 21 days. The trial was set up in a randomised block design with two repetitions per treatment, each repetition consisting of 25 plants per treatment..

Figure 8: *P. halstedii* treated with different inducers and experimental setup. (A) inoculated plants and **B**. non-inoculated plants

Table 3: List of treatments and concentrations of the inducers used in the single treatment experiment against different isolates of *P. halstedii*. (What means the I, pl add this information)

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)*)

Assessment of disease

Plants were evaluated twice during the examination period: immediately after sporulation (10 dpi) and then at 21 dpi. Nine days after inoculation (9 dpi), the seedlings were sprayed homogeneously with bi-distilled water to induce sporulation and then covered with dark bags overnight (19°C). Disease assessment was performed according to the sporulating and damped-off plants at 10 dpi and chlorotic and damped-off plants at 21 dpi Assessment of sunflower downy mildew disease severity using a 0-4 scale at 10 and 21 days post-inoculation. where 0: No visible sporangiophores on leaves, 1: Limited sporulation, sporadic sporangiophores present (≤25% of leaf disc surface covered), 2: Sporulation covering ≤50% of cotyledon area or 25–50% of leaf disc surface, 3: Sporulation covering >50% of cotyledon area or 50–75% of leaf disc surface, 4: sporulation covering more that 75% of the lcotyledon.. Plant height was measured twice during the experiment (at 10 and 21 dpi), as *P. halstedii* caused dwarfing on susceptible, non-treated sunflowers.

3.1.5 Combination experiment: Treatment with inducers and design of the in planta experiments against multiple isolates of *P.halstedii*

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 *Plasmopara hlastedii*.

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% (NeemAzal® 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 (figure 9). Plants were watered daily and fertilized twice during the experment with commercial general purpose fertilizer NPK(i.e. 10-10-10) during the experiment. The fertilizer was diluted to a concentration of 0.001% per liter for each tray when grown in perlite. 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 0-5 for chlorosis at 10 and 21 days after inoculation (dpi). A randomised block design was used, and the treatments are listed in Table 4.

Table 4. Treatments and concentrations used in the combination experiments against six *P. halstedii* isolates

Treatment's	**Concentrations**			
	AZA %	**BTH-ppm**	**MX g/kg**	***T. asperellum***
BW	-	-	-	-
Inoculated (I)	50.000sporangia/ml			
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	
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)*)

Figure 9: *P. halstedii* combination experimental setup - Inoculated plants (A) Non-inoculated plants (B)

3.1.6 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 performed using the R statistical software.

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

3.2.1 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 to other sunflower genotypes. In this study, we used our most virulent pathotype (710). Sunflower leaves infected with this pathotype were stored in a deep freezer at -70℃ 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.

3.2.2 *In vitro* examination: Influence of BTH and *T. asperellum* on sporangia morphology and zoosporangia release of sunflower downy mildew

Infected sunflower leaves were collected and stored in a deep freezer at 70°C. To release sporangia, the leaves were submerged in 100 mL of distilled water. A 10 mL falcon tube was filled with 5 mL of a sporangial suspension containing 50,000 sporangia/mL. Two different solutions were prepared by mixing BTH 80 ppm and *T. asperellum* $3x10^8$ conidia/ml with 5 mL of the sporangial suspension to make a total of 10 mL for each solution. In addition, a combination of BTH 40 ppm and *T. asperellum* $3x10^7$ conidia/ml was added to 5 mL of 50,000 sporangia/mL. 5 mL of 50,000 sporangial suspension was mixed with 5 mL of bi-distilled water were used as control for this experiment. The sporangia were incubated overnight at 16±1°C and gently mixed to prevent them from bursting and ensure uniform mixing. The samples were then placed overnight in a phytotron at 16°C in the dark. After 24 h, the samples were examined at 200x magnification under a microscope to assess how BTH, *T. asperellum*, and their combination affected sporangia shape and zoosporangia release. The first 50 sporangia in each treatment were microscopically counted, and each treatment was tested five times. A germination scale from 0 to 1 was constructed to evaluate sporangial morphology by microscopic inspection, assuming that every zoospore may infect the host plant.

3.2.3 Evaluation of the Efficacy of BTH and *T. asperellum* in controlling sunflower downy mildew (*P. halstedii*) pathotype 710 and reduce the disease rate in plants: *in vivo* experiments

In this experiment, we expanded our investigation based on the initial findings from different concentrations of resistance inducers. To evaluate their effectiveness, we developed combinations using half and the lowest effective doses of both biological and chemical inducers. This was performed as a comparison to the full effective dose used in the individual 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.

T. asperellum. conidia were obtained by flooding 7-day-old PDA-cultured *T. asperellum* with 10-15 mL BW, and the resulting conidial suspension was adjusted to 3×10^7 and 3×10^8 conidia per mL using a haemocytometer. The conidial solution contained 5% gum arabic to enhance the seed adherence. Pre-germinated sunflower seeds were immersed in *T. asperellum*. solution at 25°C for 3 h and then transferred to a growth chamber at 19°C for 3 days for germination.

The sunflower seedlings were then 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. Plants were watered daily and fertilized twice during the experment with commercial general purpose fertilizer NPK(i.e. 10-10-10) during the experiment. The fertilizer was diluted to a concentration of 0.001% per liter for each tray when grown in perlite. 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 where 0: No visible sporangiophores on leaves, 1: Limited sporulation, sporadic sporangiophores present (≤25% of leaf disc surface covered), 2: Sporulation covering ≤50% of cotyledon area or 25–50% of leaf disc surface, 3: Sporulation covering >50% of cotyledon area or 50–75% of leaf disc surface, 4: sporulation covering more that 75% of the lcotyledon.

. 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 inoculated with *P. halstedii*.

Figure 10: *P. halstedii* enzyme activity experimental setup for pathotype 710. Inoculated plants (A) Non-inoculated plants (B)

3.2.4 Measurement of antioxidant activity

3.2.4.1 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-Na_2 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 enzymes assay. The enzyme activity was measured using a Bio-Rad Smart Spec Plus spectrophotometre at room temperature.

3.2.4.2 Guaiacol-dependent peroxidase activity measurement (POX)

The method described by Rathmell and Sequeira (1974) was used to measure guaiacol-dependent peroxidase (POX) activity, with minor modifications. A volume of 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 rate of absorbance at 470 nm was measured using a spectrophotometre. At 25°C, the absorbance of 1 mg of enzyme increases by 1.0 per minute.

3.2.4.3 Polyphenol oxidase activity measurement (PPO)

The PPO activity was assessed based on the initial quinone production rate. 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 used was a modification of the approach described by Fehrmann and Dimond in 1967, and involved measuring the increase in absorbance at 400 nm. The enzyme activity was measured using a Bio-Rad Smart Spec Plus spectrophotometre at room temperature.

3.2.4.4 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*DA_{240} nm per minute (Beauchamp & Fridovich, 1971).

3.2.5 Statistical analysis

The experiments were conducted at the Plant Protection Department in Gödöllő, Hungary, with two replicates for each experiment and five replicates for each treatment. 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 performed using the Minitab-18 software (Minitab, LLC, Pennsylvania).

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

3.3.1 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 to 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 subsequently 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 sporangia/ml using a haemocytometer following the method of Cohen and Sackston (1973).

3.3.2 Preparation of treatments

3.3.2.1 Seed coating with mefenoxam

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

3.3.2.2 BTH

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

3.3.2.3 Preparation of different concentrations of azadirachtin (AZA)

The commercial product, NeemAzal® 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 pre-treated with 0.01%, 0.1%, or 0.2% azadirachtin solution for 2 h. To obtain the desired concentrations, one, ten, and 20 mL of 1% azadirachtin was dissolved in 100 mL of distilled water.

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

In this experiment, we expanded our investigation based on the initial findings from different concentrations of resistance inducers. To evaluate their effectiveness, we developed combinations using half and the lowest effective doses of both botanical and chemical inducers, as well as the fungicide. This was performed as a comparison to the full effective dose used in the individual 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 pathotype 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

- Figure 11 illustrates treated sunflower seedlings grown in 5 cm pots (10 plants per pot) with five repetitions 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 d post-inoculation. Macroscopic observations were scored on a scale of 0-4 for disease severty. where 0: No visible sporangiophores on leaves, 1: Limited sporulation, sporadic sporangiophores present (≤25% of leaf disc surface covered), 2: Sporulation covering ≤50% of cotyledon area or 25–50% of leaf disc surface, 3: Sporulation covering >50% of cotyledon area or 50–75% of leaf disc surface, 4: sporulation covering more that 75% of the lcotyledon.

The experiment was conducted using a randomised block design and included the following treatments (Table 5):

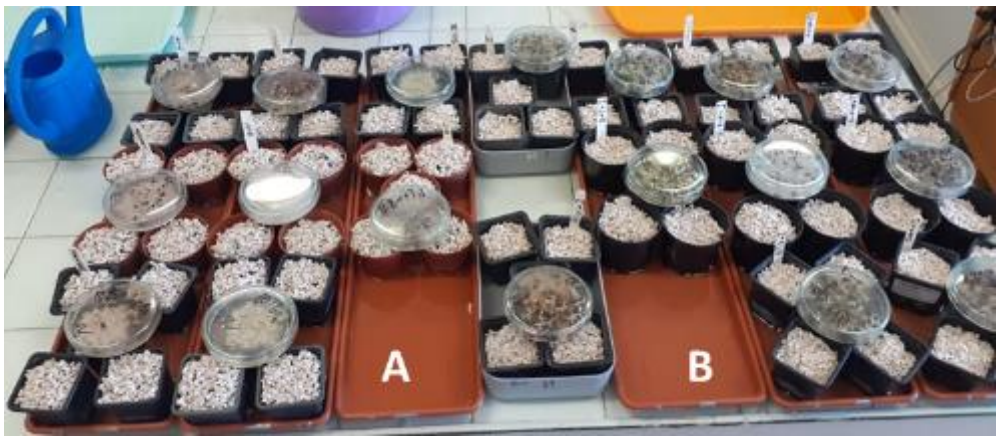


Figure 11: *P. halstedii* enzyme activity experimental setup for pathotype 710. (A) Non-inoculated plants B. inoculated plants.

Table 5: Treatments and concentrations used in the combination experiment against *P. halstedii* pathotype 710.

Treatment's	**Concentrations**		
	AZA %	**BTH - PPM**	**MX g/kg**
BW	-	-	-
Inoculated (I)	50.000sporangia/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))

3.3.4 Effects of botanical and chemical combinations for antioxidant enzymes activity induction against *P. halstedii* pathotype 710

3.3.4.1 Enzyme extraction

Following pathogen inoculation, samples were collected at designated time intervals, including 0, 3, 9, and 15 days post-inoculation (dpi), from all treated plants. 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.

3.3.4.2 Enzyme activity measurement of POX, PPO, and CAT

Following enzyme extraction, the supernatant was stored at low temperature on ice box 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 spectrophotometre (Budapest, Hungary). Guaiacol-dependent POX activity was determined using the modified approach reported by Rathmell and Sequeira (1974), whereas 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*$DA_{240\,nm}$ per minute (Beauchamp & Fridovich, 1971).

3.3.5 Statistical analysis

The experiments were carried out at the Godollo Plant Protection Department in Hungary and were repeated twice, with five duplicates in each trial. The data were subjected to one-way ANOVA and factorial analysis with a confidence interval of 0.05 utilized for mean separation. Statistical analyses were performed using Minitab-18 statistical software (Minitab, LLC, Pennsylvania). All experiments were repeated twice.

Chapter 4: RESULTS

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

4.1.1 The effects of BTH, azadirachtin, and *T. asprellum* on sporangia morphology and zoosporangia release in *P. halstedii*: A microscopic examination

Under *in vitro* conditions, both botanical and biological activators suppressed empty sporangia compared to chemical inducer and fungcide. The fraction of empty sporangia decreased linearly as activator concentration increased. Figure 13 demonstrates that the highest doses of BTH and both highest concentrations of azadirachtin, along with both concentrations of *T. asperellum*, exhibited the most notable and effective reduction in sporangia germination. Compared with the control bi-distilled water, BTH and azadirachtin did not affect zoospore release at the lowest concentrations. Mefenoxam suppressed empty sporangia in the isolates I2, I3, and I4 at a dose of 3 mg/kg but moderately inhibited the isolates I1, I5, I6, and I7.

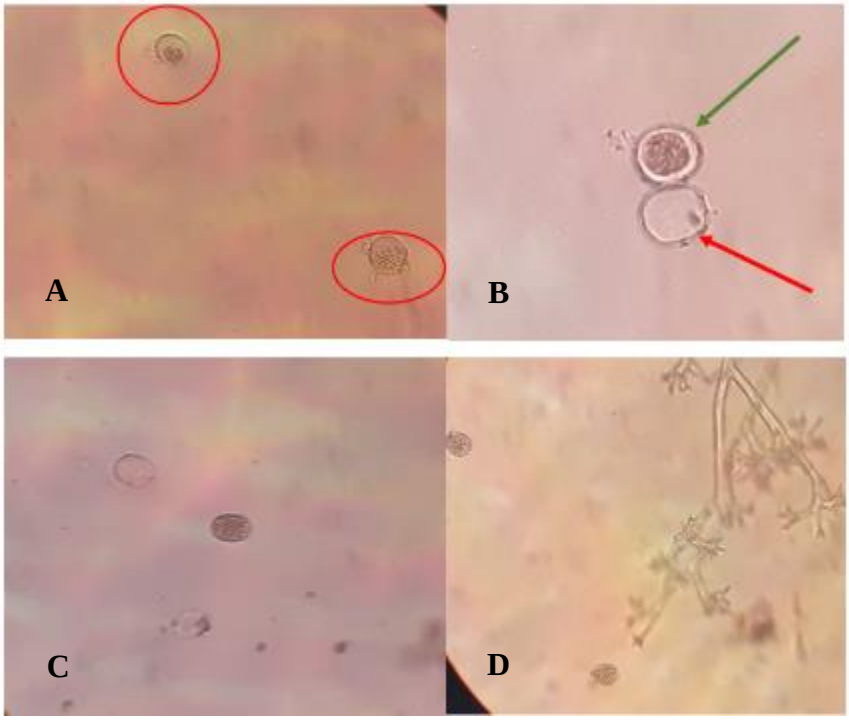


Figure 12: Effect of *T. asperellum*, azadirachtin, BTH, and mefenoxam on zoospore release from *P. halstedii* sporangia at 16°C under a microscope at 200x magnification. (A) half-empty sporangia (B,C) comparison between empty and full sporangia, and (D) full sporangia in the sporangiophore.The number of emptys porangia were counted from the first 50 on the slide.

Azadirachtin (AZA) applied to a sporangial suspension of *P. halstedii* at 16°C *in vitro* prevented the release of zoospores from sporangia at 0.1 and 0.2% concentrations against all

isolates. The lowest concentration (0.01%) resulted in high inhibition for all isolates, except for I1 and I5, which did not perform significantly (Figure 13).

Sporangial were treated with chemical inducers at concentrations of 20, 40, and 80 ppm. BTH had no noticeable influence on sporangial germination. The lowest dose of BTH (20 ppm) was less significant against the isolates I1, I2, I5, I6, and I7, while providing considerable inhibition I3, I4. A moderate concentration of BTH 40ppm performed not significant against the isolates I1, I5, and I7; moreover, BTH 40 had a moderate inhibition rate against the isolates I2, I3, I4, and I6. The highest dose of BTH (80 ppm) significantly suppressed sporangial discharge against all isolates, except I1, which had a modest inhibition rate. At both doses, *T. asperellum* significantly decreased sporangial discharge in all tested isolates (Figure 13).

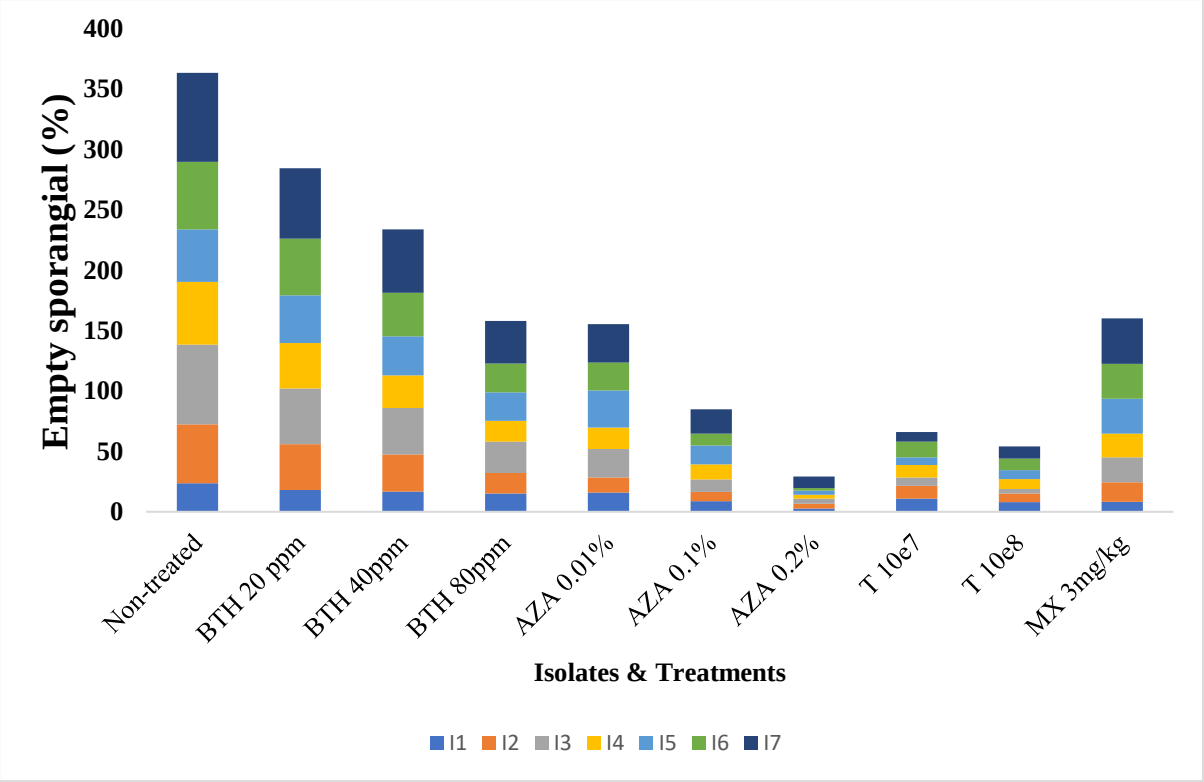


Figure 13: Empty sporangia of *P. halstedii* cultured at 16°C in water containing various doses of *Trichoderma* spp., azadirachtin, BTH, and mefenoxam and analysed microscopically for zoospore release after 24 h against seven isolates of *P. halstedii*. was assessed using a haemocytometer slide under a microscope at 200x magnification. The standard deviation represents the ±SDM of the five biological replicates. The mean values of five replicates are shown (n = 5). Significant changes between treatments were determined at P = 0.05, using Fisher's post hoc test.

4.1.2 Effects of BTH, azadirachtin, and *T. asprellum* on sunflower downy mildew disease severity and plant growth

Figure 14 shows the average disease rate 1 sporulation and damping off of several *P. halstedii* isolates on mefenoxam-treated and non-treated sunflowers. In mefenoxam-treated and *P. halstedii*-inoculated sunflower plants, five of seven isolates generated relatively high disease rates

(ranging from 12 to 100%) (Figure 14). pathotypes I7 (Borsod pathotype 704), I2 (Körösladány pathotype 710), and I1 had the highest infection rates (pathotype 704 from Abony). Mefenoxam was effective against I5 (pathotype 730 from Csanytelek) and I6 downy mildew isolates (pathotype 730 from Tiszaforet). In contrast, the infection rate was reduced by isolates I3 (Doboz pathotype 704) and I4 (Trkeve pathotype 700) (Figure 14).

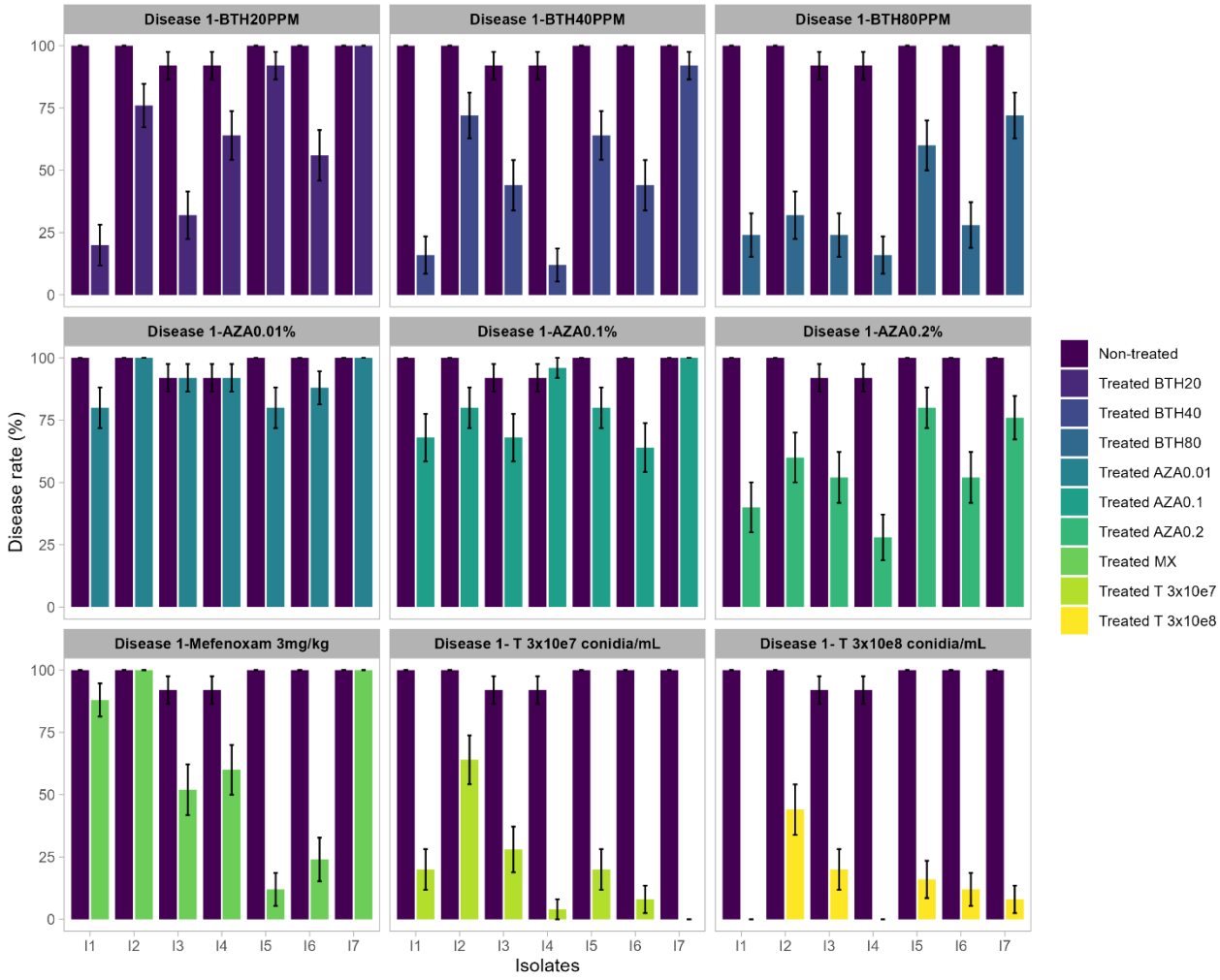


Figure 14: Disease rate 1(%) of sunflowers treated with different doses of BTH, *T. asperellum*, AZA, and mefenoxam on sporulation and damping off against seven isolates of *P. halstedii* as determined by the WSI technique. The standard deviation represents the ±SDM of the five biological replicates. The mean values of five replicates are shown (n = 5). Significant differences between treatments were determined at P = 0.05, using Fisher's post hoc test.

