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MASTER'S THESIS

The use of different methods for isolation of entomopathogenic and mycoparasitic fungi from different environmental conditions

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Declaration

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

In České Budějovice on ....

Signature

Abstrakt

Hlavním cílem této práce bylo otestovat metody izolace entomopatogenních hub z různých podmínek prostředí. V prním experiment byly inokulovány entomopatogenní houby *B. bassiana M. brunneum, I. fumosorosea* a *L muscarium* ve dvojích do sterilního půdního substrátu a inkubovány po dobu 50 dnů. Analýza kompatibility byla prováděna po 14 a 50 dnech inkubace. Výsledky byly porovnávány s výsledky v době 0, kdy byly z kontrolních suspenzí založeny testy, při kterých se hodnotil počet tvořících se kolonií (CFU, colony-forming unists). Výsledky ukázaly, že lze z půdy snadno izolovat entomopatogenní houby za pomoci rutinní metody použití selektivní živné půdy dodine. Zároveň byla z fotografické dokumentace zaznamenána interakce mezi jednotlivými druhy. Druhý experiment byl zaměřen na aplikaci entomopatogenní houby *I. fumosorosea a L muscarium* a mykoparazitické houby *T. virens* na rostliny okurek. Z výsledků tohoto experimentu též vyplývá, že jak entomopatogenní houby, tak mykoparazitickou houbu lze i z fyloplánu snadno izolovat entomopatogenní houby pomocí selektivní živné půdy na bázi dodine a mykoparazitickou houbu *T. virens* pomocí selektivní živné půdy TSM (*Trichoderma* selektive medium). Zároveň bylo prokázáno, že relativní vzdušná vlhkost je důležitá pro růst a vývoj entomopatogenních hub po preventivní aplikaci na rostliny okurky seté.

Klíčová slova: soil substrate, cucumber leaves, entomopatogenní houby, mykoparazitická houba*, I fumosorosea, L muscarium, M. brunneum, B. bassiana, T. virens,* vlhkost.

Abstract

The main objective of this work was to test methods for isolating entomopathogenic fungi from different environmental conditions. In the first experiment, entomopathogenic fungi *B. bassiana, M. brunneum, I. fumosorosea* and *L. muscarium* were inoculated in pairs into sterile soil substrate and incubated for 50 days. Compatibility analysis was performed after 14 and 50 days of incubation. The results were compared with those on day 0 when the control suspensions were used to establish tests to assess the number of colony-forming units (CFU). The results showed that entomopathogenic fungi can be easily isolated from soil using the routine method of dodine selective nutrient soil. At the same time, the interaction between species was recorded from photographic documentation. The second experiment focused on the application of the entomopathogenic fungi *I. fumosorosea* and *L muscarium* and the mycoparasitic fungus *T. virens* to cucumber plant leaves. The results of this experiment also show that both entomopathogenic fungi and mycoparasitic fungus can be easily isolated even from the phylloplane entomopathogenic fungi using dodine-based selective medium and mycoparasitic fungus *T. virens* using *Trichoderma* selective medium (TSM). It was also shown that relative humidity is important for the growth and development of entomopathogenic fungi after preventive application to cucumber plant leaves.

Keywords: entomopathogenic fungi, mycoparasitic fungi, *I fumosorosea, L muscarium, M. brunneum, B. bassiana, T. virens,* humidity.

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David Sandala

Content

1 PREFACE

A lot of entomopathogenic fungi were examined in the late 20th centuries for possible use against pests and pathogens. Although not much had been considered until in recent times people have reconsidered fungi for protection because of the increase in insecticide resistance and other human/environmental problems caused by insecticide use in the ecosystem. As a result, there has been a process going on such as identification of native fungi isolation from a variety of hosts/medium. Awareness about problems caused by pesticide use, has also helped in finding alternative solutions and control that are environmentally friendly. Solutions such as the IPM, solutions that can help us contain a host while considering the effects they possess on the environment and the consumer. The farmers of the old days relied on organic manure and cultural method as a way of eradicating or managing pests which were good in the view of biocontrol agents. A variety of fungal species occur on pests and pathogens in different ecosystems, which means maintaining biotic tolerance to keep the pest or host population limited and below the economic damage level. Entomopathogenic and mycoparasitic fungi have been recommended as a biological control agent for over 100 years now, but their use remains very limited. If considered in natural conditions of entomopathogenic and mycoparasitic fungi are very and usually important in natural mortality factor in the insect population. Some species such as *Metarhizium* spp. And *Beauveria* spp. From the order Hypocreales are facultative generalist pathogen, but most of the other species which belong to the order Entomophthorales, are obligate pathogens and they are quite specific. Facultative generalist Entomopathogenic fungi have frequently a wide range of host even-though, there is typical genetic diversity within species. Most entomopathogenic fungi are quite common and usually influence epizootics and are an important factor in regulating pest population. Unlike other bio-control agents such as entomopathogenic viruses and bacteria, entomopathogenic fungi do not need to be ingested to infect their host. They can penetrate the body of the host directly through the cuticle and this shows how they can be effectively useful against all pests including sucking pests. The main species from order *Beauveria* spp., *Metarhizium* spp. and *Isaria* spp. Naturally occur in the soil. On the other hand, *Lecanicillium* spp. and *Isaria* spp. Occur in the tropical and subtropical areas mostly on the canopy, where they cause natural epizootics in pest populations.

The aim of the thesis is focused on the use of isolation methods of entomopathogenic and mycoparasitic fungi from the soil and plants using selective and non-selective media.

2 INTRODUCTION

2.1 IPM Defination

IPM is defined according to Directive 2009/128/EC of the European Parliament and of the Council of 21 October 2009 as “Integrated pest management” means careful consideration of all available plant protection methods and subsequent integration of appropriate measures that discourage the development of populations of harmful organisms and keep the use of plant protection products and other forms of intervention to levels that are economically and ecologically justified and reduce or minimize risks to human health and the environment. ‘Integrated pest management’ emphasizes the growth of a healthy crop with the least possible disruption to agro-ecosystems and encourages natural pest control mechanisms (Anonymous 1).

2.2 Monitoring

Pest identification is the most important technique in IPM. Correct identification of pests helps choose proactive or reactive methods to eliminate pest populations. Identification of pests could be obtained from university personnel or private consultants, who are professionals in plant protection. The misidentification of the pest can cause large problems and it can be harmful to crops and cost money. Monitoring is a process that assesses the occurrence of harmful factors in growing crops. During monitoring, they systematically check the status of the pest population and it is routinely done at chosen intervals or periods. Monitoring aims to obtain information on the occurrence of pests, their developmental stages, population density, and the effectiveness of natural enemies. Or monitoring, various tools such as yellow or blue glue plates can be used in the greenhouse, but the identification of pest “in situ” in plant cover is essential (Stukenberg, 2018).

For small pests, the magnifying glass is used for identification. Pheromone or light catchers can be used in the field to tell us about the presence of the pest and how often it occurs in natural conditions. Information on the status of the pest population or the presence of natural enemies is helpful to select a suitable method of plant protection and it allows to predict the probability of the damage in the plants (Kundu et al., 2003; Zhou Z et al., 2010).

2.3 Biological control

Hussey et al., (1977) reported that the first case of biological control was culturing and realising of the parasitoid *Encarsia inaron* in greenhouses against greenhouse whitefly *T. vaporariorum*, but the introduction of this parasitoid was not successive in the control against targeted pest in the greenhouse.

The first successful attempt occurred in the year 1926 when the *Ecarsia formosa* was introduced against whiteflies, which caused diseases on the tomato crops (Eilenberg et al., 2001). Biological control methods are methods used on living organisms to maintain the population of that organism. The agents used in biological controls are known as predators, parasitoids and pathogens. They are used for population suppression of targeted pests with the reason to keep populations of pests below economical damage levels. All agents can decrease the pest populations by parasitism or predation, and after that, they reduce the damage that would be caused on crops (Osborne et al., 1992a).

2.3.1 Natural enemies

Natural enemies are organisms that kill or decrease the reproductive potential of, or otherwise reduce the numbers of another organism. Natural enemies are divided into two groups. The first group is formed by micro-organisms including parasites, parasitoids and predators. The second group includes different kinds of microorganisms, especially viruses, bacteria, nematodes and fungi (Hajek et al., 2018).

2.3.2 Introduction of Natural enemies

Biological agents are applied to crops to fight against pests or diseases by different strategies of biological control. Several different ways in which biological control can be applied, namely, classical biological control, Inoculative biological control, Augmentative biological control (Inundative control, Seasonal inoculative control), and Conservation control.

2.3.3 Strategies of biological control

Strategies of biological control are based on the introduction of natural enemies into the agroecosystem of interest against harmful organisms (pests, plant pathogens, weeds). The natural enemies are defined as basic methodological equipment available for biological control. The methods used in the framework of biological control are classical biological control - inoculation biological control, inundative biological control, seasonal inoculation biological control and conservation biological control (Eilenberg et al., 2001).

Classical biological control

Generally, classical biological control is a strategy that involved importing an alien from a region where the pest species originated from. Once the alien is released, it is self-sustainable and mostly used at the regional or national level by government agencies working together with research institutions.

This strategy aims to eradicate the exotic pest before it settles in the new agroecosystem after its colonisation. The natural enemy is released only for the necessary time until it eradicates the exotic pest population (Eilenberg et al., 2001). The first successful example of this strategy is the release of the Vedalia beetle Rodolia cardinalis Mulsant *(Coleoptera: Coccinellidae)* against the cottony cushion scale, Icerya purchases Mask. *(Homoptera: Margarodidae)*, in California in the late 1800s (Caltagirone et al., 1989).

Inoculative biological control

In some publication, Classical biological control is included as a part of a strategy named Inoculation biological control. Due to certain historical aspects, the term "Classical biological control" has become synonymous with this strategy. Classical biological control is considered in this case where concrete species of the natural enemy is intentionally introduced into a new area "behind the exotic pest" that have been unknowingly spread into the area. In this narrow concept, the inoculum introduction strategy requires the active participation of national and international institutions, including quarantine facilities Intentional inoculum introduction aims to ensure a long-term effect in the agro-ecosystem on targeted pests. Positive effects can be achieved in case

of successful attachment of the newly introduced or reintroduced onto the natural enemy, which can adapt, reproduce and spread in the new area and finally eliminate the population of pests (Landa, 2002; Sanda et al., 2014).

Augmentative biological control

This strategy aims to periodically release natural enemies that do not multiply in enough quantities to eliminate pests below the economic threshold. The practice of augmentation is based on knowledge or assumption, there are not enough natural enemies to provide optimal biological control in some crop systems. However, the bigger number of individuals of the natural enemy is increased by releasing them into the agroecosystems (Landa 2002). Augmentative strategies cannot be applied to all pest species. So far, there are quite a few examples applicable in practice. However, those that are implemented are highly effective. One of the most common ways of enlargement is the protection of greenhouse crops. In Europe, commercial use of natural enemies was applied more than 30 years ago. From the time, when insecticide resistance in greenhouse pests has been reported, the strategy was intentionally established (Sanda et al., 2014)

In the augmentative strategy, two basic methodological approaches are distinguished: Inundative biological control and Seasonal inoculative biological control.

Inundative biological control

This release aim is single or repeated introductions of a large number of natural enemies to achieve immediate regulation of pest population or suppression of the development of plant pathogens. Inundative introductions are predominantly used in the biological control of annual crops against univoltine pests (1 generation per year) or bivoltine pests (2 generations in the year). The principle of this method is to “overlap” the pest population with an effective number of natural enemies and achieve immediate, i.e. bio-insecticidal effect.

Seasonal inoculative biological control

Seasonal inoculation involves the release of a small number of natural enemies at regular intervals to control pests throughout the growing season. The introduction of natural enemies starts at a time when a low population of pests is present. It is expected that natural enemies will immediately begin to reproduce in the system and consequently establish their populations to ensure long-term control of targeted pests. During the growing season, the monitoring of targeted pest populations and natural enemies is important. If it is necessary, the introduction of the next adequate number of bioagents is considered (Landa 2002).

Conservative biological control

The strategy is focused on supporting and conserving autochthonous populations of natural enemies. The strategy can be divided into two ways, active and passive. Passive conservation is based on natural enemy support to avoid unfavourable processes that would harm natural enemies (the application of broad-spectrum pesticides). Active conservation is the advancement of natural enemies by intentional support and ensures resources that beneficial organism species need in natural ecosystems.

The establishment of various agro-ecological and agronomic operations (e.g. the intentional establishment of stable bio corridors, diversification of crops grown in the field, minimisation of agro-technical interventions, establish of "beetle banks" and sowing of flowering plants near the cultivated field crops) (Landa, 2002; Eilenberg et al., 2001; Eilenberg, 2006)

2.4 Entomopathogenic fungi

Entomopathogenic fungi are a group of phylogenetically diverse, heterotrophic, eukaryotic, unicellular or multicellular (filaments) microorganisms, which reproduce through asexual and sexual spores (Mora et al., 2017)

Table 2.4.1 shows the taxonomy of entomopathogenic fungi families and orders (Sung et al. 2007)

	Family		
	Clavicipitaceae	**Cordycipitaceae**	**Ophiocordycipitaceae**
Teleomorfa	*Hypocrella, Metacordyceps, Regiocrella, Torrubiella*	*Cordyceps s.str., Torrubiella*	*Ophiocordyceps, Elaphocordyceps*
Anamorfa	*Aschersonia, Metarhizium, Nomuraea, Paecilomyces, Pochonia, Rotiferophthora, Verticillium*	*Beauveria, Engyodontium, Isaria, Lecanicillium, Mariannaea, Microhilum, Simplicillium*	*Haptocillium, Harposporium, Hirsutella, Hymenostilbe, Paecilomyces,Paraisaria, Sorosporella, Synglіocladium, Tolypocladium, Verticillium*

Entomopathogenic fungi are potentially the most adaptable biological control agents, because of their wide range of hosts. They result in natural epizootics; it is easy to recognize the infection because fungi are visibly outgrown. The feature that makes these fungi attractive is the contact way with infected cadaver (Nadeau et al., 1996). Entomopathogenic fungi are heterogeneous and belong to many orders in a different division of Fungi kingdoms. Approximately 750 species, reported from different insect families. Besides, several fungi from order Hypocreales have great potential in pest management systems. But the most important pathogens are *Metarhizium* spp. *Beauveria* spp. *Isaria* spp. *Nomuraea rileyi, Lecanicillium* spp. and *Hirsutella* spp. (Inglis et al., 2001).

2.4.1 Life cycle

Pathogenesis is the sequence of processes in disease development from the time of infection to the final reaction in the host (Kudela, 1989). Differing from bacteria and viruses, fungal infection is visible, and pathogenesis occurs in insects through a series of integrated, systematic events progressing upon spore attachment on the insect cuticle, germination, penetration into the host, the production of blastospores, proliferation within the body of the host, utilisation of insect organs and tissues. All these

processes are in interaction with an insect defence mechanism. The finally the fungus re-emergence on the cadavers and the fungus sporulates (Zimmermann, 2007a; Thomas et al., 1996; Nadeau et al., 1996).

