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PLANT PRODUCTION

Bachelor thesis

Efficacy of mycoparasitic and entomopathogenic fungi against fungal agents of plant diseases

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Declaration

I declare that I am the author of this graduation thesis and that I used only sources and literature displayed in the list of references in its preparation.

In České Budějovice on 13. 04. 2023 .....

Signature

Abstrakt

Entomopatogenní a mykoparazitické houby mají potenciál být využívány na místní i mezinárodní úrovni jako účinná a ekologicky šetrná strategie boje proti škůdcům a chorobám rostlin způsobeným houbovými patogeny. V bakalářské práci byla testována účinnost užitečných hub v laboratorních podmínkách. Metodika zahrnovala izolaci a popis užitečných druhů hub a následné testování užitečných hub s potencionálními mykoparazitickými a antagonistickými účinky. Účinnost hub byla testována na patogenech, které byly převážně odizolovány z osiva nebo nemocných rostlin luskovin. V rámci pokusů bylo zjištěno, že všechny kmeny *Trichoderma* izolované z půd ČR prokázaly mykoparazitický efekt na vybrané patogeny. Nicméně, účinnost kmenů závisela na druhu houbového patogenu. Hodnocena byla i produkce spor kmenů Trichoderma jak v kontrolní variantě, tak i ve vzájemné kombinaci s patogeny. Výsledky opět prokázaly rozdíly v produkci spor u jednotlivých kmenů *Trichoderma*. Zároveň byl hodnocen mykoparazitický účinek entomopatogenních hub *Isaria fumosorosea* a *Akanthomyces attenuatus* proti patogenu *Botrytis cinerea*. Výsledky ukázaly, že ačkoli *I. fumosorosea* a *A. attenuatus* vykazovaly významný mykoparazitický účinek proti *Botrytis cinerea*, nejvyšší účinnost proti patogenu prokázala mykoparazitická houba *T.virens*. Zjištění naznačila, že jak mykoparazitické, tak entomopatogenní houby jsou udržitelnou alternativou umělých pesticidů v boji proti houbovým původcům chorob rostlin.

Klíčová slova: biologická ochrana, mykoparazitické houby, entomopatogenní houby, *Trichoderma* spp., *Akanthomyces attenuatus*, *Isaria fumosorosea*, patogeny rostlin

Abstract

Entomopathogenic and mycoparasitic fungi have the potential to be used both locally and internationally as an effective and environmentally friendly strategy for the management of pests and plant diseases caused by fungal pathogens. In this bachelor thesis, the efficacy of beneficial fungi was tested under laboratory conditions. The methodology involved isolation and description of useful fungal species and subsequent testing of useful fungi with potential mycoparasitic and antagonistic effects. The efficacy of the fungi was tested on pathogens that were mainly isolated from seeds or diseased legume plants. The experiments revealed that all *Trichoderma* strains isolated from soil of the Czech Republic showed mycoparasitic effect on selected pathogens. However, the efficacy of the strains depended on the type of fungal pathogen. Spore production of *Trichoderma* strains was also evaluated both in the control variant as well as the mutual interactions with pathogens. The results again showed differences in spore production among the *Trichoderma* strains. The mycoparasitic effect of the entomopathogenic fungi *Isaria fumosorosea* and *Akanthomyces attenuatus* against the pathogen *Botrytis cinerea* was also evaluated. The results revealed that although *I. fumosorosea* and *A. attenuatus* showed a significant mycoparasitic effect against *Botrytis cinerea*, the mycoparasitic fungus *T. virens* showed the highest efficacy against the pathogen. The findings indicated that both mycoparasitic and entomopathogenic fungi are sustainable alternatives to artificial pesticides in the fight against fungal agents of plant diseases.

Keywords: biological control, mycoparasitic fungi, entomopathogenic fungi, *Trichoderma* spp., *Akanthomyces attenuatus*, *Isaria fumosorosea*, plant pathogens

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Content

1 Introduction

Agriculture plays a crucial role in providing food for a country's population, but it can be severely impacted by plant diseases. These diseases can result in substantial crop losses, with estimates ranging up to 30%, posing a significant threat to the world's food supply (Pokhrel et al. 2022). Certain plant diseases can cause more severe losses in certain regions or for certain crops. To address this issue, integrated pest management (IPM) has been developed as a holistic solution to managing pests (Abhishek & Dwivedi 2021). IPM considers the interplay of pathogens, hosts, and the environment, and utilizes a variety of strategies, including biological control, to minimize the impact of pests on crops.

One of the most dangerous types of pests in agriculture are soil-borne pathogens, particularly fungi, which can be highly aggressive and cause substantial losses (Benítez et al. 2004). To combat these fungal pathogens, researchers have turned towards the use of biological control agents, such as entomopathogenic and mycoparasitic fungi. These types of fungi can naturally control the growth and spread of fungal pathogens on crops, providing a more sustainable alternative to traditional chemical fungicides.

This bachelor thesis evaluates the anti-parasitic or antagonistic effect of useful fungal species on selected fungal pathogens causing plant diseases, using laboratory techniques on samples isolated from different economically important plant species.

2 Aim of thesis

The aim of this work is to evaluate the mycoparasitic or antagonistic effect of useful fungal species on selected fungal plant pathogens isolated from different crops under laboratory conditions.

1. Spore production from the centre cultures of different fungal species of the genus *Trichoderma*.
2. Evaluation of mycoparasitic effect in the mutual interaction between mycoparasitic fungi of genus *Trichoderma* and plant pathogens.
3. Comparison of spore production of fungi of the genus *Trichoderma* after mutual interaction with fungal plant pathogens.
4. Evaluation of the mycoparasitic effect of the entomopathogenic fungi *Isaria fumosorosea* and *Akanthomyces attenuatus* against the plant pathogen *Botrytis cinerea*, including evaluation of spore production of fungal species.

3 Integrated pest management

Integrated Pest Management (IPM) is a comprehensive approach to managing pests and diseases in agriculture (Monte 2001) that takes into account the social, economic, and environmental factors of the farming system. The goal of IPM is to maintain pest populations below levels that cause economic damage while minimizing the potential harm to human health and the environment (Tang et al. 2004). This approach uses a combination of control methods, including physical, chemical, and biological methods (Timprasert et al. 2014), with a preference for natural methods. The IPM approach incorporates three basic techniques: inspection, identification and treatment (Mahato & Paikaray 2021). These techniques are used in combination with a range of other compatible tools, such as monitoring, trapping, the use of resistant varieties, crop rotation and biological control, to effectively manage pests while reducing the reliance on synthetic pesticides (Heuskin et al. 2011). IPM is a dynamic and adaptable strategy, with definitions ranging from those that focus on the basic principles of pest management in agriculture to more broadly focused definitions that take into account sustainable development, alternative farming practices, and environmental considerations.

Integrated Pest Management (IPM) is a widely accepted approach to controlling pests that has its roots in the late 1950s and early 1960s (Gui-ming et al. 2001). It was developed as a response to the negative consequences of over-reliance on pesticides and has since been widely adopted for many years. Over the past 50 years, IPM has been the preferred method for pest control and today it remains a dominant concept in the global pest management industry (Vreysen et al. 2007).

3.1 Biological Control

Biological control, also known as bio-control, is the use of natural or modified organisms, genes or gene products to reduce the impact of pests and diseases (Sharma et al. 2013). This can be achieved by applying biocontrol agents (BCAs), such as fungi, bacteria, viruses, nematodes, or natural compounds to the plant or soil or by using entomopathogenic microorganisms to infect insects (O'Brien 2017, Goettel et al. 2005). BCAs act by inhibiting the growth or reproductive ability of pests or modifying the plant's anatomy and physiology (Ghorbanpour et al. 2018). *Trichoderma, Gliocladium, Coniothyrium* and *Aspergillus* are among the most frequently utilised BCAs for the treatment of various plant diseases (Gafur 2020, Tripathi et al. 2010). These

organisms are used as the active ingredient in several commercially available BCAs that can be used to control a variety of pests, pathogens, and weeds, with methods and agents differing based on the target organism. BCAs offer several benefits compared to synthetic pesticides, such as increased safety and minimal environmental impact (Brimner et al. 2003). However, their efficacy can be influenced by environmental factors (Ghorbanpour et al. 2018) and the availability of BCAs can vary depending on regulations and location. Nevertheless, they are seen as a better alternative to chemical insecticides and pesticides (Singh et al. 2017). The use of BCAs provides opportunities for improved disease control in situations where conventional approaches are limited (Collinge et al. 2022). However, to ensure the effectiveness of using biological control methods, a deep comprehension of the relationships between the pest, the organisms that naturally control it, and the surroundings is crucial.

Biological control plays a key role in integrated pest management (IPM) (Heuskin et al. 2011). A significant part of IPM involves the use of biological control agents such as entomopathogenic fungi, which are utilized to target insect pests and other arthropods (Kidanu & Hagos 2020), as well as the use of fungal biocontrol agents, specifically *Trichoderma* spp., in order to reduce the usage of synthetic pesticides (Kumar et al. 2020).

4 Mycoparasitic and antagonistic fungi

Mycoparasitic fungi are a type of fungi that feed on other fungi (Speckbacher & Zeilinger 2018). They attach themselves to another fungi as a parasite, whether it be a hypha that is actively growing or a sclerotia that is at rest (Mukherjee et al. 2022). Fungal mycoparasites play a crucial role as biocontrol agents in controlling plant pathogenic fungi (Steyaert et al. 2003). These natural enemies of plant pathogens are able to infect and kill their host fungi, thus inhibiting the spread of plant diseases (Benítez et al. 2004). Early studies of mycoparasitic fungi can be traced back to the 1800s, with the first observations of these organisms being made by pioneering mycologists such as De Bary (Veselá 1986, Manjunatha et al. 2020). However, it was only during the later years that mycoparasites and other microorganisms began to be recognised as a potential biocontrol strategy for plant pathogens (Nesrsta 1991).

The use of antagonistic fungi as environmentally friendly controls can help reduce the occurrence and intensity of plant diseases while fostering plant growth (Berg et al. 2005). These fungi are naturally occurring, mostly soil microorganisms that possess traits enabling them to interfere with pathogen growth and infection (Chernin & Chet 2002). Fungal antagonists such as *Trichoderma* and other genera like *Gliocladium virens*, saprophytic *Fusaria*, and *Aspergilli* are extensively researched and compromise several fungal strains that are commonly used as biological control agents worldwide (Chaube et al. 2004). Among several other genera, *Trichoderma* stands out as a highly significant fungal genus due to its antagonistic properties against disease-causing fungal pathogens (Adnan et al. 2019).

4.1 Fungi genus *Trichoderma*

Fungi of the genus *Trichoderma* thrive in a variety of environments, including field and forest soils, as well as on decaying wood and plant litter (Pandaya et al. 2011). They can be commonly found in both temperate and tropical regions (Waghunde et al. 2016), where they play a crucial role in the ecosystem. *Trichoderma* species are known for their ability to combat soil-borne plant diseases, and they do so by colonizing and penetrating plant root tissues and by absorbing essential nutrients from the rhizosphere soil environment (Saba et al. 2012). In addition, mycoparasitic fungi like *Trichoderma* are known to inhibit the growth of target fungi or kill them through parasitism, and can also promote plant growth and induce systemic resistance (Martínez-Medina et al.

2017). These fungi also excel at breaking down organic matter, particularly cellulose, making them important decomposers (Zaldívar et al. 2001).

The history of *Trichoderma* fungus is extensive, with the first documentation and description recorded by Person in 1794, who named *T. viride* as the first species (Persoon 1794). Initially, it was thought that the genus *Trichoderma* was monotypic (Hawksworth 2001). According to Bisby in 1939, *T. viride* was the only species of *Trichoderma* (Jaklitsch & al. 2006). However, this claim was challenged in 1969 when Rifai conducted research and identified nine species aggregates (Rifai 1969, Samuels 2006). Rifai's findings marked a significant shift in our understanding of the genus *Trichoderma* and sparked further research into its species diversity. Numerous species of *Trichoderma* have since been discovered, and by 2022, 375 species of *Trichoderma* have been documented including *T. viride, T. reesei, T. atroviride, T. harzianum* and more (Kamil et al. 2022). Fungi belonging to the *Trichoderma* genus have been widely recognized for their effectiveness in combating plant pathogens since the 1920s (Harman 2006), and their ability to control plant diseases has made them an important tool in agriculture and horticulture. *Trichoderma*-based biocontrol agents are used to suppress various plant pathogens, including *Fusarium*, *Rhizoctonia*, and *Sclerotinia*. These agents can be applied as seed treatments, soil drenches, or foliar sprays. They can also be utilized as a component of an integrated pest management (IPM) program, which incorporates various control strategies to manage pests while minimizing the use of synthetic chemicals. In addition to their environmental benefits, *Trichoderma*-based biocontrol agents are also economical and advantageous for the environment over time, as they can establish in the environment and provide control without the need for repeated applications. The genus *Trichoderma* continues to be the subject of ongoing research to this day, as scientists and researchers work to better understand its properties and potential uses.

Trichoderma species are part of the diverse kingdom of Fungi and are classified under the phylum Ascomycota (Alfiky & Weisskopf 2021). This diverse group of fungi includes species that can be found in both asexual (anamorphic) and sexual (teleomorphic) stages (Srivastava et al. 2014). While some species of *Trichoderma* do reproduce sexually, the majority of the species within the genus primarily reproduce asexually (Cumagun 2012). In the past, *Trichoderma* and *Hypocrea* were considered separate genera because of their morphological differences. However, recent studies have shown that they are actually the same genus, and their differences are due to their

reproductive modes. As a result, the sexual and asexual forms are now referred to as different morphs or states of the same organism, and they are both classified under the genus *Trichoderma* (Samuels 2006). The sexual form of *Trichoderma* is now classified under the subgenus *Hypocrea* and the asexual form is classified under the subgenus *Trichoderma* (Srivastava et al. 2014).