The lowest dose of BTH 20 ppm did not significantly affect the three *P. halstedii* isolates I2, I5, and I7 and provided considerable protection against two isolates I4 and I6. For the two isolates I1 and I3, protection was segnificant compared to the control . The BTH 40ppm, on the other hand, performed not significant affect the two isolates I2, I7 and provided only moderate protection to the isolate I5. The isolates showed the lowest infection rates I4, I1, I3, and I6. Except

for two isolates I5 and I7 with modest infection rates, the highest dose of BTH (80 ppm) considerably reduced the infection rate of all isolates except for isolate I5 and I7 (Figure 14).

For all *P. halstedii* isolates, the lowest dose of the botanical inducer azadirachtin 0.01% (Azadirachtin1%) was not significant. Furthermore, azadirachtin 0.1% did not significantly affect all isolates, except for I1 and I6, which provided considerable protection. In contrast, the highest dose of azadirachtin (0.2%) considerably reduced the infection rate of all isolates except for two I5 and I7, which had the highest infection rates. and a modest resistance to isolate I2 (Figure 14). Furthermore, the biotic inducer, *T. asperellum*, was highly protective against all isolates and provided moderate protection against isolate I2 (Figure 14).

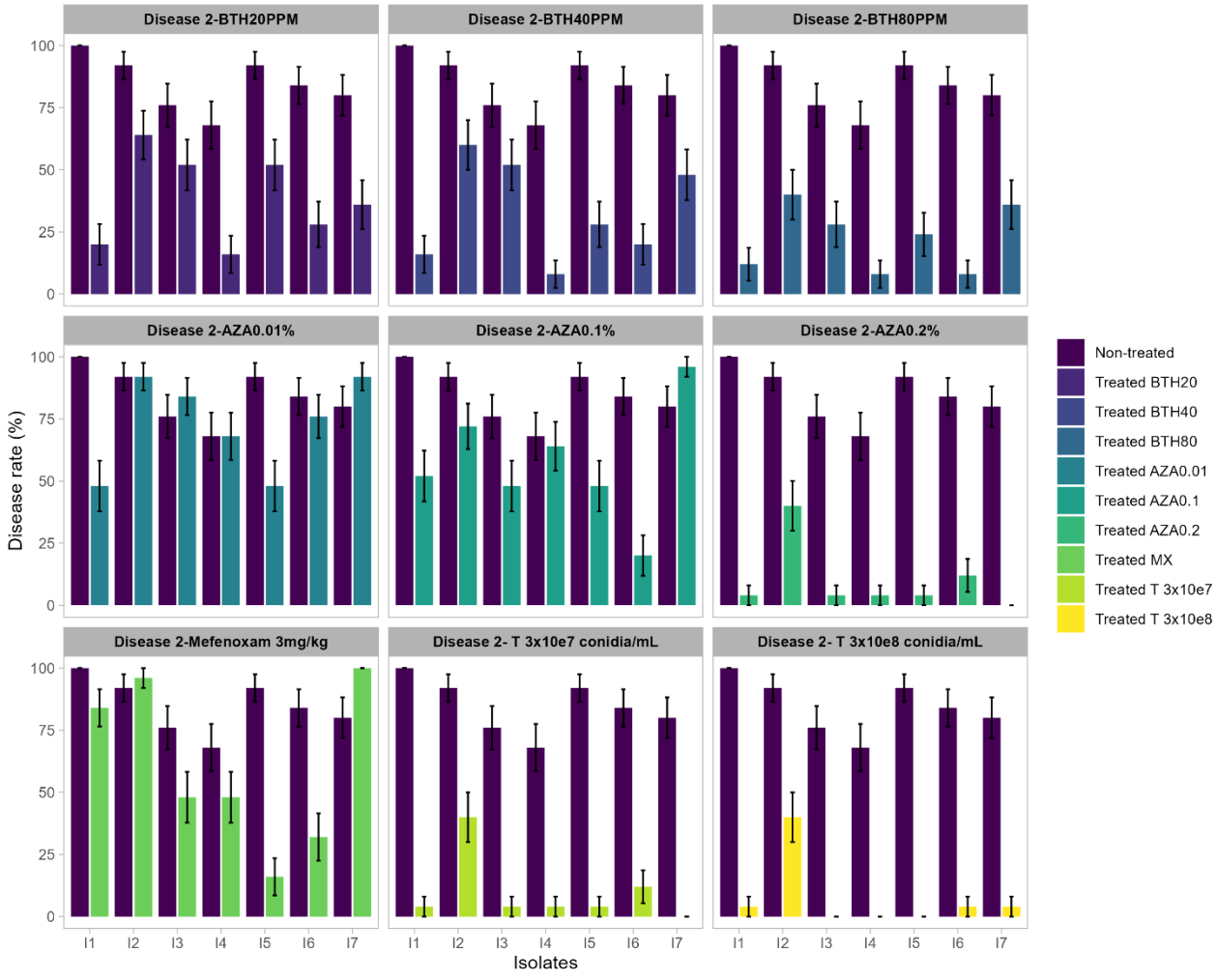


Figure 15: Disease rate 2 (%) of sunflowers treated with different doses of BTH, *T. asperellum*, AZA, and mefenoxam on chlorosis and damping off against seven isolates of *P. halstedii* as determined by the WSI technique. The standard deviation represents the ±SDM of the five biological replicates. The mean values of five replicates are shown (n = 5). Significant differences between treatments were determined at P = 0.05, using Fisher's post hoc test.

Figure 15 shows the average disease rate 2 (percentage) (chlorosis and damping-off) of several *P. halstedii* isolates on mefenoxam-treated and untreated sunflowers. In mefenoxam-treated and *P. halstedii*-inoculated sunflower plants, five of seven isolates generated high disease rates (ranging from 16 to 100%). Infection rates were higher for I7, I2, I1, and I4. The mefenoxam-sensitive downy mildew isolates were identified I5 and I6. The infection rate of Isolate I3 was moderate (Figure 15).

The lowest dose of BTH 20 ppm did not significantly affect one *P. halstedii* pathotype I2, and provided modest protection against two isolates I3 and I5. Protection was adequate for four isolates I1, I4, I6, and I7. The BTH 40ppm was not significant for either isolate I2, I3. and demonstrated moderate protection against the isolate I7. Isolates I4, I1, I5, and I6 had the lowest infection rates. The infection rates for all isolates were considerably lower at the maximum dose of BTH (80 ppm) (Figure 15).

Except for I1 and I5, the lowest dose of the botanical inducer azadirachtin 0.01% acted up on all *P. halstedii* isolates. Furthermore, azadirachtin 0.1% did not significantly affect all isolates except I1, I3, and I5, which provided moderate and high protection against isolate I6. In contrast, the highest dose of azadirachtin (0.2%) considerably reduced the infection rate of all isolates, except isolate I7, which had higher infection rates (Figure 15). Furthermore, the biotic inducer *T. asperellum* was highly protective against all isolates and provided moderate protection against isolate I2 (Figure 15).

Due to the essential symptoms of *P. halstedii*, which include stunting of the infected plant, plant height was measured twice for each isolate throughout the investigations (Figure16). There was no significant difference in height between non-inoculated, mefenoxam-treated, and non-inoculated, non-treated plants at any data collection point in any of the tests. In addition, during the initial assessment, the mefenoxam-treated and infected sunflowers I4, I5, and I6 developed similarly to the uninfected sunflowers (Figure 16).

Plant heights were considerably more significant for treated sunflowers inoculated with all *P. halstedii* isolates, except for I1, I2, and I7, which were significantly shorter than those of untreated and infected plants. Plants inoculated with isolates I4, I5, I6, and I7 and treated with the lowest dose of the chemical inducer BTH 20 ppm were considerably shorter or equivalent to non-treated inoculated plants. Plants treated with BTH 20 ppm and inoculated with isolates I1, I2, and I3 performed significantly better than the non-treated and inoculated plants. In the case of BTH 40 ppm, all treated and inoculated plants were considerably higher than non-treated inoculated plants, except for plants infected with isolates I5, I6, and I7, which were not significantly different from

non-treated and inoculated plants. The highest BTH dose (80 ppm) effectively reduced the stunting signs in all plants treated and inoculated with all isolates (Figure 16).

The lowest dose of the botanical fungicide azadirachtin (AZA 0.01%) did not affect plant stunting in any of the isolates. The plants treated with 0.1% and inoculated with the isolates I1, I3, and I4 were not statistically different from the untreated and inoculated plants. In contrast, the plants inoculated with isolates I2, I5, I6, and I7 were much taller than the untreated and inoculated plants. Plants treated with the highest concentration of azadirachtin 0.2% and inoculated with all isolates significantly higher than non-treated and inoculated plants (Figure 16). Both concentrations of *T. asperellum* considerably reduced the signs of stunting in plants treated with various *P. halstedii* isolates. However, compared with untreated and inoculated plants, a higher *T. asperellum* of 3×10^8 was the most effective in increasing plant height (Figure 16).

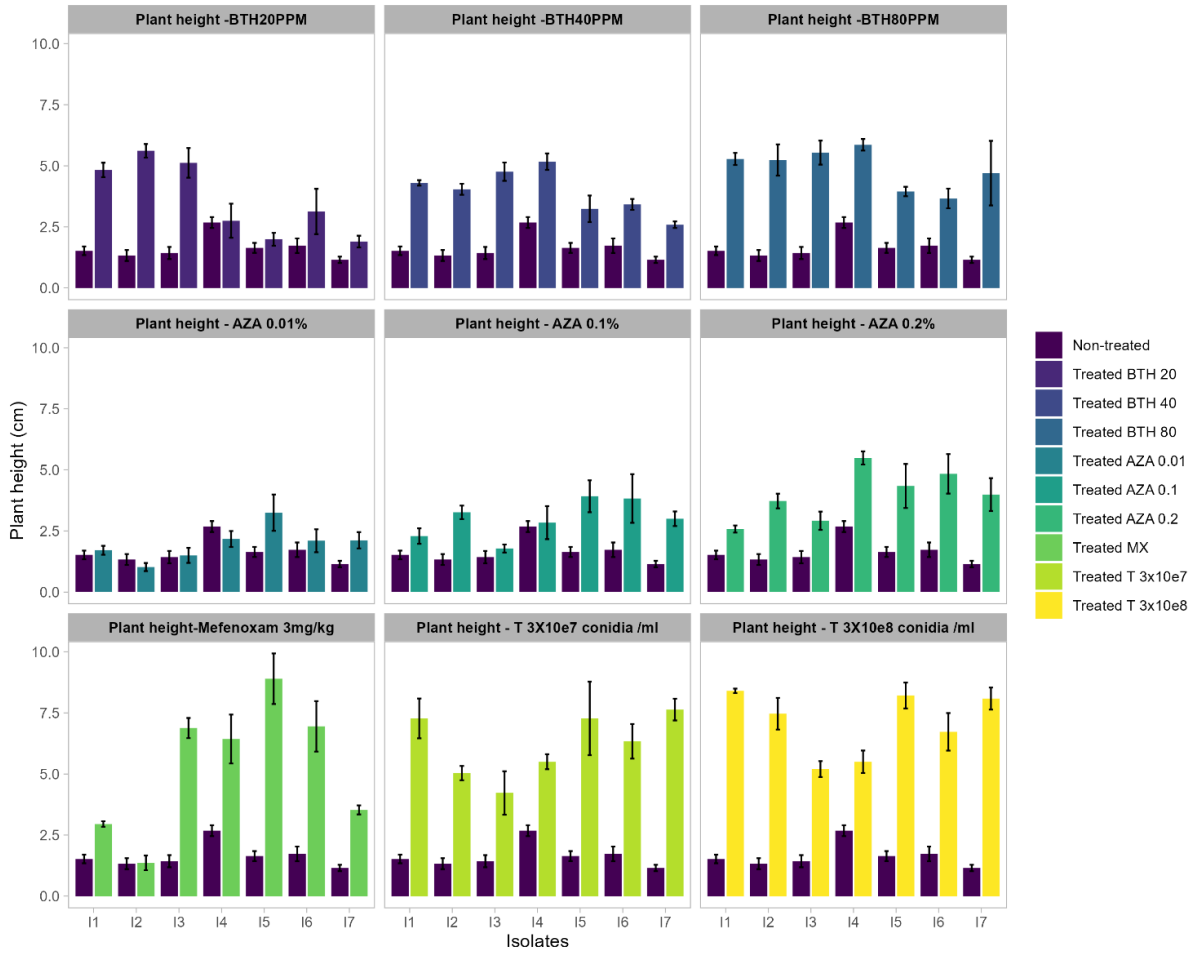


Figure 16: Dose-dependent effectiveness of BTH, AZA, *T. asperellum*, and mefenoxam on plant height against seven isolates of *P. halstedii* as determined using the WSI technique. 21 days post-infection. The standard deviation represents the ±SDM of the five biological replicates. The mean values of five replicates are shown (n = 5). Significant differences between treatments were determined at P = 0.05, using Fisher's post hoc test.

A two-factor analysis of variance without repeated measures was conducted to test whether there was a difference between the groups of the independent variable isolate concerning the dependent variable average of disease 1, disease 2 and average plant height, and whether there was an interaction between the two variables isolate and treatment concerning the dependent variables. Results showed a significant difference between the two groups, with a significant difference of P=<.001 and an interaction of P=0.001(Figures 17-18-19) (table 7 annex).

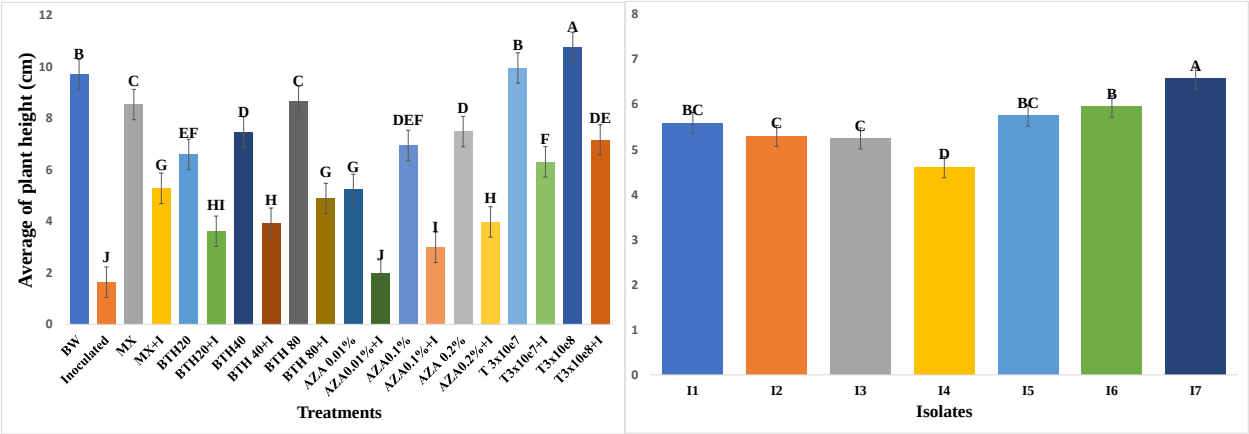


Figure 17: Effects of BTH, azadirachtin, and *T. asperellum*. on plant height. Data were subjected to factorial analysis (Factor 1= treatments) (Factor 2= isolates).
Bars represents standard deviations of five replicates.
I1-I7: *Plasmopara halstedii* isolates (see in Table 2).

The two-factor analysis of variance without repeated measures showed that there was a significant difference between the groups of the independent variable isolate concerning the dependent variable's average of sporulation and damping off (Disease 1) and chlorosis and damping off (Disease 2) ($P \leq 0.001$) and interaction between the two variables isolate and treatment concerning these averages. This difference was significantly higher than that between the two groups of the dependent variable treatment, which showed a significant difference of P<0.001(Figure 18-19).

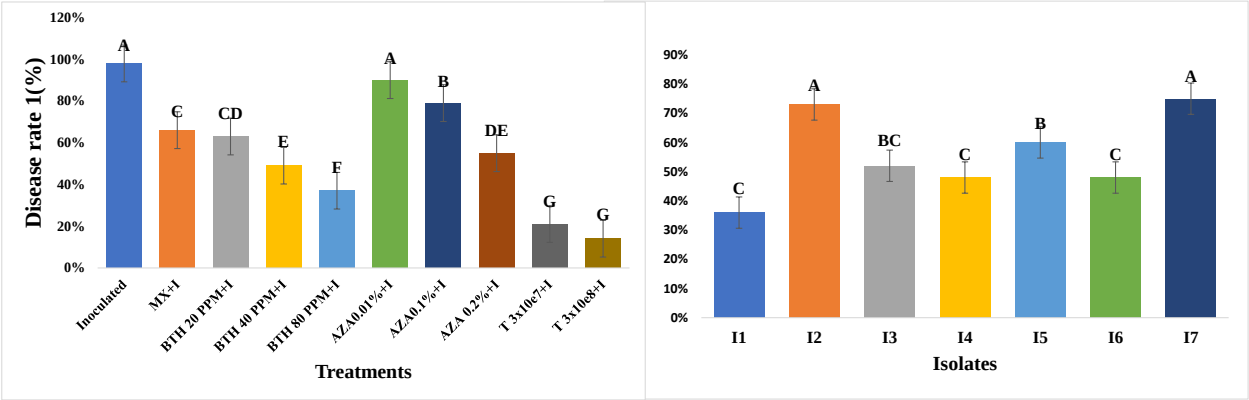


Figure 18: Disease rate of 1(%) of the (frequency of sporulation and damping off) in susceptible sunflower seedlings inoculated with downy mildew (multiple pathotypes) and treated with azadirachtin *T.asperellum*, (AZA), BTH, and mefenoxam (MX).
Disease rate 1 (%) was determined as the ratio of diseased (sporulating or damped-off plants) to healthy plants. Bars represents standard deviations of five replicates. I1-I7: *Plasmopara halstedii* isolates (see in Table 2)

Additionally, there was a significant difference in the interaction between isolate and treatment, with the two variable isolates and treatments showing two significant differences (P =.001). The two-factor analysis of variance without repeated measures showed that there was a significant difference between the groups of the independent variable Factor1 isolates and Factor 2 treatments concerning the dependent variable plant height (P ≤.001), and that there was an interaction between the two variables and the dependent variable average of plant height (Figure 17).

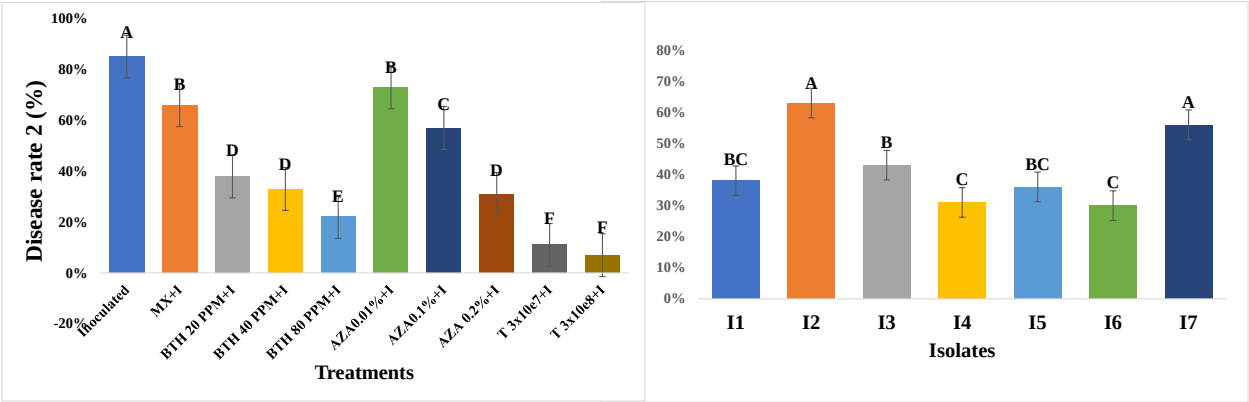


Figure 19: Disease rate 2 (%) of the (frequency and damping off) in susceptible sunflower seedlings inoculated with downy mildew (multiple pathotypes) and treated with azadirachtin (AZA), BTH, *T.asperellum* and mefenoxam (MX).
Disease rate 2 (%) was determined as the ratio of diseased (chlorotic or damped-off plants) to healthy plants. Bars represents standard deviations of five replicates.
I1-I7: *Plasmopara halstedii* isolates (see in Table 2)

Finally, all biological and chemical inducers were significantly more effective than other treatments in reducing disease symptoms (disease 1, disease 2, and plant height). Interestingly, whereas Azadirachtin at greater concentrations proved very effective in lowering disease symptoms, azadirachtinat the lowest dosage did not differ much from untreated diseased plants

4.1.3 Combination experiment: Treatment with inducers and design of the *in planta* experiments against different isolates of *P. halstedii*

There was no significant difference in height between non-inoculated, mefenoxam-treated, and non-inoculated, non-treated plants at any data collection point in any of the tests. In addition, during the initial assessment, the mefenoxam-treated and infected sunflowers I3, I4, I5, and I6 developed similarly to the uninfected sunflowers (Figure 20). Plant heights were considerably more significant for treated sunflowers inoculated with all *P. halstedii* isolates, except for I7, which was significantly shorter than that of untreated and infected plants. Plants inoculated with isolates I3 and I7 and treated with the combination of MX+AZA were significantly shorter or equivalent to the untreated inoculated plants. Plants treated with the combination of MX+AZA and inoculated with isolates I3, I4, I5, and I6 performed significantly better than the non-treated and inoculated plants.