The first step in fungus development is crucial and important. The vital and virulent spore that is attached to the body of the host by adhesive substances (mucilaginous) form a firm bond with the cuticle of the host during the first contact (e.g. fungi *Lecanicillium lecanii, Aschersonia aleyrodis, Hirsutella thompsonii*) (Boucias et al., 1991; Wraight et al., 1990; Vestergaard et al., 1999). Other species of entomopathogenic fungi (e.g. *Beauveria bassiana, Isaria fumosorosea, Metarhizium anisopliae*) produce dry, highly hydrophobic conidia with a diverse structured surface. Primary adhesion of such conidia direct interaction between two hydrophobic surfaces, electrostatic forces, or molecular interactions between substances available on the surface of the conidia and the cuticle of the host (eg haemagglutinin, N-acetyl glucosamine, glycoproteins, sterols, polar lipids) (Sosa-Gomez et al., 1997; Altre et al., 1999).

After the invasion of hyphae into the body of the host, the pathogen rapidly colonises the body cavity to form either single or multicellular hyphal bodies (blastospores) that lack a cell wall but are covered with a thin fibrillar layer on the plasma membrane. Before the pathogen grows in the haemolymph, it overcomes the immune defence mechanisms of insects by producing toxins and other secondary metabolites. Insects can respond to toxin production through humoral (e.g. phenoloxidase, lectins, defensive proteins, and peptides) or cellular (e.g., phagocytosis, cellular or humoral encapsulation) defence mechanisms (Vilcinskas et al., 1999). The killing of the host by a pathogen using a combination of several processes, including invasion of the pathogen into the host body tissues and their destruction ("Use of hyphomycetous fungi for managing insect pests", (Inglis et al., 2001).

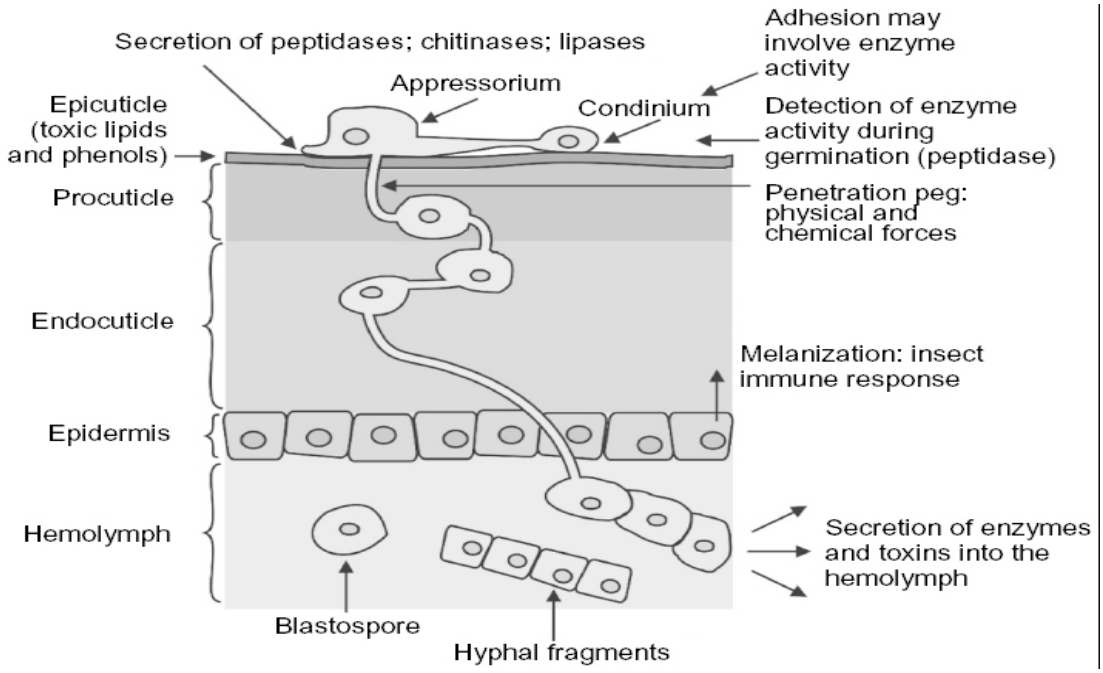


Figure 2.4.1.1 Parasitic phase of the life cycle of entomopathogenic fungi. according to (Samuels et al., 2016)

After the death of the host, the fungus colonizes the cadaver and during 2 to 3 days forms aerial hyphae and sporulates depending on the appropriate conditions. Fungi from order Hypocreales (Ascomycota) produce only asexual spores while fungi from order Entomophthorales (Zygomycota) produce two types of spores asexual (primary conidia) and sexual (zygo- or azygospores) called resting spores. The conidia of Hypocreales and the primary spores of Entomophthorales are produced externally on the surface of the insect after its colonization Besides, Conidia of Hypocreales fungi can spread by wind, water, or by another insect (zoochory). Hypocreale fungi can survive in nature without a new host as preserved cadavers or as conidia in the soil or generally saprotrophically on organic matter (Hajek et al., 1994).

2.4.2 Factors affecting the effectiveness of entomopathogenic fungi

Insect infections caused by entomopathogenic fungi are divided into biotic (e.g. physiological conditions of the host and pathogen, host plant) and abiotic factors (temperature, relative atmospheric humidity, solar radiation) (Tumuhaise et al., 2018; Cory et al., 2006; Cory et al., 2009; Jaronski, 2009). One of the critical factors in the effective use of microbial agents as insecticides is their relatively short persistence on leaf surfaces and the soil surface (Gindin et al., 2000).

Keller et al. (1989) mentioned many entomopathogens from the order Hypocreales are have been taken into consideration as soil-borne microorganisms having demonstrated as potentials against soil pests. Although the soil has extremely multiple conditions and factors, including soil moisture (i.e. water availability) and the presence of viable soil microflora, can influence the persistence and/or efficacy of entomopathogenic fungi of the order Hypocreales, type (i.e. Texture, cation exchange capacity, organic matter content, ph, etc.) Besides Inglis et al., (2001) reported a variety of strategies (e.g. formulation and application methods, such as tillage) have been tested in an attempt to enhance the success of entomopathogenic Hypocreales in soil.

2.4.3 Abiotic factors

Abiotic or environmental factors such as temperature, humidity and sunlight play an important role in the field persistence of entomopathogenic fungi. Abiotic factors that influence the virulence of entomopathogens to be considered for the successive development of the fungus as a biocontrol agent ("Use of hyphomycetous fungi for managing insect pests",) (Inglis et al., 2001).

Temperature

The optimum temperature for the entomopathogenic fungi ranges from 20 °C to 25 °C (Osborne et al., 1992). Hywel-Jones et al., (1990) examined spore germination of *M. brunneum* and *B. bassiana* at 20 - 30 °C and found higher germination levels in *M. brunneum* compared to *B. bassiana.* Rangel et al. (2008) published that most *Metarhizium* species, considered to be mesophilic fungi, have severely restricted growth below 10 °C and above 37 °C. Critical environmental factors can have a serious impact not only on growth and development but also on the pathogenicity of the fungal pathogen against the targeted pest (Faria et al., 2001).

The entomopathogens that were chosen are *Beauveria bassiana, Metarhizium brunneum* and *I. fumorosea,* the reason being there are among the most important fungi and play a major role in the fight against Pests (Zimmermann, 2008). The commercially registered mycoinsecticide, particularly the versatile *Lecanicillium lecanii* (Zimm.) (Gams et al., 2001). Therefore, the broad host range mycoparasite *T. virens* because of their potential in bio-control (Harman, 2000).

Relative humidity

Humidity is one of the key factors in spore germination and fungi sporulation CFU development in the entomopathogenic fungi, especially relative air air air humidity (RH%). As mentioned by Allen et al., (1971) Of all the important cofactors, that influences the epizootics of phytopathogen, none is more critical for sporulation, germination, and invasion of the host than high humidity. However, pathogenesis occurs at much lower surrounding values of temperature and humidity (Ferron, 1978; Butt, 2002; Ramoska, 1982). Relative atmospheric atmospheric atmospheric humidity is necessary for germination having most conidia of entomopathogenic fungi germinate at a humidity of more than 90% (Hall, 1981). If the humidity is high on the insect cuticle, it enables the micro-climate of the micro-climate the parasitic phase of its development. If the humidity is low on the insect cuticle, the fungus never causes the disease of the pest. The external sporulation never occurs on the killed insect, if the relative humidity is too low. In the natural environment, these humidity humidity humidity conditions occur during the day several times, with decreases and increases? Rises in temperature, whereby each living organism produces a water-saturated layer on the surface of the cuticle (Arthurs et al., 2001).

2.4.4 Soil conditions

Most of the entomopathogenic fungi can withstand extreme and variable temperatures, as well as conditions of high moisture and drought stress, in soils (Ansari et al., 2011; Roberts et al., 1976), and several studies have demonstrated that conidia applied on soil surfaces or incorporated into the soil following application exhibit considerable persistence in temperate climates (Müller-Kögler et al., 1986; Inglis et al., 1997). Soil moisture has indicated to adversely influence the persistence of *B. bassiana, B. brongniartii, M. anisopliae* and *L. lecanii* and others in soil. In most situations, limited vertical movement of conidia occurs in soils. But, in other circumstances, the loss of inoculum may occur (Storey et al., 1987; Storey et al., 1988). The texture and organic matter of the soil appear to be the most important factors determining the vertical movement of fungal propagules in water, and sandy-textured and soils low in organic matter tend to retain fewer propagules than clay-textured and organic soils (Ignoffo et al., 1977; Keller et al., 1989; Fornallaz, 1992). Though the conditions of temperature

in the top few layers of soil may range to over 40°C between daylight and night hours, and temperatures above 50°C can occur (Carruthers et al., 1988). Therefore, the incorporation of propagules into the soil to intensify the persistence will depend not only on climatic factors (e.g. Ambient temperatures and vegetation) but also on other factors, such as shading or the depth to which propagules are incorporated. In addition to injections, the encapsulation of propagules is another formulation strategy that may facilitate penetration and persistence in soil, but this has not been extensively tested with entomopathogenic of the order Hypocreales (Inglis et al., 2001).

2.4.5 The most important entomopathogenic fungi

Isaria fumosorosea WIZE

This cosmopolitan species *I. fumosorosea (Hypocreales: Cordycipitaceae)*, known by the older name *Paecilomyces fumosoroseus*, is very polyphagous. On the infected host first, it forms a white cottony mycelium, which is later a mauve colour when full sporulation and the cottony character of this mycelium becomes dusty. Conidiophores formed on aerial mycelium are in the whorls on the hyphae. Conidia having character cylindrical shape consists of chain-shaped formations, each chain consists of up to 50 individual conidia (Samson, 1974). Under optimal conditions, a rapid infection can cause a pathogen to cause the death of whitefly at all developmental stages (Osborne et al., 1992). Temperatures between 30 and 40 °C are very limiting for the growth of the pathogen than temperatures 8 -11 °C. The optimum temperature for the growth of this mycoparasitic fungus is in the range of 20 - 30 °C (Vidal et al., 1997).

Lecanicillium lecanii R. ZARE et W. GAMS

This species *Lecanicillium lecanii (Hypocreales: Cordycipitaceae)*, first described in 1861 and often mentioned in earlier works under the synonymous name *Verticillium lecanii (ZIMMERMAN.)* VIERGAS is widely polyphagous entomopathogenic, used in biological protection to fight thrips, moths and aphids in horticulture. The species also occurs in natural conditions as a pathogen in populations of various species of beetles, butterflies and two-winged insects (Landa, 1998)The main determining feature of this species is the typical form of sporulation. On airborne mycelium forms long, typical flasks during conidiogenesis cells at the end of which ellipsoidal conidia form. There

are conidiophores on the mycelium formed in whorls and 2 to 4 conidiophores grow opposite one zone. In the end, hyphae can also be formed by a larger number of conidiophores. A newly created conidiophore pushes the previously formed cluster into a compact shape (Askary et al.,2007). Germination of the pathogen begins only at high relative humidity (Osborne et al., 1992), the optimum for germination of spores is 97%, for growth than 85-90%. Temperatures should be between 20-25 ° C (Subramaniam et al., 2021).

Metarhizium anisopliae METSCH. SOROKIN

This species of entomopathogenic fungus *Metarzizium brunneum (*formarly *Metarhizium anisopliae (*Bischoff et al. 2009)*) (Hypocreales: Clavicipitaceae)* was first described by Metschnikoff in 1879 (Zimmermann et al., 1995) as an entomopathogen against the species Anisoplia austriaca *(Coleoptera, Scarabaeidae).* The created mycelium completely covers the body of the infected host, the conidiophores are in compact clusters, individual conidiophores branch widely. Conidiophores are quite common they cluster and form sporodochia that are simple or branched (Watanabe, 2010). Zimmermann, (1993) states that this fungus can infect more than a hundred species of insects, mainly from the orders O*rthoptera, Hemiptera, Coleoptera, Diptera, Lepidoptera* and *Hymenoptera*. Individual strains have a wide temperature valence for growth. It is stated that some grow at temperatures above 35 ° C and some even at temperatures below 12 ° C, however, the optimum temperature for growth is between 25 and 30 ° C (Boucias et al., 1988).

Beauveria bassiana (BALSAMO-CRIV.) VUILLEMIN

The genus *Beauveria (Hypocreales: Cordycipitaceae)* currently includes three important species of entomopathogenic hub: *B. bassiana*, *B. tenella* and *B. brogniartii*. *B. bassiana* belongs to the species Most important (Zimmermann, 2007b). The entomopathogenic fungus *B. bassiana* was discovered about 170 years ago. It was discovered in 1834 by the Italian scientist Agostino Bassi (1773- 1856). It was determined by Giuseppe Balsamo Crivelli and then described as *Botrytis paradox*, but later changed its name to *Botrytis bassiana* in honour of her discoverer (Dirlbeková, 1991). In 1912,

Vuillemin conducted a revision systematic classification and created the currently respected genus *Beauveria* into which also belongs to the species *B. bassiana* (Zimmermann, 2007).

Beauveria brongniartii (SACCARDO) PETCH

This entomopathogenic fungus *Beauveria brongniartii* (*Hypocreales: Cordycipitaceae*) is rarer in nature than the more well-known *B. bassiana,* however, it is also cosmopolitan on insects as well as in various other environments. Its occurrence has been recorded in alpine forest areas and other places. This fungus is characterized by the formation of first white and later yellowish, but sometimes even reddish colonies. Ellipsoidal conidia are arranged separately or in small groups (Zimmermann, 2007b). The pathogen sporulates and grows at temperatures of 2 to 33 °C, preferably growing at temperatures range between 22 - 33 °C (Schwartz, 1967).