4.2 Identification and morphology of *Trichoderma*

Trichoderma species have been a subject of interest for mycologists for several decades, with efforts to accurately identify and classify the genus dating back to the late 1960s. In 1969, Rifai attempted to establish the first classification system based on morphological features, recognizing the morphology of individual species and the concept of species aggregates (Samuels et al. 2012, Druzhinina & Kubicek 2005). Rifai's classical identification method, which relied on nine species, involved grouping together morphologically similar but genetically diverse aggregate species (Singh et al. 2005). In a follow-up examination in 1991, Bissett revised and analysed Rifai's findings and went on to divide the *Trichoderma* species into five sections and also identify additional species within each of the five sections (Jangir et al. 2017). Bissett and Rifai's contributions to the field have been instrumental in advancing our understanding of *Trichoderma* species and have provided a foundation for future studies in this area. Despite these efforts, accurate identification of *Trichoderma* species has remained a challenge due to the difficulty in distinguishing morphological structures and variability of the species (Siddiquee et al. 2007). These days, identifying *Trichoderma* species has become easier with the availability of modern molecular techniques, but some challenges still persist.

Trichoderma species can be identified using a combination of molecular and morphological methods. Molecular techniques involve the use of universal PCR (Polymerase Chain Reaction) primers, UP-PCR (Universally Primed-Polymerase Chain Reaction) product of cross-hybridization, and ITS1 (internal transcribed spacer region) ribotyping (Gomez-Mendez et al. 2020) to establish detailed interspecies relationships. These molecular methods used to identify *Trichoderma* species rely on the analysis of specific regions of their DNA. Morphological studies of these fungi involves the observation of both macroscopic and microscopic characters. Macroscopic observations include examining features such as color and texture of the colony surface verse and

reverse, presence or absence of pigmentation, and pattern of growth and sporulation (Filizola et al. 2019). On the other hand, microscopic observations focus on more detailed characteristics, such as the branching patterns of conidiophores, size and disposition of the conidia and the phialides and their color (Kumar et al. 2020). While morphology is an important tool for identifying species, it may not always be sufficient, as some species of *Trichoderma* can display morphological variability. Environmental conditions can also cause variations in the morphological characteristics of some *Trichoderma* species (Venkataramanamma et al. 2022). Nonetheless, despite these limitations, the use of morphology remains an essential method for identifying and distinguishing different *Trichoderma* species. Understanding the ecology, diversity, and potential applications of *Trichoderma* species in biological control and other environmental areas depends on accurate identification.

4.3 Morphological structures

Trichoderma is a genus of filamentous fungi with a diverse range of morphological characteristics, including fast growth and the ability to produce colonies of various pigments. Their branching hyphae often form dense mycelial networks that allow them to obtain nutrients and minerals from their surrounding environment, including those that may not be easily accessible to other microorganisms (Qiu et al. 2018, Benítez et al. 2004).

In addition to their characteristic colony morphology, *Trichoderma* can produce a range of asexual spores, including chlamydospores and conidia, which are instrumental in their dispersal and survival (Chaverri et al. 2003). Conidia can have a range of colors, including colorless, green, grey, or brownish tints (Blaszczyk et al.2014). Conidiophores are specialized structures (hyphae) on which *Trichoderma* spores are produced, and these structures are often branched with phialides that produce single or multiple conidia (Esposito & Silva 1998).

When environmental conditions are favorable, *Trichoderma* spores can germinate and form new colonies. Under certain conditions, such as nutrient depletion or adverse environmental factors, *Trichoderma* can undergo sporulation to produce survival structures such as chlamydospores that allow them to persist in adverse conditions until favorable growth conditions return (Hjeljord et al. 1998). The ability of *Trichoderma* to produce a diverse range of spores and sporulation structures, along with their

efficient mycelial network, makes them important contributors to ecosystem functioning and biotechnological applications particularly in agriculture. Their biotechnological applications in agriculture include biocontrol agents against plant pathogens and growth promotion of crops.

4.4 Life cycle of mycoparasitic *Trichoderma* spp.

Mycoparasitic species of *Trichoderma* genus exhibit a complex life cycle that includes several stages (Druzhinina et al. 2011), such as spore germination, hyphal growth, and mycoparasitism. These fungi have the ability to act as mycoparasites, parasitizing the hyphae of other fungal pathogens that cause plant diseases (Gruber & Zeilinger 2014). They also produce antibiotics and compete with other microorganisms in the environment (Blaszczyk et al. 2014), which helps to suppress the growth of harmful pathogens.

Mycoparasitic *Trichoderma* species use chemical signals to identify and bind to the surface of the host pathogen's cell (Sood et al. 2020), which is a crucial first step in the process of parasitism. Once attached, *Trichoderma* hyphae coils around the host's hyphae or attaches using hook-shaped structures, enabling *Trichoderma* to invade and destroy the host by secreting enzymes including powerful chitinases, and other molecules that break down the cell walls of the host and consume its nutrients (Brotman et al. 2010, Elad et al. 1983). These nutrients support the formation of new conidia, which are responsible for the spread of these mycoparasitic fungi in the environment (Adnan et al. 2019), and can germinate to continue the mycoparasitic cycle.

4.5 Mechanisms of action

The term "mechanisms of action" describes the wide range of intricate and complex methods through which antagonistic fungi, like *Trichoderma*, can affect harmful pathogens, either through direct or indirect means. These mechanisms include mycoparasitism, competition for space and nutrition, antibiosis and induced resistance, among others (Infante et al. 2009). The effectiveness of these mechanisms depends on various factors such as the *Trichoderma* strain, the target fungus, the crop plant, and environmental conditions including pH, temperature, nutrient availability, and iron concentration (Benítez et al. 2004). Understanding the different mechanisms of action employed

by *Trichoderma* can provide insights into how these fungi can be optimally utilized as biocontrol agents against phytopathogenic fungi.

4.5.1 Mycoparasitism

Mycoparasitism is a type of symbiotic relationship between fungi, in which one fungus feeds on another fungus (Moreno-Ruiz et al. 2020). Mycoparasitism is a form of antagonism (Kishan et al.2017). Antagonism is a mechanism of action in which one organism actively opposes or inhibits the growth or survival of another organism. During mycoparasitism, the mycoparasite releases enzymes and secondary metabolites (volatile, nonvolatile, diffusable) that break down the host's cell walls and extract nutrients (Steyaert et al. 2003, Patil et al. 2016). Mycoparasitism occurs in several steps, including host identification, penetration, attack initiation, and host death (Benítez et al. 2004).

Trichoderma species are prominent mycoparasites that can infect and kill a variety of plant pathogenic fungi (Verma et al. 2007). During mycoparasitism, *Trichoderma* fungi secrete hydrolytic enzymes such as chitinases, glucanases and proteases (Mukhopadhyay & Kumar 2020). These enzymes degrade the host's cell wall, enabling *Trichoderma* to penetrate and grow inside the host's hyphae (Brotman et al. 2010). Once inside, *Trichoderma* steals nutrients from the host, outcompeting it and ultimately leading to its death (Monte 2001).

Barnett and Binder (1973) first described two groups of mycoparasites, namely biotrophic mycoparasites and necrotrophic mycoparasites (Herrera-Estrella & Chet 1999, Barnett & Binder 1973). Biotrophic mycoparasites rely on living hosts for their nutrition, while necrotrophic mycoparasites kill their hosts with secondary metabolites or enzymes (saprophytic phase) before feeding on their remains (Jeffries 1995, Barnett 1963). *Trichoderma* species are generally categorized as necrotrophic mycoparasites (Viterbo & Horwitz 2010) and are well-known for their effectiveness in biological control of plant diseases.

4.5.2 Antibiosis

Antibiosis is a biological mechanism of action in which one microorganism suppresses or reduces the growth and functions of another microorganism by producing antimicrobial compounds that are toxic to other microorganisms (Ghorbanpour et al. 2018). This mechanism is commonly observed in microorganisms, such as *Trichoderma* (Halifu et al. 2020), which are used as biocontrol agents in agriculture. These biocontrol agents can produce a wide range of antimicrobial compounds, as well as enzymes, peptides, and secondary metabolites, which can directly target and control the growth of plant pathogens (Verma et al. 2007). The specific ways in which these compounds work can vary depending on the type of compound and the specific pathogen involved.

Antibiosis is one of the most effective mechanisms employed by *Trichoderma* to antagonize other microorganisms (Hasan et al. 2014). This process involves the production of a range of volatile and non-volatile metabolites, including antibiotics, which act to inhibit the growth and spread of pathogenic microorganisms (Benítez et al. 2004). An example of these antibiotics is trichodermin, which has been shown to have potent antimicrobial effects against both bacteria and fungi (Chen et al. 2021). *Trichoderma* also produces low molecular weight diffusible compounds that directly impede the colonization of targeted microorganisms (Benítez et al. 2004). The synergistic effect of antibiotics and hydrolytic enzymes produced by *Trichoderma* results in greater effectiveness of antagonism than that obtained by either mechanism alone (Gajera et al. 2013). As such, the antibiosis of *Trichoderma* is an important mechanism for controlling the growth and spread of pathogenic microorganisms and has significant potential for use as biocontrol agents.

4.5.3 Competition for space and nutrition

Competition is a mechanism that describes the interaction between organisms involving a contest for resources such as nutrients and space. It is an important factor in the interactions between *Trichoderma* species and pathogenic fungi (Blaszczyk et al. 2014, Saba et al. 2012). *Trichoderma* has proven to be an effective competitor against soil-borne fungal pathogens due to its ability to rapidly colonize and absorb nutrients in the rhizosphere, where it competes with pathogens for space (infection sites) and key exudates from plant roots (Howell 2003, Shahriar et al. 2022). Moreover, *Tricho-*

derma can outcompete other microorganisms by producing antibiotics or other compounds that inhibit the growth of other microbes (Gupta et al. 2019), or simply by being better adapted to the conditions in the rhizosphere than other organisms.

In biocontrol systems, the competition for micronutrients (such as iron) and macronutrients (such as carbon, nitrogen) plays a pivotal role (Sood et al. 2020, Vinale et al. 2008). Iron is a key component of many enzymes involved in cellular respiration and other metabolic processes, so fungi need it in order to survive and grow. *Trichoderma* has a superior capacity to mobilize soil nutrients and absorb them compared to other microbes, which enables it to produce several siderophores (small molecules) that bind to iron and remove it from the environment (Ghazanfar et al. 2018, Contreras-Cornejo et al. 2016), thereby reducing the availability of iron to pathogenic microorganisms and giving *Trichoderma* an advantage in the competition for nutrients.

4.6 Root colonization

Colonization refers to a microorganism's ability to attach to plant roots, infiltrate the plant, and withstand the toxic compounds produced by the plant in response to foreign invaders (Benítez et al. 2004). *Trichoderma* spp. are known to efficiently colonize plant roots, leading to improved growth, development, and resistance to a variety of abiotic stresses (Zaidi et al. 2014). During colonization, *Trichoderma* strains release compounds that stimulate plant growth and activate defense mechanisms (Benítez et al. 2004). Certain strains of *Trichoderma*, such as *Trichoderma virens*, have been shown to promote root growth in plants by producing the plant hormone auxin, which has been proven to promote lateral root growth through an auxin-dependent mechanism in *Arabidopsis* (Contreras-Cornejo et al. 2009). Other strains of *Trichoderma* can produce enzymes that break down complex organic compounds, such as cellulose and chitin, into simple nutrients that plants can use (Mukhopadhyay & Kumar 2020, Zaldívar et al. 2001). Additionally, some *Trichoderma* strains establish long-lasting colonization of plant roots and penetrate the epidermis, producing compounds that induce plant resistance responses (Benítez et al. 2004). For example, Yan et al. (2021) showed that *T. harzianum* induced the production of compounds in tomato plants that protected against root-knot nematodes. However, the success of colonization depends on the strain's ability to tolerate the antimicrobial compounds that plants produce to protect themselves (Hermosa et al. 2012). *Trichoderma*'s ability to break down plant defense

compounds can lead to the alteration of the plant's growth and defense pathways, resulting in improved growth, increased yield, and enhanced resistance to pathogens (Shahriar et 2022, Chakraborty et al. 2020).

4.7 Some important fungal species within the genus *Trichoderma*

Trichoderma virens

Preferred name: *Trichoderma virens* (J.H. Miller, Giddens & A.A. Foster) Arx.

Other scientific names: *Gliocladium virens* (J.H. Miller, J.E. Giddens & A.A. Foster)

Trichoderma virens is a soil-inhabiting fungus known for its ability to suppress a wide range of plant pathogens, such as *Rhizoctonia solani*, *Pythium* spp., and *Fusarium oxysporum*, through various mechanisms, including competition and mycoparasitism (Mukherjee et al. 2003, Mishra et al. 2004). Despite primarily reproducing asexually, T. *virens* also has a sexual or teleomorphic stage known as *Hypocrea virens* (Chaverri et al. 2001). In addition, *Trichoderma virens* exhibits characteristics typically associated with different species of symbiotic or pathogenic microorganisms, enabling it to interact with plants in complex ways that establish beneficial relationships while providing protection against plant pathogens (Gupta et al. 2021). This prevalent fungus is found worldwide and produces various antibiotic compounds (eg, gliotoxin), which increase its ability to thrive in soil (Lumsden & Knauss 2007, Lumsden et al. 1992). *T. virens* has been extensively studied and serves as the active ingredient in SoilGard, a commercial product formerly known as GlioGard (Monte 2001). SoilGard offers protection against fungal diseases, including damping-off, root rot and wilt, caused by soil-borne pathogens (Eyal et al. 1997, Christopher et al. 2010).