In the case of BTH+MX, all treated and inoculated plants were significantly higher than non-treated inoculated plants, except for plants infected with isolates I4, I5, and I6, which were not significantly different from non-treated and inoculated plants. The combination of BTH +AZA significantly reduced stunting signs in all plants treated and inoculated with all isolates, except I1 (Figures 20). The BTH+AZA+MX mixture significantly reduced stunting symptoms, except that the plants infected with isolate I3 were significantly shorter than the other plants (Figure 20). In contrast, the plants treated with a combination of BTH + to *T. asperellum* were significantly higher than non-inoculated plants.

Azadirachtin worked synergistically with mefenoxam, BTH, and both, to provide significantly greater disease control than anticipated. This indicates that a modest dose of azadirachtin delivered in a mixture is as effective as the full dose alone. Synergy was observed when azadirachtin was present in low concentrations (0.01%). Accordingly, it was evident from multiple experiments that the reaction of different pathogen isolates had different responses to *T. asperellum*, and BTH the combination of reduced rates with a full rate of *T. asperellum* and BTH was as effective as the full rate of *T. asperellum*. This was true, even though the lowest dose of BTH was not significantly effective when applied alone. This suggests that *T. asperellum* synergises with BTH.

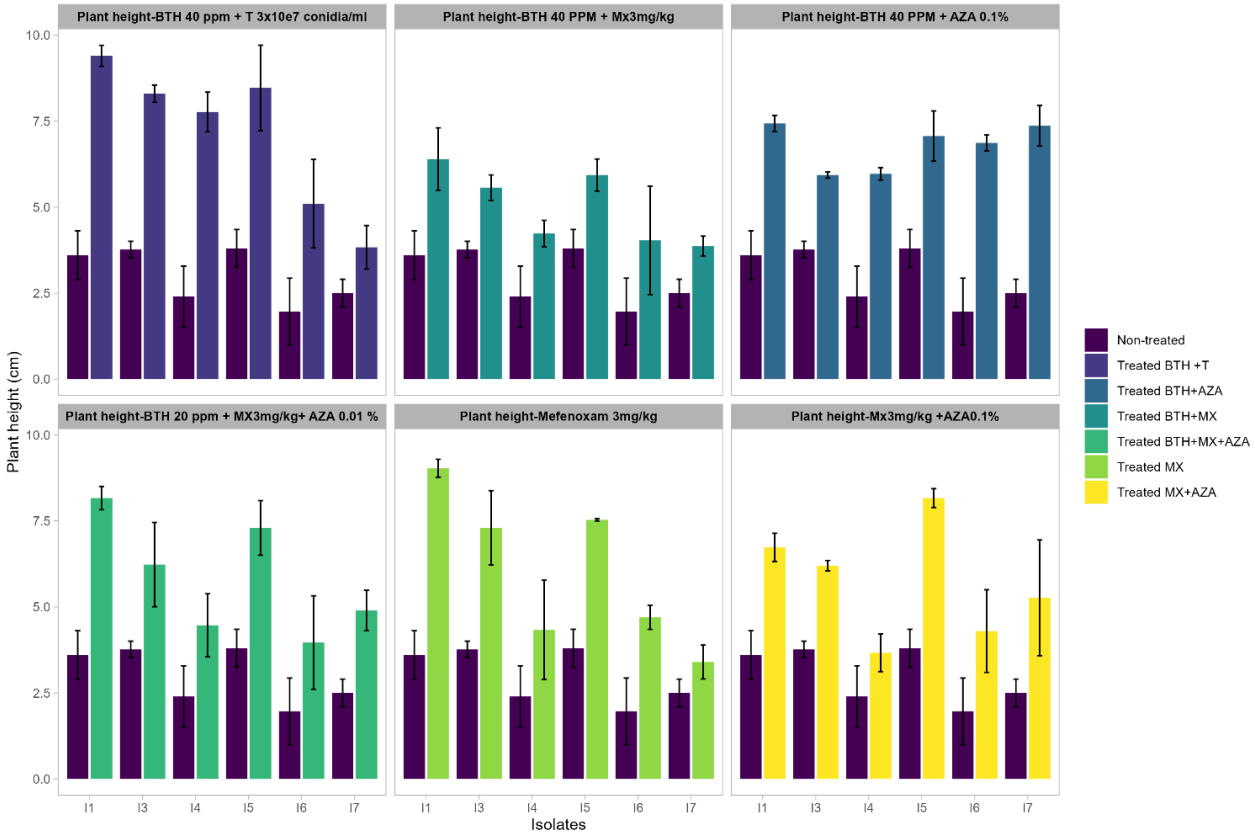


Figure 20: Average of Initial and final plant height in susceptible sunflower seedlings inoculated and non-inoculated with downy mildew (multiple pathotypes) and treated with azadirachtin (AZA), BTH, *T.asperellum* and mefenoxam (MX) and their combinations. The standard deviation represents the ±SDM of the three biological replicates. The means of three replicates are shown (n = 5). Significant differences between treatments were determined at P = 0.05, using Fisher's post hoc test.

Figure 21 shows the average disease rate 1(percentage) (sporulation and damping off) of several *P. halstedii* isolates on mefenoxam-treated and untreated sunflowers. In mefenoxam-treated and *P. halstedii*-inoculated sunflower plants, most of the seven isolates generated relatively lower disease rates, Except the plants inoculated with isolates I7 > I4, which showed significantly higher infection rates. In comparison, all other inducer mixtures significantly reduced sporulation in treated and infected plants (Figure 21).

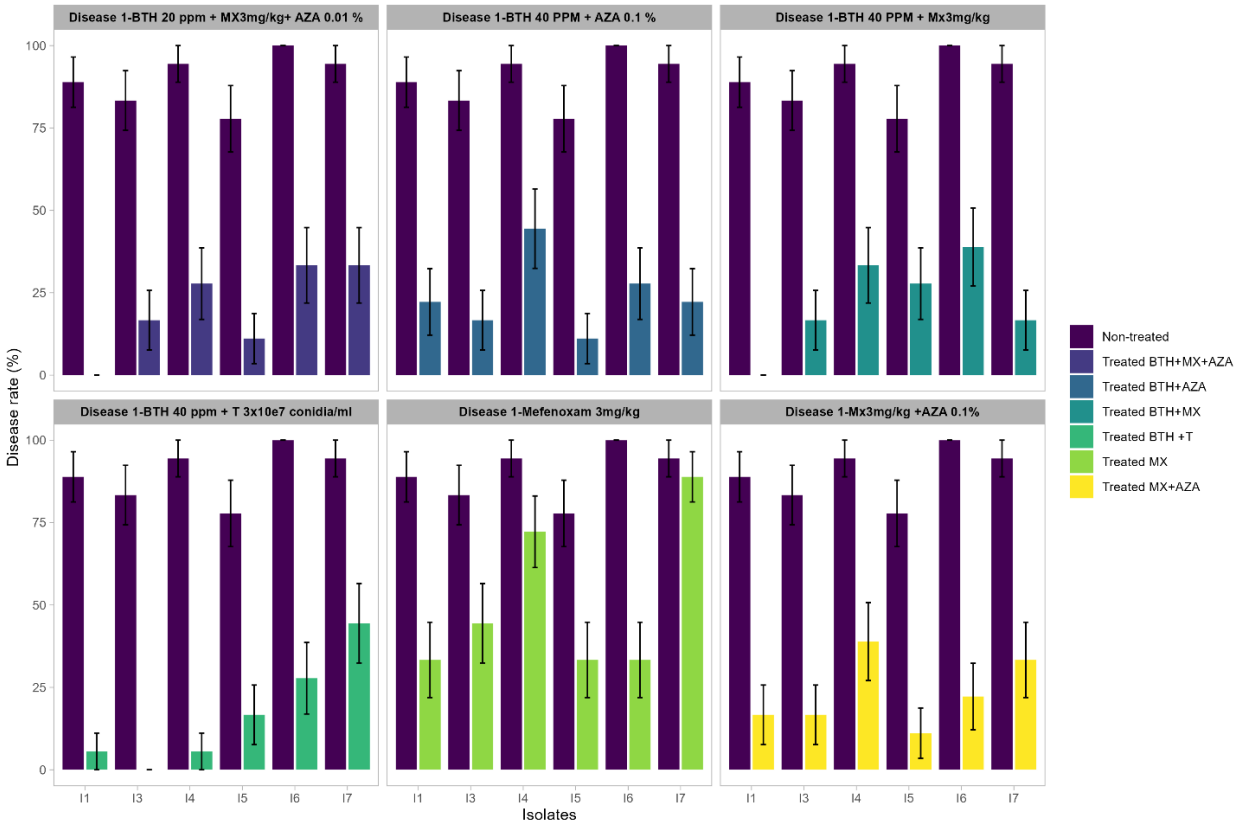


Figure 21: Disease rate 1(%) of the (frequency of sporulation and damping off) in susceptible sunflower seedlings inoculated with downy mildew (multiple pathotypes) and treated with azadirachtin *T. asperellum* (AZA), BTH and mefenoxam (MX) and/or their two or three combinations. The standard deviation represents the ±SDM of the three biological replicates. The means of three replicates are shown (n = 5). Significant differences between treatments were determined at P = 0.05, using Fisher's post hoc test.

Figure 22 shows the average disease rate 2 (percentage) (chlorosis and damping-off) of several *P. halstedii* isolates on mefenoxam-treated and untreated sunflowers. In mefenoxam-treated and *P. halstedii*-inoculated sunflower plants, six isolates generated varied disease rates, ranging from the lowest to the highest. Infection rates were significantly higher for isolate I7. Mefenoxam-sensitive downy mildew isolates were identified I1, I5, and I6, which showed the lowest infection rates. The infection rates of isolates I3 and I4 were less significant (Figure 22).

The combination of MX+AZA (Mefenoxam and Azadirachtin) did not significantly affect two *P. halstedii* isolates I6 and I7. In addition, for the other four isolates I1, I3, I4, and I5, the protection rate was highly significant. On the other hand, all the different inducer mixtures were effective against multiple *P. halstedii* isolates with respect to decreasing chlorosis and damping-off signs. In addition, it demonstrates a highly effective protection rate. (Figure 22).

Our investigations have shown that lowered azadirachtin or BTH rates are inferior to total rates. However, combining decreased doses with a full dose of Azadirachtin, BTH, and MX was

as effective as a full dose of mefenoxam or BTH. This was true, even though AZA and MX were unsuccessful when used individually. This indicates that azadirachtin interacts well with BTH, MX, or both.

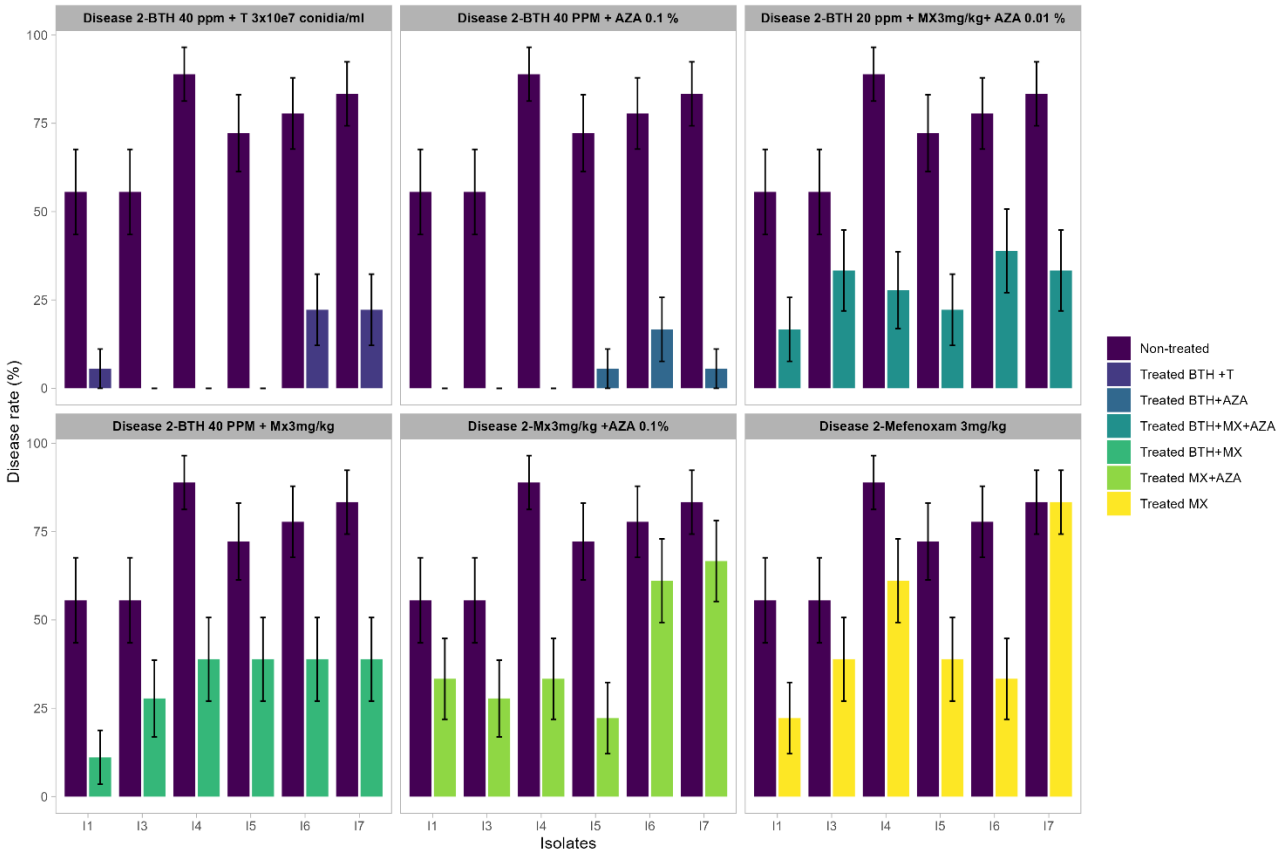


Figure 22: Disease rate 2 (%) (frequency of chlorosis and damping off) in susceptible sunflower seedlings inoculated with downy mildew (multiple pathotypes) and treated with azadirachtin (AZA), BTH, *T.asperellum* and mefenoxam (MX) and/or their two or three combinations. The standard deviation represents the ±SDM of the three biological replicates. The means of three replicates are shown (n = 5). Significant differences between treatments were determined at P = 0.05, using Fisher's post hoc test.

4.2 Interaction of biotic and abiotic inducers benzothiadiazole and *T. asperellum* to activate plant resistance against sunflower downey mildew pathotype 710

4.2.1 *In vitro* examination: Influence of BTH and *T. asperellum* on sporangia morphology and zoosporangia release of sunflower downy mildew

Both activators reduced sporangial discharge and release zoospores *in vitro*. There was no significant difference between treatments that inhibited empty sporangial. The biotic inducer had the most significant effect in delaying sporangial discharge. In contrast, the combination was less effective in reducing sporangial discharge at 48 h after incubation, as shown in (Table 6).

Table 6: Effects of biotic and chemical inducers on sporangial discharge under *in vitro* conditions.

	Empty sporangia %	
Treatments	Frequency	Mean ± STD
Control (BW)	5	20.2±4.15 A
BTH 80 PPM	5	6±1.58 B
T 10^8	5	4.8±1.48 BC
BTH 40 ppm+ T 10^7	5	6.2±1.3 B

*Different letters indicate whether or not there are significant changes between treatments at = 0.05. against seven *P. halstedii* isolates.

4.2.2 Evaluation of the Efficacy of BTH and *T. asperellum* in controlling sunflower downy mildew (*P. halstedii*) pathotype 710 and reduce the disease rate in plants: *in vivo* experiments

Macroscopic assessment of BTH 80 PPM and bioagents on sunflower downy mildew symptoms across two experimental trials in the laboratory: Untreated and injected sensitive Iregi plants showed signs of downy mildew such as sporulation, chlorosis, and stunting. *Trichoderma* spp. treatment decreased *P. halstedii* sporulation and chlorosis signs in susceptible Iregi sunflower seedling cotyledons and enhanced plant development. In contrast, BTH administered at doses of 80 ppm or greater yielded equivalent inhibition results for Diseases 1 and 2 (percentage) (Figures. 23 & 24). Compared with non-inoculated susceptible sunflowers, inoculated sunflowers treated with biotic or chemical inducers or both showed significantly lower sporulation signs than inoculated untreated seedlings. In contrast, the combination of *T. asperellum* and BTH was not significantly different from other treatments in lowering symptom intensity, and recorded the highest efficacy across the two experimental repetitions, followed by *T. asperellum* alone and BTH alone. According to the data shown in fingers 23 and 24, we can conclude that the various treatments that generated resistance in sunflower plants, as opposed to untreated controls, considerably reduced the disease severity of downy mildew. Data also showed that, compared to other treatments, biological inducer significantly lowered the severity of downy mildew infection

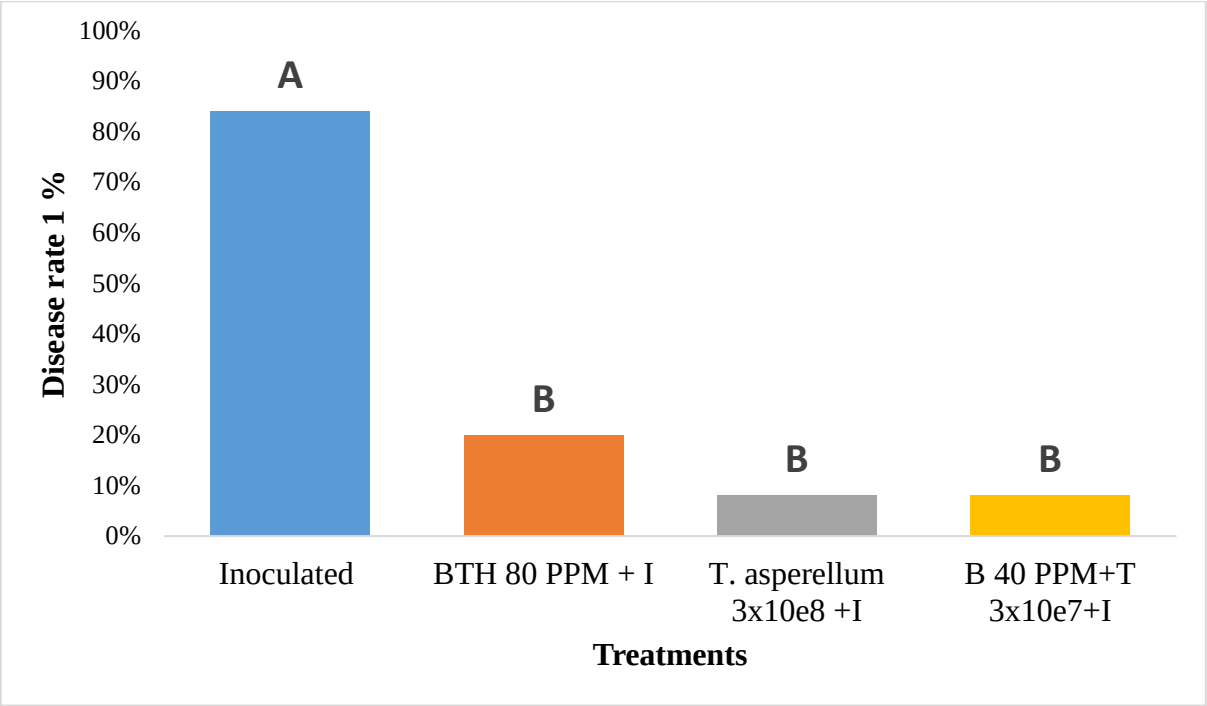
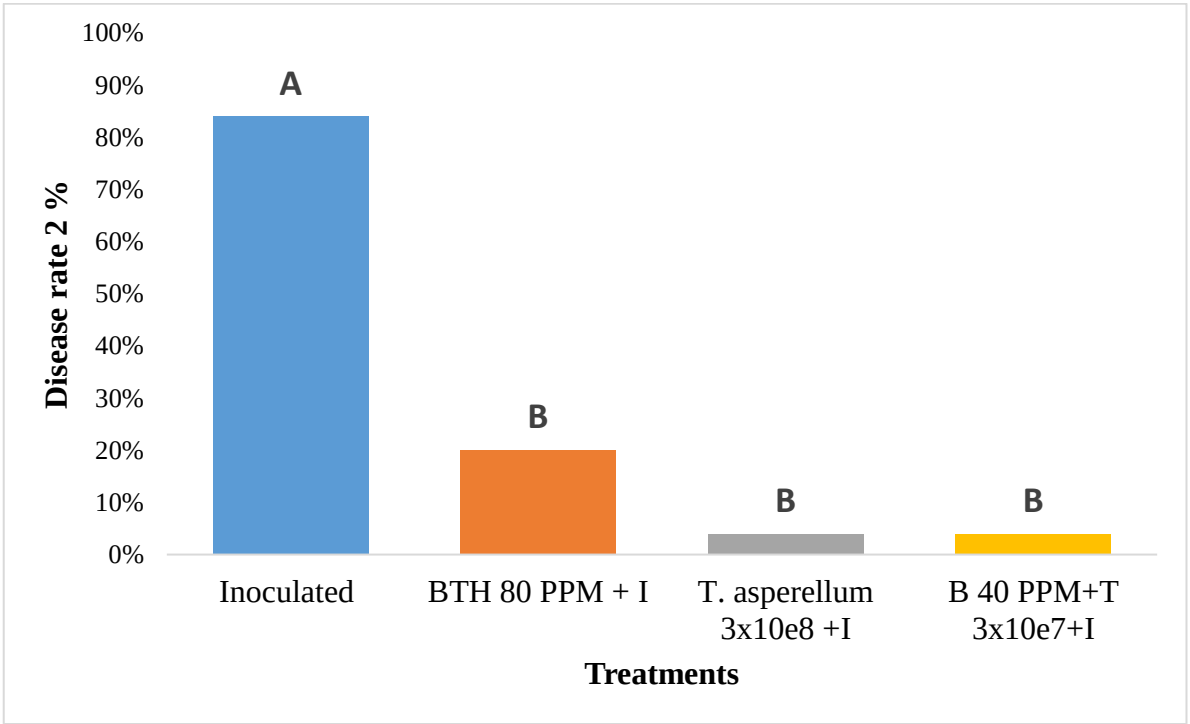


Figure 23: Effect of single and combined treatments with biotic and chemical resistance inducers to inhibit sporulation and damping-off caused by *P. halstedii*, respectively. The mean values of five replicates are shown (n = 5). Different uppercase letters above the columns indicate significant differences between treatments at $P < 0.05$, according to Fisher's post hoc test.