2.5 Mycoparasitic fungi

Biological control against plant pathogens can be well defined as a decrease in the amount of inoculum or pathogenic activities of a pathogen by one or more microorganisms with mycoparasitic or antagonistic activities (Landa, 2002).

Fungi in the same substrate will compete with each other for control of that nutrient source and space, (Kolesidis et al., 2019), and this competition can be so high that the most successful competitive fungi will specialize in parasitizing other fungi.

The biocontrol by natural antagonists relies on various mechanisms, including mycoparasitism, competition for substrate and space, antibiosis, enzymatic activity, and induced resistance (Kant et al., 2011). Biocontrol activities are applied either directly by antagonism of soil-borne pathogens or indirectly by inducing an induced systemic resistance response in plants (Ram et al., 2018)

Mycoparasitism is the most significant form of antagonism that involves direct physical contact with the host mycelium (Pal et al., 2006). It involves tropical growth of the fungal mycelium for biological control directed towards the target pathogen, which is followed by extensive coiling and secretion of various hydrolytic enzymes resulting

in the dissolution of the pathogen cell wall or membrane. Mycoparasitism can be categorized as a four-stage process. An initial step comprises the chemotropic growth of an antagonistic fungal mycelium towards the phytopathogenic fungus, followed by detection.

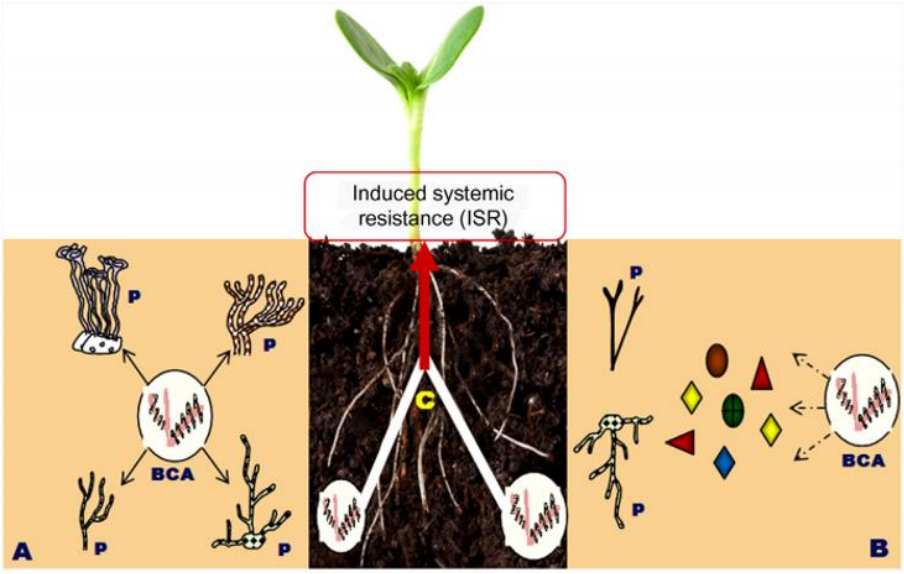


Figure 2.5.1 Schematic representation of (A) mycoparasitism of various fungal pathogens (P) by BCA, (B) antibiosis of pathogens by antimicrobial secondary metabolites secreted by BCA, and (C) elicitation of induced systemic response in host plant by BCA according to (Tumuhaise et al., 2018).

The third and fourth steps involve direct adherence and degradation of the cell wall of the phytopathogenic fungus, after which penetration into the host fungus cell occurs. This is one of the major mechanisms used by *Trichoderma sp*. To eliminate phytopathogenic fungi.

2.5.1 Fungi of the genus *Trichoderma*

The genus *Trichoderma* was introduced into the mycological literature by Persoon (1794), species. Fungi of the genus *Trichoderma spp*. Are actively used against several plant pathogens since the 1920s. *Trichoderma* reproduce both sexually and asexually. Telemorphs belong to the genus Hypocrea *(Ascomycota, Ascomycetes, Hypocreales)* and the asexual form resp. The anamorphic stage belongs to the genus *Trichoderma*

(Mitosporic fungi, Hyphomycetes, Moniliales) (Chaverri et al., 2003). They create perfect stages belonging to the Ascomycetes class and imperfect stages were classified in the class Hyphomycetes (Chaverri et al., 2003).

Trichoderma is efficient in improving the vegetative growth of plants and nutrient content of soil through decomposition and biodegradation (Woo et al., 2014). The limiting factors are humidity and also the temperature of the soil. The species *T. harzianum* is especially found in warmer conditions, and in contrast, *T. polysporum and T. viride* are found in cooler areas (Danielson et al., 1973). The ranges of temperatures in which these species have the potential to grow are wide.

Table 2.5.1.1 List of Biocontrol Agents Displaying Mycoparasitism According (Ram et al. 2018)

Biocontrol agents	**Target pathogens**
Coniothyrium minitans	*Sclerotinia sp.*
Sporidesmium sclerotivorum	*Sclerotinia minor*
Trichoderma sp.	*R. solani, Fusarium sp., sclerotinia sclerotiorum rolfsii, Uncinula necator*
Pythium nunn	*Pythium sp*
Aspergillus niger	*Macrophomina phaseolina*
Ampelomyces quisqualis, Acrodontium crateriforme	*Powdery mildew fungi (Erysiphe sp., Uncinula sp*

2.6 Selective medium

Soil is regarded as the natural habitat of fungi because they deposit their infectious spores there and stay there for a defined period of their life cycle (Medo et al., 2011). Selective media are used for the cultivation of only selected microorganisms. For example, if a microorganism is resistant to a particular antibiotic such as ampicillin or tetracycline, this antibiotic can be incorporated into the medium to suppress the growth of other cells that do not have such resistance. Selective growth media are also used in cell culture to enable cells with certain characteristics, such as antibiotic resistance or the ability to synthesise a particular metabolite, to survive or proliferate. Typically, the presence of a specific gene or allele of a gene endows a cell with the ability to grow in selective media (Anonymous 2)

Soil is an essential reservoir of insect pathogenic fungi and is widely considered to be a suitable habitat for microbial control of fungi because it provides a shelter from extreme environmental influences, which increases the persistence of conidia and their ability to thrive (Ekesi et al., 2007; Jaronski et al., 2007).

Selective media are usually solid, liquid or semi-liquid substance generally used to isolate or identify particular microorganism. But on the other hand, they are used for qualitative or quantitative analysis of entomopathogenic fungi in field conditions (Landa, 2008). The most known feature about them is the ability to effectively suppress the development of species that are "non-targets". But at the same time not hinder the growth of the targeted species of microorganisms. In some case e.g a relatively narrow species of entomopathogenic fungi (Landa, 2008). A broad range of antibiotics and fungicides can be used to isolate entomopathogenic fungi such as *Metarhizium* and *Beauveria* from the soil. Veen et al., (1966) wrote that the first selective media was composed of or rather contained chloramphenicol and cycloheximide (actidione) which was taken from a selective medium that was used to isolate fungus from clinical (Liu et al., 1993). Which was later simplified according to Milner et al., (1976) and it was used for the isolation of *Metarhizium* with the medium being named " Veen's medium". In the year 1982, it was discovered that the medium containing active ingredients would make it possible to isolate *Metarhizium* from the soil in Brazil compared to other mediums (Beilharz et al., 1982).

2.6.1 Selective media for entomopathogenic and mycoparastic fungi

The doses of selective medium mentioned below correspond to 1000 ml of the final selected selective medium, all media must be sterilized by autoclaving for a period of 20 minutes at a temperature of 121 °C and pressure around 18-20 Pa.

Selective agarized medium (Strasser et al., 1996)

10 g peptone, 20 g glucose and 18 g bacteriological agar, 0.5 ml streptomycin, 0.5 ml (0.05 g / ml) tetracycline, 0.5 ml (0.05 g / ml) cyclohexamide and 0.5 ml (0.1 g / ml) a.i. Dodine.

Veen's agar medium (Veen et al., 1966)

35 g of mycological agar or 10 g of a soy peptone, 10 g of dextrose, 15 g bacteriological agar, 1 g chloramphenicol and 0.5 g cycloheximide.

Dodine (Landa, 2008)

The foundation of this method is based on the selective effect of a fungicide, the active ingredient dodine (N-dodecylquanidine monoacetate), which harms the growth rate, which includes cell wall transformations, conidial induction and germination of a vast number of saprotrophic and phytopathogenic fungi. The secondary effects are usually manifested in the soil or the phytoplast of plants, impacting the most important species of entomopathogenic fungi.

Table 2.6.1.1 Variants of selective medium i.e. Dodine

	Variant A	Variant B	Variant C
selective media ingredients	1000 ml	1000 ml	1000 ml
bramboro-dextrose agar	39,00 g	39,00 g	39,00 g
cycloheximid (actidione)	0,25 g	0,25 g	0,25 g
chloramfenikol	0,50 g	0,50 g	0,50 g
dodine	0,10 g	0,05 g	0,01

The doses of selective medium mentioned below correspond to 1000 ml of the final selected selective medium, all media must be sterilized by autoclaving for a period of 20 minutes at a temperature of 121 °C and pressure around 18-20 Pa.

Trichoderma selective media (TSM)

Trichoderma selective medium (TSM) is recognized for quantitative isolation of *Trichoderma* spp. From the soil. It is composed of a low glucose level for rapid growth and sporulation of the fungus. Chloramphenicol is used to inhibit the growth of bacteria, while pentachloronitrobenzene, *p*-Dimethylaminobenzenediazo sodium sulfonate (DABDS), and rose bengal are used as selective fungal inhibitors (Elad et al., 1981). 0.2 g of mgso4·7H2O, 0.9 g of K2HPO4. 0.15 g of KCL, 1.0 g of NH4NO3, 3.0 g of glucose, 0.15 g of rose Bengal, 20 g of agar, 0.25 g of chloramphenicol, 0.3 g of p-dimethylaminobenzenediazo sodium sulfonate, 0.2 g of pentachloronitrobenzene. The

recipe is dissolved in 1000 ml distilled water and autoclaved at 121°C, 1.4 kg cm−1 for 15 min. When the medium is cooled at the temperature of 45 – 50 °C the ingredients 0.25 g chloramphenicol and 0.2 g pentachloronitrobenzene into the solution are added.

Trichoderma harzianum selective medium (THSM)

The selection of THSM enables comparison between aggressive and non-aggressive *Trichoderma* groups. The antimicrobials chloramphenicol, streptomycin, quintozene, and propamocarb are added to the medium to highly select *T. harzianum* in compact colonies without visible contamination (Shah et al., 2019).

Recipe for THSM, 0.2 g of mgso4·7H2O, 0.9 g of K2HPO4, 1.0 g of NH4NO3, 0.15 g of KCL, 0.15 g of rose Bengal, 3 g of glucose, 20 g of agar, 950 ml of distilled water. When the medium is cooled at the temperature of 45 – 50 °C the ingredients 0.25 g of chloramphenicol, 9.0 ml of streptomycin, 1.2 ml of propamocarb, and 0.2 g of quintozene are added.

Rose Bengal Agar (RBA)

RBA is a nonselective medium for isolation of *Trichoderma* which is developed by (King et al., 1979), for the enumeration of moulds and yeasts from food. The medium is suitable for protein foods and tolerates high temperatures. Chloramphenicol or chlortetracycline is added to suppress the growth of bacteria.

Recipe for Rose Bengal Agar Mycological peptone 5.0 g, rose bengal 0.05 g, glucose 10.0 g, chloramphenicol 0.1 g, dipotassium phosphate 1.0 g, agar 15.0 g, mgso4.7 H20 0.5 g.
When the medium is cooled at the temperature of 45 – 50 °C the ingredients 0.25 g of chloramphenicol or 0.25 g chlortetracycline.

Selective media for *G. virens* and *G. roseum* from the soil.

Recipe for *G. virens* and *G. Roseum* 3.0 g of glucose, 0.2 g of mgso4.7H2O, 1.0 g of K2HPO4, 0.5 g of KCL, 1.0 g of nano3, 0.01 g of feso4.7H2O, 50 mg of chloramphenicol, 50 mg of rose Bengal, 50 mg of streptomycin sulfate, 500-mu-g of benomyl (50WP), 500 mg of sodium propionate, and 20 g of Bacto-agar. The medium was adjusted to ph 6.0 with 25% phosphoric acid before autoclaving (Park et al,.1992).

2.7 Isolation of entomopathogenic fungi

The other method widely used to detect entomopathogenic fungi in the soil is the bait method. Bait methods use Galleria mellonella L. (*Lepidoptera: Pyralidae*) or other insects and are comparatively simple and sensitive (Zimmermann, 1986).

Observing the presence of entomopathogenic fungi in the soil using the "bait method"

The basic principle of this experiment is to see the virulence of the strains of the entomopathogenic fungi in the soil, by causing an infection on a sensitive living host. In this instance, the larvae of certain species of insects are used most often of the Wax moth (*Galleria mellonella*) or the mealybug (*Tenebrio molitor*). The larvae of these species are very suitable for monitoring the presence of entomopathogenic fungi in the soil. Because they allow the selective isolation of the most important entomopathogenic fungi, most importantly the fungi like *B.bassiana* and *M. anisopliae* var. Are spores are easily spotted on the surface body of the Larvae (Zimmermann, 1986). And platinum methods using various selective media (Fernandes et al., 2010; Veen et al., 1966; Shin et al., 2010) are especially useful when there is a need for quantification or when the input material is not soil (e.g. air and some plant parts) (Medo et al., 2016; Steinwender et al., 2014).

3 MATERIAL AND METHODOLOGY

3.1 Entomopathogenic fungi

Metarhizium anisopliae

The entomopathogenic fungus *Metarhizium anisopliae*, strain Bio 1020 was re-isolated from bioproduct MET52 (Novozyme®, Denmark) using an artificial medium PDA (Potato-dextrose agar, Himedia laboratories Pvt. Ltd., India) (PDA).

Lecanicillium muscarium

The entomopathogenic fungus strain *Lecanicillium muscarium* strain Lmu 01 was isolated from adult of bark beetle *Ips typographus* captured by pheromone traps in the Sumava National Park in 2001. The selective medium based on dodine was used for reisolation of strain from the adult.

Isaria fumosorosea

The entomopathogenic fungus ***Isaria fumosorosea*** strain PRF 97, was re-isolated firstly from bioproduct PFR 97 TM 20% WDG (Certis USA, Llc., USA). Strain PFR 97 was used extensly in experiments in Florida in the year 1997. The reference strain is stored in the American Collection of Microorganisms under ATCC 20874. In Europe, the strain PFR 97 is distributed by company Biotest, Belgium under the name PreFeRal® WG.

Beauveria bassiana

The entomopathogenic fungus *Beauveria bassiana* strain CCM 8382 was isolated from adult of bark beetle *Ips typographus* in the Sumava National Park in 2007 and isolated using an artificial medium PDA (Potato-dextrose agar, Himedia laboratories Pvt. Ltd., India). The strain is patented and deposit in the Czech Collection of Microorganisms, Brno, Czech Republic.