Trichoderma harzianum

Preferred name: *Trichoderma harzianum* (Rifai)

Other scientific names: *Sporotrichum narcissi* (Tochinai & Shimada), *Trichoderma narcissi* (Tochinai & Shimada), *Trichoderma lignorum var. narcissi* (Tochinai & Shimada).

Trichoderma harzianum is a fungus that is commonly found in soil and plant rhizosphere around the world. As a rhizosphere colonizer, it can grow on plant roots and penetrate the root epidermis through endophytic interactions, resulting in improved

plant growth and immune response (Chaverri et al. 2015). Moreover, *Trichoderma harzianum* produces enzymes that enable it to more effectively regulate biological pathogens, such as *Fusarium*, *Rhizoctonia*, and *Phytophthora* species (Mazrou et al. 2020, Das et al. 2019). These abilities make *T. harzianum* a key ingredient in various commercial biofungicide products, including Trianum P and Tricho D (Woo et al. 2014).

5 Entomopathogenic fungi

Entomopathogenic fungi (EPFs) such as *Akanthomyces attenuatus* (= *Lecanicillium attenuatum*) and *Isaria fumosorosea* (= *Paecilomyces fumosoroseum*), are a unique group of microorganisms that invade and kill insects and other arthropods through penetration of their tough outer coverings. Evidence suggests that EPFs can be found in over 700 species of hosts and are commonly used for microbial management of insect pests (Shin et al. 2017). The majority of EPF strains are concentrated in four orders: Hypocreales (various genera), Onygenales (*Ascosphaera* genus), Entomophthorales, and Neozygitales (*Entomophthoromycota*) (Mora et al. 2018).

EPFs are naturally present in soil and can infect a variety of insect species, with different strains targeting specific types of insects. These fungi are typically obtained from insect cadavers. Over the years, many EPF isolates have been documented and tested, with some achieving impressive results (Mantzoukas et al. 2022). The ability of EPFs to cause disease in insects largely depends on their enzymatic equipment, which includes lipases, proteases, and chitinases, responsible for breaking down the insect's outer coverings (integuments) (Sánchez-Pérez et al. 2014). EPFs differ significantly in their virulence and mode of action, and a successful infection primarily relies on the fungus's ability to adhere to and penetrate the host integuments (Shahid et al 2012).

The mode of action of EPFs typically involves attaching spores to the insect cuticle, germinating, penetrating the cuticle, and spreading inside the insect (Mora et al. 2018). Once inside, the fungus colonizes the insect's body tissues and hemolymph, ultimately leading to the insect's death (Pedrini 2018). The life cycle of EPFs varies depending on the fungus species, and involves the following steps leading to the infection and death of the host insect: attachment, germination, penetration, colonization, and sporulation.

EPFs have been found to have a dual biocontrol function against both insect pests and plant diseases, as supported by several studies (Shin et al. 2017). This makes them a promising alternative to chemical pesticides, as they are environmentally friendly and have a low risk of developing resistance in pests. Among the significant species, *Beauveria bassiana*, *Metarhizium brunneum*, *Lecanicillium* spp., and *Isaria fumosorosea* (Shin et al. 2017) stand out as effective biocontrol agents. *Lecanicillium* and *Isaria* species are commonly used EPFs against greenhouse pests and diseseas such as

powdery mildew (Folorunso et al. 2022). EPFs offer a sustainable and effective method of pest and disease control in agriculture, and their use in combination with other management practices can further improve crop health and yield.

5.1 Some important entomopathogenic species

Lecanicillium attenuatum (Akanthomyces attenuatus)

The genus *Lecanicillium* is a group of entomopathogenic fungi that are recognized as important pathogens of insect pests. They are able to parasitize various hosts including arthropods, nematodes, plants, and fungi (Zhou et al 2022). Some of the species within this genus have been developed as commercial biopesticides, particularly for controlling sucking insect pests such as aphids and whiteflies (Folorunso et al. 2022). *Lecanicillium* spp. not only employ mechanical forces and hydrolytic enzymes to infiltrate the integument of insects and the cell wall of fungal plant pathogens but also colonize host plant tissues, thereby inducing systemic resistance (Goettel et al 2008). The genus *Lecanicillium* was previously considered a single species known as *Verticillium lecanii*. However, subsequent molecular and morphological studies revealed significant differences between different strains, leading to the recognition of multiple species within this genus. *Lecanicillium* is now established as a distinct group of fungi belonging to the order Hypocreales.

The genus *Lecanicillium* includes numerous fungal species (Nicoletti & Becchimanzi 2020), including *Lecanicillium attenuatum*, which is a species of entomopathogenic fungus with significant commercial and scientific importance. This species is also known as *Akanthomyces attenuatus,* which was initially described as a separate species. However, subsequent research determined it to be the same organism as *Lecanicillium attenuatum*, which is now the accepted name for this species.

Fungus *A. attenuatus* is known for its rapid germination rate and high persistence, making it a promising candidate for the development of biopesticides to manage several insect pests in agricultural systems such as cotton aphid nymphs and whiteflies (Folorunso et al. 2022, Kim & Kim 2008). In particular, studies conducted by Kim & Kim (2008) have shown that isolates of *A. attenuatus*, such as the highly pathogenic isolate CS625, are effective against cotton aphid nymphs. This fungus has also been found to exhibit insecticidal properties against other agricultural pests such as thrips (Zhou et al 2020).

Furthermore, recent studies have also shown that *A. attenuatus* can parasitize fungi, including the plant pathogen *Botrytis cinerea* (Shin et al. 2017), which opens up new possibilities for its use in disease control. *A. attenuatus* is an excellent choice for integrated pest management plans and a useful source for creating new biocontrol agents.

Isaria fumosorosea (Paecilomyces fumosoroseum)

The *Isaria* genus is a group of fungi within the family Cordycipitaceae that plays an important role in controlling insect pests. These entomopathogenic fungi are geographically widespread, and can infect a variety of insects at all stages of development, making them useful for pest management. They are frequently found in soil and have been extensively studied due to their ability to produce various secondary metabolites (Sabareesh et al. 2007, Paschapur et al. 2021). The *Isaria* genus is highly virulent to several orders of insects (Zimmermann 2008), making it a powerful tool for biological pest control. This genus comprises a large number of entomopathogenic species, which highlights its potential for managing pest populations. The *Isaria* genus is a valuable resource in the fight against insect pests and is worth further investigation for its potential applications in pest management strategies.

Isaria fumosorosea, previously known as *Paecilomyces fumosoroseum*, is a type of polyphagous fungus belonging to the *Isaria* genus that shows promise as a biological control agent for insect pests. Studies have shown that it can infect and parasitize various insect pests, including aphids, whiteflies, thrips (Zimmermann 2008), several species of caterpillars, mites, and nematodes. In addition to its insecticidal properties, I. *fumosorosea* has also been found to be effective in controlling fungal diseases like cucumber powdery mildew (Kavková & Čurn 2005), indicating its potential as a viable option for disease management.

Isaria fumosorosea is a versatile fungus with applications in various settings. It is effective against both soil-dwelling and foliar-feeding pests, making it a useful tool in greenhouse, landscape, and turf management (Toscano 2015). This rapidly growing fungus forms white mycelial colonies that can shift to a purple or pink coloration over time (Brunner-Mendoza et al. 2017). In agriculture, the conidia (spores) of I. *fumosorosea* are employed to control insect pests (Angel-Cuapio et al 2015), and the surface of infected insects is usually covered with a multitude of these spores. As research on

Isaria fumosorosea continues to expand, it is possible that new applications and formulations may emerge, further increasing its potential as a sustainable and environmentally-friendly alternative to synthetic pesticides.

6 Methodology

Native strains of mycoparasitic fungi from genus *Trichoderma*

Native strains of mycoparasitic fungi from the genus *Trichoderma* were isolated from soil samples collected from the localities of Zvikov (South Bohemia), Sobekury (West Bohemia), and Uhrineves (Prague). To isolate the fungi, 20 g of soil samples were diluted in 100 ml of distilled water and placed in a shaker for 30 minutes. The resulting leachate was further diluted (1:10), and 0.5 ml was inoculated on the surface of a selective medium (*Trichoderma selected medium* - TSM) or on the surface of PDA with antibiotics (*dilution plate technique* - DPT). Some strains were obtained using the live trap method. After 14 days, the *Trichoderma* strains were isolated, and pure cultures were used for subsequent experiments. The pure cultures were cultivated on PDA and incubated at 25°C. For the experiments, 7-day-old cultures were used.

DPT (dilution plate technique)

The principle of the method is to apply a leachate obtained from the analyzed soil samples onto the surface of a standard nutrient medium that is supplemented with broad-spectrum antibiotics. First, the soil leachate must be gradually diluted, and then a descending dilution series should be applied to the surface of the nutrient medium enriched with antibiotics to inhibit bacterial growth and development. This procedure is mainly advantageous for the detection of fast-growing antagonistic and mycoparasitic fungal species capable of quickly growing on the surface of the nutrient soil or subsequently developing on other fungal species also present in the soil.

TSM (Trichoderma selective medium),

The principle of this method is similar to the previous method, but Bengal rose and a fungicidal active ingredient are added to the standard PDA medium in addition to broad-spectrum antibiotics. In this case, the active substance propamocarb was added to the medium. The selective nutrient medium inhibits the growth of the vast majority of soil fungi and bacteria, but it does not completely inhibit the growth of some species of mycoparasitic fungi from the genus *Trichoderma*.

SBT (Sclerotia bait technique)

The principle of this method is to detect the presence of mycoparasitic fungi through their basic property of parasitizing a suitable host species. In this study, the objectives were implemented using the sclerotia of the phytopathogenic fungus *S. sclerotiorum*.

Table 1. The localities of isolation of strains of *Trichoderma* spp.

Localities	Strain	Method of isolation*
Zvikov Ceske Budejovice, South Bohemia	Tri 905	DPT
	Tri 906	DPT
	Tri 908	DPT
	Tri 909	TSM
	Tri 910	SBT
Farm Hodan, Sobekury, West Bohemia	Tri 901	TSM
	Tri 903	DPT
	Tri 911	TSM
	Tri 946	SBT
	Tri 948	SBT
Uhrineves, Prague	Tri 904	DPT
	Tri 912	TSM
	Tri 913	SBT
	Tri 914	DPT
	F5-SNA-G1	TSM

*TSM (*Trichoderma selective medium*), SBT (*Sclerotia bait technique*), DPT (dilution plate technique)
**preliminary species identification was based on the phenotypic expression of fungal species of the genus *Trichoderma*

Commercial beneficial fungi

The entomopathogenic fungus used in the experiments in the interaction with the plant pathogen *Botrytis cinerea* was *Isaria fumosorosea*, strain PFR 97. The strain was re-isolated from the commercial bioproduct PreFeRal (Certis Biologicals, USA). The entomopathogenic fungus *Akanthomyces attenuatus*, strain CCM 9195, was isolated from the adult bark beetle *Ips typographus* in Sumava National Park in 2008, and the strain is currently in patent procedure. The mycoparasitic fungus *Trichoderma virens*, isolate GL-21, was re-isolated from the bioproduct SoilGard 12G of the company Certis Biologicals, USA. Mature cultures of all the strains were maintained at the laboratory of the Department of Plant Production, Faculty of Agriculture, the University of

South Bohemia in Ceske Budejovice. Pure cultures of beneficial fungi were cultivated on PDA and incubated at 25 °C. For the experiments, 10-day-old cultures were used.

Plant pathogens

The plant pathogens *Sclerotinia sclerotiorum, Rizoctonia solani, Fusarium culmorum, Fusarium oxysporum* and *Fusarium sporotrichoides,* used in the experiments were isolated from legume germplasm and from soil samples. Pure cultures of the plant pathogens were cultivated on PDA and incubated at 25 °C. For the experiments, cultures that were 14 days old were used.

Radial growth

The suspension of each strain of *Trichoderma* sp. was inoculated in the middle of PDA plate. After the suspension was applied, the Petri dishes were incubated at 25±1 °C. The radial growth was measured at 24-hour intervals until the strains reached the edge of Petri dish. The size of the culture was determined by measuring two perpendicular diameters, from which the diameter of the culture was calculated at daily intervals, or the mycelial increment over time.

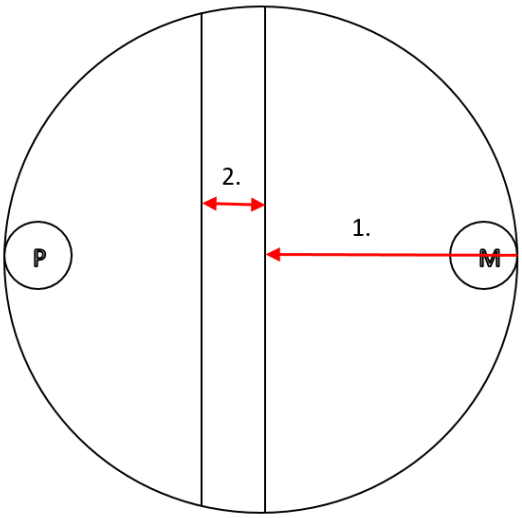
Spore production of fungi

The aim of the test is to determine the amount of spores produced during the cultivation of mycoparasitic fungi on PDA artificial medium. Spore production was determined from centre cultures incubated for 14 days in a thermostat at 25±1 °C. The culture of mycoparasitic fungi in Petri dishes were homogenized together with the PDA medium in a blender with an adequate amount of water (200 ml per petri dish). Using a counting chamber hemocytometer (Neubauer's improved chamber), spore production was determined, expressed per Petri dish, and then recalculated to spore production per 1 mm^2.