Figures 24: Effect of single and combined treatments with biotic and chemical resistance inducers to inhibit the chlorosis and damping-off caused by *P. halstedii*, respectively. The mean values of five replicates are shown (n = 5). Different uppercase letters above the columns indicate significant differences between treatments at $P < 0.05$, according to Fisher's post hoc test.

Due to the severe stunting of downy mildew in susceptible plants, the ability of the activator to reduce stunting is a glaring example of how host plants might be resistant. The highest results with suceptable plants were treated with *T. asperellum*, and the combination of *T. asperellum* and BTH 40 PPM; BTH 80 PPM alone also decreased stunting compared to the infected control. Plants treated with BTH 80 PPM were significantly shorter than those treated with *T. asperellum*. This effect is illustrated in Figure. 25.

In contrast, the combination of *T. asperellum* and BTH was not significantly different from other treatments, except BTH+ I, which was significantly shorter than the plants treated with the combination in lowering the dwarfing symptom across the two experimental repetitions. During the two repetitions of the experiment, there were substantial differences in the height and growth of sunflower seedlings compared to infected, untreated plants. Based on this statement, we can say that besides *T. asperellum* biocontrol activities, it can improve plant growth and reduce the disease rate .

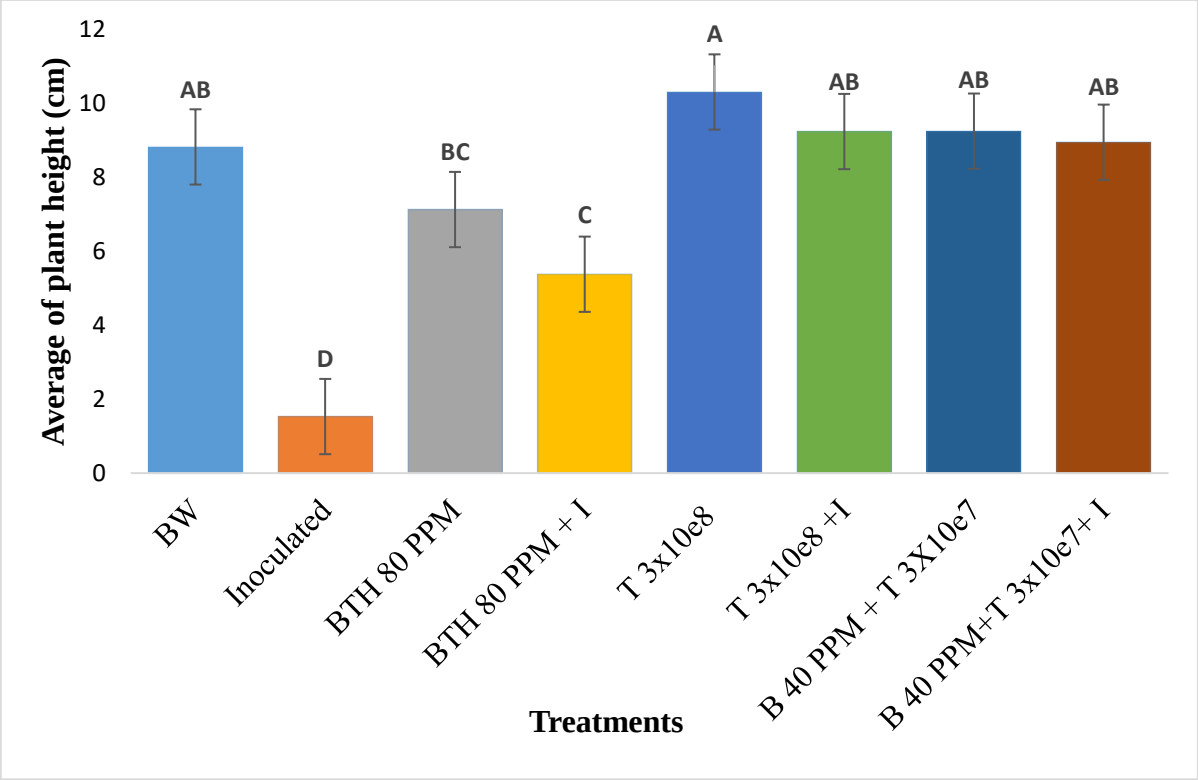
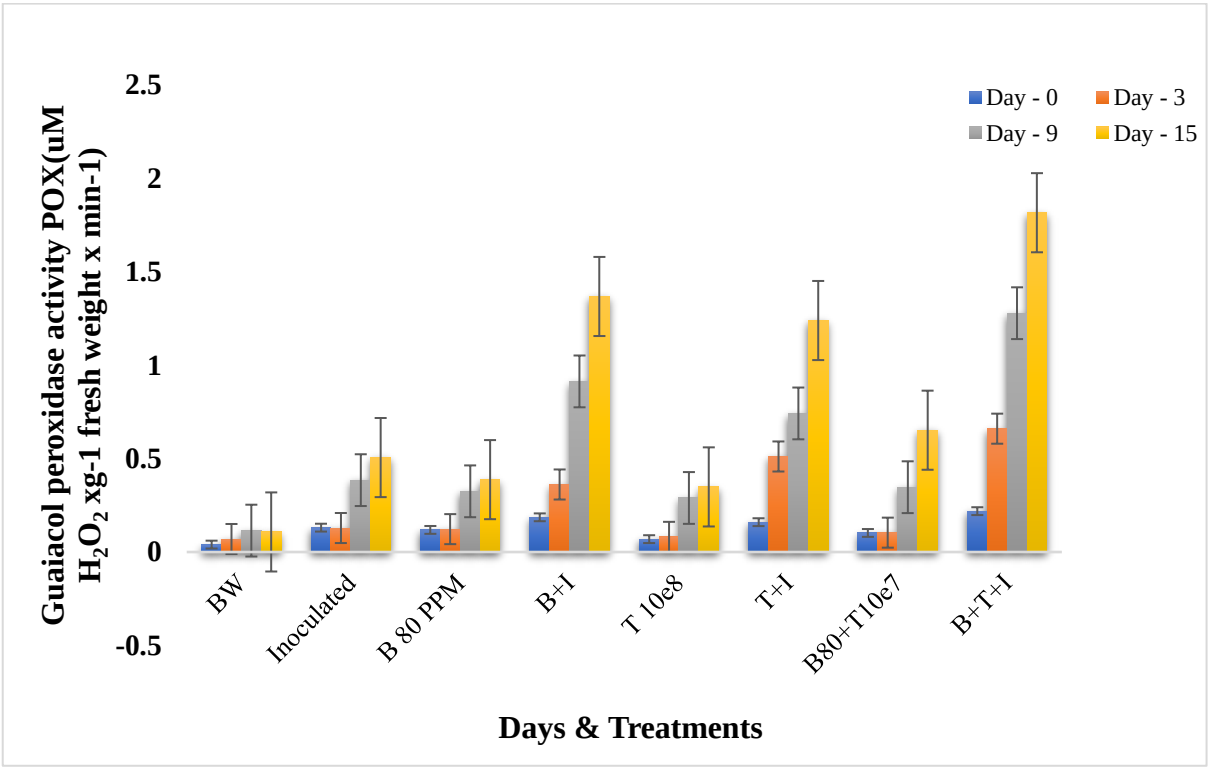


Figure 25: Effect of single and combined treatments with BTH and *T. asperellum* on stunting symptoms. The mean values of five replicates are shown (n = 5). Different uppercase letters above the columns indicate significant differences between treatments at $P < 0.05$, according to Fisher's post hoc test.

4.2.3 Measurement of antioxidant activity

Concerning the formation of oxidative enzymes in sunflowers, the data in Figures 26 and 27 indicate that the lowest values of oxidative enzymes were detected in the non-inoculated non-treated control. Generally, either individually or in combination, the chemical inducer BTH and

the biological inducer (*T. asperellum*) increased enzyme activity. In this respect, the highest activity in all determined enzymes were induced by a mixture of *T. asperellum* with BTH 40 PPM compared to any other treatment, rather than a chemical inducer. The data also showed that when applied alone, BTH was more effective than *T. asperellum in* increasing oxidative enzyme activities. In contrast, this combination was more effective. When the chemical inducer was evaluated for oxidative enzymes, BTH showed the highest effect on peroxidase (POX). The combination of BTH and *T. asperellum* was the highest for polyphenol oxidase (PPO) as a single treatment. In contrast, a mixture of these compounds, due to synergistic effects, increased oxidative enzyme activities during the time interval over days, as shown in Figures 26 and 27.



Figures 26: The graphs show The effect of the inducers BTH and *T. asperellum* on peroxidase activity on different days during the experiment. The means of three replicates are shown (n = 3). Significant differences between treatments were determined at P = 0.05, using Fisher's post hoc test.

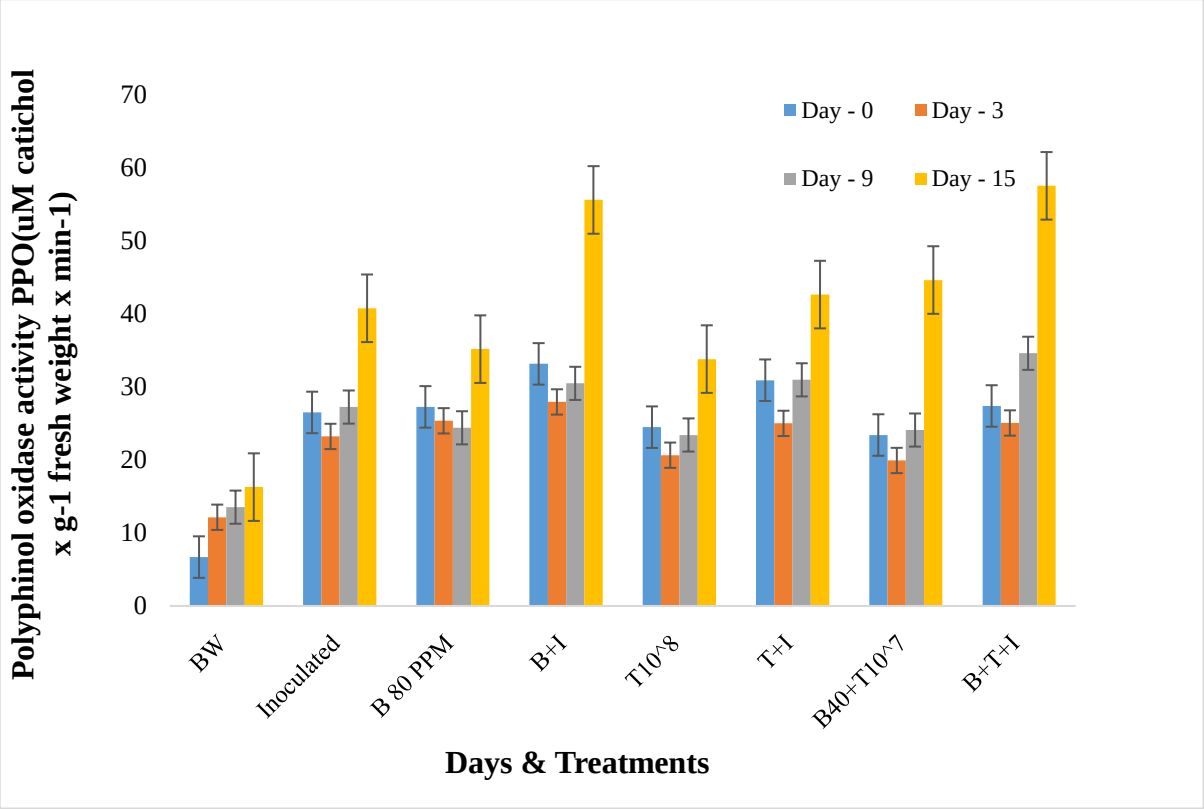
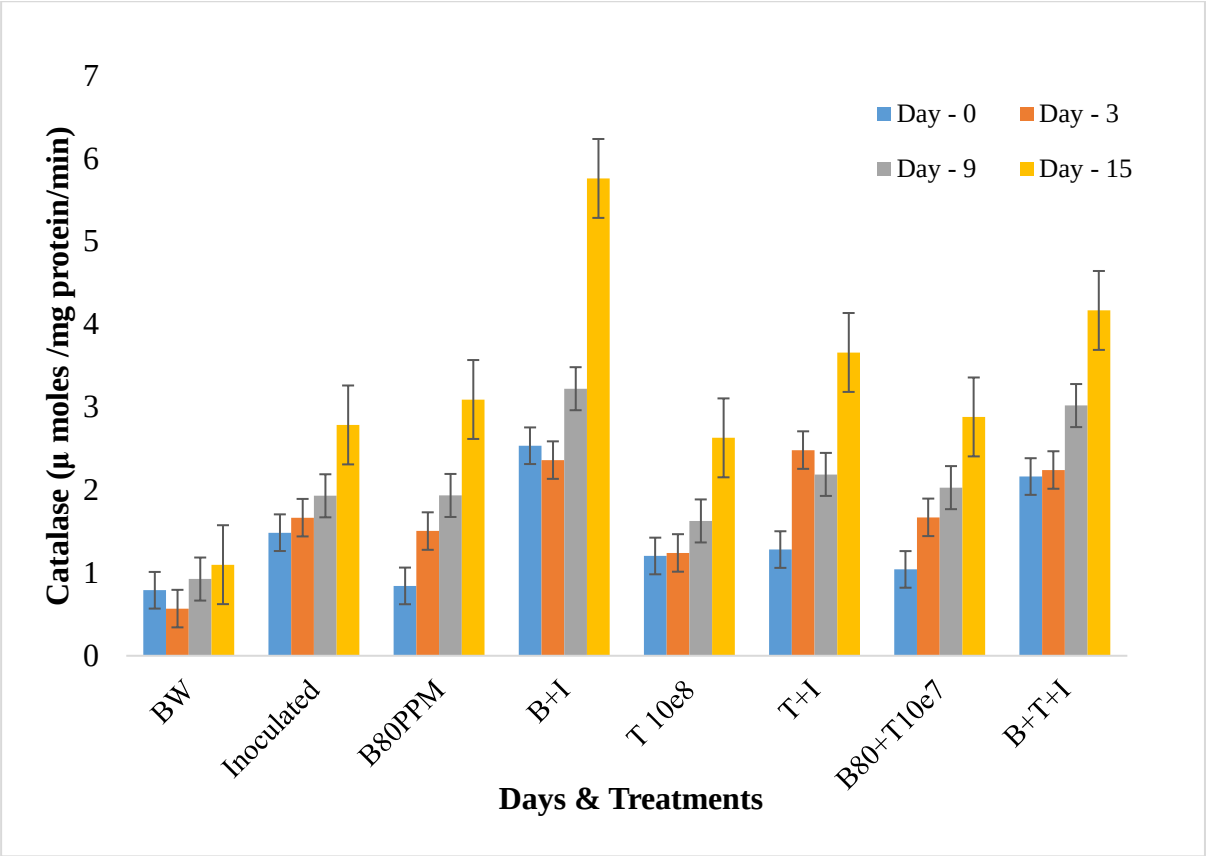


Figure 27: Effect of resistance inducers BTH and *T. asperellum* on increase in polyphenol oxidase activity (PPO) in sunflower susceptible variety (Iregi) infected with *P. halstedii* pathotype 710. The means of three replicates are shown (n = 3). Significant differences between treatments were determined at P = 0.05, using Fisher's post hoc test.

One-way ANOVA showed a significant difference between the days and decrease in H_2O_2 ($F = 4.35$, $P < 0.012$), and a significant difference between treatments concerning the decrease in H_2O_2 ($F = 3.72$, $P < 0.007$). Catalase (CAT) activity increased from 0 to 15 d. p. i.. BTH was more effective than *T. asperellum* alone for CAT activity. In this respect, a mixture of *T. asperellum* and BTH resulted in enzyme activities equivalent to those of the full dose of BTH during the two experimental repetition (Figure 28).

In conclusion, BTH, *T. asperellum*, and their combination may produce systemic resistance to downy mildew in sunflowers, and their impact is comparable to that of BTH. The POX, PPO, and CAT enzyme activities increased during the resistance reactions. Nonetheless, the activation of these defense-related proteins did not correspond with the level of resistance, but rather with the severity of necrotic symptoms in the resistant tissues.



Figures 28: Effect of resistance inducers BTH and *T. asperellum* on increase catalase activity in susceptible sunflower variety (Iregi) The means of three replicates are shown (n = 3). Significant differences between treatments were determined at P = 0.05, using Fisher's post hoc test.

4.3 Efect of azadirachtin on the *P. halstedii*-sunflower interaction

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

Sunflower dwarfism is one of the most consistent signs of *a P. halstedii* infection. Using multiple inducers and their two or three combinations significantly reduced the stunting symptoms produced by an aggressive isolate of pathotype 710. The treated plants was much higher than that of the untreated inoculated plants. Plants were treated but not infected with various combinations for phytotoxicity testing and to determine the effect of these combinations on plant growth and development. All non-inoculated plants were significantly more abundant than the inoculated control plants and exhibited no signs of phytotoxicity (Figure 29).

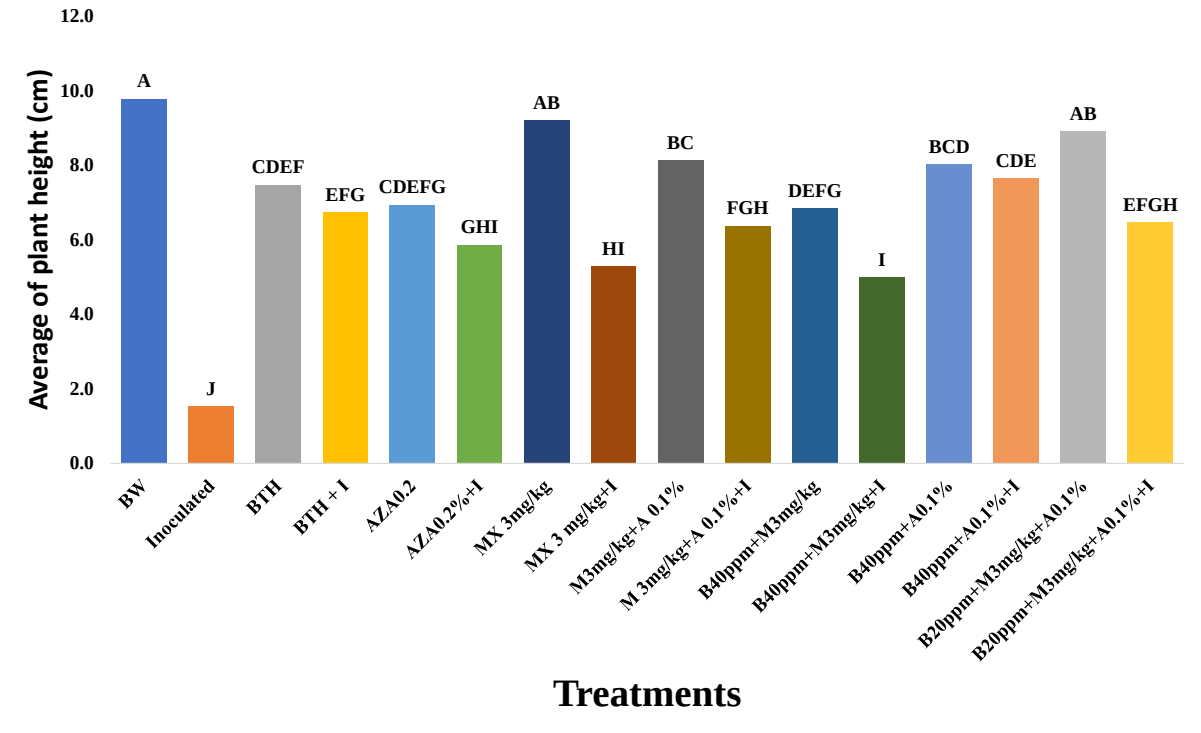


Figure 29: Effect of single and combined treatments with resistance inducers on stunting symptoms. The mean values of five replicates are shown (n = 5). Different uppercase letters above the columns indicate significant differences between treatments at $P < 0.05$, according to Fisher's post hoc test.

Regarding macroscopic indications of *P. halstedii*, all treated plants showed significantly decreased sporulation and chlorosis (Disease 1 and Disease 2 (percentage)) symptoms. Compared with the chemical control treated with mefenoxam, the treatments significantly reduced the signs and symptoms. This indicated that the isolates developed resistance to chemical fungicides. Plants inoculated and treated with mefenoxam were not significantly different from inoculated untreated plants (Figures 30 and 31).