3.2 Mycoparasitic fungus

Trichoderma virens

Mycoparasitic fungus *Trichoderma virens*, strain GL-21 was re-isolated from bio-product SoilGard 12G (Certis USA Llc., USA).

3.3 Immobilization of strains to alginate pellets

All strains of entomopathogenic fungi and mycoparasitic fungus *T. virens* are stored in the mycological collection in the Department of Plant Production, Faculty of Agriculture, the University of South Bohemia, Czech Republic.

The alginate pellets are made by mixing of strain suspension with wheat bran and Na-alginate (sodium alginate). The biomass is consequently slowly dropped into the $CaCl_2$ solution where pellets are formed. The pellets are left to harden for 1 to 2 hours and then rinsed with water used sieve and alginate pellets are dried. After drying, the alginate pellets of each strain were transferred into the sterile small plastic bottle and they were marked. The bottles are stored in the freezer at a temperature of – 20 °C until they were used for experiments

3.3.1 Activation of alginate pellets

The at least 4 alginate pellets of each strain were separately placed on the surface of the 2 % water agar in small Petri dish (60 mm) and incubated for 7 to 10 days in the thermostat at 25 °C. During incubation, the pellets absorbed water from the water agar while increasing in size and strain is activated and start to overgrowing on the surface of pellets and pellet is covered by mycelium and strain start to sporulate. After sporulation, the conidia were transferred using inoculate loop on the surface of PDA and on this medium the strains were maintained. For experiments, 10 days old culture of entomopathogenic fungi and 7 days old culture of *T. virens* were used for experiment.

3.3.2 Preparation of suspensions

The suspensions where obtained from the fully sporulated cultures of each strain. The culture was poured using sterilized 0.05% Tween 80 solution over the mycelium and spores were scraped using inoculate loop the solution. The obtained suspensions were then filtered using a sterilized gauze to get homogeneous spore suspension without

cluster of mycelia of the strains. For each strain, the spore concentration in the suspension of each strain was counted using a counting hemocytometer (Neubauer's improved chamber), which was counting under the microscope. So the concentration of spores was determined by averages from two replicates. The density of the spore was determined by recalculated.

Suspension of each strain was adjusted for concentration 2.00 x 10^5 for the experiment where the entomopathogenic fungi we inoculated into the soil substrate. For control variants, the suspension 2.00 x 10^5 was mixed with 0.05 % sterile solution of Tween 80 in ratio 1:1 to obtain the concentration of 1.00 x 10^5. The combinations of fungi were prepared. Each fungus was in pairs with another entomopathogenic fungus. One strain (fungus concentration 2.00 x 10^5) was mixed with another strain (fungus 2.00 x 10^5) in ratio 1:1 to obtain the suspension in combination 1.00 x 10^5 spores of each fungus in 1 ml.

For greenhouse experiment, the spore suspension of *L. lecanicillium*, *I. fumosrosea* and *T. virens* were adjusted for concentration 1.00 x 10^7 spores per 1 ml. The suspensions were prepared immediately before applying the fungi on the cucumber plants planted in the greenhouse. The application was repeated every 7 days. The presence and spore survival of fungi on the leaves was evaluated after 7 days before next application.

3.4 Media used for re-isolation of fungi

The non-selective and selective media used for these experiments was chosen based on the species of fungus was used. In this case, the chosen ones are Potato-dextrose agar with antibiotics chloramphenicol, selective medium based on dodine, and Trichoderma selective medium (TSM). For activation of strains from alginate pellets were used 2 % water agar.

Water Agar *(2%)*

The mixture of water agar was made of 20 g of agar to 1000 ml of distilled water. This media-created was used to activate pellets of individual entomopathogenic fungi and mycoparasitic fungus *T. virens*.

PDA - Potato Dextrose Agar

Potato dextrose agar (PDA) was used for strain cultivations to obtain the suspensions for experiments and also for reisolation of strains from the environments. PDA was prepared from the semi-finished product and water agar. The 24 g of PDB (Potato Dextrose Broth, HiMedia Laboratories Pvt. Ltd., Mumbai, India) and 16 g of agar (Dr. Kulich Pharma s. R. O., Hradec Králové) were added into 1000 ml of distilled water. For fungi reisolation, the antibiotic chloramphenicol was added to the PDA. The medium was sterilized at 121 °C for 45 minutes and then poured into the Petri dishes (90 mm).

Selective medium based on dodine

The basis of this method is the selective activities of the fungicide respectively on i.e. dodine (N-dodecylquanidine monoacetate), which negatively affects the growth rate which includes the cell wall transformation, indicative action of conidia and germination of huge number of saprotrophic and phytopathogenic fungi. Two antibiotics chloramphenicol and cycloheximide are added to also to suppress bacteria or another saprotrophic fungi. The 24 g of PDB, 16 g of agar, 50 mg of dodine, 0.50 g of chloramphenicol, 0.25 g of cyclohemiximede is added to 1000 ml of distilled water and sterilized.

Trichoderma selective medium

This medium is used to isolated fungi from the genus Trichoderma from a given host, from the canopy or soil. The ingredients suppress all the another saprotrophic fungi and bacteria. The growth of Trichoderma is slower in compare when the Trichoderma fungi are isolated on PDA. The 24 g of PDB, 16 g of agar, 0.2 g of $MgSO4 \cdot 7H2O$, 0.9 g of K2HPO4, 1.0 g of NH4NO3, 0.15 g of KCl, 0.15 g of rose Bengal, 950 ml of distilled water. Medium was autoclaved at 121°C, 45 min. When the medium was cooled to 45-50°C, 0.25 g of chloramphenicol, 9.0 mL of streptomycin, 1.2 mL of propamocarb, and 0.2 g of quintozene are added.

3.5 Inoculation of fungi soil

The sterile soil substrate was used for experiment. The 20 g of sterile soil substrate was added to Petri dish (90 mm) and the fungi were inoculated. The suspension of each strain were used in concentration $1x10^5$ spores per 1 ml to inoculate soil substrate. Into each Petri dish 1 ml of suspension of four strains of entomopathogenic fungi was inoculated separately. Final concentration of suspension in prepared Petri dish was $5x10^3$ spores per 1 g of sterile substrate.

Also the combinations of entomopathogenic fungi were prepared. Each entomopathogenic fungus was combined in pairs with another entomopathogenic fungus. A total of 6 combinations were prepared. The combinations were: *Beauveria bassiana* and *Metarhizium anisopliae; Beauveria bassiana* and *Lecanicillium muscarium; Beauveria bassiana* and *Isaria fumosorosea; Metarhizium anisopliae* and *Isaria fumosorosea; Metarhizium anisopliae* and *Lecanicillium muscarium; Isaria fumosorosea* and *Lecanicillium muscarium.* Totally with control variants, 10 combinations were tested. For each variant, 3 repetitions were prepared. The Petri dishes were incubated at 25 °C for 50 days. Re-isolation of strain of entomopathogenic fungi were done on selective media. The results were expressed as a colony forming units (CFU) per one gram. The evaluation was done after 14 days and 50 days. These results CFU were compared to results which were obtained immediately at day 0.

3.5.1 Colony forming units (CFU)

Control variant on Day 0

Prepared suspension of each strain which were prepared for soil inoculation were used also for inoculation of media, potato dextrose agar with antibiotics (PDA+A) and selective medium based on dodine (dodine). The suspensions of control variants and also the combinations were diluted three times to the final concentration $1.00x10^2$ spores per one 1 ml. The 0.5 ml of each variant was inoculated on the surface of both media. Using spatula, the suspension was spread around all the surface to allow the suspension to suck into the media. For each variant, three replications were done. The Petri dishes were incubated in the thermostat at 25±1 °C. The colonies of each fungus were counted separately and CFU was determined. The concentration spores per 1 ml in the suspension was recounting. These results were used for comparison with the other results obtained after 14 and 50 days.

Re-isolation from the soil

14 days after incubation of fungi in the soil substrate, the all amount of substrate was transferred into the 250 ml Erlenmeyer flask and 100 ml of 0.05 % solution of Tween 80 was added. The content was mixed by hand and consequently the Erlenmeyer flasks of all variants were placed on the laboratory orbital shaker and flasks were shaken at speed 150 rpm for 30 minutes. After shaking, the elution of each variant was diluted two, three and 4 times. From each dilution, 3 Petri dishes with PDA+A and selective medium based on dodine were inoculated by 0.5 ml. The dilute elution was spread around the surface using inoculate spatula. After the suspension was suck, the media were incubated in the thermostat at 25±1 °C. The colonies of each fungus were again counted separately and CFU was determined. The results from adequate dilution were used for comparison with the results obtained in Day 0. The same procedure was prepared after 50 days of incubation.

3.6 Greenhouse experiments

This experiment took place in a aquaponics greenhouse located at the University of South Bohemia in České Budějovice. The owner of the greenhouse is the Faculty of Fisheries and Protection of Waters, USB.

Application in the greenhouses

For the plant experiment, the cucumber (Cucumis sativus) plants were used. The suspension of *Lecanicillium muscarium, Isaria fumosorosea* and *Trichoderma virens* in the concentration $1.00x10^7$ in 1 ml were used. The 1000 ml of each suspension was prepared. Plants in the control variant were treated by 0.05 % solution of Tween 80. The 2 drops of detergent were added to the suspension. The detergent serves to thoroughly moisturise the leaf surface(s) to promote the conidia dispersion of the fungus species in water. The 6 plants for each variant were spread by suspensions. The suspension was spread properly on the underside and upper side of each leaf per plant. The application was repeated every 7 days. After application, two leaves of plant of each variant were after suspension drying covered by plastic bags to keep high humidity, which is important factor for fungi growth and development.

Sample collection

After 7 days of application (before new application), leaf disks were collected. The samples were cut from the cucumber leaves by using core borer 2 cm in diameter. Each leaf disk of each variant (fungi, humidity) was placed into the sterile plastic box separately. The samples were taken to the lab for analysis.

Re-isolation of fungi from the leaves

Leaf disks were transferred into the 250 ml Erlenmeyer flask and 50 ml of 0.05 % solution of Tween 80 was added. The content was mixed by hand and consequently the Erlenmeyer flasks of all variants were placed on the laboratory orbital shaker and flasks were shaken at speed 150 rpm for 45 minutes. After shaking, the elution of each variant was diluted one time. Three Petri dishes with PDA+A and 3 plates of selective medium based on dodine were used for the entomopathogenic fungu *L. muscarium* and *I. fumosorosea*. Three Petri dishes with PDA+A and 3 plates of Trichoderma selective medium were used. From dilution and for stock elution of each variant, 0.5 ml was inoculated per plate. The elution was spread around the surface using inoculate spatula. After the suspension was suck, the media were incubated in the thermostat at 25±1 °C. The colonies of each fungus were again counted separately and CFU was determined. The colonies counting for *T. virens* variant was done after 2 (PDA+A) respectively 4 days (TSM) of incubation. The same procedure was prepared every week during 42 days. The comparison of CFU in optimal and suboptimal condition for fungi growth and development was done for first two weeks.

3.7 Statistics analysis

The differences in colony forming units was evaluated using one-way analysis of variance (ANOVA, $p<0.05$, was considered significant). CFU data were log10 (x+1) - transformed. Data are expressed as the mean ± SE. Statistical analyses were performed using StatSoft software StatSoft Inc. (2013)

4 RESULTS

The interaction of entomopathogenic fungi after their incubation in the soil substrate

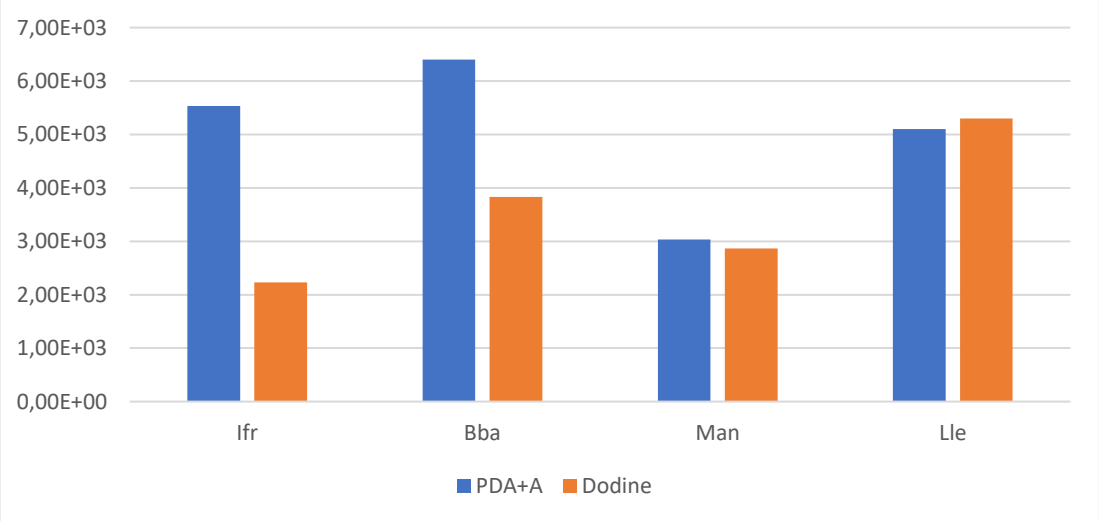
4.1 Colony-forming units from basic suspensions

- Suspensions of *B. bassiana. M. brunneum. I. fumosorosea* a *L. muscarium.* Concentration - $1x10^2$ per 1 ml
- 0.5 ml of suspension inoculated on PDA+A and dodine selective medium
- Spreading of suspension around the medium surface by spatula
- Incubation at 25±1 °C for 4 or 7 days
- CFU determination (colony forming units)

Table 4.1.1: Observing Colony-forming units (CFU) of entomopathogenic fungi from basic suspensions on different media

	PDA+A	Dodine
Ifr	$5.53x10^3$	$2.23x10^3$
Bba	$6.40x10^3$	$3.83x10^3$
Man	$3.03x10^3$	$2.87x10^3$
Lle	$5.10x10^3$	$5.30x10^3$

Graph 4.1.1: Observation of CFU of entomopathogenic fungi on a different medium



The graph shows the colony-forming units of entomopathogenic fungi after application of their basic suspension on the surface of standard medium PDA with antibiotics (PDA) with antibiotics and selective medium based on i.e. dodine. The results show

baseline data for the experiment. Where the prepared suspensions of entomopathogenic fungi were applied to a sterile substrate individually.

The evaluation on those media used indicated that entomopathogenic fungi *B. bassiana* and *I. fumosorosea* were suppressed on selective medium i.e. dodine. Compared to a standard medium PDA. While *M. brunneum* and *I. muscarium* were able to grow well on both mediums. And there was no suppression observed.