Interaction "in vitro" tests on agar plates (*Trichoderma* vers. plant pathogen)

The aim of interaction tests is to determine and quantify the relationship between mycoparasitic fungus and plant pathogen. In this experiment, the effect of fungal strains of the genus *Trichoderma* on the suppression of different plant pathogenic fungal species was evaluated. The mycoparasitic effect was determined. Five species of plant pathogenic fungi, *Sclerotinia sclerotiorum, Rhizoctonia solani, Fusarium culmorum*

strain CCF 1834, *Fusarium oxysporum* strain 55-D1 and *Fusarium sporotrichoides* strain 55-L3-Ca, were evaluated for the mycoparasitic activity of *Trichoderma* spp. strains.



M = strain of mycoparasitic fungus

P = pathogen (plant pathogen)

1. = touch zone (ZT) - measured from the edge of Petri dish with mycoparasite to the point of contact with the plant pathogen

2. = mycoparasitic zone (ZM) - zone of overgrowth of the plant pathogen by the mycoparasitic fungus

Figure 1. Measurement of the touch zone and the mycoparasitic zone

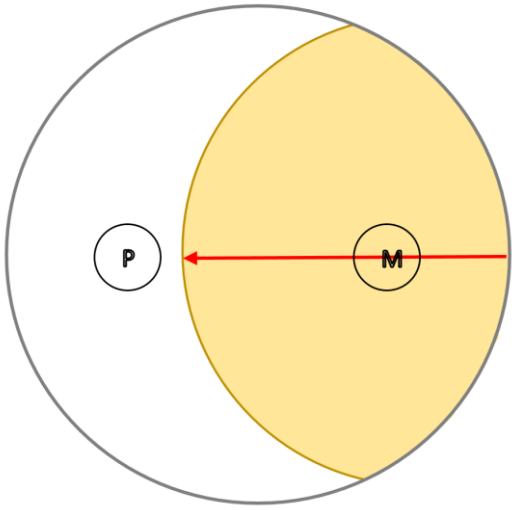
In the interaction tests, a 10 mm diameter disk of the plant pathogen was placed on one edge of the Petri dish (90 mm in diameter, inner diameter of 83 mm) and a 10 µl drop of the tested mycoparasitic fungal strain of the genus *Trichoderma* was applied to the opposite edge using an inoculation loop. In the control, discs of the pathogen were placed on opposite edges of the Petri dish, and drops of *Trichoderma* suspensions were applied to the opposite edge. The evaluation of the mycoparasitic effect between *Trichoderma* species and plant pathogen was performed after 7 days of incubation.

Interaction "in vitro" tests on agar plates (beneficial fungi vers. *Botrytis cinerea*)

The aim of the interaction was to evaluate the mycoparasitic effect of the entomopathogenic fungi *I. fumosorosea* and *A. attenuates* against the plant pathogen *B. cinerea* which causes gray mold. The mycoparasitic fungus *T. virens*, which is typical mycoparasitic fungus, was used as a control.

In this interaction test, a 10 mm diameter disk of the plant pathogen was placed 2 cm from one edge of the Petri dish (90 mm in diameter, inner diameter of 83 mm), and a 10 µl drop of the suspension of the tested entomopathogenic/mycoparasitic fungal

strain was applied to the opposite edge using an inoculation loop. The drop was also inoculated 2 cm from the edge of Petri dish. In the control, disks of the pathogen were placed on opposite sides, 2 cm from the edges of the Petri dish, and drops of beneficial fungi suspensions were placed opposite each other, 2 cm from the edges of Petri dish. The efficacy of beneficial fungi against the plant pathogen *B. cinerea* by the measurement of the touch zone and mycoparasitic zone was evaluated after 14, 21, 28, and 35 days. The spore production in the fungi combination was also evaluated.



M = strain of mycoparasitic fungus

P = pathogen (plant pathogen)

yellow zone = beneficial fungus overgrowing the pathogen *B. cinerea*

Figure 2. Measurement of mycoparasitic zone between beneficial fungi and *B. cinerea*

7 Results

Morphological markers of mycoparasitic fungi of the genus *Trichoderma*

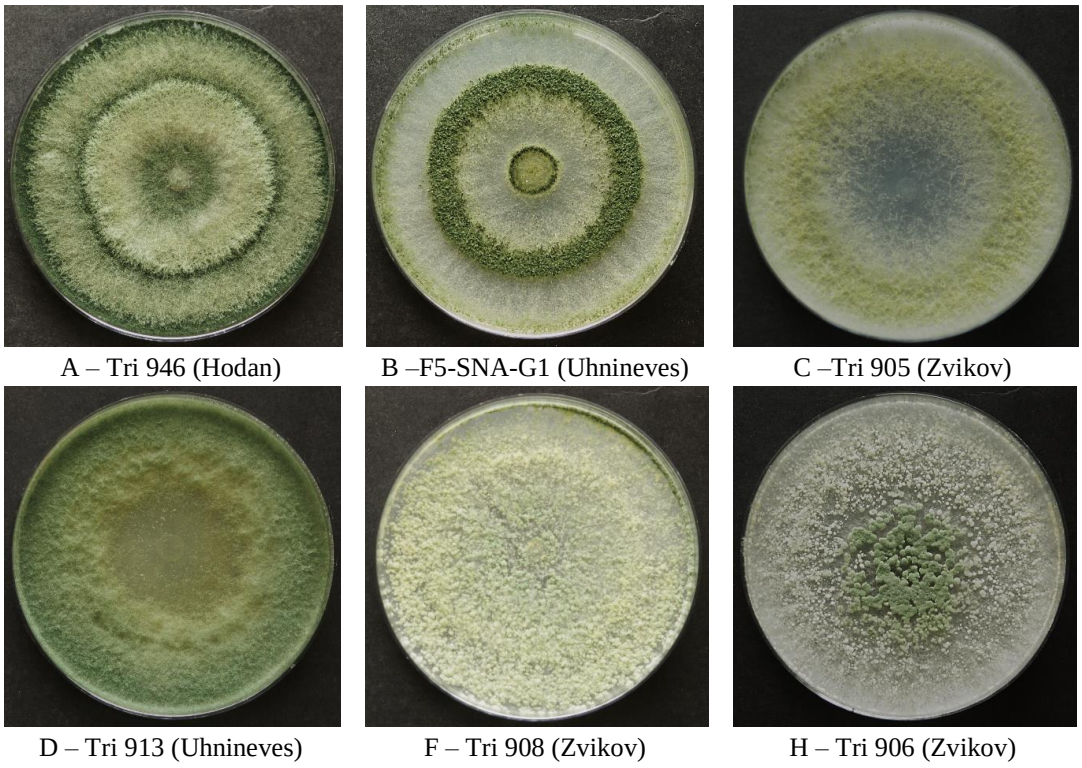
The phenotypic expression of cultures was evaluated for individual strains of mycoparasitic fungi based on the centre cultures grown on PDA artificial medium. For selected strains of the genus *Trichoderma*, the mid-culture was first documented photographically, and then assessed visually in detail. The parameters observed were culture colour, culture sporulation type, and the presence of pustules (a cluster of conidiophores on the mycelium). Currently, individual isolates are identified using molecular genetic methods.

Table 2: Summary table of individual characters in selected isolated strains of fungi of the genus *Trichoderma*

Morphotype	Strain	Isolation*	Marker of phenotypes			
			Structure	Pustules	Color	Species**
A	Tri 901	TSM	filamentous	-	dark green	*T. virens*
	Tri 946	SBT	filamentous	-	dark green	*T. virens*
	Tri 948	SBT	filamentous	-	dark green	*T. virens*
B	Tri 914	DPT	compact	-	dark green	*T. atroviride* like
	F5-SNA-G1	TSM	compact	-	dark green	*T. atroviride* like
C	Tri 905	DPT	filamentous	-	light green/yellow	*T. harzianum* like
	Tri 909	TSM	filamentous	-	light green/yellow	*T. harzianum* like
D	Tri 913	SBT	filamentous	-	light green	*T. harzianum* like
F	Tri 908	DPT	filamentous	-	green/yellow	*T. viride* like
	Tri 911	TSM	filamentous	-	green/yellow	*T. viride* like
G	Tri 912	TSM	compact	+	dark green	*T. polysporum* like
H	Tri 903	DPT	compact	+	light green	*T. polysporum* like
	Tri 904	DPT	compact	+	light green	*T. polysporum* like
	Tri 906	DPT	compact	+	light green	*T. polysporum* like
	Tri 910	SBT	compact	+	light green	*T. polysporum* like

*TSM (*Trichoderma selective medium*), SBT (*Sclerotia bait technique*), DPT (dilution plate technique)
**preliminary species identification was based on the phenotypic expression of fungal species of the genus *Trichoderma*

Figure 1: Examples of morphotypes of isolated fungi of genus *Trichoderma*



A – Tri 946 (Hodan) B –F5-SNA-G1 (Uhnineves) C –Tri 905 (Zvikov)

D – Tri 913 (Uhnineves) F – Tri 908 (Zvikov) H – Tri 906 (Zvikov)

The faster growth rate of central cultures was observed after 24 hours in strains Tri 948 and Tri 946, classified in morphotype A, species *T. virens*. The diameter of the central cultures ranged from 14.5 to 22.0 mm after 24 hours. After 48 hours, the growth dynamics were maintained by strains classified in morphotype A. However, strain Tri 906 achieved the highest radial growth. In contrast, strains Tri 908 and Tri 901 showed the lowest colonization of the Petri dish, which was confirmed after 72 hours, when almost all strains except Tri 908 and Tri 901 had reached the edge of the Petri dish. After 4 days, all tested strains had reached the edge of the Petri dish.

Table 3: Radial growth of fungi of the genus *Trichoderma* on PDA artificial medium (continuous measurement of strain cultures at 24-hour intervals)

Morphotype	Strain	Radial growth (mm)			
		24 h	48 h	72 h	96 h
A	Tri 901	17,38 ± 0,74	47,75 ± 1,28	79,88 ± 1,36	83,00 ± 0,00
	Tri 946	20,88 ± 2,17	56,63 ± 0,52	83,00 ± 0,00	83,00 ± 0,00
	Tri 948	22,00 ± 0,93	56,89 ± 1,76	83,00 ± 0,00	83,00 ± 0,00
B	Tri 914	18,00 ± 0,76	58,13 ± 1,81	83,00 ± 0,00	83,00 ± 0,00
	F5-SNA-G1	14,50 ± 0,76	56,38 ± 2,00	83,00 ± 0,00	83,00 ± 0,00
C	Tri 905	14,63 ± 1,06	59,25 ± 1,67	83,00 ± 0,00	83,00 ± 0,00
D	Tri 913	17,88 ± 0,64	50,50 ± 2,33	83,00 ± 0,00	83,00 ± 0,00
F	Tri 908	15,25 ± 2,55	37,88 ± 0,99	61,50 ± 1,20	83,00 ± 0,00
H	Tri 903	17,50 ± 1,60	54,13 ± 3,91	83,00 ± 0,00	83,00 ± 0,00
	Tri 904	18,00 ± 1,41	52,00 ± 1,20	83,00 ± 0,00	83,00 ± 0,00
	Tri 906	18,25 ± 1,67	61,50 ± 0,93	83,00 ± 0,00	83,00 ± 0,00

Figure 2: Radial growth of fungi of the genus *Trichoderma* on PDA artificial medium (continuous measurement of strain cultures at 24-hour intervals)

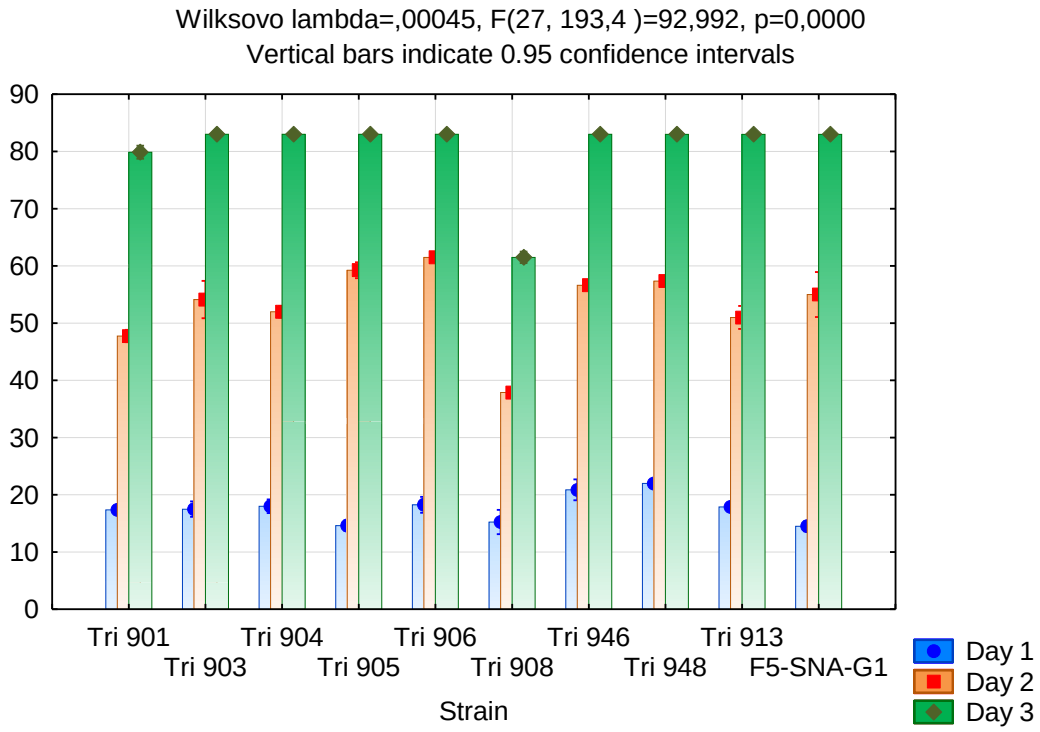


Figure 3: Comparison of spore production of *Trichoderma* fungi on PDA medium after 10 days of cultivation