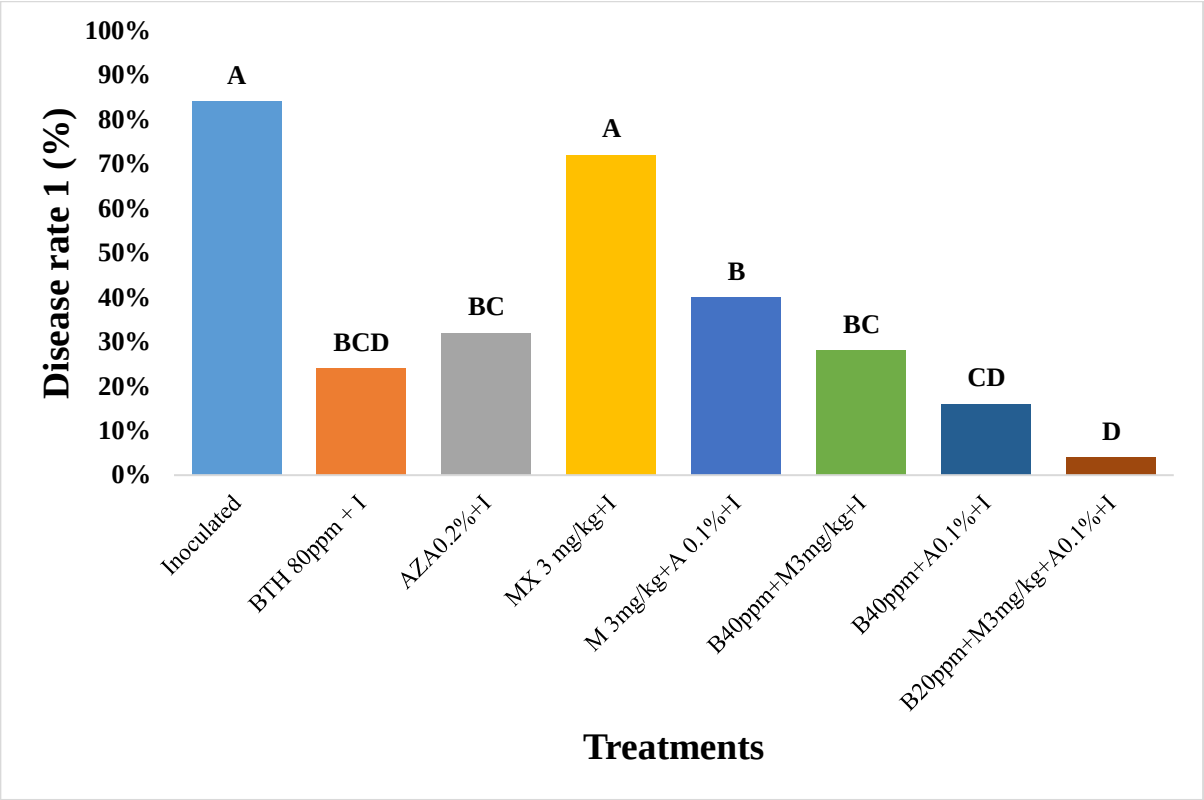


Figure 30: Effect of single and combined treatments of botanical and chemical resistance inducers to inhibit sporulation and damping off caused by *P. halstedii*, respectively. The mean values of five replicates are shown (n = 5). Different uppercase letters above the columns indicate significant differences between treatments at $P < 0.05$, according to Fisher's post hoc test.

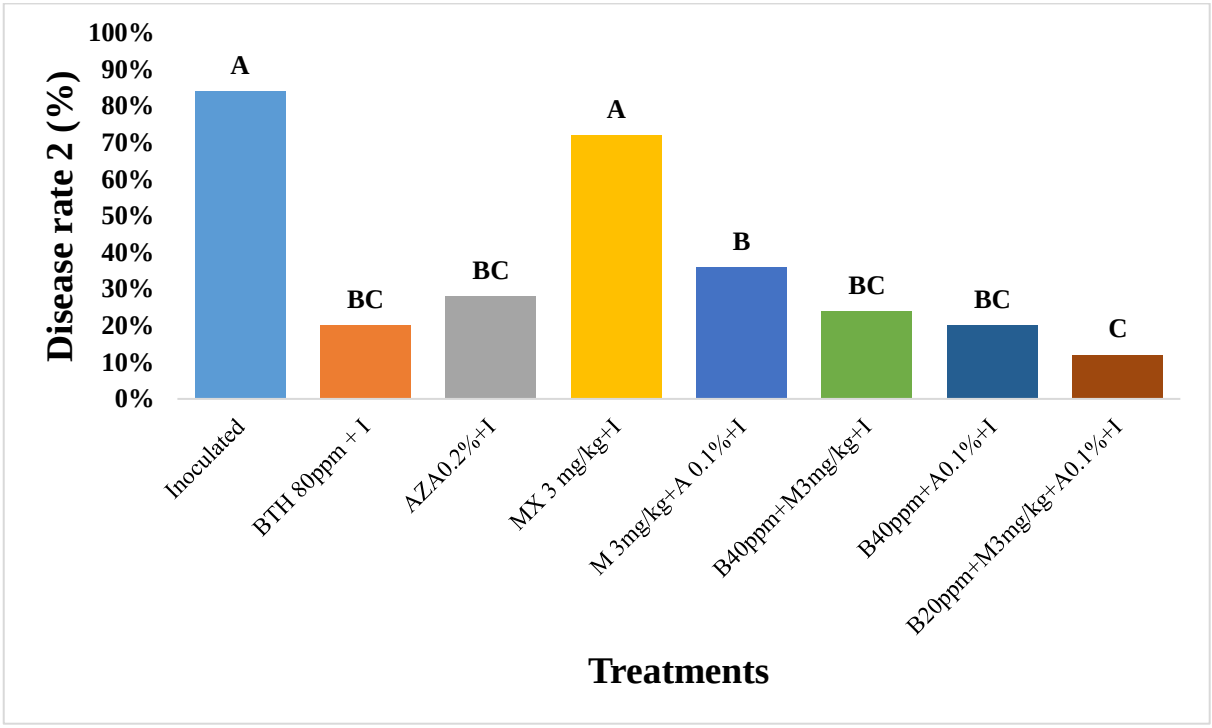


Figure 31: Effect of single and combined treatments with botanical and chemical resistance inducers to inhibit chlorosis and damping-off caused by *P. halstedii*, respectively. The mean values of five replicates are shown (n = 5). Different uppercase letters above the columns indicate significant differences between treatments at $P < 0.05$, according to Fisher's post hoc test.

We used both approaches in this investigation, which was conducted in growth chambers. Except for mefenoxam, we combined azadirachtin with BTH and MX and decreased its percentage in the mixes to approximately half of the full dose used in the treatment. For each combination, mefenoxam was administered at the recommended dosage of 3 mg/kg.

The seeds were treated with varying concentrations of azadirachtin, BTH, mefenoxam (Apron), or their two- or 3-way combinations. The plants that grew from these seeds were infected with several isolates of *P. halstedii*, and their disease susceptibility was measured in comparison with untreated seeds. All combinations considerably decreased the symptoms of the disease in sunflower seedlings. None of the mixtures exhibited phytotoxicity in the positive controls.

4.3.2 Effects of botanical and chemical combinations for antioxidant enzymes activity induction against *P. halstedii* pathotype 710

The POX, PPO, and CAT enzyme activity of sunflower seedlings treated with an inducer were significantly higher than those of untreated, uninoculated controls (Figure 32,33 & 34). All enzyme activities were greater in seedlings infected with pathogens than in those that were not inoculated. POX, PPO, and CAT enzyme activities increased considerably from 0 to 15 days post-inoculation (dpi) in susceptible sunflower seedlings treated and inoculated with the pathogen. The maximum enzyme activity per day post-inoculation was observed on day 15. Treated-inoculated seedlings had the highest enzyme activity, followed by inducer-treated and control-inoculated seedlings. Azadirachtin or its combinations may establish systemic resistance against downy mildew in sunflowers, and their impact is comparable to that of BTH. The POX, PPO, and CAT enzyme activities increased during the resistance reactions. Nonetheless, the activation of these defense-related enzymes did not correspond with the level of resistance, but rather with the severity of necrotic symptoms in the resistant tissues.

POX activity was considerably higher on day 15 in the treated seedlings (BTH+MX+AZA+I and BTH+AZA+I and BTH+I and AZA+I, respectively) than in the inoculated control seedlings. Compared with the control water, the plants that were treated and infected, as well as those that were treated but not inoculated, exhibited considerably enhanced enzyme activity from day 0 to day 15 after infection. Compared with untreated plants that had not been inoculated, plants that had been treated and infected exhibited greater enzyme activity. As shown in Table 7, the combinations showed an increase in peroxidase enzyme activity that was either substantially similar to that of a single treatment or did not significantly vary from that of the single treatment.

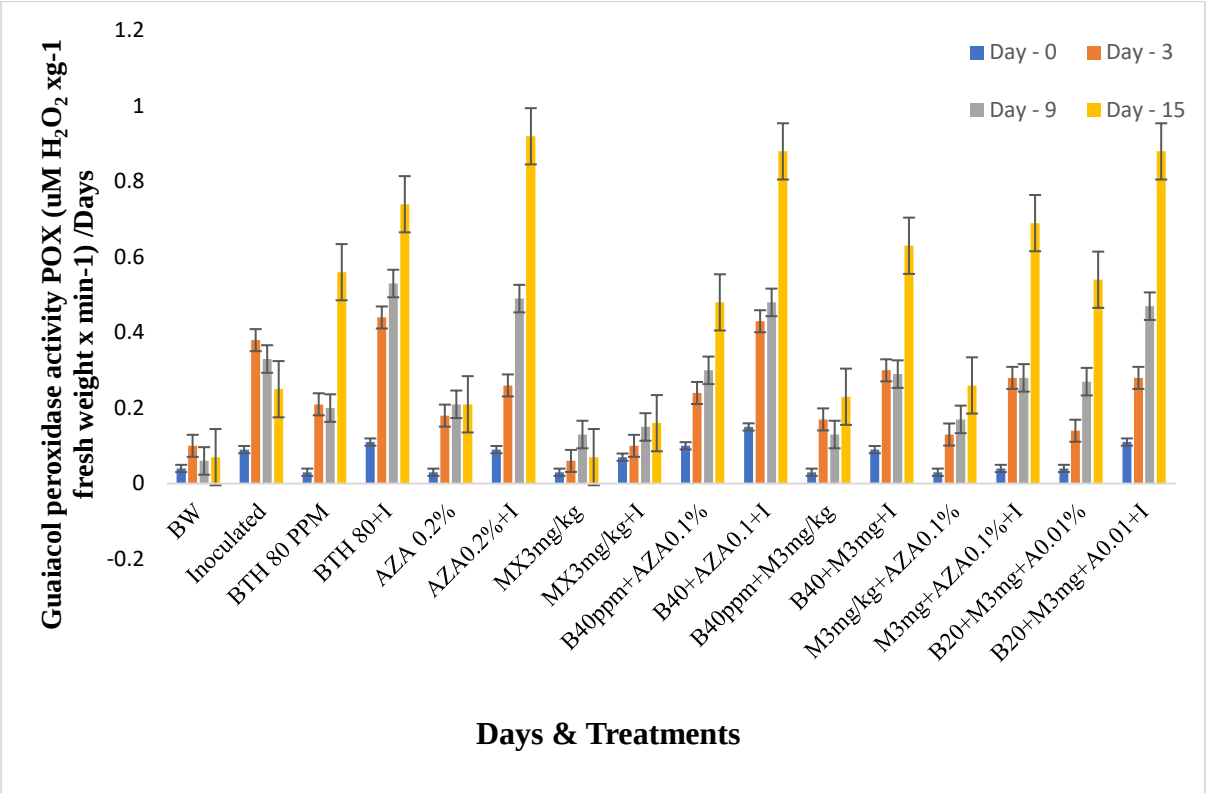


Figure 32: the guaiacol peroxidase activity (µm H_2O_2/g fresh weight/min) in a susceptible sunflower line after treatment and *P. halstedii* inoculation at different time intervals following inoculation (0, 3, 9, and 15 d). The letters beside the decimal numbers indicate the differences between the treatments on different days post infection. The mean values of three replicates are shown (n = 3).

As shown in Figure 33, on day 15, the susceptible sunflower seedlings treated with inducers and inoculated had the highest levels of PPO enzyme activity. The seedlings that had been treated with the inducer and those that had not been inoculated had the lowest levels of PPO enzyme activity. Treatment-inoculated seedlings showed the highest PPO enzyme activity. PPO and activity increased in BTH+MX+AZA+I, BTH+AZA+I, BTH+I, and AZA+I-treated seedlings, with the highest values compared to susceptible inoculated and uninoculated untreated controls. There was no significant difference in the duration or PPO activity between the single full treatment and the combinations with half doses of various inducers.

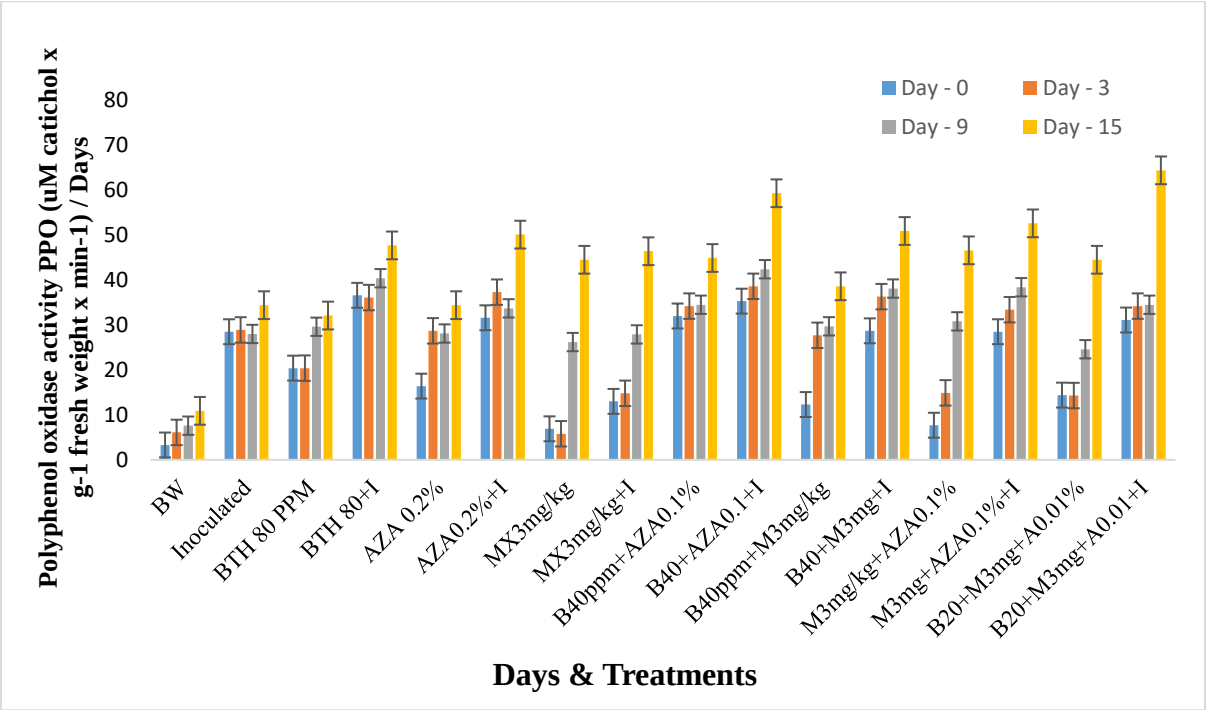


Figure 33 shows the polyphenol oxidase activity in susceptible sunflower lines (µm catechol per gram fresh weight per minute) following treatment and *P. halstedii* inoculation at various time intervals at 0, 3, and 15-days post-inoculation. The letters beside the decimal numbers indicate the differences between the treatments on different days post infection. The mean values of three replicates are shown (n = 3).

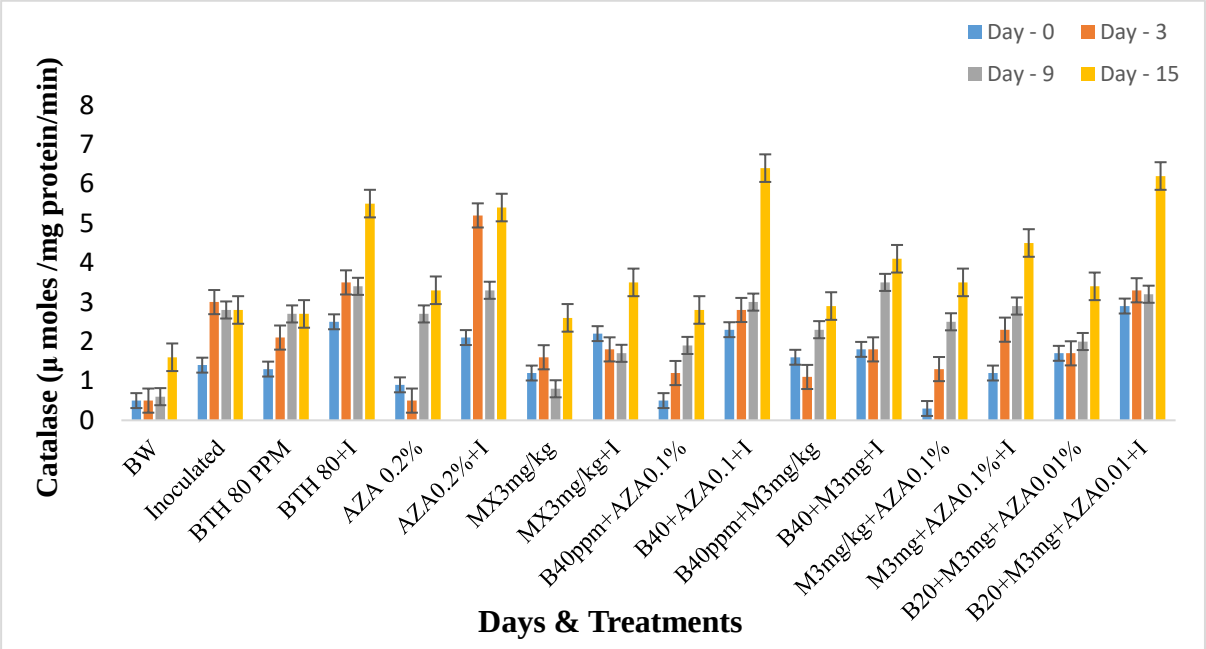


Figure 34: Catalase activity (µm catalase per gram fresh weight per minute) in susceptible sunflower lines following treatment and *P. halstedii* inoculation at various time intervals of 0, 3, 9 and 15-days post-inoculation. The letters beside the decimal numbers indicate the differences between the treatments on different days post infection. The mean values of three replicates are shown (n = 3).

On day 15, susceptible sunflower seedlings treated with inducers and inoculated were reported to have the greatest levels of catalase activity. This information is given in Figure34,

which can be seen above. The seedlings that had been treated with an inducer and those that had not been infected had the lowest levels of catalase enzyme activity. Catalase activity was the highest in seedlings that had been treated and inoculated. Compared to susceptible inoculated and uninoculated untreated controls, BTH+MX+AZA+I, BTH+AZA+I, BTH+I, and AZA+I-treated seedlings showed the highest catalase activity. When comparing the effects of a single complete treatment to those of combinations, including half doses of several different inducers, there was no noticeable difference in catalase activity or duration.

Chapter 5: DISCUSSION

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

5.1.1 The effects of BTH, azadirachtin, and *T. asprellum* on sporangia morphology and zoosporangia release in *P. halstedii*: A microscopic examination

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. This study investigated the effects of various treatments on the inhibition of germination of *P. halstedii* sporangia under *in vitro* conditions. All the concentrations of both botanical and biological activators effectively prevented sporangial discharge compared to the chemical inducer and fungcide. The activator concentration showed a linear relationship with the fraction of empty sporangia. Compared with BTH, mefenoxam, and untreated sporangia, azadirachtin successfully reduced sporangial germination, and the highest dosage was more effective. However, the response to the chemical inducer and the number of empty sporangia differed across the seven isolates due to isolate sensitivity.

Metalaxyl is a systemic fungicide extensively used to protect delicate cultivars against downy mildew caused by Peronosporales species 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. The concentration and mode of application of metalaxyl affects the infectiousness of sporangia (Singh and Shetty, 1990). Metalaxyl has been found to be a potent inhibitor of mycelial growth and sporangial development (Farih et al. 1981), although it has reduced efficacy in inhibiting encysted zoospore germination (Matheron and Porchas, 2000).

Benzo (1,2,3)-thiadiazole7-carbothermic acid S-methyl ester (BTH) activates plant defense mechanisms with minimal antifungal activity *in vitro* or in planta (Körösi et al. 2009). In this study, while each activator delayed sporangial germination, the sporangia produced only marginally decreased at the highest dosages. Unlike Kessmann et al. (1994) and Körösi et al. (2009), who reported no direct toxic effect of BTH on diverse oomycete and fungal infections, this study's data indicate that BTH has no direct harmful influence on such infections. The results of this investigation demonstrate that the novel botanical fungicide azadirachtin (AZA) is highly effective against numerous races of *P. halstedii*, causing sunflower downy mildew. It inhibits zoospore release from sporangia at low concentrations ranging from 0.1% to 0.2%. A similar efficacy was observed by Rashid et al. (2004) when comparing different neem products against two *P. infestans* isolates, as all neem products significantly inhibited various developmental stages

of the oomycete. These results also corroborate the findings of Mboussi et al. (2016), who assessed the effects of aqueous neem extracts on *P. infestans* and noted similar effects to Ridomil Gold Plus, a chemical fungicide containing mefenoxam and copper, against *Phytophthora megakarya* Brasier & M.J. Griffin.

Trichoderma spp. are common plant rhizospheres and essential decomposers of decaying wood (Chaverri et al. 2011). It produces various enzymes that have widespread applications in agriculture, such as plant growth-promoting fungi (PGPF) or biocontrol agents (BCA), to regulate nematodes or other fungal pests (Monfil & Casas-Flores, 2014). The complex mechanisms of mycoparasitism, such as *Trichoderma* spp., directed development toward target fungi, *Trichoderma* spp. attachment and coiling on target fungi, and the manufacture of a range of antifungal extracellular enzymes, have been discovered (Chet, 1987). Secondary metabolites (SMs) in *Trichoderma* spp. culture filtrate have been found to inhibit sporangiophore formation in sporangia under *in vitro* conditions, and sporicidal activity against pearl millet downy mildew (Nandini et al. 2021). Based on the findings of this study, the novel biological PGPF *T. asperellum* is highly effective against numerous pathotypes of *P. halstedii*, causing sunflower downy mildew. It suppresses zoospore discharge from sporangia at a very low dose, ranging from 3×10^7 to 3×10^8 conidia/ml.