4.1.1 Interaction between *B. bassiana* in combination with another species of entomopathogenic fungi

- Suspensions of *B. bassiana* in combination in pairs with *M. brunneum*. *I. fumosorosea* a *L. muscarium. The* concentration of each fungus $1x10^5$ per 1 ml
- Inoculation of soil substrates and incubation 25±1 °C for 50 days
- Elution of soil substrate of all variants and 2.3.4 times dilution was done
- 0.5 ml of eluting from each dilution was inoculated on PDA+A and dodine selective medium
- Spreading of suspension around the medium surface by spatula
- Incubation at 25±1 °C for 4 or 7 days
- CFU determination of each fungus and recalculated per 1 g of soil

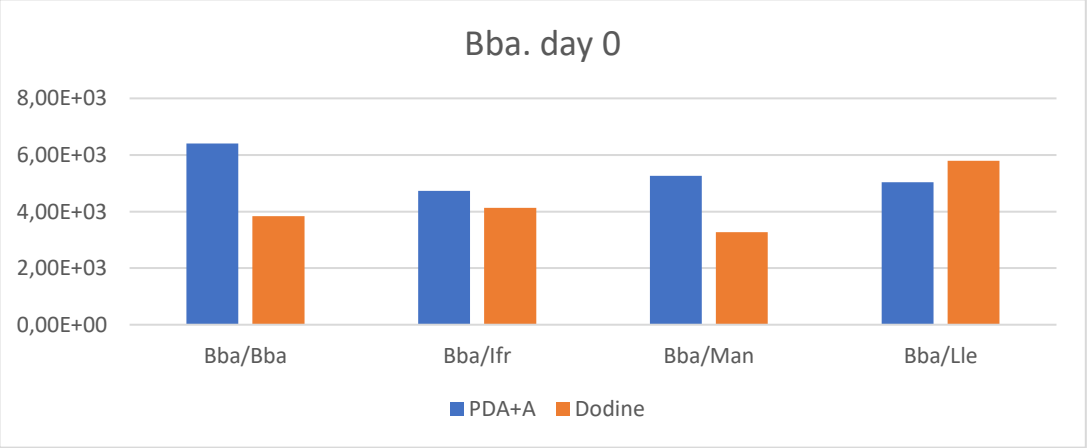
Table 4.1.1.1: Statistic analyses of the interaction between entomopathogenic fungus *B. bassiana* in combination with another species of entomopathogenic fungi

	SD	DF	SS	F value	P value
Effect	1491.990	1	1491.990	73129.99	0.000000
Variant	20.546	3	6.849	335.68	0.000000
Time	28.768	2	14.384	705.03	0.000000
Medium	8.671	1	8.671	425.02	0.000000
Variant*Time	22.821	6	3.803	186.43	0.000000
Variant*Medium	12.824	3	4.275	209.52	0.000000
Time*Medium	5.725	2	2.863	140.31	0.000000
Variant*Time*Medium	18.653	6	3.109	152.38	0.000000
Error	0.979	48	0.020		

Table 4.1.1.2: Evaluating the influence of different entomopathogenic fungi on *B. bassiana's* colony (CFU) forming units on different media

	Bba with	PDA + A		Dodine	
		Mean ± SE	Tukey HSD	Mean ± SE	Tukey HSD
Day 0	Bba/Bba	$6.40x10^3±6.08x10^2$	hi	$3.83x10^3±8.02x10^2$	i
	Bba/Ifr	$4.73x10^3±1.44x10^3$	i	$4.13x10^3±8.33x10^2$	i
	Bba/Man	$5.27x10^3±5.13x10^2$	hi	$3.27x10^3±8.02x10^2$	i
	Bba/Lle	$5.03x10^3±4.93x10^2$	i	$5.80x10^3±7.55x10^2$	hi
Day 14	Bba/Bba	$4.60x10^5±0.00$	abcd	$1.37x10^5±1.44x10^5$	g
	Bba/Ifr	$1.93x10^5±1.15x10^4$	defg	0.00±0.00	j
	Bba/Man	$9.47x10^5±9.61x10^4$	ab	$4.07x10^5±1.07x10^5$	bcde
	Bba/Lle	$3.30x10^5±5.57x10^4$	cdef	$1.17x10^6±2.32x10^5$	a
Day 50	Bba/Bba	$3.63x10^5±4.16x10^4$	bcde	$1.67x10^5±6.81x10^4$	efg
	Bba/Ifr	$1.17x10^5±1.53x10^4$	g	$1.67x10^4±1.15x10^4$	h
	Bba/Man	$5.80x10^5±3.61x10^4$	abc	$9.67x10^4±3.79x10^4$	g
	Bba/Lle	$1.20x10^5±1.00x10^4$	fg	$1.20x10^5±1.00x10^4$	fg

Graph 4.1.1.1: shows the evaluation of colony density of *B. bassiana* in control. and when combined with other different entomopathogenic fungi on Day 0

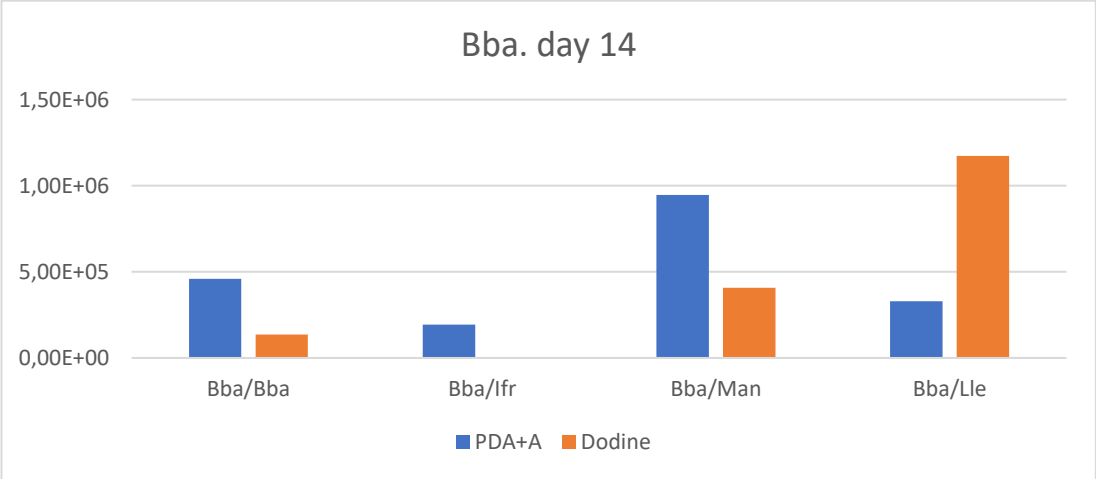


On Day 0 we can observe that the CFU for *B. bassiana* is good on both media. Especially the CFU on standard medium PDA was high in every experiment on every Petri dish. Except when *B. bassiana* was combined with *L. muscarium.* In this variant, the CFU of *B. bassiana* was high on the selective medium i.e., dodine compared to when combining with other entomopathogenic fungi.

This observation was evaluated immediately after applying suspensions on selective medium i.e., dodine and standard medium PDA. So, we cannot see much difference here except that the results were used as a baseline to the possible outcomes after 14 and 50 days of incubation, respectively.

After 14 days of incubation of both fungi in the soil substrate. It can be observed that the CFU for *B. bassiana* forms more colonies when combined with all entomopathogenic fungi on standard medium PDA. But in combination with *Isaria fumosorosea.* The species *B. bassiana* did not produce a high level of CFU because the spore germination was suppressed by *Isaria fumosorosea.* While on selective media i.e., dodine. Spores of *B. bassiana* did not germinate when combined with *Isaria fumosorosea.*

Graph 4.1.1.2: Evaluating the influence of different entomopathogenic fungi on *B. bassiana's* colony (CFU) forming units on different media after 14 days

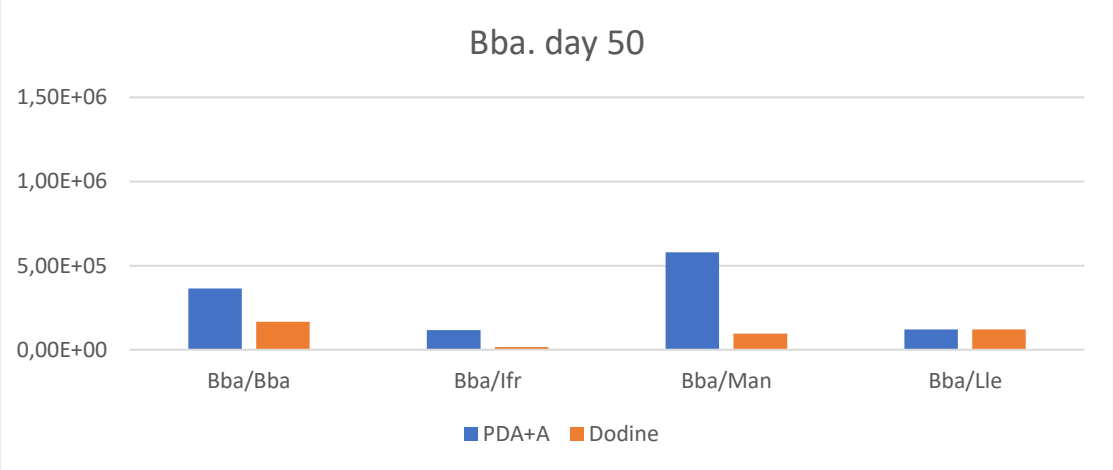


During the 14 days of incubation. *B. bassiana* produced new generations of spores after several developmental cycles when combined with all the fungi including control. In combination with *M. brunneum* and *L. muscarium*, the highest amount of spore were recorded. During the next incubation time. spores in the soil substrate were reduced. a

change was observed after 50 days of incubation. when the fungi were again re-isolated. The less CFU was detected in all combinations. The fungus *B. bassiana* spores were either degraded or the spores were not viable compared to the results attained after 14 days especially in the combination with *M. brunneum* and *L. muscarium*. The degradation of *B. bassiana* was also observed in combination with *Isaria fumosorosea*. but this degradation was not so intensive. The major difference was that *B. bassiana* produce colonies on selective media i.e. dodine in comparison to 14 days where colonies were not observed. However. the number of spores per 1 g of soil substrate after 50 days was 1.67 x 10^4.

From these results with data presented above. it was determined that the colony-forming units of *B. bassiana* are much higher on standard medium PDA when combined with *M. brunneum*. On the other hand. on selective medium i.e. dodine *B. bassiana* produced more CFU in combination with *L. muscarium*. The interaction in time between *B. bassiana* and each variant on different media is statistically significant (F=152.38; df=6,48; p=0.0000).

Graph 4.1.1.3: evaluating the influence of different entomopathogenic fungi (Colony forming units) on *B. bassiana's* colony (CFU) forming units on different media after 50 days.



4.1.2 Interaction between *M. brunneum* in combination with another species of entomopathogenic fungi

- Suspensions of *M. brunneum* in combination in pairs with *B. bassiana*. *I. fumosorosea* a *L. muscarium*. *the* concentration of each fungus 1x10^5 per 1 ml

- Inoculation of soil substrates and incubation 25±1 °C for 50 days
- Elution of soil substrate of all variants and 2.3.4 times dilution was done
- 0.5 ml of eluting from each dilution was inoculated on PDA+A and dodine selective medium
- Spreading of suspension around the medium surface by spatula
- Incubation at 25±1 °C for 4 or 7 days
- CFU determination of each fungus and recalculated per 1 g of soil

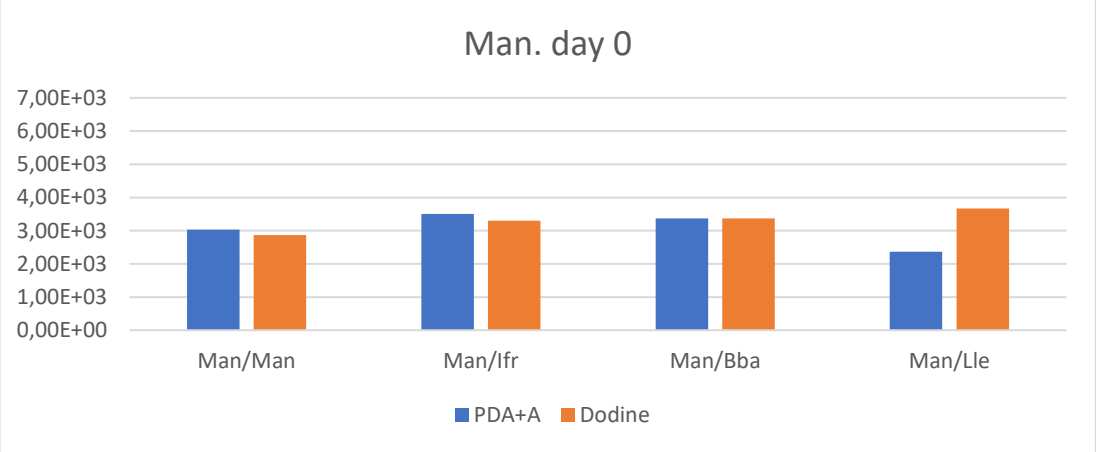
Table 4.1.2.1: Evaluating the influence of different entomopathogenic fungi (Colony forming units) on *M. brunneum* colony (CFU) forming units on different media

	Man with	PDA + A		Dodine	
		Mean ± SE	Tukey HSD	Mean ± SE	Tukey HSD
Day 0	Man/Man	$3.03x10^3±6.43x10^2$	j	$2.87x10^3±5.69x10^2$	j
	Man/Ifr	$3.50x10^3±6.00x10^2$	j	$3.30x10^3±6.08x10^2$	j
	Man/Bba	$3.37x10^3±7.23x10^2$	j	$3.37x10^3±2.31x10^2$	j
	Man/Lle	$2.37x10^3±1.53x10^2$	j	$3.67x10^3±1.15x10^2$	j
Day 14	Man/Man	$4.50x10^5±3.61x10^4$	abc	$5.30x10^5±3.61x10^4$	ab
	Man/Ifr	$3.67x10^4±2.31x10^4$	hi	$5.67x10^4±2.08x10^4$	gh
	Man/Bba	$1.43x10^5±4.62x10^4$	def	$2.00x10^5±1.73x10^4$	cdef
	Man/Lle	$2.00x10^4±1.00x10^4$	i	$4.00x10^4±1.00x10^4$	ghi
Day 50	Man/Man	$1.01x10^6±6.03x10^4$	a	$8.53x10^5±7.51x10^4$	a
	Man/Ifr	$3.67x10^4±5.77x10^3$	ghi	$9.00x10^4±1.00\ x10^4$	efg
	Man/Bba	$2.60x10^5±7.94x10^4$	bcd	$2.23x10^5±2.52x10^4$	bcde
	Man/Lle	$2.67x10^4±5.77x10^3$	hi	$9.00x10^4±2.65x10^4$	fg

Table 4.1.2.2: Statistic analyses of the interaction between entomopathogenic fungus *M. brunneum* in combination with another species of entomopathogenic fungi