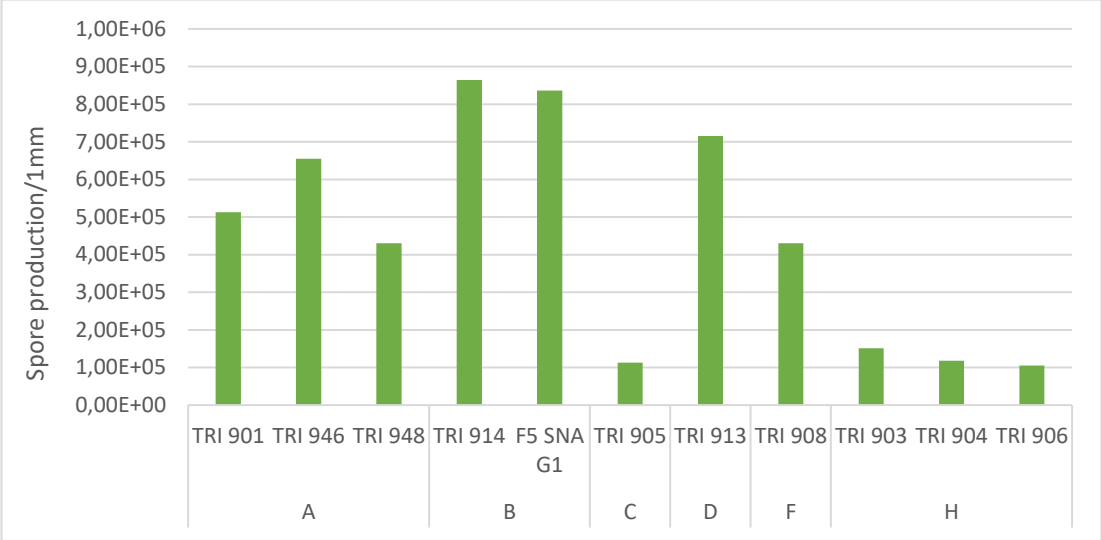


Table 4: Spore production of fungi of the genus *Trichoderma* from the medium cultured on PDA medium after 10 days of cultivation

Morphotype	Strain	Mean of spore production per 1 mm^2
A	Tri 901	5,13 x 10^5
	Tri 946	6,55 x 10^5
	Tri 948	4,30 x 10^5
B	Tri 914	8,64 x 10^5
	F5-SNA-G1	8,36 x 10^5
C	Tri 905	1,13 x 10^5
D	Tri 913	7,16 x 10^5
F	Tri 908	4,30 x 10^5
H	Tri 903	1,51 x 10^5
	Tri 904	1,18 x 10^5
	Tri 906	1,05 x 10^5

The difference in spore production between the same strains of mycoparasitic fungi was evaluated after 10 days of cultivation under controlled conditions at 25 °C. The spore production showed statistically significant differences among the strains (F=383,546; df=10,11; p=0,0000). The strains Tri 914 (8.64 x10^5) and F5-SNA-G1 (8.36 x$10^{5)}$, classified in morphotype B (*T. atroviride* like), produced the highest

amount of spores. The least productive strains were Tri 903, Tri 904 and Tri 906 classified in morphotype H (*T. polysporum* like), which produce spores in so-called pustules. The spore production of these strains ranged from 1.05 $x10^5$ to 1.51$x10^5$. Tri 905 (morphotype C) also achieved significantly lower spore production (1.13 $x10^5$) compared to the other tested strains.

Interaction between *Trichoderma* sp. vers. plant pathogen

Table 5: Evaluation of the mycoparasitic zone in the interaction of fungi of the genus *Trichoderma* with selected plant pathogenic species (measured on Day 7 after inoculation)

Morphotype	Strain	Mycoparasitic zone in interaction *Trichoderma* with plant pathogens (mm)				
		S. sclerotiorum	*R. solani*	*F. culmorum*	*F. oxysporum*	*F. sporotrichoides*
A	Tri 946	12,75±0,50	43,25±1,71	31,00±2,16	21,00±0,82	12,50±1,00
	Tri 948	14,25±0,96	49,00±1,15	25,25±0,50	22,25±1,50	28,50±0,50
B	F5-SNA-G1	8,50±1,73	52,25±2,22	26,50±1,29	11,25±0,96	3,50±0,58
C	Tri 905	14,50±0,58	36,00±2,45	0,75±0,50	2,50±0,58	2,75±0,50
	Tri 909	10,25±0,50	36,25±4,79	7,00±0,82	25,75±1,26	7,75±0,50
D	Tri 913	8,25±1,26	51,50±1,29	30,50±0,58	12,25±0,50	27,75±1,26
F	Tri 911	20,00±2,45	3,75±0,50	2,00±0,00	25,75±1,71	1,25±0,50
G	Tri 912	2,50±0,58	1,50±0,58	0,00±0,00	10,50±0,58	0,00±0,00
H	Tri 903	7,25±0,96	8,50±0,58	1,75±0,50	8,50±0,58	2,25±0,50
	Tri 904	7,00±1,41	8,25±1,50	6,50±1,29	3,00±0,82	2,75±0,50
	Tri 906	11,75±0,96	1,75±0,50	4,75±0,26	2,75±0,50	1,50±0,58
	Tri 910	4,00±0,82	6,25±0,50	2,75±0,50	2,00±0,00	2,25±0,50

In the control variant, both cultures of *S. sclerotiorum* touched in the middle of the petri dish, i.e. at a distance of 41.5 mm from the edge of the Petri dish. The strains Tri 948 and F5-SNA-G1 of *Trichoderma* fungi showed the fastest growth rate among all variants. The touch zone in the interaction with the plant pathogen *S. sclerotiorum* was formed at a distance of 52.5 mm (Tri 948) and 45.0 mm (F5-SNA-G1), respectively. The other strains colonized the Petri dish in a similar manner, with values ranging from 42.25 to 44.75 mm. The mycoparasitic zone was also measured for all variants. The strain Tri 911 demonstrated the best mycoparasitic ability with a width of 20 mm, while the strain Tri 912 showed the least ability with a mycoparasitic zone of only 2 mm. Differences in the touch zone ($F=7.55$, $df=12;39$, $p=0.0000$) and mycoparasitic

zone (F=90.090, df=12;39, p=0.0000) among all strains in interaction with *S. sclerotiorum* were statistically significant.

Figure 4: Evaluation of the touch zone (ZT) and mycoparasitic zone (ZM) between species/strains of *Trichoderma* spp. and the plant pathogen *Sclerotinia sclerotiorum*

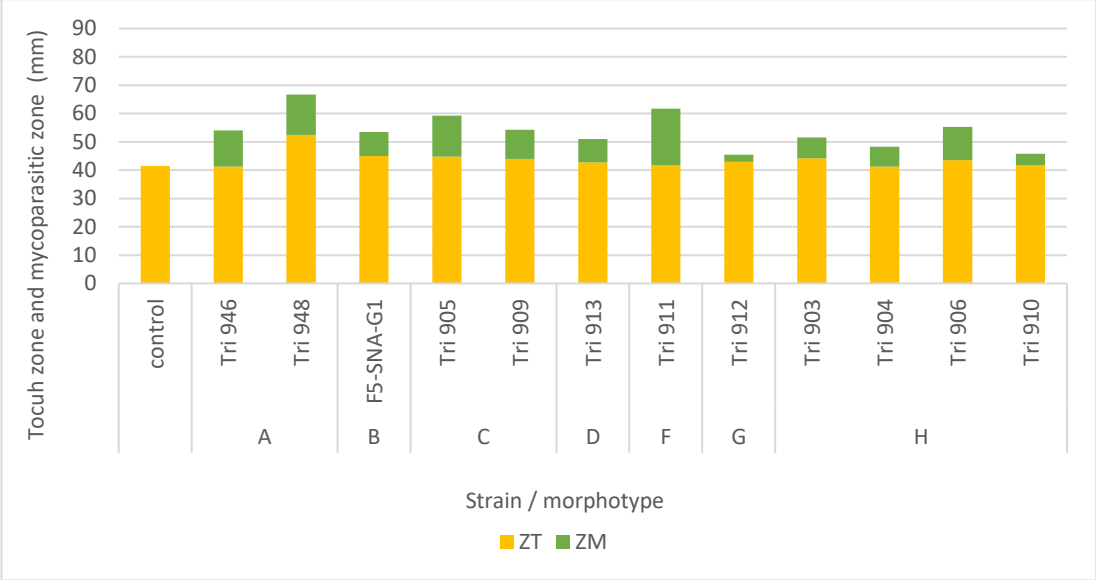
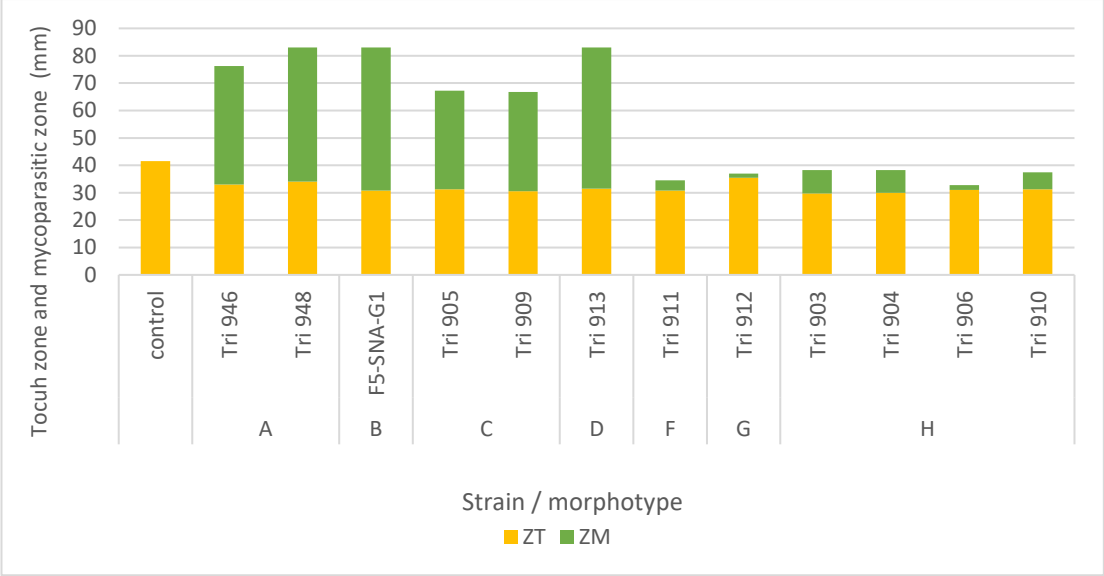


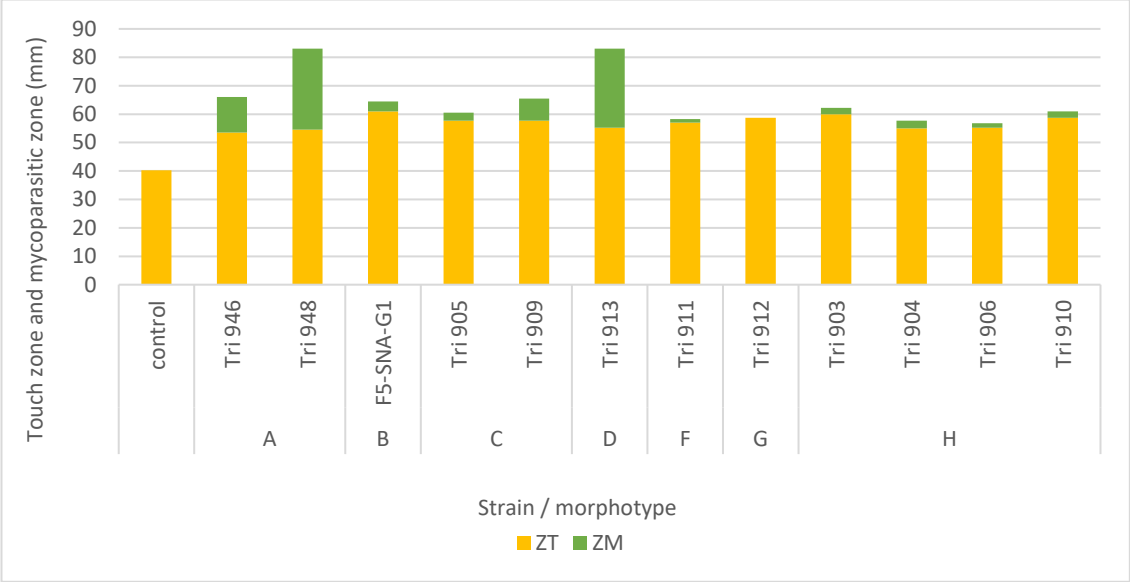
Figure 5: Evaluation of the touch zone and mycoparasitic zone between species/strains of fungi of the genus *Trichoderma* and the plant pathogen *Rhizoctonia solani*



In the control variant, the two cultures of *R. solani* touched in the center of the Petri dish. The distance of interaction between *Trichoderma* fungi and *R. solani* varied

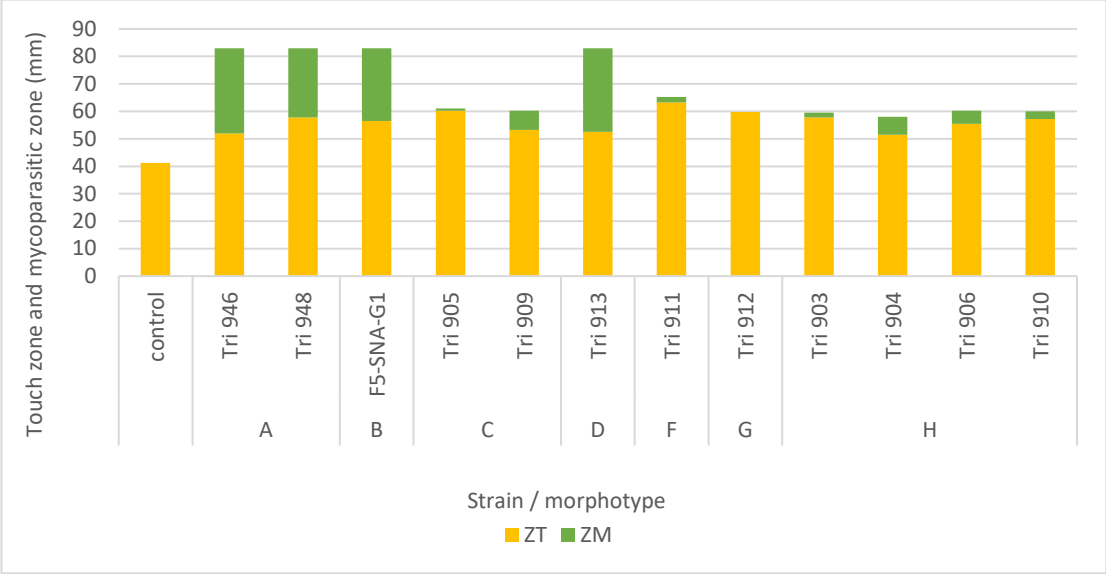
among the tested strains, ranging from 29.75 to 35.50 mm. Strain Tri 912 showed the fasted colonization of the Petri dish, followed by strains Tri 948 and Tri 946, while strain Tri 903 was the slowest. The widest mycoparasitic zone of 52.25 mm was observed in the interaction between *Trichoderma* strain F5 SNA G1 and *R. solani*. Strain Tri 912 showed the lowest efficacy against *R. solani*, parasitizing *R. solani* mycelium only up to a distance of 1.50 mm. Overall, strains classified in morphotypes F, G, and H were the least effective in interacting with the *R. solani* pathogen. Statistical analysis showed that the differences in touch zone (F=24.61, df=12;39, p=0.0000) and mycoparasitic zone (F=560.936, df=12;39, p=0.0000) among all the strains in interaction with *R. solani* were significant.