5.1.2 Effect of BTH, azadirachtin, and *T. asprellum* on sunflower downy mildew disease severity and plant growth

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). The phenomenon of induced resistance in plants has been studied for many years, and benzothiadiazole, a chemical plant resistance activator, has been shown to offer sufficient protection against wheat powdery mildew (Sticher et al. 1997) and increase sunflower downy mildew resistance in field and greenhouse studies (Tosi et al. 1998; Bán et al. 2004). In this study, we demonstrated the effectiveness of azadirachtin (AZA) as a botanical fungicide against numerous races of *P. halstedii* that cause sunflower downy mildew. It was found to be highly efficient in preventing downy mildew infection in seedlings and is ideal for treating sunflower seeds against downy mildew. 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).

Trichoderma spp., a microparasitic microorganism, has also been found to control plant diseases through their ability to act as an antibiosis and competitor for resources and space. It can also boost nutrient availability, induce resistance, and promote healthy root growth and plant development (Harman 2006). Products containing *Trichoderma* spp. are commercially available and some have been found to increase plant productivity and prolong plant life. This study found that the novel biological *T. asperellum* is highly efficient against numerous races 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 the hypocotyl length, outperforming both the untreated control and fungicide (ai: Metalaxyl-m) treatments (Özer et al. 2021).

Moreover, *T.harzianum* (TRIC8) effectively reduced pathogen sporulation density. Metabolite analysis revealed an increase in specific compounds, including heptanoic acid, methyl palmitate, and 10,12 octadecadiynoic acid, in the roots of TRIC8-treated sunflowers. Seventeen metabolites unique to *T. harzianum* (TRIC8) treatment were identified, and these compounds are believed to contribute to enhanced hypocotyl length and protection of sunflowers against *P. halstedii* (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). In field trials, four *Trichoderma* spp. isolates were used to induce systemic resistance against powdery mildew in grapevines. Among these, the combination of 5R and NAIMCC-F-01812 exhibited the greatest reduction in disease severity. Phylogenetic analysis confirmed that all four isolates belonged to *T. asperelloides* (Sawant et al. 2020).

Metalaxyl and metalaxyl-M have been investigated for their efficacy in controlling downy mildew on sunflowers, but their potency against *P. halstedii* has been found to decrease over time (Albourie et al. 1998; Gulya, 2000; Körösi et al.2020). The use of resistant sunflower varieties and fungicides such as mefenoxam as seed coatings has been found to be the most effective method for managing sunflower downy mildew (Molinero-Ruiz et al. 2008). 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. 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.

5.1.3 Combination experiment: Treatment with inducers and design of the *in planta* experiments against different isolates of *P. halstedii*

In recent years, there have been significant advancements in eco-friendly plant disease-control methods. Biotic agents have been explored for their potential to enhance plant growth and induce resistance against sunflower downy mildew through the activation of various signalling pathways at the histological, biochemical, and molecular levels (Pánek et al. 2022). This study aimed to assess the efficacy of different treatments in managing plant pathogens that affect susceptible plant populations, thereby reducing fungicide dependence. Among the treatments tested, the combination of BTH and *T. asperellum* demonstrated the highest effectiveness in reducing sunflower downy mildew disease and enhancing plant characteristics, such as length in different isolates. These findings align with those of previous studies, including those by Ban et al. (2004) and Körösi et al. (2011), who highlighted the effectiveness of BTH in reducing sunflower downy mildew severity.

Additionally, this study provides further support for prior research conducted by Alkahtani et al. (2011) and Nagaraju et al. (2012), indicating the role of *Trichoderma* spp. in promoting plant growth, inducing pathogen resistance, and improving crop yields. Furthermore, in agreement with the findings of Sharma et al. (2004) 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 seedlings infected with multable isolates of *P. halstedii*, thereby offering insights into the use of biotic agents for seed quality improvement and disease control in sunflower production. These results suggest that catalase, peroxidase, and polyphenol oxidase play essential roles in conferring resistance to pathogens after infection, and utilising PGPF (*T. asperellum*) as a seed treatment for integrated disease management could be beneficial, given their anti-pathogenic properties and growth-promoting capabilities. *T. harzianum*, particularly the T39 isolate, exhibited efficacy in inducing plant immunity against downy mildew, underscoring its potential for disease control in different plant systems, as supported by previous research (Perazzolli et al. 2011).

The results of this study are consistent with those reported by Mirza et al. (2000), indicating the effectiveness of various neem products against different stages of the oomycete *Phytophthora infestans* (Mont.) de Bary, thereby reducing mycelial growth, sporangial germination, and sporangium production. Azadirachtin, a component of the Neem Azal extract, exhibited a unique ability to hinder pathogen progression in pea leaves by reducing the formation of germ tubes, haustoria, branches, and colonisation. It also induces a hypersensitive response leading to protein accumulation in intercellular fluids (Singh & Prithiviraj, 1997). Further experiments demonstrated

that a combination of tebuconazole and azadirachtin, applied as a mixed foliar spray, yielded promising results in controlling Alternaria blight in cumin, surpassing individual applications of fungicides or botanical treatments as well as untreated controls (Shekhawat et al. 2013). A similar combined approach of difenaconazole and azadirachtin, when applied as a foliar spray, proved highly effective in managing purple blotch disease caused by *Alternaria porri* in onions. This strategy outperformed the individual applications of fungicides, botanical treatments, and untreated controls (Jhala et al. 2017).

In the case of leaf and bud nematodes (LBN), especially *Aphelenchoides fragariae*, a preventative approach utilising azadirachtin, abamectin, spirotetramate, and ASM, either as single products or in combination with ASM, resulted in a reduction in nematode populations (Rotifa et al. 2021). The application of ASM and ASM + mefenoxam through foliar spraying was evaluated for the control of *Phytophthora capsici* in pepper. ASM + mefenoxam treatment demonstrated superior efficacy compared to ASM alone in both bioassays (Ji et al. 2011). These findings are consistent with previous reports, such as those by Ngadze (2014), who highlighted the effectiveness of *Azadirachta indica* (Neem) against *P. infestans* under both *in vitro* and *in vivo* conditions. The application of mefenoxam and Azadrachtin and BTH or their combinations resulted in significantly greater disease control against different isolates of *P. halstedii*, over their individual applications as well as over untreated controls. It would therefore, be desirable to apply integrated management practices to reduce the chances of development of resistance against fungicides, for effective and sustainable management of sunflower downy mildew.

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

5.2.1 *In vitro* examination: Influence of BTH and *T. asperellum* on sporangia morphology and zoosporangia release of sunflower downy mildew

The use of *Trichoderma* spp-based treatments has significant economic and agricultural implications, including the reduced use of fungicides, improved crop length and yield, and sustainable agricultural practices. *Trichoderma asperellum* (isolate CGMCC 6422) exhibited strong antagonistic activity against *Phytophthora capsici*, effectively breaking down the pathogen's hyphae into fragments in dual culture tests. Furthermore, *T. asperellum* was able to enter *P. capsici* oospores, developing hyphae, and causing the disintegration of the oospores(Jiang et al. 2016). Alfiky et al. (2023) characterized cell-free filtrates (CFF) from the diffrent *Trichoderma* spp pathotypes, assessing their anti-Phytophthora activities and biochemical stability. The results confirmed the chemical stability of CFF of *Trichoderma* spp., which exhibited

strong suppression of *P. infestans* mycelial growth, zoospore motility, and viability. Moreover, Alfiky et al. (2023) found that *Trichoderma* spp. are highly compatible with copper-based fungicides, suggesting the potential of combining these protective agents in integrated pest management programs.

Application of *Trichoderma* spp in variable culture based on diffusible and volatile metabolites *in vitro* reduced the growth of *Pythium* species isolated from *Lycopersicon esculentum*. Thus, it has been found that volatile metabolites of the three selected species of to *T. asperellum* showed broad-spectrum inhibition of Pythium compared to diffusible metabolites (Patil et al. 2012). Based on the information provided above, it can be inferred that the combination of *T. asperellum* with BTH and their associated mechanisms as biocontrol agents appears to be highly effective in controlling pathogens, such as *P. halstedii, in vitro*. Studies have highlighted the strong antagonistic activities of *Trichoderma* spp against various plant pathogens, their ability to break down pathogen hyphae, suppress mycelial growth, inhibit zoospore motility, and promote the disintegration of oospores. Additionally, the compatibility of *Trichoderma* spp. with other protective agents, such as resistance inducers, suggests that these combinations have potential for use in integrated pest management programs.

5.2.2 Evaluation of the Efficacy of BTH and *T. asperellum* in controlling sunflower downy mildew (*P. halstedii*) pathotype 710 and reduce the disease rate in plants: *in vivo* experiments

Reviving plant defense mechanisms against sunflower downy mildew caused by *P. halstedii* is a key objective for researchers and farmers. One promising solution is the use of biotic and abiotic inducers to activate the plant's natural defense mechanisms. Among these, benzothiadiazole and *T. asperellum* have shown great potential in enhancing plant resistance. In this context, the interaction between the two inducers is of particular interest. This synergistic effect can lead to an even greater activation of plant resistance, providing a more effective solution for combating sunflower downy mildew. The treatments in the current study, especially *T. asperellum* combined with BTH, resulted in sustained improvement in physiological features (oxidative enzyme activities) throughout the two growing seasons. The severity of downy mildew infections might be reduced by using this combination, and an effective strategy to enhance the efficacy of chemical-induced resistance agents is their synergistic application in combination with other agents. Multiple studies have demonstrated that the concurrent use of chemical-induced resistance agents and biological control agents (BCAs) yields heightened disease control outcomes. BCAs represent naturally occurring assemblages inherently antagonistic to particular plant pests and pathogens, while displaying limited non-target effects, as elucidated by Cook

(2000) These BCAs constitute an integral element of Integrated Pest Management (IPM) approaches.

The extensively studied biological control agents (BCAs) in this context primarily encompass *Trichoderma* spp., which are known for their chemotropic growth towards the roots of numerous crop varieties. Within the root environment, they synthesise diverse metabolites that foster plant growth by augmenting nutrient accessibility. Moreover, *Trichoderma* spp. activates plant defense mechanisms, leading to the suppression of plant pathogens (Mukherjee et al. 2013). In bread wheat (*Triticum aestivum* L.) plants, the co-application of MeJA and *Trichoderma harzianum* UBSTH-501 led to a marked reduction in spot blotch (*Bipolaris sorokiniana*) symptoms, surpassing the effects of either treatment alone. This enhanced efficacy of the combined treatment correlated with the increased production of indole acetic acid, a promoter of plant development and growth, in the plant rhizosphere (Singh et al. 2019).

In a separate investigation, individual treatments with MeJA, SA, and *T. harzianum* conferred protection against *Fusarium oxysporum* f. sp. *lycopersica* wilt disease in tomatoes. Remarkably, their combined application synergistically enhanced tomato antioxidant defense against *F. oxysporum*, as documented by Zehra et al. (2017). Similarly, the combined application of *T. harzianum* and ASM significantly enhanced control over *Botrytis fabae* disease severity in faba bean plants, surpassing the effectiveness of individual treatments (Abd El-Rahman and Mohamed, 2014). In addition to enhancing protective efficacy, the combination of biological control agents (BCA) and chemical-induced resistance (IR) agents has been demonstrated to promote growth. In bread wheat plants, the combined application of MeJA and *T. harzianum* significantly increased the biomass, regardless of the presence of *B. sorokiniana* (Singh et al. 2019). In tomatoes, the combination of MeJA and SA with *T. harzianum* exhibited greater protection against *F. oxysporum* f.sp. *lycopersica* disease incidence than SA or MeJA alone. Additionally, enhanced protection corresponded to a significant increase in biomass in plants subjected to combined treatment (Zehra et al. 2017). In addition, the recommendation of the Fungicide Resistance Action Committee (FRAC) to reduce pathogen exposure to selection pressure to slow the emergence of resistance would be supported using combinations (Ons et al. 2020).

5.2.3 Synergistic effect of chemical and biological inducers to enhance antioxidant activity in plants infected with (*P. halstedii*) pathotype 710

According to Hafez et al. (2018), oxidative enzymes are positively correlated with resistance. Hydrogen peroxide poisons several microorganisms that also produce peroxidase. In addition to polyphenol oxidase, increasing its activity can prevent the spread of the illness by

forming phenolic barriers surrounding the infection (Elsisi, 2019). Heba (2021) reported that *Trichoderma* spp. could also increase antioxidant activity by oxidising phenolic compounds to quinines, which are essential in the defined mechanism against pathogens, causing an increase in antimicrobial activity (El-Khallal, 2007). BTH increases the activity of oxidative enzymes (Körösi et al. 2011). As a result, they can actively participate in hindering pathogen creation by hastening cell death around the infection site and preventing disease transmission by establishing a toxic environment that limits pathogen development in plants (Khalil & Ashmawy, 2019).

According to a recent study, the plant defence activator benzothiadiazole (BTH) suppresses the formation of downy mildew on sunflowers. Following BTH treatment, the concentrations of three gene transcripts in the sensitive sunflower genotype increased: glutathione S-transferase (GST), defensin (PDF), and catalase (CAT) (Körösi & Virányi, 2008). Salicylic acid and *T. harzianum*, as Yousef (2021) claimed, provide access to peroxidase and polyphenol oxidase, respectively. Infected plants are affected in two ways by oxidative enzymes: first, the pathogen is slowed down in its life cycle owing to the enzyme's direct action; second, the pathogen's range is narrowed and biocontrol activity is increased owing to the chemicals generated in response to the enzyme's activity (Mayer, 2006; Saleem et al. 2012). Several other biological control agents (BCAs) have demonstrated synergy with chemical-induced resistance (IR) agents. For example, the saprophytic yeast-like fungus *Aureobasidium pullulans* CG163, when combined with ASM, led to a significant reduction in leaf spot incidence compared with untreated plants. Notably, the CG163 + ASM combination treatment outperformed individual treatments. Furthermore, substantial upregulation of defense-related genes, including PR1, Class IV chitinase, and β-1,3-glucosidase, was observed in plants treated with both agents. This change in gene expression was positively correlated with treatment efficacy, with the highest expression being observed in plants treated with CG163 + ASM (de Jong et al. 2019).

Our research revealed that *T. asperellum* could be a greener alternative to commercial fungicides for the treatment of downy mildew. The application of these techniques has resulted in the development of systemic resistance against various plant diseases (Mmbaga et al. 2016). This study showed that *T. asperellum*, a bioagent, effectively reduced the severity of downy mildew disease in sunflowers during both seasons. These findings were consistent with those reported by Oyoo-Okoth et al. (2011). Due to its abundance, ease of extraction and culture, rapid growth on different media, effects on various pathogens, mycoparasite role, competent competition for location and food, production of antibiotics, and enzyme mechanisms that can attack a wide range of plant pathogens (Islam et al. 2008). *Trichoderma* spp. are a promising model for studying biocontrol (Abd El-Moity, 2001). Sharma et al. (2004) showed that *T. harzianum,* or BTH stimulates the activity of Phenylalanine Ammonia Lyase (PAL) in cauliflower plants sprayed with

T. harzianum and BTH as a combined treatment, they noticed a significant increase in PAL activity against downy mildew.

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

5.3.1 Effects of botanical and chemical inducers and their combination *in planta* experiments

The 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. Our study used azadirachtin with BTH and MX to reduce disease severity in sunflower seedlings and found that plants that had only been subjected to the combination treatments did not exhibit any evidence of phytotoxicity. This indicates that the mixtures of different ingredients were not toxic to the plants and could be applied as part of the integrated management of sunflower downy mildew.

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) on sunflower seedlings can decrease *P. halstedii-induced* downy mildew. Plenaries were found to have a synergistic effect when used in conjunction with bion (acibenzolar-S-methyl) and apron (mefenoxam). Although Bion and Apron were somewhat effective in reducing the illness, the use of plenaries as a seed coating was dose dependent and more effective. When combined with reduced doses of plenaries and full doses of Bion and/or Apron, the illness was fully controlled owing to their synergy (Cohen et al. 2019). In another study conducted by Cohen et al. (2018), the combination of oxathiapiprolin and mefenoxam was found to be more effective in reducing mefenoxam-insensitive isolate-induced late blight outbreaks caused by *Phytophthora infestans* pathotypes than other fungicides.

Lower doses of azadirachtin or BTH were less effective than the full doses in this study, but the combination of lower doses with a full dose of azadirachtin, BTH, and MX was found to be as protective as the full dose of BTH alone. Although AZA and MX were not effective when used individually, a combination of azadirachtin with BTH, MX, or both was found to be effective. Haq et al. (2014) conducted a study to evaluate the effectiveness of four fungicides and three plant

extracts against purple blotch disease in onion under field conditions. The results showed that mancozeb had the highest efficacy, followed by dorazole and dora. Among the plant extracts, *Moringa oleifera* was the most effective, followed by *Azardirachta indica* and *Allium sativum*. Similarly, Jhala et al. (2017) investigated the efficacy of six fungicides and the neem component azadirachtin against *A. porri* in onions. This study found that difenaconazole completely inhibited mycelial growth at various concentrations, followed by tebuconazole. However, the combination of difenaconazole and azadirachtin as a foliar spray was most effective in controlling the disease.

5.3.2 Effect of botanical and chemical combinations on antioxidant enzyme activity in induced resistance against *P. halstedii*) pathotype 710

According to Guleria and Kumar (2006), neem leaf extract (1:2 dilution) protects sesame plants against Alternaria leaf spot pathogen (*Alternaria sesami*) and triggers metabolic alterations in the host plant. Moreover, neem extract did not affect the spores of *A. sesame*, indicating that the natural defense response of plants is involved in the protection afforded by neem extract. Moreover, the neem leaf extract increased the levels of phenylalanine Ammonia Lyase (PAL), peroxidase (POX), and phenols in sesame cv. LTK-4. 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, Lipoxygenase (LOX), and β-1,3-glucanase activities, which supported the findings of studies on disease prevention. This response was characterised by the production of PR proteins with -1,3-glucanase activity, and it was observed that the synthesis of this specific isoenzyme was a direct outcome of the antagonistic treatment (Ezziyyani et al. 2017).

The combination of the botanical pesticide azadirachtin and the chemical inducer BTH has shown great promise for significantly enhancing the efficacy of azadirachtin. Our results clearly demonstrated that this combination effectively induces plant resistance and boosts the activity of antioxidant enzymes (POX, PPO, and CAT) in sunflower seedlings against *P. hlastedii*. Importantly, we observed no signs of phytotoxicity in the treated plants, confirming the safety of this approach for plant health. Paul and Sharma (2000) observed an aqueous neem leaf extract-induced time-dependent increase in PAL, POX), and phenolic activities in barley. They observed a decline in the concentrations of PAL, POX, and total phenols in barley leaves as the duration of elicitor application increased. After 48 h of treatment with the elicitor, maximal levels were recorded. Infection with *Fusarium oxysporum* f.sp *lycopersica.* induced morphological and biochemical changes in tomato seedlings, including reduced shoot and root growth, increased lipid peroxidation, and elevated activity of antioxidant enzymes (POX, CAT, and superoxide Dismutase SOD). Electrophoretic analysis of peroxidase (POX) isoenzymes indicated upregulation caused by

Fusarium oxysporum f.sp *lycopersica*. The application of neem and willow extracts promoted the growth of tomato seedlings, whether infected or not. Additionally, the extracts reduced lipid peroxidation levels and enhanced the activity of antioxidant enzymes, observed three and seven days after infection (Hanaa et al. 2011).

The aqueous extract of neem leaves effectively controlled the leaf stripe pathogen (*Drechslera graminea*) on barley, performing as well as the fungicide bavistin (carbendazim). Treated leaves displayed increased activity of phenylalanine ammonia lyase (PAL) and tyrosine ammonia lyase (TAL), accompanied by a rapid accumulation of fungitoxic phenolic compounds (Paul and Sharma,. 2002). Most phylloplane mycoflora species were unaffected, although a few were inhibited by the neem leaf extract(Paul and Sharma,. 2002). Neem extract did not significantly hinder germination of *D. graminea* spores. These findings suggest that neem extract may indirectly induce plant defense mechanisms, and could be valuable in the integrated management of leaf stripe disease in barley (Paul and Sharma,. 2002). Similarly, time-dependent variations in PAL activity in pea leaves treated with aqueous neem extracts have been documented (Singh & Prithviraj, 1996).

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, but 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 minimise 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 6: CONCLUSION

- Due to fungicide resistance by *P. halstedii* and pathotype development, it is essential to conduct frequent evaluations of commercial active ingredients against different pathotypes of *P. halstedii* to ensure the efficacy of the ingredient against multiple races of the pathogen. Developing novel eco-friendly management strategies that are effective against various *P. halstedii* pathotypes is critical. Some natural materials (plant extracts and biocontrol agents) can be used as control agents against this disease with varied modes of action. These natural components might be more effective than chemicals because they act on different plants, such as antifungals, extracellular enzyme synthesis, plant growth promoters, or resistance inducers. These plant disease-control natural components were equivalent to our study's chemical inducers and fungicides. Thus, they may be promising plant disease control agents in the future.
- *In vitro*, both botanical and biological activators effectively prevented sporangial discharge compared to the chemical inducer and fungcide. The fraction of empty sporangia decreased linearly with the activator concentration. Compared with BTH, mefenoxam, and untreated sporangia, all azadirachtin doses successfully reduced sporangia germination, although the highest dosage was more efficient. Due to isolate sensitivity, the chemical inducer response and number of empty sporangia differed across the seven isolates.
- The current study demonstrates that the novel botanical fungicide azadirachtin is highly efficient against numerous races of *P. halstedii* that cause sunflower downy mildew. It inhibits zoospore release from sporangia at low concentrations (0.1-0.2%). When used as a WSI, the minimal inhibitory concentration (MIC) values for various isolates ranged from 0.1% to 0.2%. It is efficient for preventing and inducing antioxidant enzyme use (WSI). These characteristics make azadirachtin and its combination with BTH and MX ideal for treating downy mildew in sunflower seeds.
- According to the findings of this investigation, the novel biological (PGPF) *T. asperellum* is highly efficient against numerous races of *P. halstedii* that cause sunflower downy mildew. It suppresses zoospore discharge from sporangia at very low doses of 3×10^7–3×10^8 conidia/ml. When used as a WSI, its minimal inhibitory concentration (MIC) values for various isolates ranged from 3×10^7 to 3×10^8 conidia/ml. It is beneficial not only for prevention, but also for inducing antioxidant enzymes (WSI). *T. asperellum* and its combination with BTH are ideal candidates for seed treatment, because they can operate synergistically against downy mildew in sunflowers.
- In this study, we used both tactics in the growth chambers. We mixed azadirachtin with BTH and MX and reduced its proportion in the mixtures to approximately one-tenth of the recommended dose, with the exception of mefenoxam. We used a recommended dose of 3 mg/kg for all combinations. Seeds were treated with various doses of azadirachtin, BTH, mefenoxam

(Apron), or their 2- or 3-way mixtures. The plants developed from these seeds were inoculated with various isolates of *P. halstedii*, and the disease was monitored relative to untreated seeds. All combinations significantly reduced the disease severity in sunflower seedlings. A positive control experiment was conducted for each treatment combination. We found that plants subjected only to combination treatments did not exhibit any evidence of phytotoxicity.