	SD	DF	SS	F value	P value
Effect	1495.756	1	1495.756	93883.72	0.000000
Variant	8.238	3	2.746	172.37	0.000000
Time	41.144	2	20.572	1291.22	0.000000
Medium	0.390	1	0.390	24.45	0.000010
Variant*Time	4.365	6	0.727	45.66	0.000000
Variant*Medium	0.365	3	0.122	7.63	0.000285
Time*Medium	0.110	2	0.055	3.45	0.039794
Variant*Time*Medium	0.165	6	0.028	1.73	0.135207
Error	0.765	48	0.016		

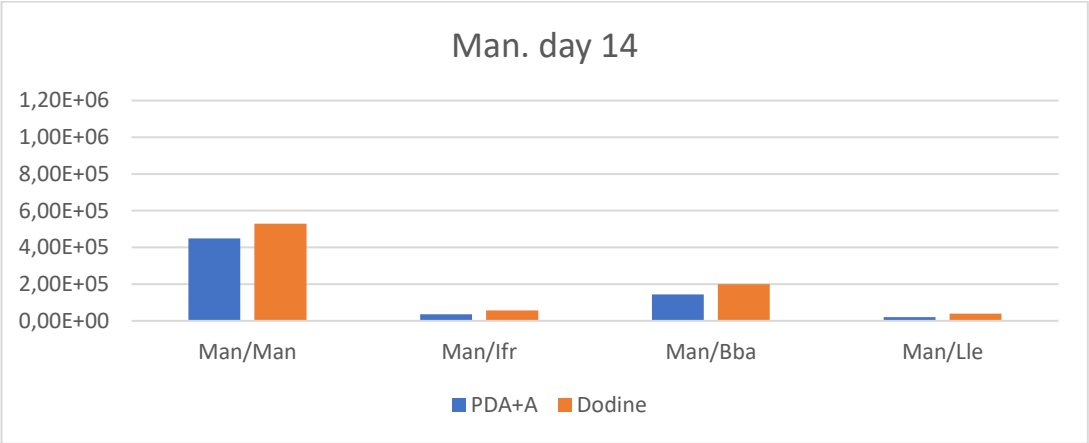
Graph4.1.2.1: Shows the evaluation of colony density of *Metarhizium brunneum* in control. and when combined with other different entomopathogenic fungi on day 0



On Day 0 we can observe that the colony-forming unit for *M. brunneum* was good on both media used and the number were of spores per 1 g of soil substrate was almost similar. A much difference was observed when *M. brunneum* was combined with *L. muscarium* on selective media i.e., dodine. The colony-forming units increased because selective media is suitable for *M. brunneum* growing.

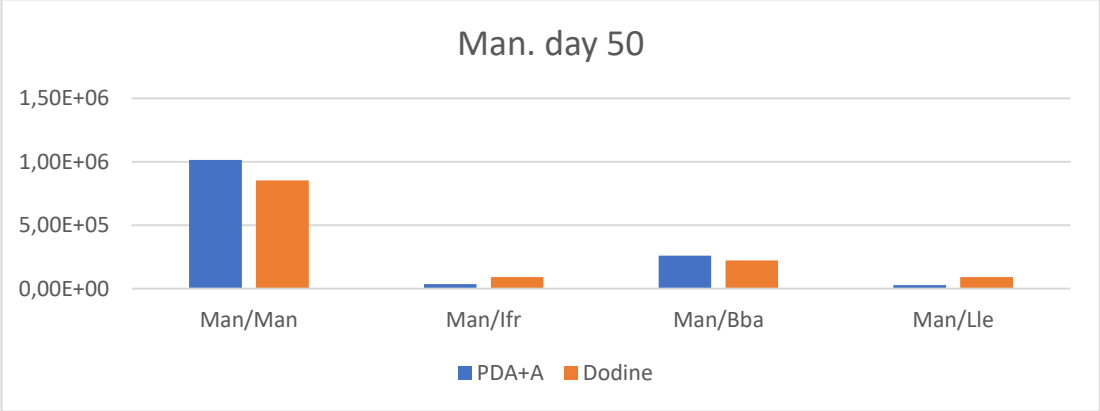
This observation was done immediately after applying suspensions on selective medium i.e., dodine and standard medium PDA. So, we cannot see any differences here except that results were used as a baseline to possible outcomes after 14 and 50 days of incubation, respectively.

Graph 4.1.2.2: evaluating the influence of different entomopathogenic fungi (Colony forming units) on *M. brunneum's* colony (CFU) forming units on different media after 14 days.



The results obtained on Day 14. the spores of *M. brunneum* germinated from all soil substrates. the results exhibited above show that the CFU of *M. brunneum* was less in combinations with other fungi compared to control. A significant difference was observed in the combination of *M. brunneum* and *B. bassiana* on selective media i.e. dodine. *M. brunneum* had a higher number of colony-forming units because the selective media i.e. dodine suppresses *B. bassiana* which slows the rate of spore germination while giving an advantage to *M. brunneum* which was not under suppression.

Graph 4.1.2.3: evaluating the influence of different entomopathogenic fungi (Colony forming units) on *M. brunneum's* colony (CFU) forming units on different media after 50 days



From the graphs of 14 and 50 days is evident. that *M. brunneum* is still developed in the control variant. The results from the fungi combination are similar for

both days. There is no degradation of decreasing in spore viability of *M. brunneum*. The differences are just observed variant when *M. brunneum* is combined with *B. bassiana*, where the expression of colonies is changed. After 14 days *M. brunneum* produced more CFU on selective medium i.e., dodine on the other hand after 50 days higher CFU was observed on PDA. The interaction in time between *M. brunneum* and each variant on different media is not statistically significant (F=1.73; df=6,48; p=0.1352).

4.1.3 Interaction between *I. fumosorosea* in combination with another species of entomopathogenic fungi

- Suspensions of *I. fumosorosea* in combination in pairs with *B. bassiana*. *M. brunneum* a *L. muscarium. the* concentration of each fungus $1x10^5$ per 1 ml
- Inoculation of soil substrates and incubation 25±1 °C for 50 days
- Elution of soil substrate of all variants and 2.3.4 times dilution was done
- 0.5 ml of eluting from each dilution was inoculated on PDA+A and dodine selective medium
- Spreading of suspension around the medium surface by spatula
- Incubation at 25±1 °C for 4 or 7 days
- CFU determination of each fungus and recalculated per 1 g of soil

Table 4.1.3.1: Statistic analyses of the interaction between entomopathogenic fungus *Isaria fumosorosea* in combination with another species of entomopathogenic fungi

	SD	DF	SS	F value	P value
Effect	2115.329	1	2115.329	678342.0	0.000000
Variant	2.509	3	0.836	268.2	0.000000
Time	122.605	2	61.302	19658.4	0.000000
Medium	0.378	1	0.378	121.2	0.000000
Variant*Time	1.842	6	0.307	98.4	0.000000
Variant*Medium	0.161	3	0.054	17.2	0.000000
Time*Medium	0.099	2	0.050	15.9	0.000005
Variant*Time*Medium	0.203	6	0.034	10.9	0.000000
Error	0.150	48	0.003		

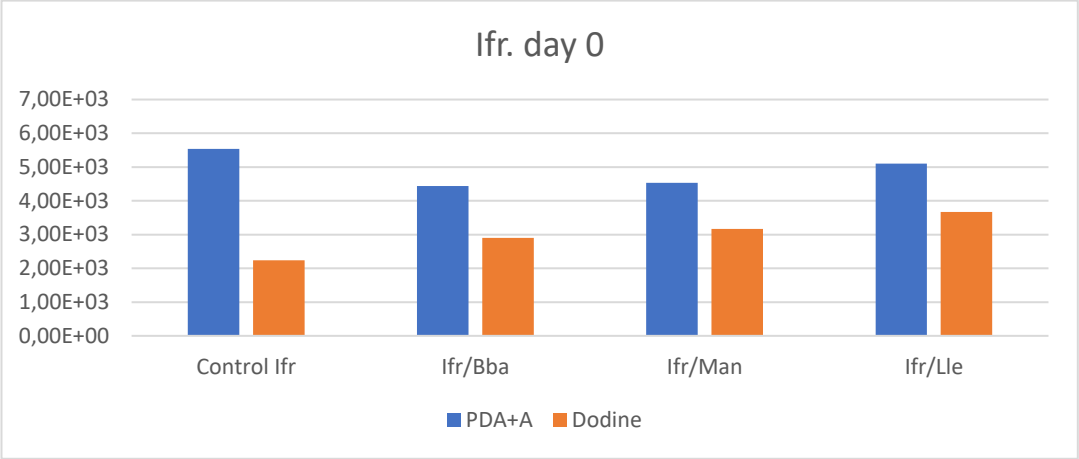
Table4.1.3.2: Evaluating the influence of different entomopathogenic fungi (Colony forming units) on *B. bassiana's* colony (CFU) forming units on different media

Day	Ifr with	PDA + A		Dodine	
		Mean ± SE	Tukey HSD	Mean ± SE	Tukey HSD
Day 0	Ifr/Ifr	$5.53x10^3±6.11x10^2$	i	$2.23x10^3±5.77x10^2$	m
	Ifr/Bba	$4.43x10^3±2.52x10^2$	ijk	$2.90x10^3±9.17x10^2$	lm
	Ifr/Man	$4.53x10^3±3.21x10^2$	ijk	$3.17x10^3±4.04x10^2$	klm
	Ifr/Lle	$5.10x10^3±4.58x10^2$	ij	$3.67x10^3±1.15x10^2$	jkl
Day 14	Ifr/Ifr	$3.93x10^6±1.89x10^5$	cd	$1.94x10^6±7.94x10^4$	ef
	Ifr/Bba	$6.19x10^6±4.98x10^5$	a	$5.99x10^6±8.20x10^5$	ab
	Ifr/Man	$1.66x10^6±1.54x10^5$	ef	$1.96x10^6±1.27x10^5$	ef
	Ifr/Lle	$1.38x10^6±1.87x10^5$	fg	$5.43x10^5±4.16x10^4$	h
Day 50	Ifr/Ifr	$3.34x10^6±1.29x10^5$	d	$3.36x10^6±5.79x10^5$	d
	Ifr/Bba	$4.09x10^6±7.83x10^5$	bcd	$5.10x10^6±2.16x10^5$	abc
	Ifr/Man	$2.06x10^6±3.61x10^4$	e	$1.62x10^6±5.86x10^4$	ef
	Ifr/Lle	$9.60x10^5±1.57x10^5$	g	$6.30x10^5±5.20x10^4$	h

Table 4.1.3.3: Statistic analyses of the interaction between entomopathogenic fungus *Isaria fumosorosea* in combination with another species of entomopathogenic fungi

	SD	DF	SS	F value	P value
Effect	2115.329	1	2115.329	678342.0	0.000000
Variant	2.509	3	0.836	268.2	0.000000
Time	122.605	2	61.302	19658.4	0.000000
Medium	0.378	1	0.378	121.2	0.000000
Variant*Time	1.842	6	0.307	98.4	0.000000
Variant*Medium	0.161	3	0.054	17.2	0.000000
Time*Medium	0.099	2	0.050	15.9	0.000005
Variant*Time*Medium	0.203	6	0.034	10.9	0.000000
Error	0.150	48	0.003		

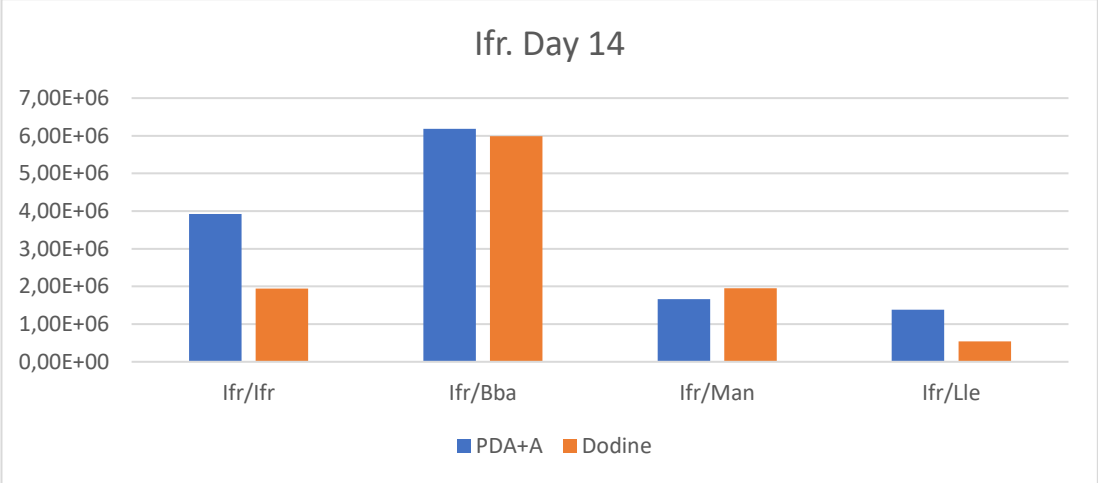
Graph 4.1.3.1: Shows the evaluation of colony density of *I. fumosorosea* control. and when combined with other different entomopathogenic fungi on day 0.



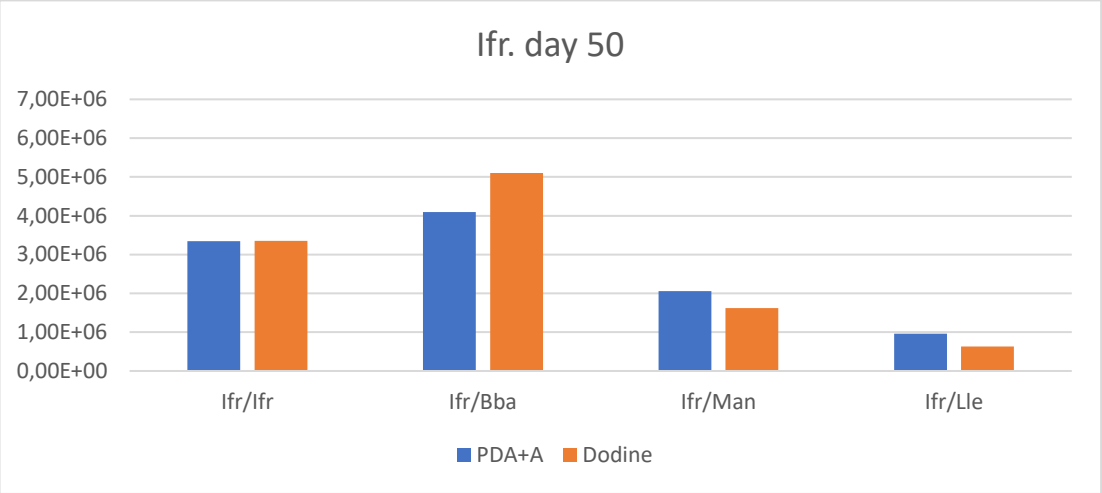
On Day 0 it can be observed that the colony-forming unit for *I. fumosorosea* was well developed on both media. Especially colony-forming units on standard medium PDA with antibiotics increased compared to those on a selective media i.e., dodine in every petri dish.