Figure 6: Evaluation of the touch zone and mycoparasitic zone between species/strains of fungi of the genus *Trichoderma* and the plant pathogen *Fusarium sporotrichoides* 55-L3-Ca



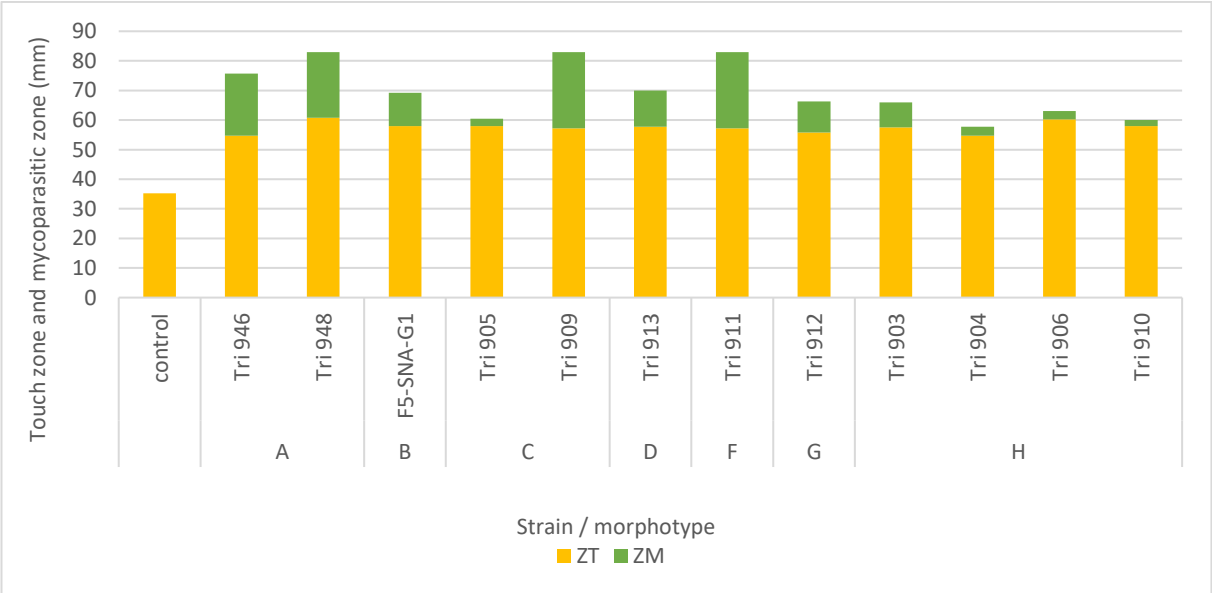
Similar results were obtained in the interactions between fungal strains of *Trichoderma* spp. and plant pathogens of the genus *Fusarium*. However, the efficacy of mycoparasitic *Trichoderma* spp. against different *Fusarium* spp. varied. In particular, the weakest mycoparasitic effect of *Trichoderma* spp. was demonstrated in the interaction with the pathogen *F. sporotrichoides* (F=560.936, df=12;39, p=0.0000), as well as *F. culmorum* (F=227.257, df=12;39, p=0.0000). On the other hand, *Trichoderma* spp. species were more effective against *F. oxysporum* (F=443.472, df=12;39, p=0.0000). *Trichoderma virens* and *T. harzianum* species generally appeared to be the best mycoparasitic fungi against *Fusarium* spp.

Figure 7: Evaluation of the touch zone and mycoparasitic zone between species/strains of fungi of the genus *Trichoderma* and the plant pathogen *Fusarium culmorum* CCF 1834



Differences in touch zone among *F. culmorum* CCF 1834 (F=59.34, df=12;39, p=0.0000), *F. sporotrichoides* 55-L3-Ca (F=24.61, df=12;39, p=0.0000), and *F. oxysporum* 55-D1 (F=14.76, df=12;39, p=0.0000) in interaction with all *Trichoderma* spp. strains are statistically significant.

Figure 8: Evaluation of the touch zone and mycoparasitic zone between species/strains of fungi of the genus *Trichoderma* and the plant pathogen *Fusarium oxysporum* 55-D1



Spore production of *Trichoderma* spp. in interaction with plant pathogens

Table 6a: Influence of plant pathogen on spore production of *Trichoderma* spp. in their mutual interaction

Strain	*F. sporotrichoides* 55-L3-Ca		*F. culmorum* CCF 1834		*F. oxysporum* 55-D1	
TRI 903	$7.85\pm0.42\times10^{8}$	def	$3.20\pm0.10\times10^{8}$	g	$9.89\pm0,44\times10^{8}$	c
TRI 904	$6,58\pm0,39\times10^{8}$	efg	$2.04\pm0.09\times10^{8}$	g	$4,11\pm0,55\times10^{8}$	d
TRI 905	$1,24\pm0,12\times10^{8}$	g	$1,84\pm0,02\times10^{8}$	g	$2,11\pm0,37\times10^{8}$	d
TRI 906	$8,04\pm0,94\times10^{8}$	def	$9.13\pm0,71\times10^{8}$	de	$1,15\pm0,05\times10^{9}$	c
TRI 909	$2,03\pm0,28\times10^{8}$	fg	$4.98\pm0.60\times10^{8}$	fg	$3,60\pm1,80\times10^{8}$	d
TRI 910	$7,66\pm0,44\times10^{8}$	def	$8.28\pm0.21\times10^{8}$	ef	$8,79\pm1,01\times10^{8}$	c
TRI 911	$2,65\pm0,00\times10^{8}$	fg	$1.38\pm0.35\times10^{8}$	g	$1,31\pm0,34\times10^{8}$	d
TRI 912	$1,55\pm0,25\times10^{9}$	bc	$1.25\pm0.07\times10^{9}$	cd	$1,85\pm0,00\times10^{9}$	b
TRI 946	$1,13\pm0,12\times10^{9}$	cde	$1.45\pm0.17\times10^{9}$	c	$1,16\pm0,14\times10^{9}$	c
TRI 948	$1,28\pm0,04\times10^{9}$	bcd	$1.87\pm0.20\times10^{9}$	b	$1,83\pm0,28\times10^{9}$	b
TRI 913	$1,81\pm0,19\times10^{9}$	b	$1.98\pm0.10\times10^{9}$	b	$1,95\pm0,08\times10^{9}$	b
F5-SNA-G1	$4,61\pm0,38\times10^{9}$	a	$4.22\pm0.14\times10^{9}$	a	$4,88\pm0,02\times10^{9}$	a
	F=125,856; df=11,12; p=0.0000		F=283,116; df=11,12; p=0.0000		F=251,652; df=11,12; p=0.0000	

Table 6b: Influence of plant pathogens on spore production of *Trichoderma* spp. in their mutual interaction

Strain	*S. sclerotiorum*		*R. solani*	
TRI 903	$7,79\pm0,19\times10^{8}$	bcd	$7,11\pm0,16\times10^{8}$	gf
TRI 904	$8,19\pm0,73\times10^{8}$	bcd	$5,19\pm0,09\times10^{8}$	g
TRI 905	$1,19\pm0,58\times10^{8}$	d	$3,80\pm0,07\times10^{8}$	gh
TRI 906	$3,20\pm0,35\times10^{9}$	cd	$1,21\pm0,10\times10^{9}$	ed
TRI 909	$1,14\pm0,23\times10^{8}$	d	$1,35\pm0,18\times10^{8}$	h
TRI 910	$4,75\pm0,78\times10^{8}$	cd	$9,91\pm0,02\times10^{8}$	fe
TRI 911	$6,75\pm3,18\times10^{7}$	d	$1,13\pm0,28\times10^{8}$	h
TRI 912	$1,47\pm0,58\times10^{9}$	bc	$1,48\pm0,18\times10^{9}$	cd
TRI 946	$1,54\pm0,50\times10^{9}$	bc	$1,34\pm0,05\times10^{9}$	cd
TRI 948	$1,89\pm0,04\times10^{9}$	b	$1,69\pm0,15\times10^{9}$	c
TRI 913	$1,72\pm0,23\times10^{9}$	b	$2,15\pm0,06\times10^{9}$	b
F5-SNA-G1	$3,90\pm0,69\times10^{9}$	a	$4,61\pm0,15\times10^{9}$	a
	F=25,6126; df=11,12; p=0.0000		F=384,288; df=11,12; p=0.0000	

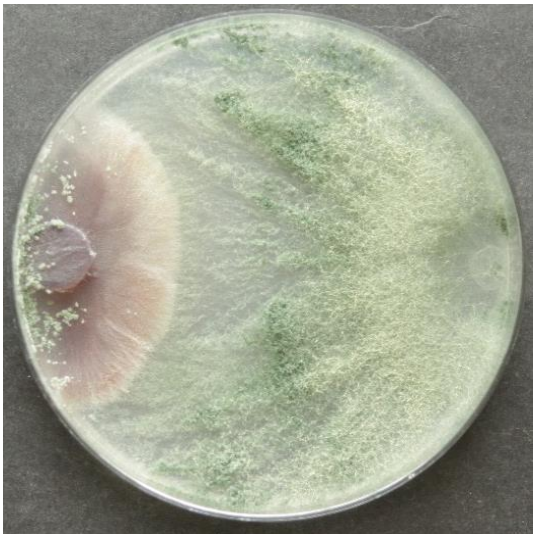
The most productive strain in interaction with plant pathogens *Fusarium* spp. is strain F5-SNA-G1, isolated from the soil from the locality Uhrineves, Prague. The strains

were assigned to the morphotype group B, *T. atroviride*-like. The strain is characterized by compact mycelium and produces dark green spores. Strains of *T. virens* (TRI 646 and TRI 648) are also highly productive in combination with fungi of the genus *Fusarium*. These strains showed high levels of mycoparasitism in combination with all Fusarium spp. Their efficacy was higher compared to strain F5-SNA-G1, which also showed a high mycoparasitic effect only on species *F. culmorum*. A lower mycoparasitic effect of this strain was recorded in interaction with *F. oxysporum* and the lowest with *F. sporotrichoides*.

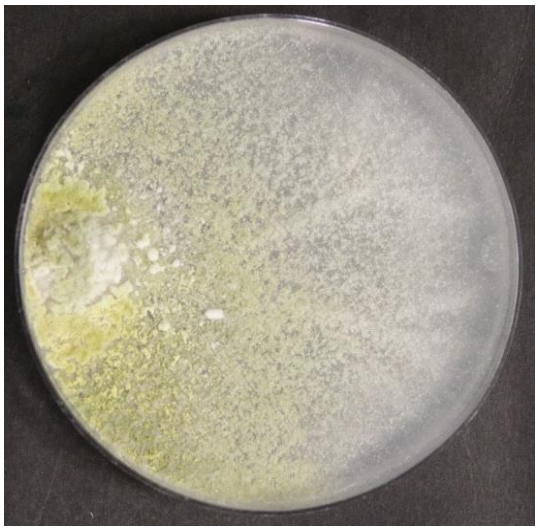
The highest spore production of the strain F5-SNA-G1 and strains of *T. virens* was also determined in the interaction with plant pathogens *S. sclerotiorum* and *R. solani*. In the interaction with *S. sclerotiorum,* strain TRI 911 produced the lowest amount of spores but the mycoparasitic effect was significant. The strain TRI 906 also produced a high amount of spores with all the plant pathogens.