- It was evident from multiple experiments that reduced rates of azadirachtin or BTH were less effective than the total rate. However, the combination of reduced rates with the full rate of azadirachtin, BTH and MX was as effective as the full rate of AZA or BTH. This was true, even though AZA and MX were ineffective when applied alone. This suggests that azadirachtin synergises with BTH, MX, or both.
- It was evident from multiple experiments that the different isolates of the pathogen had different responses to the treatments of *T. asperellum*, BTH, or the combination of reduced rates with a full rate of *T. asperellum*, and BTH was as effective as the full rate of *T. asperellum*. This was true, even though the lowest dose of BTH was not significantly effective when applied alone. This suggests that *T. asperellum* synergises with BTH .
- The current study also determined the effectiveness of the tested biotic agent *T. asperellum* in inducing resistance against downy mildew in sunflowers through seed treatment. Thus, the present study combines the knowledge of applying a biotic agent for seed quality improvement and disease management in a plant-pathogen system in sunflower production. The current results imply that catalase, peroxidase, and polyphenol oxidase are effective resistance measures against pathogen infection. The use of PGPF (*T. asperelluim*) as a seed treatment could be useful for integrated disease control. In addition to their anti-pathogenic properties, these fungi are also effective growth boosters, which are beneficial for any practical agricultural system.
- According to our research, both resistance inducers were substantially more efficient than untreated plants in reducing disease severity and inducing antioxidant activity in CAT, POX, and PPO. Both inducers induced resistance in distinct ways. The chemical inducer BTH was substantially more efficient than the biological inducer *T. asperellum in* generating antioxidant activity. However, when combined, chemical and biological inducers function synergistically, creating a far more significant effect than when applied alone. Owing to its numerous mechanisms of action, the natural inducer *T. asperellum* appears to be more effective in reducing the symptoms of downy mildew on sunflowers, as determined by macroscopic evaluation.
- Azadirachtin and BTH or MX may establish systemic resistance against downy mildew in sunflowers, and their impact is comparable to that of BTH. The POX, PPO, and CAT enzyme activities increased during the resistance reactions. Nonetheless, the activation of these defense-

related enzymes did not correspond with the level of resistance, but rather with the severity of necrotic symptoms in the resistant tissues.

- In conclusion, BTH, *T. asperellum,* and their mixture could induce systemic resistance in sunflowers against downy mildew, and their effects were similar to those caused by BTH. POX, PPO, and CAT enzyme activities were elevated during the resistance reactions. However, activation of these defense-related proteins did not correlate with the degree of resistance, but with the extent of necrotic symptoms in resistant tissues.

Chapter 7: SUMMARY

Plasmopara halstedii (Farl.) Berl. *et* de Toni is the oomycete that causes sunflower downy mildew (SDM). Traditional means of controlling this pathogen are using resistant hybrids, crop rotation and seed coating with fungicides. Disease control strategies that use a variety of approaches are becoming increasingly essential aspects of pest management. We conducted this exploratory investigation to evaluate whether specific plant resistance inducers might function against *P. halstedii*. In this study, we used azadirachtin (AZA), a botanical insecticide benzothiadiazole (BTH), and *T. asperellum* TGAM-G16. Three-day-old susceptible sunflower seedlings were pre-treated with different doses of inducers for two hours. Seedlings were immediately inoculated with seven different pathotypes of *P. halstedii*. Metalaxyl-M, a systemic fungicide, was used as the control. Nine-day-old sunflower plant leaves were sprayed with bidistilled water to stimulate the sporangial growth. *In vivo* experiments showed that BTH, *T. asperellum*, and the highest doses of azadirachtin significantly reduced the downy mildew symptoms. The various pathotypes of the pathogen significantly affected the plant height and disease symptoms under experiments. Our findings suggest the use of such inducers to reduce pathogen selection pressure for field seed treatment.

Genetic resistance to fungicides can control sunflower downy mildew (*P. halstedii*). New pathogen genotypes may re-establish the disease. *The T. asperellum* rhizosphere fungal isolate improved sunflower development and resistance to *P. halstedii* downy mildew pathotype (710). Biotic inducer spores ($3x10^8$ conidia/ml-1) with 5% gum arabic accelerated sunflower seed growth and reduced illness. *T.asperellum* ($3x10^7$ conidia/ml^{-1}) and BTH 40 ppm, as well as a single treatment of 3×10^8 spores ml^{-1}, induced resistance of 96%-100% and 92%, respectively, providing 72% disease protection in the laboratory settings. This study examined whether *T. asperellum* and benzothiadiazole (BTH) or their combination can activate antioxidant enzymes and produce downy mildew resistance in sensitive sunflowers. These biotic or abiotic inducers reduce downy mildew symptoms (sporulation, chlorosis, and stunting) and increase enzymatic activity in susceptible seedlings. This inducer boosts plant peroxidase, catalase, and polyphenol oxidase activity. Plant height expanded with plant growth-promoting fungal (PGPF) isolates in the experiments.

This exploratory study also examined whether specific plant inducers could inhibit *P. halstedii* either alone or in combination. Azadirachtin, BTH, and mefenoxam were used as resistance-inducing agents in this study. Three-day-old seedlings were pre-treated with different concentrations of azadirachtin (0.01%, 0.1%, and 0.2% of azadirachtin, 20, 40, and 80 ppm of BTH, and their combinations for two hours. Mefenoxam (3 mg/kg) was used for seed counting.

The seedlings were promptly injected with 50 000 sporangia/ml of the seven *P. halstedii* pathotypes overnight at 16°C. Mefenoxam (3 mg/kg) systemically controlled downy mildew. BTH and the highest azadirachtin dosage significantly reduced downy mildew symptoms *in vivo*. The disease pathotypes greatly influenced the germination % *in vitro*, plant height, and signs *in vivo*. Azadirachtin, BTH, and mefenoxam, and their combinations in different ratios could induce systemic downy mildew resistance in sunflowers against the isolate (710), comparable to BTH. Resistance inducers increased POX, PPO, and CAT enzyme activities. Our findings suggest that treating agricultural seeds with these inducers can reduce selective pathogen pressure.

Key words:

P. halstedii, azadirachtin, BTH, mefenoxam, antioxidants enzymes, resistance inducer, combinations, *T.asperellum*

Chapter 8: NEW SCIENTIFIC FINDINGS

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 effeciant than using the full dosage of the chemical fungicide mefenoxam when used alone against multable 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.

Chapter 9: APPENNDIX

Table 7 The result of the two-factor ANOVA

Disease 1		Type III Sum of Squares	df	Mean Squares	F	p
	Corrected Model	168.79	69	2.45	15.6	<.001
	Intercept	346.77	1	346.77	2211.39	<.001
	Isolate	24.15	6	4.02	25.67	<.001
	treatment	95.99	9	10.67	68.02	<.001
	Isolate x treatment	48.65	54	0.9	5.75	<.001
	Error	263.44	1680	0.16		
	Total	779	1750			
	Corrected total variation	432.23	1749			
Disease 2		**Type III Sum of Squares**	**df**	**Mean Squares**	**F**	**p**
	Corrected Model	173.61	69	2.52	16.12	<.001
	Intercept	384.23	1	384.23	2462.25	<.001
	Isolate	30.4	6	5.07	32.46	<.001
	treatment	99.69	9	11.08	70.98	<.001
	Isolate x treatment	43.52	54	0.81	5.17	<.001
	Error	262.16	1680	0.16		
	Total	820	1750			
	Corrected total variation	435.77	1749			
Plant height		**Type III Sum of Squares**	**df**	**Mean Squares**	**F**	**p**
	Corrected Model	5684.23	139	40.89	28.44	<.001
	Intercept	26623.37	1	26623.37	18515.64	<.001
	Isolate	83.23	6	13.87	9.65	<.001
	treatment	4514.86	19	237.62	165.26	<.001
	Isolate x treatment	1086.14	114	9.53	6.63	<.001
	Error	805.22	560	1.44		
	Total	33112.82	700			
	Corrected total variation	6489.45	699			

Chapter 10: 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.Viranyi, F., & Spring, O. (2011). Advances in sunflower downy mildew research. *European Journal of Plant Pathology*, *129*, 207-220.

Agarwal, A., Tvrda, E., Mulgund, A. (2015). Oxidative stress in pre-eclampsia. In *Handbook of Fertility* (pp. 283-290). Academic Press.

Agrios, G.N., 2005. Disease caused by oomycetes. Plant Pathol. 5th Ed Acad. Press N. Y. 409–428

Ahmad, S., GORDON-WEEKS, R., Pickett, J., Ton, J., 2010. Natural variation in priming of basal resistance: from evolutionary origin to agricultural exploitation. Mol. Plant Pathol. 11, 817–827.

Albourie, J.-M., Tourvieille, J., De Labrouhe, D.T., 1998. Resistance to metalaxyl in isolates of the sunflower pathogen *Plasmopara halstedii*. Eur. J. Plant Pathol. 104, 235–242.

Alexander, D., Goodman, R.M., Gut-Rella, M., Glascock, C., Weymann, K., Friedrich, L., Maddox, D., Ahl-Goy, P., Luntz, T., Ward, E., 1993. Increased tolerance to two oomycete pathogens in transgenic tobacco expressing pathogenesis-related protein 1a. Proc. Natl. Acad. Sci. 90, 7327–7331.

Alfiky, A., Abou-Mansour, E., De Vrieze, M., L'Haridon, F., & Weisskopf, L. (2023). Newly isolated *Trichoderma* spp. show multifaceted biocontrol strategies to inhibit potato late blight causal agent Phytophthora infestans both in vitro and in planta. Phytobiomes Journal, (ja).

Alkahtani, M., Omer, S. A., El-Naggar, M. A., Abdel-Kareem, E. M., & Mahmoud, M. A. (2011). Pathogenesis-related protein and phytoalexin induction against cucumber powdery mildew by elicitors. International journal of plant pathology, 2(2), 63-71.

Aziz, A., Trotel-Aziz, P., Dhuicq, L., Jeandet, P., Couderchet, M., Vernet, G., 2006. Chitosan oligomers and copper sulfate induce grapevine defence reactions and resistance to grey mould and downy mildew. Phytopathology 96, 1188–1194.

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.

Bán, R., Kovács, A., Körösi, K., Perczel, M., Turóczi, G., Zalai, M., Pálinkás, Z., Égei, M., 2018. 283901. First Report on the Occurrence of a Globally New Pathotype, 724, of *Plasmopara halstedii* Causing Sunflower Downy Mildew in Hungary. Plant Dis. 102, 1861.

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.

Bán, R., Kovács, A., Perczel, M., Körösi, K., Turóczi, G., 2014. First report on the increased distribution of pathotype 704 of *Plasmopara halstedii* in Hungary. Plant Dis. 98, 844–844.

Bán, R., Virányi, F., Komjáti, H., 2004. Benzothiadiazole-induced resistance to *Plasmopara halstedii* (Farl.) Berl. et de Toni in sunflower, in Advances in Downy Mildew Research—Volume 2. Springer, pp. 265–273.

Barceló, A. R., Pomar, F., López-Serrano, M., & Pedreno, M. A. (2003). Peroxidase: a multifunctional enzyme in grapevines. *Functional Plant Biology*, *30*(6), 577-591.

Barzman, M., Ba`rberi, P., Birch, A.N.E., Boonekamp, P., Dachbrodt-Saaydeh, S., Graf, B.,Hommel, B., Jensen, J.E., Kiss, J., Kudsk, P., Lamichhane, J.R., Messean, A., Moonen, A.-C., Ratnadass, A., Ricci, P., Sarah, J.-L., Sattin, M., 2015. Eight principles of integrated pest management. Agron. Sustain. Dev. 35, 1199–1215.

Basavaraj, G. L., Murali, M., Lavanya, S. N., & Amruthesh, K. N. (2019). Seed priming with biotic agents invokes a 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.

Belimov, A. A., Safronova, V. I., Sergeyeva, T. A., Egorova, T. N., Matveyeva, V. A., Tsyganov, V. E., ... & Stepanok, V. V. (2001). Characterization of plant growth-promoting rhizobacteria isolated from polluted soils and containing 1-aminocyclopropane-1-carboxylate deaminase. *Canadian Journal of Microbiology*, *47*(7), 642-652.

Benitez, T., Limon, C., Delgado-Jarana, J., & Rey, M. (1998). Glucanolytic and other enzymes and their genes. *Trichoderma and gliocladium*, *2*, 101-127.

Bhuvaneshwari, V., Paul, P., 2012. Transcriptional and translational regulation of defence enzymes induced by neem fruit extract in tomato. Arch. Phytopathol. Plant Prot. 45, 1374–1385.

Bolar, J. P., Norelli, J. L., Harman, G. E., Brown, S. K., & Aldwinckle, H. S. (2001). Synergistic activity of endochitinase and exochitinase from *Trichoderma atroviride* (*T. harzianum*) against the pathogenic fungus (Venturia inaequalis) in transgenic apple plants. *Transgenic Research*, *10*(6), 533-543.

Bostock, R.M., 2005. Signal crosstalk and induced resistance: straddling the line between cost and benefit. Annu Rev Phytopathol 43, 545–580.

Campos, E.V., de Oliveira, J.L., Pascoli, M., de Lima, R., Fraceto, L.F., 2016. Neem oil and crop protection: from now to the future. Front. Plant Sci. 7, 1494.

Chaverri, P., Gazis, R. O., & Samuels, G. J. (2011). *Trichoderma amazonicum*, a new endophytic species on Hevea brasiliensis and H. guianensis from the Amazon basin. Mycologia, 103(1), 139-151.

Chet, I. (1987). Trichoderma: application, mode of action, and potential as biocontrol agent of soilborne plant pathogenic fungi. Innovative approaches to plant disease control, 137-160.

Cohen, Y., Gisi, U., 1994. Systemic translocation of 14C-DL-3-aminobutyric acid in tomato plants in relation to induced resistance against Phytophthora infestans. Physiol. Mol. Plant Pathol. 45, 441–456.

Cohen, Y., Rubin, A. E., & Galperin, M. (2018). Oxathiapiprolin-based fungicides provide enhanced control of tomato late blight induced by mefenoxam-insensitive Phytophthora infestans. PLoS One, 13(9), e0204523.

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.

Colbach, N. & Cordeau, S. 2018. Reduced herbicide use does not increase crop yield loss if compensated by alternative preventive and curative measures. Eur. J. Agron. 94,67–78.

Conrath, U., Thulke, O., Katz, V., Schwindling, S., Kohler, A., 2001. Priming is a mechanism that induces systemic resistance in plants. Eur. J. Plant Pathol. 107, 113–119.

Cook, R. J. (2000). Advances in plant health management in the twentieth century. Annual review of phytopathology, 38(1), 95-116.

Daayf, F., Adam, L., Fernando, W., 2003. Comparative screening of bacteria for biological control of potato late blight (strain US-8) using in vitro detached leaves and whole-plant testing systems. Can. J. Plant Pathol. 25, 276–284.

Daayf, F., Schmitt, A., Belanger, R., 1995. The effects of plant extracts of Reynoutria sachalinensis on powdery mildew development and leaf physiology of long English cucumber. Plant Dis. 79, 577–580.

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.

de Jong, H., Reglinski, T., Elmer, P. A., Wurms, K., Vanneste, J. L., Guo, L. F., & Alavi, M. (2019). Integrated use of Aureobasidium pullulans strain CG163 and acibenzolar-S-methyl for management of bacterial canker in kiwifruit. Plants, 8(8), 287.

Devaiah, S.P., Mahadevappa, G.H., Shetty, H.S., 2009. Induction of systemic resistance in pearl millet (Pennisetum glaucum) against downy mildew (Sclerospora graminicola) by Datura metel extract. Crop Prot. 28, 783–791.

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

El Assiuty, E., Abd-El-Moity, T., El-Shafey, H., 1986. Mycoparasitic effect of *Trichoderma harzianum* Rifai against Cephalosporium acremonium Corda. Egypt. J. Microbiol. 21, 111–116.

Elad, Y., Chet, I., & Henis, Y. (1982). Degradation of plant pathogenic fungi *by Trichoderma harzianum. Canadian journal of Microbiology*, *28*(7), 719-725.

El-Khallal, S. M. (2007). Induction and modulation of resistance in tomato plants against Fusarium wilt disease by bioagent fungi (arbuscular mycorrhiza) and/or hormonal elicitors (jasmonic acid & salicylic acid): 1-Changes in growth, some metabolic activities and endogenous hormones related to defence mechanism. Aust J Basic Appl Sci, 1(4), 691-705.

Ezziyyani, M., Hamdache, A., Egea-Gilabert, C., Requena, M. E., Candela, M. E., & Mater, J. (2017). Production of Pathogenesis-Related proteins during the induction of resistance to Phytophthora capsici in pepper plants treated with Burkholderia cepacia and *Trichoderma harzianum* in combination compatible. J. Mater. Environ. Sci, 8, 4785-4795.

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

Flint, M. L. (2012). *IPM in practice: principles and methods of integrated pest management* (Vol. 3418). University of California Agriculture and Natural Resources.

Furuya, S., Mochizuki, M., Aoki, Y., Kobayashi, H., Takayanagi, T., Shimizu, M., Suzuki, S., 2011. Isolation and characterization of Bacillus subtilis KS1 for the biocontrol of grapevine fungal diseases. Biocontrol Sci. Technol. 21, 705–720.

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

Girijamba, R., Hariprasad, P., Brijeshsingh, S., & Niranjana, S. R. (2014). The antimildew activity of methanolic extract from selected medicinal plants against Plasmopara halstedii (Farl.) Berl. And De Tonic incitant of sunflower downy mildew disease. Int. J. Pharm. Bio. Sci, 5(4), 1010-1019.

Godard, S., Slacanin, I., Viret, O., Gindro, K., 2009. Induction of defence mechanisms in grapevine leaves by emodin-and anthraquinone-rich plant extracts and their conferred resistance to downy mildew. Plant Physiol. Biochem. 47, 827–837.

Guleria, S., & Kumar, A. (2006). Azadirachta indica leaf extract induces resistance in sesame against Alternaria leaf spot disease. *Journal of Cell and Molecular Biology*, *5*(2), 81-86.

Gulya, T., 1998. Tourvieille de Labrouhe D, Masirevic S, Penaud A, Rashid K, Virányi F. Proposal for standardized nomenclature and identification of races of *Plasmopara halstedii* (sunflower downy mildew). Presented at the ISA Symposium III Sunflower Downy Mildew. Fargo ND, pp. 130–136.

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.

Gulya, T., 2007. Distribution of *Plasmopara halstedii* races from sunflowers around the world. Adv. Downy Mildew Res. 3, 121–134.

Gulya, T., Rashid, K.Y., Masirevic, S.M., 1997. Sunflower diseases. Sunflower Technol. Prod. 35, 263–379.

Gulya, T., Sackston, W., Viranyi, F., Masirevic, S., Rashid, K., 1991. New races of the sunflower downy mildew pathogen (Plasmopara halstedii) in Europe and North and South America. J. Phytopathol. 132, 303–311.

Hammerschmidt, R., 1999. Induced disease resistance: how do induced plants stop pathogens?

Hammerschmidt, R., Kuć, J., 1982. Lignification as a mechanism for induced systemic resistance in cucumber. Physiol. Plant Pathol. 20, 61–71.

Hammerschmidt, R., Kuc, J., 2013. Induced resistance to disease in plants. Springer Science & Business Media.

Hanaa, R. F., Abdou, Z. A., Salama, D. A., Ibrahim, M. A., & Sror, H. A. M. (2011). Effect of neem and willow aqueous extracts on Fusarium wilt disease in tomato seedlings: Induction of antioxidant defensive enzymes. *Annals of Agricultural Sciences*, *56*(1), 1-7.

Harman, G. E. (2006). Overview of Mechanisms and Uses of *Trichoderma* spp. Phytopathology, 96(2), 190-194.

Harman, G. E., Howell, C. R., Viterbo, A., Chet, I., & Lorito, M. (2004). *Trichoderma* species—opportunistic, avirulent plant symbionts. *Nature reviews microbiology*, *2*(1), 43-56.

Harman, G. E., Pintor-Toro, J. A., Filippone, E., Muccifora, S., Lawrence, C. B., Zoina, A., Tuzun, S., and Scala, F. 1998. Genes from mycoparasitic fungi as a source for improving plant resistance to fungal pathogens. Proc. Natl. Acad. Sci. USA 95:7860-7865.

Heil, M., Ploss, K., 2006. Induced resistance enzymes in wild plants–do 'early birds' escape from pathogen attack? Naturwissenschaften 93, 455–460.

Héloir, M. C., Adrian, M., Brulé, D., Claverie, J., Cordelier, S., Daire, X., ... & Poinssot, B. (2019). Recognition of elicitors in grapevine: From MAMP and DAMP perception to induced resistance. Frontiers in plant science, 10, 1117.

Henry, A. W., & Gilbert, H. C. (1924). Minnesota sunflower diseases in 1923. In *Abstr. Phytopath* (Vol. 14, p. 64).

Herman, M., Restrepo, S., Smart, C., 2007. Defence gene expression patterns of three SAR-induced tomato cultivars in the field. Physiol. Mol. Plant Pathol. 71, 192–200.

J.K. Aronson MA, DPhil, MBChB, FRCP, HonFBPhS, HonFFPM, in Meyler's Side Effects of Drugs, 2016.

Jalaluddin, N. H., Norsimah, M. A., & Kesumawati, A. B. (2008). The mastery of English language among lower secondary school students in Malaysia: A linguistic analysis. *European Journal of Social Sciences*, *7*(2), 106-119.