This observation was evaluated immediately after applying suspensions on selective medium i.e., dodine and standard medium PDA with antibiotics. So, we cannot see any differences here except that we will use these results as a baseline to the possible outcomes after 14 and 50 days of incubation.

Graph 4.1.3.2: evaluating the influence of different entomopathogenic fungi (Colony forming units) on *Isaria fumosorosea* colony (CFU) forming units on different media after 14 days.



Graph 4.1.3.3: evaluating the influence of different entomopathogenic fungi (Colony forming units) on *Isaria fumosorosea's* colony (CFU) forming units on different media after 50 days



After 14 days of incubation. it can be observed that *I. fumosorosea* was well growing on both media. However. *I. fumosorosea* produce a higher number of colony-forming units when combined with *B. bassiana* compared to all variants including control.

In comparison to control, the combination with *L. muscarium* and *M. brunneum* these entomopathogenic fungi have a negative effect on the life cycle development of *I. fumosoroseus*. In combination with *L. muscarium*. *I. fumosoroseus* did not produce a high number of colonies on selective medium i.e., dodine.

But after 50 days, *I. fumosorosea* was not able to maintain the germination rate which was been observed after 14 days. Thereafter not producing new generations of spores. The spores of *I. fumosorosea* were degrading or they lost their viability. However, the CFU of *I. fumosorosea* is still the highest in combination with *B. bassiana*. The results observed, indicate that *Isaria fumosorosea* grows well on both mediums. The interaction in time between *I. fumosorosea* and each variant on different media is statistically significant (F=10.9; df=6,48; p=0.0000).

4.1.4 Interaction between *L. muscarium* in combination with another species of entomopathogenic fungi

- Suspensions of *L. muscarium* in combination *B. bassiana*. *M. brunneum* a *I. Fumosorosea in pairs. the* concentration of each fungus was $1x10^5$ per 1 ml
- Inoculation of soil substrates and incubation 25±1 °C for 50 days
- Elution of soil substrate of all variants and 2.3.4 times dilution was done
- 0.5 ml of eluting from each dilution was inoculated on PDA+A and dodine selective medium
- Spreading of suspension around the medium surface by spatula
- Incubation at 25±1 °C for 4 or 7 days
- CFU determination of each fungus and recalculated per 1 g of soil

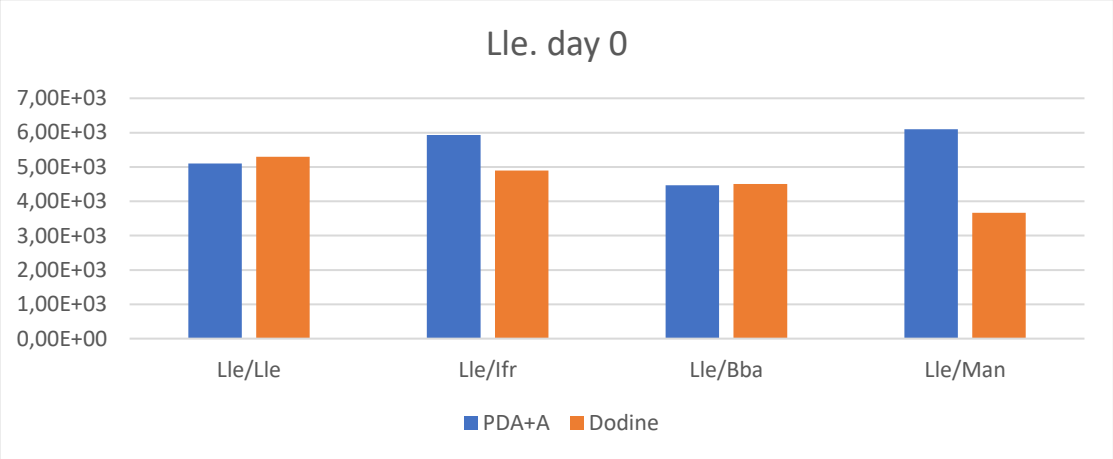
Table 4.1.4.1: Evaluating the influence of different entomopathogenic fungi (Colony forming units) on *L. muscarium's* colony (CFU) forming units on different media

Day	Lmu with	PDA + A		Dodine	
		Mean ± SE	Tukey HSD	Mean ± SE	Tukey HSD
Day 0	Lle/Lle	$5.10x10^3±5.29x10^2$	j	$5.30x10^3±3.00x10^2$	j
	Lle/Ifr	$5.93x10^3±6.81x10^2$	j	$4.90x10^3±6.56x10^2$	jk
	Lle/Bba	$4.47x10^3±2.52x10^2$	jk	$4.50x10^3±2.65x10^2$	jk
	Lle/Man	$6.10x10^3±7.55x10^2$	j	$3.67x10^3±1.05x10^3$	k
Day 14	Lle/Lle	$2.94x10^6±1.91x10^5$	bc	$2.15x10^6±9.07x10^4$	cde
	Lle/Ifr	$1.09x10^6±1.05x10^5$	hi	$9.77x10^5±9.61x10^4$	i
	Lle/Bba	$1.86x10^6±2.51x10^5$	def	$1.53x10^6±8.72x10^4$	efgh
	Lle/Man	$1.50x10^6±6.00x10^4$	fgh	$1.25x10^6±1.44x10^5$	ghi
Day 50	Lle/Lle	$4.72x10^6±1.93x10^5$	a	$2.89x10^6±2.75x10^5$	bc
	Lle/Ifr	$2.58x10^6±2.72x10^5$	bcd	$1.18x10^6±1.40x10^5$	ghi
	Lle/Bba	$2.59x10^6±2.15x10^5$	bcd	$1.45x10^6±2.25x10^5$	fgh
	Lle/Man	$3.49x10^6±1.96x10^5$	ab	$1.65x10^6±2.20x10^5$	efg

Table 4.1.4.2: Statistic analyses of the interaction between entomopathogenic fungus *L. muscarium* in combination with another species of entomopathogenic fungi

	SD	DF	SS	F value	P value
Effect	2113.078	1	2113.078	891663.4	0.000000
Variant	0.538	3	0.179	75.7	0.000000
Time	107.684	2	53.842	22719.9	0.000000
Medium	0.396	1	0.396	167.0	0.000000
Variant*Time	0.339	6	0.056	23.8	0.000000
Variant*Medium	0.031	3	0.010	4.4	0.008098
Time*Medium	0.166	2	0.083	35.0	0.000000
Variant*Time*Medium	0.049	6	0.008	3.5	0.006291
Error	0.114	48	0.002		

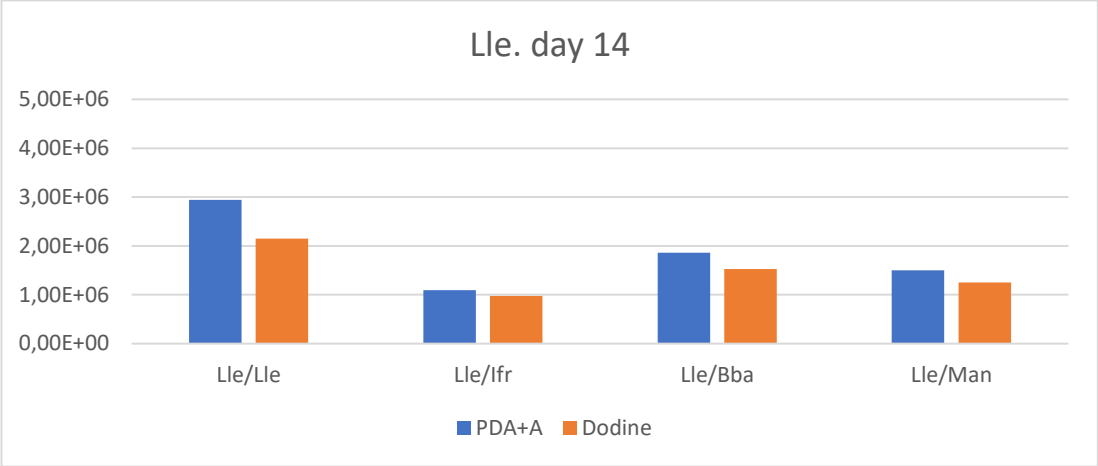
Graph 4.1.4.1: This shows the evaluation of colony density of *L. muscarium* in control, and when combined with other different entomopathogenic fungi that were chosen.



On day 0 we can observe that the colony-forming unit for *L. muscarium* was good on every petri dish and both media used. But on standard medium PDA with antibiotics, it was better when combined with *I. fumosorosea* and *M. brunneum.*

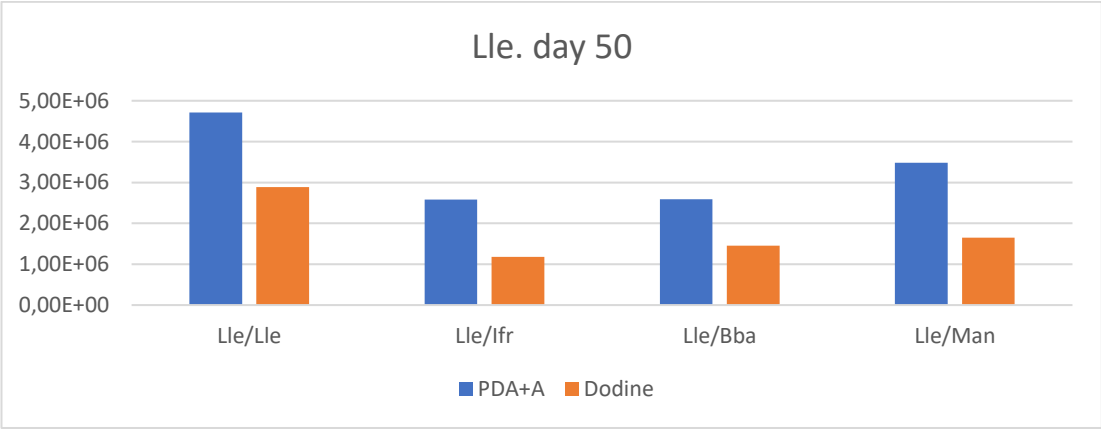
This observation was done immediately after applying suspensions on selective medium i.e., dodine and standard medium PDA with antibiotics. So, we cannot see any differences here except that we will use these results as a baseline to possible outcomes after 14 and 50 days of incubation, respectively.

Graph 4.1.4.2: evaluating the influence of different entomopathogenic fungi (Colony forming units) on *L. muscarium's* colony (CFU) forming units on different media after 14 days.



After 14 days of incubation, we can observe that the colony-forming unit for *L. muscarium* was good with a lot of colonies developing when combined with all entomopathogenic fungi on both media (standard medium PDA with antibiotics and selective media i.e., Dodine). After 14 days we cannot say much of a difference here, except when combined with *Isaria fumosorosea,* it is in some way a bit suppressed. but they can coexist in the soil.

Graph 4.1.4.3: evaluating the influence of different entomopathogenic fungi (Colony forming units) on *L. muscarium's* colony (CFU) forming units on different media after 50 days



After 50 days of incubation, the only difference observed is that *L. muscarium* produces new generations with time and this is not dependent on any other fungi. Combined or not with another fungus after 50 days it does not matter. The best germination

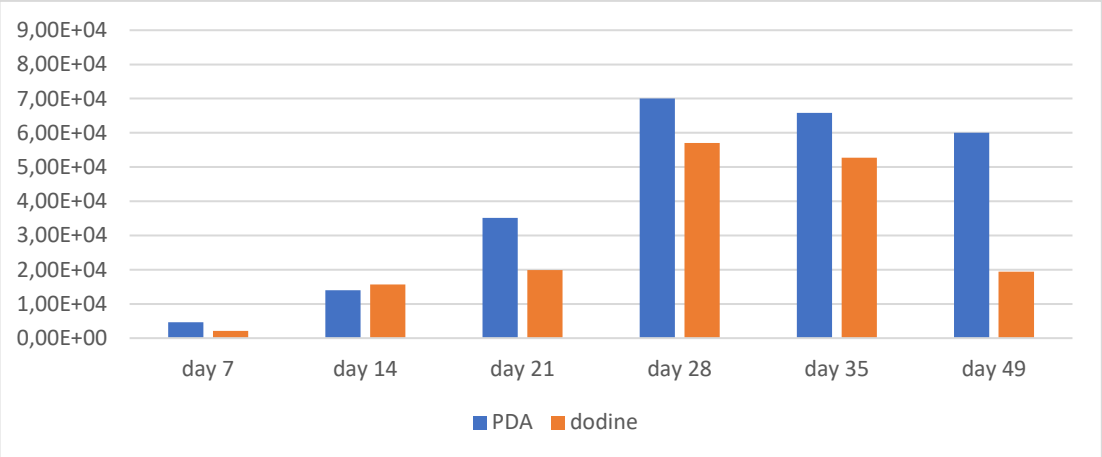
is on PDA with antibiotics, but the colony-forming units are good also on selective media i.e., dodine. The interaction in time between *L. muscarium* and each variant on different media is statistically significant (F=3.5; df=6,48; p=0.0063).

4.2 Preventive application of entomopathogenic and mycoparasitic fungi on the cucumber plants in the greenhouse

- Suspensions of *L. muscarium, I. fumosorosea* a *T. virens*, concentration - $1x10^7$ per 1 ml
- Cucumber plants, variety *Tolstoj F1* and *Verdon F1*
- Application every 7 days during the period of 42 days
- Sample of leaf disks was collected after every 7 days before the next application
- The fungi for the leaf disks were washed using Tween solution
- Entomopathogenic fungi were incubated on a selective medium i.e. dodine or PDA with antibiotic
- The mycoparasitic fungus was incubated on *Trichoderma* selective medium (TSM) and PDA with antibiotics
- Incubation at 25±1 °C for 3, 7 or 10 days depends on fungus and medium

4.2.1 Evaluation of spore presence of *Lecanicillium muscarium* on the leaf disk after preventive application on cucumber plants

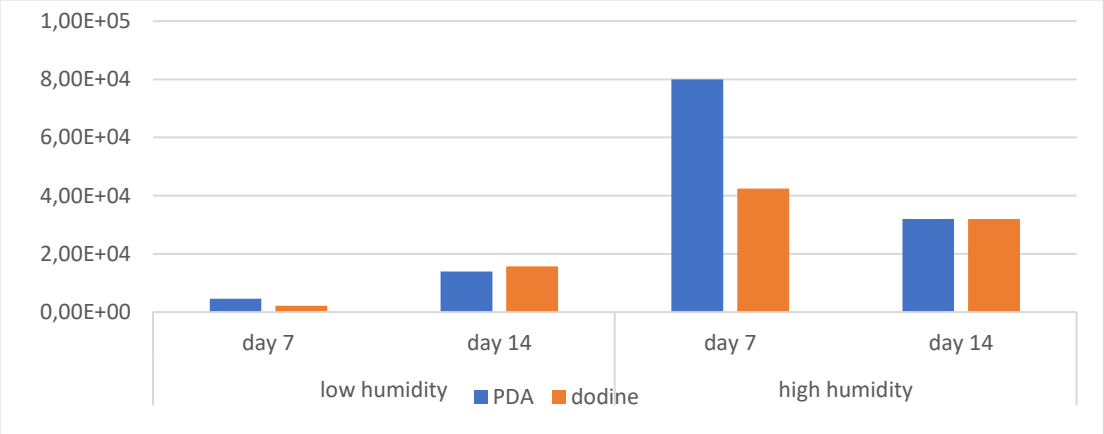
Graph 4.2.1.1: Evaluation of CFU of *L. muscarium* isolated from cucumber leaves



The results show, that both media are suitable for re-isolation of entomopathogenic fungus *L. muscarium* from leaf disks. The graph above indicates the number of colonies realized after a given period. Fungi were applied after analysis every 7-days. Hence, the number of spores isolated from a leaf disk on a particular day of observation increased. *L. muscarium* gradually grew as seen in the graph above. After the first 14-

days, the number of colonies is so much lower than the results on the 28 and 35th-day, the reason could be the number of spores accumulated after several applications of suspensions of *L. muscarium.* The results after a 42day show an extreme decrease in spore production, this was as a result of the application of pesticide 7 days before isolation.