Figure 9: Interactions of selected strains of *Trichoderma* spp. isolated from soils with selected plant pathogens

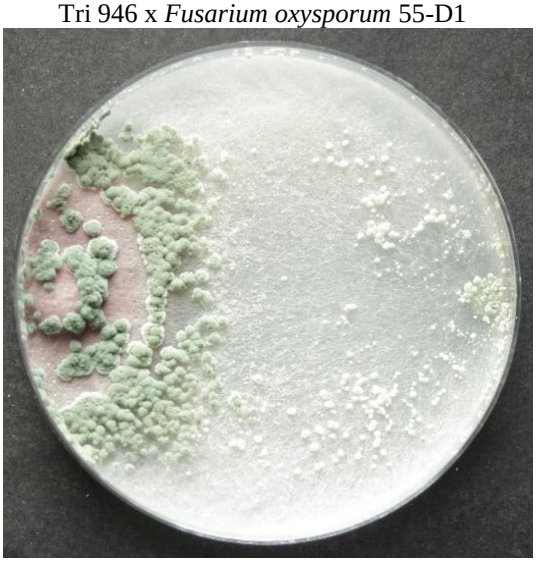
Tri 946 x *Rhizoctonia solani*



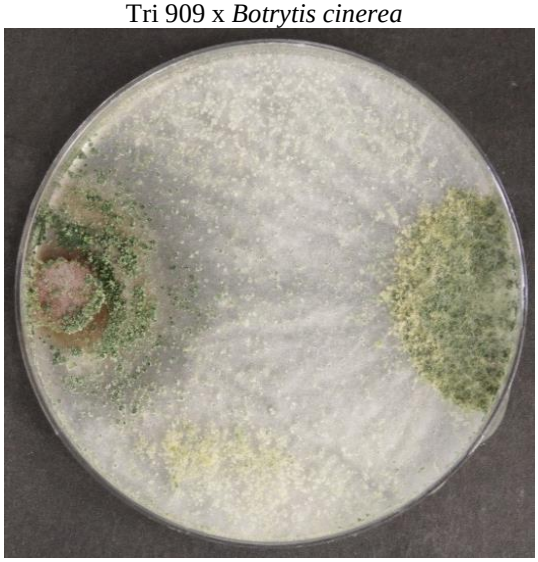
Tri 946 x *Fusarium oxysporum* 55-D1



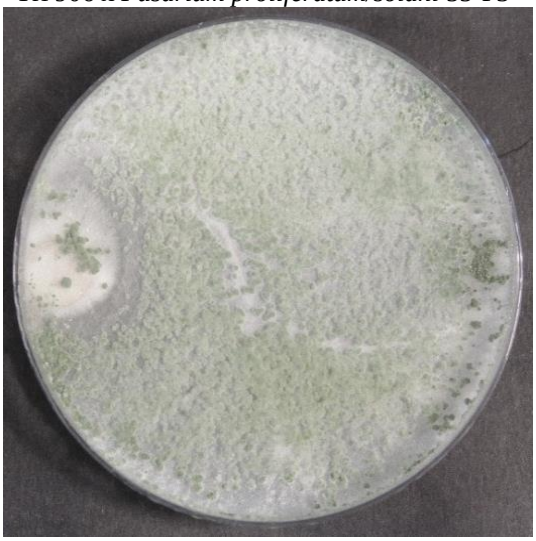
Tri 909 x *Botrytis cinerea*



Tri 906 x *Fusarium proliferatum/solani* 55-F3



Tri 912 x *Fusarium culmorum* CCF 1834

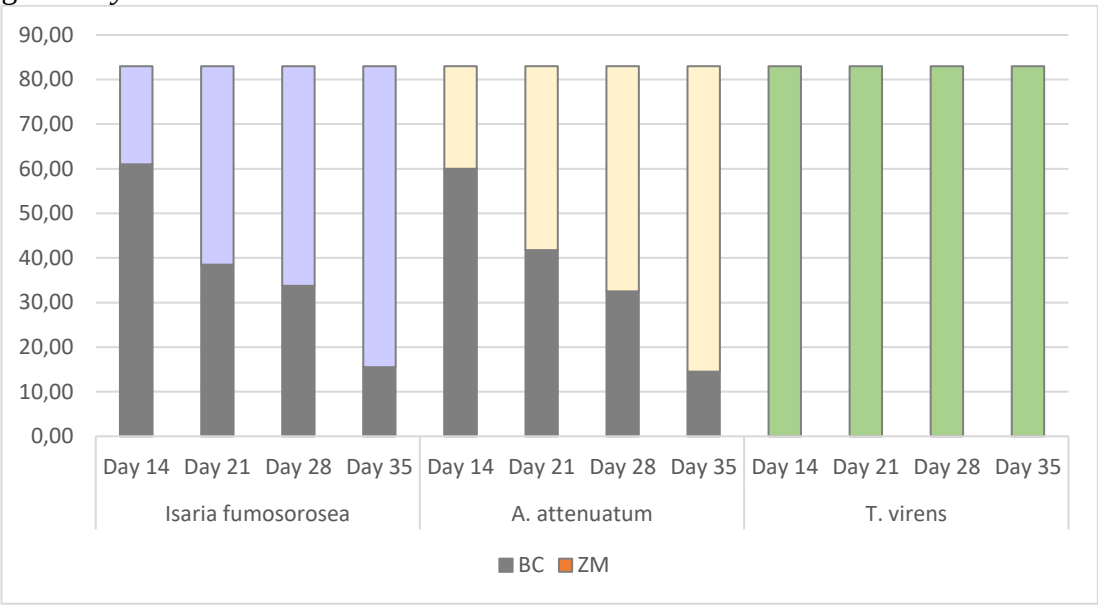


Tri 903 x *Fusarium avenaceum/tricintum* 55-L4-C

Efficacy of entomopathogenic fungi and mycoparasitic fungus *T. virens* against *B. cinerea*

The mycoparasitic zone is expressed by the area of the culture of the beneficial fungal species that has interacted with the pathogen *Botrytis cinerea*. The mycoparasitic zone is visually evident due to the color of *B. cinerea*.

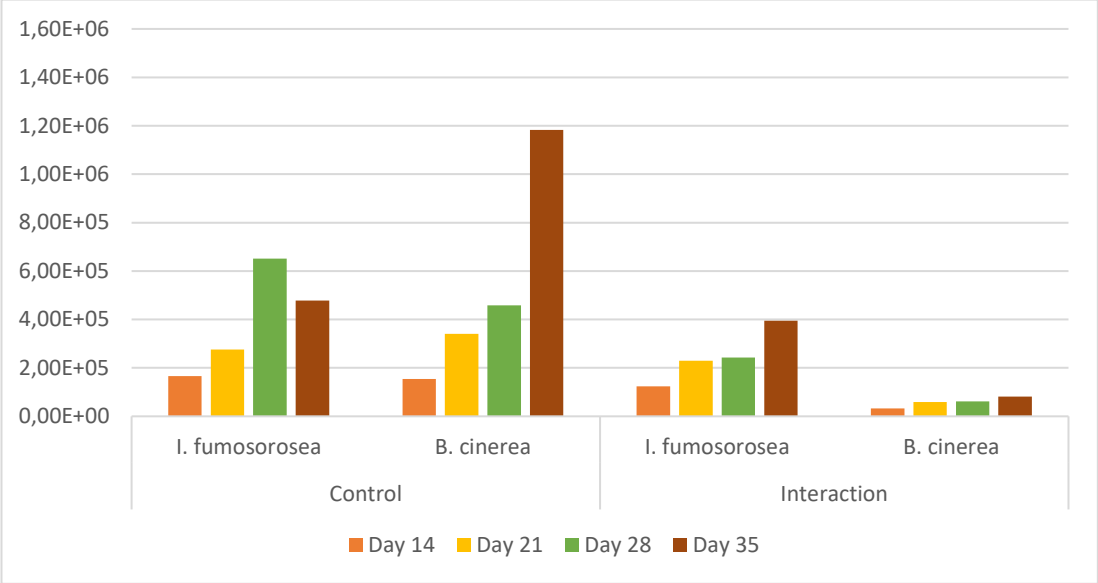
Figure 10: Mycoparasitic zone of beneficial fungi in the interaction with plant pathogen *Botrytis cinerea*



The Figure 14 shows that the mycoparasitic fungus *T. virens* is a very strong mycoparasite, which, after only 14 days of incubation, sup-pressed the growth and development of the B. cinerea pathogen. The entomopathogenic fungi *I. fumosorosea* and *A. attenuates* have demonstrated mycoparasitic status, which is also evident in the Figure 11 and Figure 12. The longer the interactions were cultured at 25 °C, the more mycoparasitic effect was observed. However, in contrast to the *T. virens* fungus, both entomopathogenic fungi were unable to completely sup-press the *B. cinerea* colony and degrade the pathogen's mycelium.

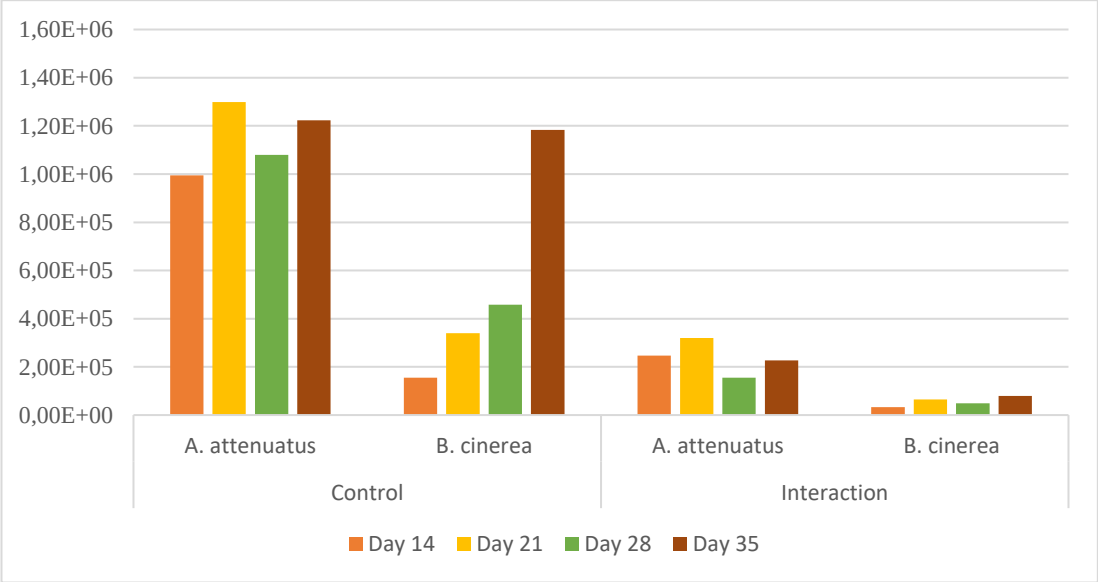
As part of the experiment, sporulation was monitored not only in the mutual interactions but also in the control variant of all the fungal species.

Figure 11: Evaluation of spore production in the interaction between entomopathogenic fungus *Isaria fumosorosea* and plant pathogen *Botrytis cinerea* in comparison with control variants



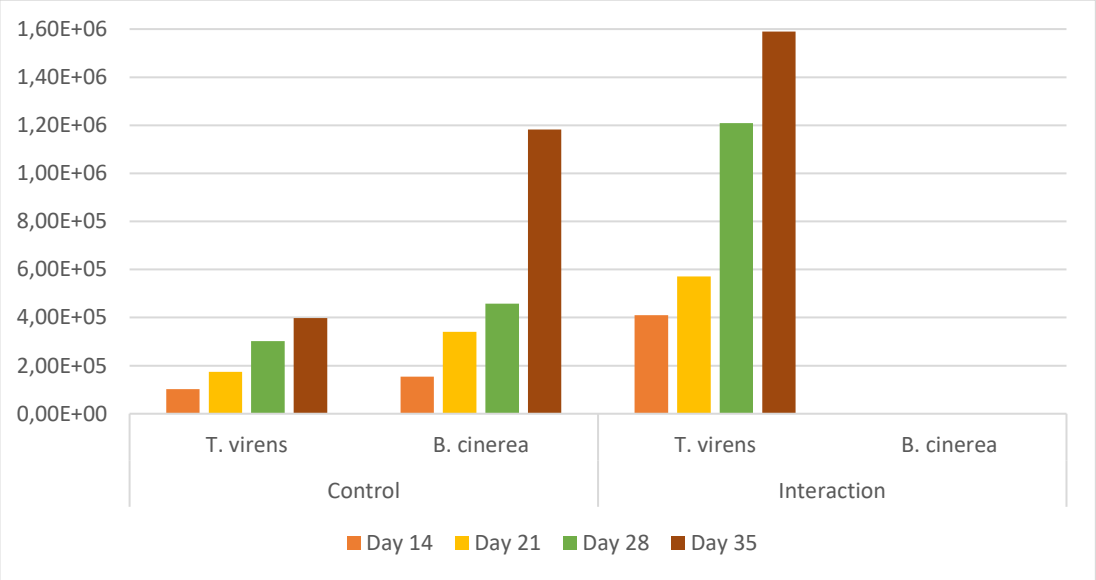
In the control variant, one colony of the entomopathogenic fungus *I. fumosorosea* produced from 1.66 x 10^5 (day 14) to 6.25 x 10^5 (day 28) spores per 1 mm^2 of area. The pathogen *B. cinerea* produced from 1.55 x 10^5 (day 14) to 1.18 x 10^6 (day 35) spores per 1 mm^2 of area. In mutual interaction, the fungus *I. fumosorosea* produced a smaller number of spores, but it suppressed the sporulation of the pathogen *B. cinerea*.