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.

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.

Ji, P., Yin, J., & Koné, D. (2011). Application of acibenzolar-S-methyl and standard fungicides for control of Phytophthora blight on squash. Crop Protection, 30(12), 1601-1605.

Jiang, H., Zhang, L., Zhang, J. Z., Ojaghian, M. R., & Hyde, K. D. (2016). Antagonistic interaction between *Trichoderma asperellum* and Phytophthora capsici in vitro. Journal of Zhejiang University. Science. B, 17(4), 271.

Jocić, S., Cvejić, S., Hladni, N., Miladinović, D., Miklič, V., 2010. Development of sunflower genotypes resistant to downy mildew. Helia 33, 173–180.

Kang, S.H., Cho, H., Cheong, H., Ryu, C.-M., Kim, J.F., Park, S.-H., 2007. Two bacterial entophytes eliciting both plant growth promotion and plant defense on pepper (*Capsicum annuum* L.). J. Microbiol. Biotechnol. 17, 96.

Kehrer, J. P., Robertson, J. D., Smith, C. V., & McQueen, C. A. (2010). Comprehensive Toxicology.

Kessmann, H., Staub, T., Hofmann, C., Maetzke, T., Herzog, J., Ward, E., Uknes, S., Ryals, J., 1994. Induction of systemic acquired disease resistance in plants by chemicals. Annu. Rev. Phytopathol. 32, 439–459.

Khirallah, W., Mouden, N., Selmaoui, K., Achbani, E., Benkirane, R., Touhami, A. O., & Douira, A. (2016). Compatibility of Trichoderma spp. with some fungicides under in vitro conditions. Int. J. Recent Sci. Res, 7(2), 9060-9067.

Kogan, M., 1998. Integrated pest management: historical perspectives and contemporary Developments. Annu. Rev. Entomol. 43, 243–270.

Komjáti, H., Walcz, I., Virányi, F., Zipper, R., Thines, M., Spring, O., 2007. Characteristics of a Plasmopara angustiterminalis isolate from Xanthium strumarium. Eur. J. Plant Pathol. 119, 421–428.

Körösi, K. A. T. A. L. I. N., Lázár, N. E. L. L. I., & Virányi, F. (2009). Resistance to downy mildew in sunflower induced by chemical activators. *Acta phytopathological et Entomologica Hungarica*, *44*(1), 1-9.

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., Bán, R., Barna, B., & Virányi, F. (2011). Biochemical and molecular changes in downy mildew-infected sunflower triggered by resistance inducers. *Journal of Phytopathology*, *159*(7-8), 471-478.

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.

Kosaka, H., Aikawa, T., Ogura, N., Tabata, K., Kiyohara, T., 2001. Pine wilt disease caused by the pine wood nematode: the induced resistance of pine trees by the avirulent isolates of nematode. Eur. J. Plant Pathol. 107, 667–675.

Lamb, C., Dixon, R.A., 1997. The oxidative burst in plant disease resistance. Annu. Rev. Plant Biol. 48, 251–275.

Linthorst, H., Meuwissen, R., Kauffmann, S., Bol, J.F., 1989. Constitutive expression of pathogenesis-related proteins PR-1, GRP, and PR-S in tobacco has no effect on virus infection. Plant Cell 1, 285–291.

Liu, D., Raghothama, K.G., Hasegawa, P.M., Bressan, R.A., 1994. Osmotin overexpression in potato delays development of disease symptoms. Proc. Natl. Acad. Sci. 91, 1888–1892.

Liu, X., & Kokare, C. (2017). Microbial enzymes of use in industry. In *Biotechnology of microbial enzymes* (pp. 267-298). Academic Press.

Loeffer, J., Kley, N., Pittius, C., Almedida, O., Hollt, V., 1986. *In vivo* and *in vitro* studies of GABAergic inhibition of prolactin biosynthesis. Neuroendocrinology 43, 504–510.

Lorito, M. 1998. Chitinolytic enzymes and their genes. Pages 73-99 in: Trichoderma and Gliocladium.Vol. 2. G. E. Harman and C. P. Kubicek, eds. Taylor & Francis, London. Lorito, M., Woo, S. L., Garcia Fernandez, I., Colucci, G.,

Lorito, M., Woo, S. L., Donzelli, B., and Scala, F. 1996. Synergistic, antifungal interactions of chitinolytic enzymes from fungi, bacteria and plants. Pages 157-164 in Chitin Enzymology II. R. A. A. Muzzarelli, ed. Atec, Grottammare (AP), Italy

Lück, H. (1965). Peroxidase. *Methods of enzymatic analysis*, 895-897.

Lyon, G., 2007. Agents that can elicit induced resistance. Induc. Resist. Plant Def. Sustain. Approach Crop Prot. Blackwell Publ. Ltd Oxf. 9–29.

Mandels, M. 1975. Microbial sources of cellulase. Biotechnol. Bioeng. Sym. 5:81-105

Markell, S. G., Harveson, R. M., Block, C. C., & Gulya, T. J. (2015). Sunflower diseases. In Sunflower (pp. 93-128). AOCS Press.

Martín-Sanz, A., Rueda, S., García-Carneros, A., Molinero-Ruiz, L., 2019. First Report of a New Highly Virulent Pathotype of Sunflower Downy Mildew 2 (Plasmopara halstedii) Overcoming the Pl8 Resistance Gene in Europe. Plant Dis. PDIS-07.

Mboussi, S., Ambang, Z., Ndogho, A., Dooh, J., & Essouma, F. (2016). In vitro antifungal potential of aqueous seeds extracts of Azadirachta indica and Thevetia peruviana against Phytophthora megakarya in Cameroon. Journal of Applied Life Sciences International, 4(4), 1-12.

Meliala, C., Vear, F., De Labrouhe, D.T., 2000. Relation between the date of infection of sunflower downy mildew (Plasmopara halstedii) and symptoms development. Helia 23, 35–44.

Mirza, J. I., Hameed, S., Ahmad, I., Ayub, N., & Strang, R. H. C. (2000). In vitro antifungal activity of neem products against Phytophthora infestans. Pakistan Journal of Biological Sciences (Pakistan).

Mirza, M., & Beg, A. (1983). Diseases of sunflower in Pakistan-1982. *Information bulletin of the FAO research network on sunflower Helia*.

Misaghi, I. J. (1982). Alterations in phenol metabolism. Physiology and Biochemistry of Plant-pathogen Interactions. Plenum Press, New York, USA, 287-289.

Mmbaga, M. T., Mrema, F. A., Mackasmiel, L., & Rotich, E. (2016). Effect of bacteria isolates in powdery mildew control in flowering dogwoods (Cornus florida L.). Crop Protection, 89, 51-57.

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.

Molinero-Ruiz, M., Cordón-Torres, M., Martínez-Aguilar, J., Melero-Vara, J.M., Domínguez, J., 2008. Resistance to metalaxyl and to metalaxyl-M in populations of *Plasmopara halstedii* causing downy mildew in sunflower. Can. J. Plant Pathol. 30, 97–105.

Monfil, V. O., & Casas-Flores, S. (2014). Molecular mechanisms of biocontrol in *Trichoderma* spp. and their applications in agriculture. In Biotechnology and biology of *Trichoderma* (pp. 429-453). Elsevier.

Mouzeyar, S., De Labrouhe, D.T., Vear, F., 1993. Histopathological studies of resistance of sunflower (Helianthus annuus L.) to downy mildew (Plasmopara halstedii). J. Phytopathol. 139, 289–297.

Mukherjee, P. K., Horwitz, B. A., Herrera-Estrella, A., Schmoll, M., & Kenerley, C. M. (2013). Trichoderma research in the genome era. Annual review of phytopathology, 51, 105-129.

Mukhtar, I. (2009). Sunflower disease and insect pests in Pakistan: A review. *African crop science journal*, *17*(2).

Murphree, D.S., 2012. Native America: A State-by-State Historical Encyclopedia. ABC-CLIO.

Musetti, R., Stringher, L., Borselli, S., Vecchione, A., Zulini, L., Pertot, I., 2005. Ultrastructural analysis of *Vitis vinifera* leaf tissues showing atypical symptoms *of Plasmopara viticola*. Micron 36, 73–80.

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.

Nagini, S. (2014). Neem limonoids as anti-cancer agents: modulation of cancer hallmarks and oncogenic signalling. In *The enzymes* (Vol. 36, pp. 131-147). Academic Press.

Nandeeshkumar, P., Sudisha, J., Ramachandra, K.K., Prakash, H., Niranjana, S., Shekar, S.H., 2008. Chitosan-induced resistance to downy mildew in sunflower caused by *Plasmopara halstedii*. Physiol. Mol. Plant Pathol. 72, 188–194.

Nelson, E.B., 2004. Biological control of oomycetes and fungal pathogens. Encycl. Plant Crop Sci. Marcel Dekker Inc 137–140.

Ngadze, E. (2014). In vitro and greenhouse evaluation of botanical extracts for antifungal activity against Phythopthora infestans. Journal of Biopesticides, 7(2), 199.

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.

Novotelnova, N., 1962. Plasmopara halstedii (Farl.) Berl. et de Toni as a cospecies. Bot. Zhurnal 47, 970–981.

Oyoo-Okoth, E., Ngugi, C. C., & Chepkirui-Boit, V. (2011). Physiological and biochemical responses of Nile tilapia (Oreochromis niloticus) exposed to aqueous extracts of Neem (Azadirachta indica). Journal of Applied Aquaculture, 23(2), 177-186.

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.

Patil, A., Laddha, A., Lunge, A., Paikrao, H., & Mahure, S. (2012). In vitro antagonistic properties of selected Trichoderma species against tomato root rot causing Pythium species. International Journal of Science, Environment and Technology, 1(4), 302-315.

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.

Perazzolli, M., Roatti, B., Bozza, E., Pertot, I., 2011. *Trichoderma harzianum* T39 induces resistance against downy mildew by priming for defense without costs for grapevine. Biol. Control 58, 74–82.

Pieterse, C.M., Van Wees, S.C., Van Pelt, J.A., Knoester, M., Laan, R., Gerrits, H., Weisbeek, P.J., Van Loon, L.C., 1998. A novel signalling pathway controlling induced systemic resistance in Arabidopsis. Plant Cell 10, 1571–1580.

Pisani, C., Patel, P., Rosskopf, E., Abbasi, M., Aime, M., 2019. First Report of Downy Mildew Caused by Plasmopara halstedii on Ageratum houstonianum in the United States. Plant Dis. 103, 2968–2968.

Pozo, M.J., Azcón-Aguilar, C., 2007. Unraveling mycorrhiza-induced resistance. Curr. Opin. Plant Biol. 10, 393–398.

Pretty, J., 2018. Intensification for redesigned and sustainable agricultural systems. Science 362, eaav0294.

Putt, E.D., 1997. Early history of sunflower. Sunflower Technol. Prod. 35, 1–19.

Qi, L., Foley, M., Cai, X., Gulya, T., 2016. Genetics and mapping of a novel downy mildew resistance gene, Pl 18, introgressed from wild Helianthus argophyllus into cultivated sunflower (Helianthus annuus L.). Theor. Appl. Genet. 129, 741–752.

Quresh, Z. and Jan, C. 1993. Allelic Relationships among Genes for Resistance to Sunflower Rust. Crop Science 33:235-238.

Ramírez-Valdespino, C.A., Casas-Flores, S. and Olmedo-Monfil, V., 2019. Trichoderma as a model to study effector-like molecules. *Frontiers in microbiology*, *10*, p.1030.

Ranganathswamy, M., Patibanda, A. K., Chandrashekhar, G. S., Sandeep, D., Mallesh, S. B., & Kumar, H. H. (2012). Compatibility of Trichoderma isolates with selected fungicides in vitro. International Journal of Plant Protection, 5(1), 12-15.

Rashid, A., Ahmad, I., Iram, S., Mirza, J. I., & Rauf, C. A. (2004). Efficiency of different neem (Azadirachta indica A. Juss) products against various life stages of Phytophthora infestans (Mont.) Debary. Pak. J. Bot, 36(4), 881-886.

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

Rotifa, I. J., & Evans, K. A. (2021). Use of acibenzolar-S-methyl and other novel products for the management of Aphelenchoides fragariae on ornamental plants in glasshouse and commercial conditions. Crop Protection, 141, 105467.

Ruiz-García, Y., & Gómez-Plaza, E. (2013). Elicitors: a tool for improving fruit phenolic content. *Agriculture*, *3*(1), 33-52.

Sackston, W. E. (1981). The Sunflower Crop and Disease: Progress, Problems, and Prospects. *Plant disease*, *65*(8), 643-648.

Sadoma, M., El-Sayed, A., El-Moghazy, S., 2011. Biological control of downy mildew disease of maize caused by Peronosclerospora sorghi using certain biocontrol agents alone or in combination. J Agric Res 37, 1–11.

Sağol, S., Özkinay, E., & Özşener, S. (1999). Impaired antioxidant activity in women with pre-eclampsia. *International Journal of Gynecology & Obstetrics*, *64*(2), 121-127.

Sawant, I. S., Wadkar, P. N., Ghule, S. B., Salunkhe, V. P., Chavan, V., & Sawant, S. D. (2020). Induction of systemic resistance in grapevines against powdery mildew by *Trichoderma asperelloides* pathotypes. Australasian Plant Pathology, 49, 107-117.

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 races 705 and 715 on sunflower from the Czech Republic–short communication. Plant Prot. Sci. 52, 182–187.

Seiler, G., 1998. Wild sunflower as a potential source of resistance to downy mildew. Presented at the ISA Symposium III. Sunflower Downy Mildew, pp. 113–121.

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.

Shekhawat, N., Trivedi, A., Kumar, A., & Sharma, S. K. (2013). Management of Alternaria burnsii causing blight of cumin. International journal of plant protection, 6(2), 280-284 .

Shivakumar, P., Geetha, H., Shetty, H., 2003. Peroxidase activity and isozyme analysis of pearl millet seedlings and their implications in downy mildew disease resistance. Plant Sci. 164, 85–93.

Siddique Mirza, M., Yasmin, A., & Aslam, M. (1984). Sclerotinia wilt and head rot of sunflower. *Plant protection bulletin*.

Siddiqui B S., Afshan F and Gulzar T., (2004). Tetracyclic triterpenoids from the leaves of Azadirachta indica. Phytochemistry65, PP.2363-2367

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.

Singh, U. B., Malviya, D., Singh, S., Kumar, M., Sahu, P. K., Singh, H. V., ... & Saxena, A. K. (2019). *Trichoderma harzianum*-and methyl jasmonate-induced resistance to Bipolaris sorokiniana through enhanced phenylpropanoid activities in bread wheat (Triticum aestivum L.). Frontiers in microbiology, 10, 1697.

Singh, U., Prithiviraj, B., 1997. Neemazal, a product of neem (*Azadirachta indica*), induces resistance in pea (*Pisum sativum*) against *Erysiphe pisi*. Physiol. Mol. Plant Pathol. 51, 181–194.

Smith, B.D., 2006. Eastern North America as an independent center of plant domestication. Proc. Natl. Acad. Sci. 103, 12223–12228.

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

Spring, O., 2000a. Homothallic sexual reproduction in *Plasmopara halstedii*, the downy mildew of sunflower. Helia 23, 19–26.

Spring, O., 2009. Transition of secondary to systemic infection of sunflower with *Plasmopara halstedii*–An underestimated factor in the epidemiology of the pathogen. Fungal Ecol. 2, 75–80.

Spring, O., 2019. Spreading and global pathogenic diversity of sunflower downy mildew–Review. Plant Prot. Sci. 55, 149–158.

Sticher, L., Mauch-Mani, B., Métraux, and J., 1997. Systemic acquired resistance. Annu. Rev. Phytopathol. 35, 235–270.

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.

Thines, M., Choi, Y.-J., 2016. Evolution, diversity, and taxonomy of the Peronosporaceae, with focus on the genus Peronospora. Phytopathology 106, 6–18.

Thoudam, R. O. M. I. L. A., & Dutta, B. K. (2014). Compatibility of *Trichoderma atroviride* with fungicides against black rot disease of tea: an in vitro study. Journal of International Academic Research for Multidisciplinary, 2(2), 25-33.

Tosi, L., Luigetti, R., Zazzerini, A., 1998. Induced Resistance Against *Plasmopara helianthi* in Sunflower Plants by DL-β-Amino-n-butyric acid. J. Phytopathol. 146, 295–299.

Trojanová, Z., Sedlářová, M., Gulya, T. J., & Lebeda, A. (2017). Methodology of virulence screening and race characterization of Plasmopara halstedii, and resistance evaluation in sunflower–a review. *Plant Pathology*, *66*(2), 171-185.

Tucci, M., Ruocco, M., De Masi, L., De Palma, M., Lorito, M., 2011. The beneficial effect of *Trichoderma* spp. on tomato is modulated by the plant genotype. Mol. Plant Pathol. 12, 341–354.

Udoh, D. I., Asamudo, N. U., Bala, D. N., & Enwongo, O. (2012). Inhibitory Effect of Varying Concentrations of Leaves' Extracts of Centella Asiatica(Gotu Kola) on Some Microorganisms of Medical Importance. *International Journal of Chemical, Environmental and Pharmaceutical Research*, *3*(2), 142-148.

ul Haq, I., Zaman, Z., Habib, A., Javed, N., Khan, S. A., Iqbal, M., & Ihsan, J. (2014). Assessment of yield losses caused by purple blotch disease in onion (Allium cepa L.) and its management. Pakistan Journal of Phytopathology, 26(2), 225-232.

Vallad, G. E., & Goodman, R. M. (2004). Systemic acquired resistance and induced systemic resistance in conventional agriculture. Crop Science, 44(6), 1920-1934.

Vallance, J., Le Floch, G., Déniel, F., Barbier, G., Lévesque, C.A., Rey, P., 2009. Influence of Pythium oligandrum biocontrol on fungal and oomycete population dynamics in the rhizosphere. Appl Env. Microbiol 75, 4790–4800.

Van Loon, L., 1997. Induced resistance in plants and the role of pathogenesis-related proteins: a review. Eur. J. Plant Pathol. Neth.

Van Loon, L., Bakker, P., Pieterse, C., 1998. Systemic resistance induced by rhizosphere bacteria. Annu. Rev. Phytopathol. 36, 453–483.

Vear, F., Serieys, H., Petit, A., Serre, F., Boudon, J.-P., Roche, S., Walser, P., de Labrouhe, D.T., 2008. Origins of major genes for downy mildew resistance in sunflower. Presented at the Proc. 17th International Sunflower Conference Cordoba, Spain, available at www. is a sunflower. Org/publications.

Vear, F., Serre, F., Roche, S., Walser, P., 2007. Recent research on downy mildew resistance is useful for breeding industrial-use sunflowers. Helia 30, 45–54.

Vincent, J. M. (1947). Distortion of fungal hyphae in the presence of certain inhibitors. Nature, 159(4051), 850-850.

viranyi, F. 1990. Downy mildew of sunflower. In: Plant Diseases of International Importance.

Virányi, F., 2008. Research progress in sunflower diseases and their management. Presented at the Proc 17th Intl. Sunflower Conf., Cordoba, Spain, pp. 8–12.

Viranyi, F., Gulya, T.J., Tourvieille, D.L., 2015. Recent changes in the pathogenic variability of Plasmopara halstedii (sunflower downy mildew) populations from different continents. Helia 38, 149–162.

Viranyi, F., Oros, G., 1991. Developmental stage response to fungicides of Plasmopara halstedii (sunflower downy mildew). Mycol. Res. 95, 199–205.

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

Walcz, i., bogár, k., virányi, f., 2000. Study on an ambrosia isolate of *Plasmopara halstedii/estudio* de un aislado del hongo *Plasmopara halstedii* en la especie ambrosia/étude d'un isolat du champignon *Plasmopara halstedii* sur l'espèce ambrosia. Helia 23, 19–24.

Walker, C.A., van West, P., 2007. Zoospore development in the oomycetes. Fungal Biol. Rev. 21, 10–18.

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., Walsh, D., Newton, A., Lyon, G., 2005. Induced resistance for plant disease control: maximizing the efficacy of resistance elicitors. Phytopathology 95, 1368–1373.

Walters, D.R., Havis, N.D., Paterson, L., Taylor, J., Walsh, D.J., 2011. Cultivar effects on the expression of induced resistance in spring barley. Plant Dis. 95, 595–600.

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.

Wehtje, G., Zimmer, D.E., 1978. Downy Mildew of Sunflower: Biology of Systemic Infection. Phytopathology 68, 1568–1571.

Weindling, R., and Fawcett, H. S. 1936. Experiments in the control of Rhizoctonia damping-off of citrus seedlings. J. Agric. Sci. Calif. Agric. Exp. Stn. 10:1-16.

Yan, Z., Reddy, M., Ryu, C.-M., McInroy, J.A., Wilson, M., Kloepper, J.W., 2002. Induced systemic protection against tomato late blight elicited by plant growth-promoting rhizobacteria. Phytopathology 92, 1329–1333.

Yousef, H. (2021). Integration of bioagents with antioxidants to control Powdery Mildew disease in sunflower. Egyptian Journal of Agricultural Research, 99(2), 158-169.

Zafrilla, P., Morillas, J., Mulero, J., Cayuela, J. M., Martínez-Cachá, A., Pardo, F., & López Nicolás, J. M. (2003). Changes during storage in conventional and ecological wine: Phenolic content and antioxidant activity. Journal of agricultural and food chemistry, 51(16), 4694-4700.

Zehra, A., Meena, M., Dubey, M. K., Aamir, M., & Upadhyay, R. S. (2017). Activation of defense response in tomato against Fusarium wilt disease triggered by *Trichoderma harzianum* supplemented with exogenous chemical inducers (SA and MeJA). Brazilian Journal of Botany, 40, 651-664.

Zimmer, D. E., & Hoes, J. A. (1978). Diseases. *Sunflower Science and Technology*, *19*, 225-262.

Zimmer, D., 1971. a serious outbreak of downy mildew in the principal sunflower production area of the United States. Plant Dis. Report.

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, abstract, and short presentations 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.) Plants inoculated with *Plasmopara halstedii* (sunflower downy mildew) and treated with neem. International Sunflower Conference, Novi Sad, Serbia, 21-24 June 2021. **Poster Sessions.**

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Conferences abstracts:

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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.

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