Figure 12: Evaluation of spore production in the interaction between entomopathogenic fungus *Akanthomyces attenuatus* and plant pathogen *Botrytis cinerea* in comparison with control variants



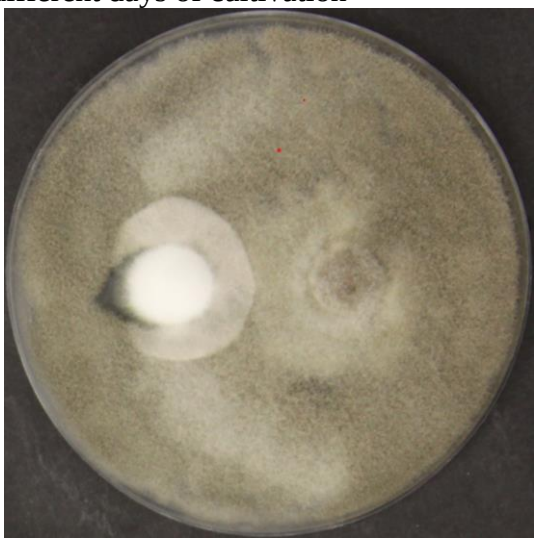
In the mutual interactions between *A. attenuatus* and *B. cinerea*, differences in spore production were observed again. In the control variant, the entomopathogenic fungus *A. attenuatus* produced a much larger number of spores than it did when it was combined with the pathogen. During cultivation, A. attenuatus produced 9.95 x 10^5 spores from the beginning of the trial until the end of the trial, where it produced up to 1.22 x 10^6 spores per 1 mm^2 of culture area. In the interaction variant, the *A. attenuatus* fungus produced far fewer spores, with a production of 2.27 x 10^5 spores per 1 mm^2 of culture area recorded at the end of the experiment. The spore production of the pathogen *B. cinerea* was again suppressed by the entomopathogenic fungus.

Figure 13: Evaluation of spore production in the interaction between mycoparasitic fungus *Trichoderma virens* and plant pathogen *Botrytis cinerea* in comparison with control variants



The mycoparasitic fungus *T. virens* demonstrated the highest efficacy against the pathogen *B. cinerea*. In comparison to the entomopathogenic species, an opposite effect was observed in terms of spore production. In the control variant, *T. virens* produced far fewer spores (ranging from 1.02 x 10^5 to 3.97 x 10^5 spores) than in mutual interaction, where spore production reached much higher values (ranging from 4.10 x 10^5 to 4.59 x 10^6 spores). In the mutual interaction, the pathogen *B. cinerea* was suppressed to such an extent that almost no spore production was recorded. The spore production of *B. cinerea* ranged from 2.54 x 10^3 to 2.31 x 10^3 over time.

Figure 14: Interactions between beneficial fungi and plant pathogen *B. cinerea* after different days of cultivation

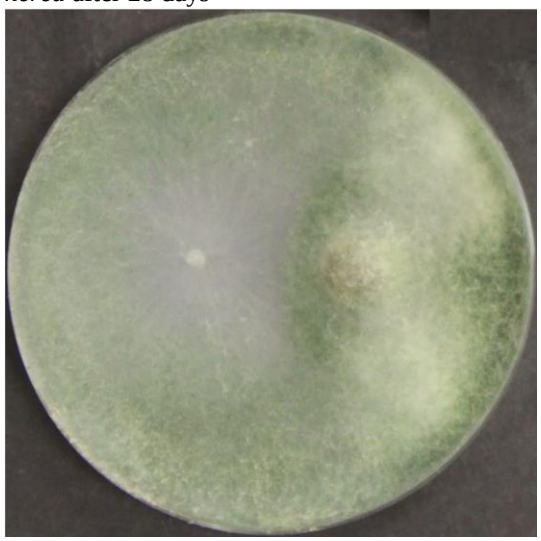


Interaction between *A. attenuates* and *B. cinerea* after 14 days

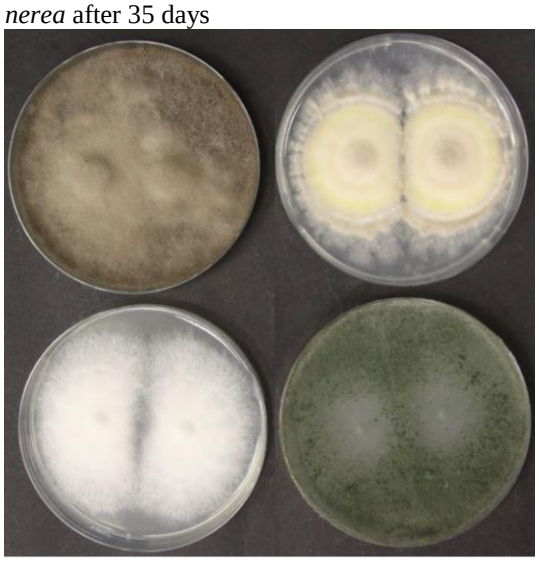
Interaction between *A. attenuates* and *B. cinerea* after 35 days

Interaction between *I. fumosorosea* and *B. cinerea* after 28 days

Interaction between *I. fumosorosea* and *B. cinerea* after 35 days



Interaction between *I. fumosorosea* and *B. cinerea* after 14 days



Control variants of beneficial fungi and *B. cinerea* after 35 days

8 Discussion

Trichoderma spp. fungi are versatile organisms that inhabit diverse environments, ranging from fields and forests to decomposing plant matter, and are essential components of both tropical and temperate ecosystems due to their ability to counteract soilborne plant diseases through penetrating plant roots and extracting vital nutrients from the soil (Pandaya et al. 2011, Waghunde et al. 2016, Saba et al. 2012). *Trichoderma* spp. are known for their diverse mechanisms of action, including competition for nutrients, secretion of lytic enzymes, and induction of systemic resistance in plants. Since the 1920s, *Trichoderma* fungi have been acknowledged for their effectiveness in fighting plant pathogens (Harman 2006), making them a valuable asset in agriculture and horticulture due to their capability to manage plant diseases.

The presented study evaluated the phenotypic expression of cultures for individual strains of mycoparasitic fungi, specifically *Trichoderma* spp., based on their growth on PDA artificial medium. Culture color, sporulation type, and the presence of pustules were observed for mid-cultures of selected *Trichoderma* strains. The particular native strains that were used for the study were collected from different localities in the Czech Republic; namely Uhrineves, Sobekury, and Zvikov (Table 1). These strains were then tested against plant pathogens that were isolated from legumes and soil samples, using different isolation techniques such as SBT, DPT and TSM. These methods involved diluting the soil samples in distilled water, placing them in a shaker, and then diluting them further before inoculating them on a selective medium or PDA with antibiotics. The use of these methods enabled the isolation and purification of *Trichoderma* strains from complex soil samples. Pure cultures were cultivated on PDA at 25°C, and 7-day-old cultures were used for the experiments. The subsequent cultivation of pure cultures and their use in the experiments allowed for the evaluation of their potential as biocontrol agents against plant pathogens.

In a study by Lui et al. (2020), the researchers evaluated the growth rate and spore production of various strains of *Trichoderma* spp. on different media. They found that *T. afroharzianum* T52 and *T. asperelloides* T57 strains had the highest growth rate on potato dextrose agar (PDA) medium (Liu et al. 2020). Similarly, Abuhena et al. (2022) reported that *T. harzianum* strain TI21 had the highest spore production on PDA medium. These findings are consistent with the results of the study mentioned in the research, which showed statistically significant differences in spore production among

the *Trichoderma* strains, with the *T. atroviride* strains Tri 914 and F5-SNA-G1 producing the highest amount of spores. The strains classified in morphotype H (*T. polysporum* like), specifically Tri 903, Tri 904 and Tri 906 had the lowest spore production. Regarding the interaction of *Trichoderma* spp. with plant pathogens, several studies have demonstrated their potential as biocontrol agents. For example, Martínez-Medina et al. (2017) reported that *Trichoderma* spp. can induce systemic resistance in plants against a wide range of pathogens. In a study by Bokhari & Perveen (2012), *Trichoderma harzianum* was shown to be effective in reducing the incidence of root rot disease caused by *Fusarium solani* in tomato plants. However, the efficacy of *Trichoderma* spp. can vary depending on the plant pathogen being targeted. In a study by Sarma et al. (2014), *Trichoderma* spp. showed varying levels of mycoparasitic activity against different fungal species. The study found that *Trichoderma* spp. had the weakest mycoparasitic effect against *Fusarium sporotrichoides* and *Fusarium culmorum*. On the other hand, the morphotypes A, B, C and D (*T. virens, T. atroviride* and *T. harzianum*) were very effective against *Rhizoctonia solani*.

The research also investigated the interactions between entomopathogenic and mycoparasitic fungi with the pathogen *B. cinerea*. Several strains of entomopathogenic fungi have been shown to have the ability to suppress different plant pathogens, as reported by Mantzoukas et al. (2022). The efficacy of entomopathogenic fungi against *B. cinerea* has been explored and demonstrated in previous studies. In a study by Sarven et al. (2020), the entomopathogenic fungus *Metarhizium anisopliae* was shown to have antagonistic effects against *B. cinerea*. Similarly, the study demonstrated that both *I. fumosorosea* and *A. attenuates* have mycoparasitic effects against *B. cinerea* although they were unable to completely suppress it. The *B. cinerea* pathogen produces dark brown cultures on which the growth of the entomopathogenic fungi *I. fumosorosea* and *A. attenuates* is clearly visible. Both entomopathogenic fungi produce light colonies, *I. fumosorosea* light grey and *A. attenuatus* white colonies. The typical colour of the mycoparasitic fungus *T. virens* is green. The entomopathogenic fungi *I. fumosorosea* and *A. attenuates* showed a mycoparasitic status, which can be seen in the Figure 14. However, in contrast to *T. virens*, both entomopathogenic fungi were unable to completely suppress the *B. cinerea* colony and degrade the mycelium of the pathogen. In comparison to the entomopathogenic species, the mycoparasitic fungus *T. virens* demonstrated the highest efficacy against the pathogen *B. cinerea*.

As part of the experiment, the sporulation of all the fungal species was observed in both the control variant as well as the mutual interactions. The results showed that the entomopathogenic fungi *I. fumosorosea* and *A. attenuatus* produced a larger number of spores in the control variant and a smaller number of spores in the mutual interaction. In contrast, an opposite effect was observed with *T. virens*, where fewer spores were produced in the control variant, while higher values were recorded in the mutual interaction.

In terms of practical use, mycoparasitic and entomopathogenic fungi are useful tools in the sustainable management of plant diseases and insect pests in agriculture. These fungi can act as natural enemies of plant pathogens and insect pests (Benítez et al. 2004, Shin et al. 2017), providing an environmentally friendly alternative to synthetic pesticides. Bioproducts based on these fungi are used and registered globally. These bioproducts have gained increasing attention as they have the advantage of being harmless for people and animals as well as the ecosystem.

Mycoparasitic fungi are parasitic on other fungi and can be used to control plant diseases caused by pathogenic fungi (Speckbacher & Zeilinger 2018). These fungi which are used as biological control agents (BCAs) offer a sustainable and effective method of pest and disease control in agriculture, and their use in combination with other management practices can further improve crop health and yield. Some wellknown bioproducts used against plant pathogens based on *Trichoderma* spp. are SoilGard 12G, Trianum-G, PlantShield HC, RootShield Plus WP, Binab TF and Prestop Mix which is based on *Clonostachys rosea* f. *catenulata* (Folorunso et al. 2021). These products have been demonstrated to improve the disease resistance, environmental adaptability, and root system development of crops, all of which can result in increased crop yield and higher quality.

Entomopathogenic fungi are parasitic on insects and can be used as a natural alternative to chemical insecticides. These fungi infect and kill insects, including pests that damage crops (Goettel et al. 2005). Entomopathogenic fungi such as *Isaria fumosorosea* and *Akanthomyces attenuates* can be applied as biopesticides and are effective against a wide range of insect pests. Common examples of such bio products are PreFeRal WG, PFR 97 20% WDG, NoFly WP based on *Isaria fumosorosea*, Mycotrol ES, BoteGHA ES, BotaniGard 22 WP based on *Beauveria bassiana*, Mycotal based on *Lecanicillium muscarium* and Met52 EC based on *Metarhizium brunneum* (Folorunso et al. 2021). These products have proven to be highly effective against a

variety of insect pests (Faria & Wraight 2007), making them an important resource for farmers and growers seeking to reduce their reliance on chemical pesticides and the environmental harm they cause.

Mycoparasitic fungi and entomopathogenic fungi are two types of fungi that have practical applications in agriculture and pest control. These fungi are valuable options in integrated pest management strategies and are effective resources for the development of new bioproducts. As research on these products continues to expand, it is possible that new applications and formulations may emerge, thereby enhancing their capacity to serve as eco-friendly and sustainable substitutes for artificial pesticides.

Conclusions

- Phenotypic expression of mycoparasitic fungi was evaluated based on center cultures on PDA medium.
- Selected strains of *Trichoderma* spp. were assessed for culture color, sporulation type, and presence of pustules.
- *Trichoderma* strains showed varying effectiveness against plant pathogens, with Tri 912 being the least effective against *R. solani* and *F. culmorum*, and Tri 911 showing the best mycoparasitic ability against *S. sclerotiorum*.
- Differences in touch and mycoparasitic zones were statistically significant among all strains in interaction with plant pathogens.
- Strains Tri 948 and Tri 946 (*T. virens*) had the fastest growth rate, with Tri 906 achieving the highest radial growth.
- Sporulation of fungal species was evaluated in both the control variant and mutual interactions, with varying effects observed among the different fungi.
- Among *Trichoderma* strains, *T. atroviride*-like strains produced the highest amount of spores and *T. polysporum*-like strains produced the lowest amount.
- The entomopathogenic fungi *I. fumosorosea* and *A. attenuatus* produced fewer spores in mutual interactions with *B. cinerea* compared to control variants.
- *I. fumosorosea* and *A. attenuatus* have mycoparasitic effects against *B. cinerea*, but the mycoparasitic fungus *T. virens* demonstrated the highest efficacy against the pathogen.
- Both mycoparasitic and entomopathogenic fungi offer eco-friendly and sustainable substitutes for artificial pesticides.
- Bioproducts based on these entomopathogenic and mycoparasitic fungi are used and registered globally, and have continued to gain increased attention due to their effectiveness.

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