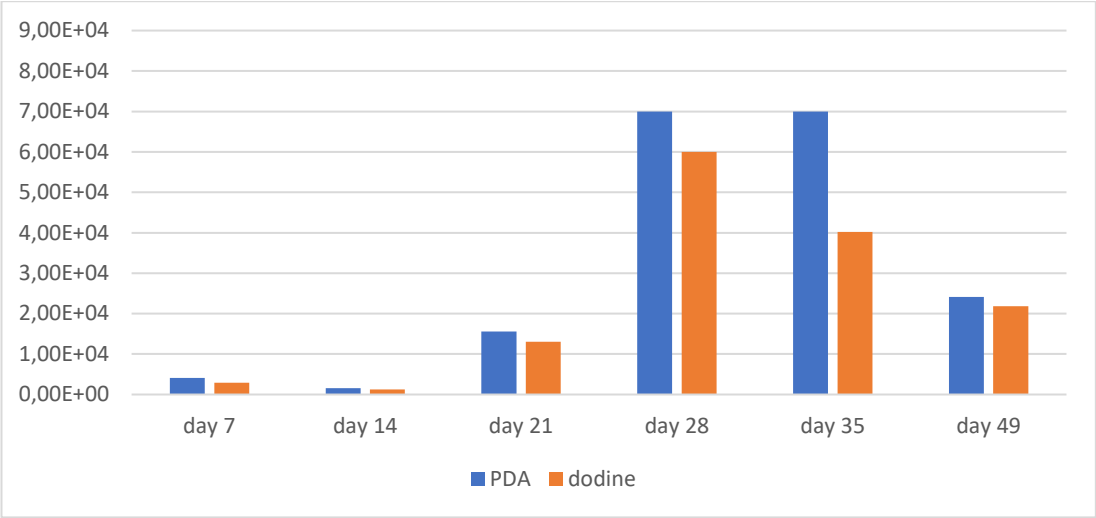
Graph 4.2.1.2: The effects of optimal and suboptimal conditions on the CFU of *L. muscarium* from the cucumber leaves



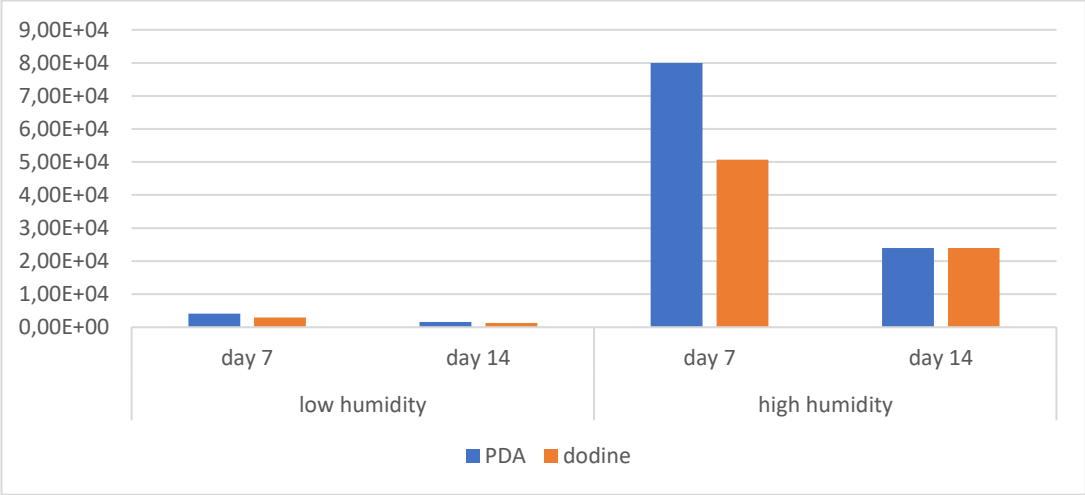
The graph shows that the fungus *L. muscarium* survives and well developed in optimal conditions when the cucumber leaves were after application covered by plastic bags. From these leaves, more spores were isolated compare to plants that were planted in greenhouse conditions, where the humidity is not so high. This is confirmed in particular by the result obtained after 7 days of incubation. After 14 days, the CFU decreased in the conditions where the high RH was ensured. This could be due to the effect that the combination of extreme temperature and very high humidity because of plastic bag can have a negative effect on the surviving of spores.

4.2.2 Evaluation of spore presence of *Isaria fumosorosea* on the leaf disk after preventive application on cucumber plants

Graph 4.2.2.1: Re-isolation of *I. fumosorosea* from the leaves of cucumbers after every 7 days of application of suspension during the period of 42 days in suboptimal conditions



Graph 4.2.2.2: Comparison of re-isolation of fungus *I. fumosorosea* from the leaf disks kept in optimal and suboptimal conditions



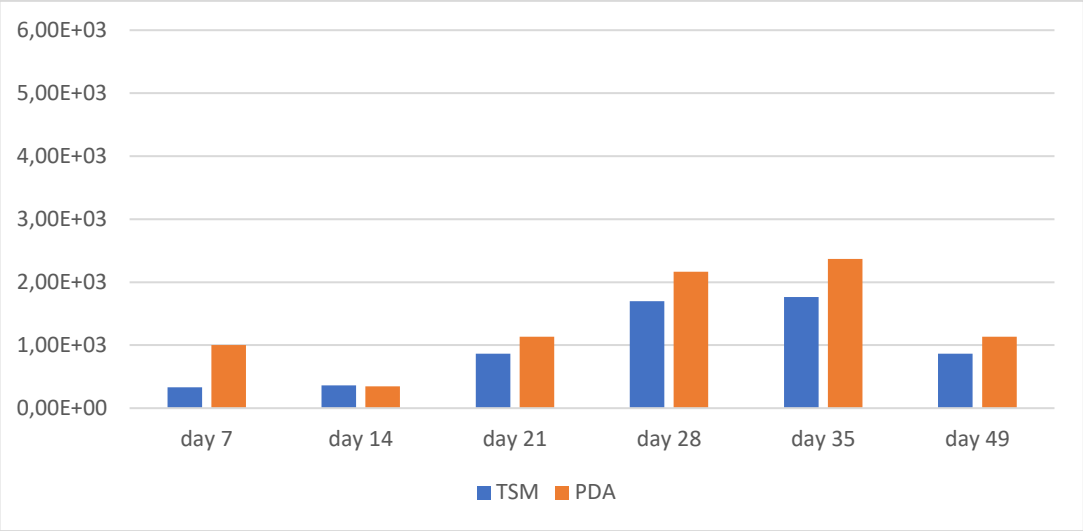
Almost the same data with *L. muscarium* were obtained when the fungus *I. fumorosorosea* was applied to the cucumber seedling in the greenhouses.

The data presented above express the results obtained after 7 and 14 days. The leaf disks collected from low humidity exposures indicated High CFU compared to the results obtained from application in high humidity. In this case, the analysis above was

understood that *L. Muscarium* grows well in high humidity. After 7days (high moisture), the results observed show the highest CFU of *L. muscarium* obtained from all the experiments while a decrease was observed on day 14. This occurrence is probably because of the high temperature from the surroundings, the plastic bags possibly had effects on spore production.

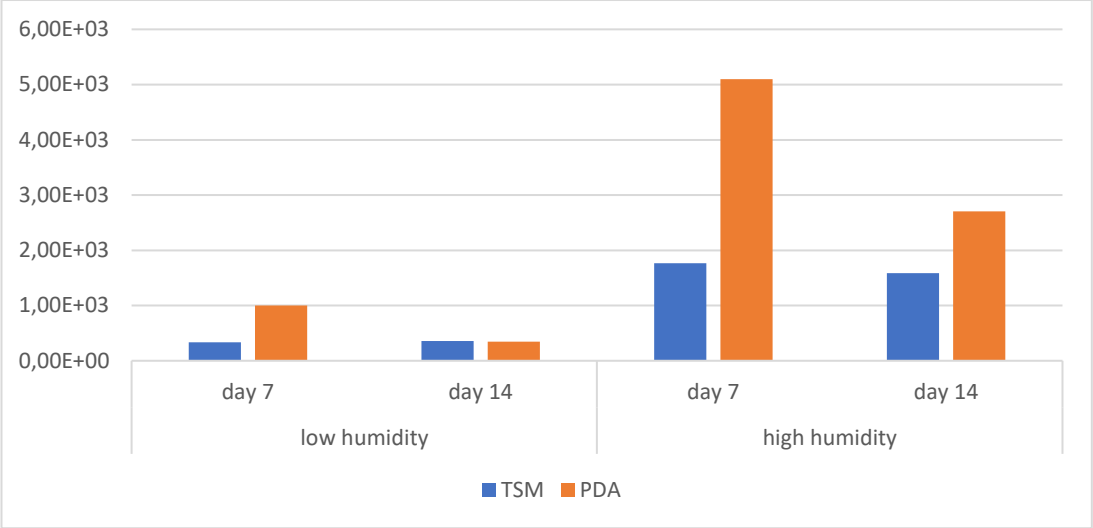
4.2.3 Evaluation of spore presence of *Trichoderma virens* on the leaf disk after preventive application on cucumber plants.

Graph 4.2.3.1: Re-isolation of *T. virens* from the leaves of cucumbers after every 7 days of application of suspension during the period of 42 days kept in suboptimal conditions



The graph above indicates the number of colonies realized after a given period. Fungi were applied after analysis every 7-days. Hence, the number of spores obtained from a leaf disk on a particular day of observation increased. *L. muscarium* gradually grew as seen in the graph above. After the first 7-days, the number of colonies is so much lower than the results on the 35th-day. The results after a 42day show an extreme decrease in spore production, this was as a result of the application of pesticide 7 days before isolation.

Graph 4.2.3.2: Comparison of re-isolation of fungus L. muscarium from the leaf disks kept in optimal and suboptimal conditions



The data presented above express the results obtained after 7 and 14 days. The leaf disks collected from low humidity exposures indicated High CFU compared to the results obtained from application in high humidity. In this case, the analysis above was understood that *L. Muscarium* grows well in high humidity. After 7days (high moisture), the results observed show the highest CFU of *L. Muscarium* obtained from all the experiments while a decrease was observed on day 14. This occurrence is probably because of the high temperature from the surroundings, the plastic bags possibly had effects on spore production.

5 DISCUSSION

Biological control with natural antagonists relies on several mechanisms, substrate, and space competition, including mycoparasitism, antibiosis, enzymatic activity, and induced resistance (Kant et al., 2011). Entomopathogenic fungi have considerable potential in controlling populations effectively of pest organisms. Nevertheless, their application can easily be impacted by various abiotic factors (temperature, solar radiation, humidity) and biotic factors (pathogen, host, host plant) (Inglis et al. 2001). There are approximately 750 naturally existing species of entomopathogenic fungi that can potentially infect insects or mites, and a wide range of fungi can be found in the insects or mites, and dozens of species of mycoparasitic fungi that can parasitize pathogens of plant diseases. The purpose of insecticides is to rapidly suppress populations of pests but resulting in contamination of the environment and progressive resistance of these insect pests (Quintela et al., 1998). Like other organisms, insects are susceptible to a variety of diseases caused by entomopathogens such as viruses, bacteria, fungi, microsporidia, or nematodes. These entomopathogenic microorganisms are used in biological control insect pests and are constantly under intensive research. Following studies, they have been different strategies for the use of beneficial microorganisms against pests under development (Ramanujam et al., 2014). For instance, studies have been initiated where the combinations of entomopathogenic fungi together with a deadly dose of chemical insecticides. This was thought to increase the efficacy and rate of control of pest populations. The combination of fungal pathogens and selected insecticides have shown increased efficacy, which could lead to a reduction in insecticide doses than when used alone. Thus, the use of a combination of fungi and insecticides could lead to preserving more abundant populations of natural enemies, minimizing pollution of the environment, and reducing the possibility of resistance to the pest (Boman, 1981, Khun et al., 2020, Litwin et al., 2020).

In biological control, there are many products based on entomopathogenic fungi, respectively. However, there are not much-known knowledge of their interaction with each other. Books have been published on different strategies on how to successively use beneficial microorganisms against pests (Usta, 2013). For example, combining entomopathogens and mycoparasite using assayon pre-colonised plates as a host range as described in detail by (Krauss et al., 1998). There have been experiments to access

the effects of combined fungal isolates and *H. bacteriophora* against larvae of RPW which was done by (Krauss et al., 2004). The occurrence of entomopathogenic fungi in the soils has been studied by different scientists in different countries. In biological control, there are many products based on entomopathogenic respectively (Faria et al., 2007). Various species of hypocrealean (*Ascomycota*) fungi live most of their life cycle in the soil. Amongst them is *Beauveria* spp., *Metarhizium* spp. *Isaria* spp. And *Lecanicillium* spp. (Zimmermann, 2007, Zimmermann, 2008, Vänninen, 1995).

The major aim of this work was to evaluate different species of entomopathogenic fungi in combinations with other entomopathogenic fungi in the soil. The species *Beauveria bassiana, Metarhizium anisopliae, Lecanicillium muscarium* and *Isaria fumosorosea* were used in the experiment. During the experiments, all the combinations of entomopathogenic fungi were used to evaluate the growth and spore production of the assessed species in interaction with each other, at temperatures of 25 °C. All these species are among the most important fungal pathogens based on which bioproducts against pests or plant pathogens (Faria et al., 2007). In 2000, sales of bioproducts, which included micro-organisms and pheromones, amounted to 97 million dollars. The sales of bioproducts include micro-organisms, macro-organisms, and pheromones. The sales figures for 2005 were $135 million and the annual sales growth was estimated at 11.7 % (Ravensberg, 2010). Lisansky (1997) even claimed that high for example sales potential increased steadily. The biopesticides market grew 24% from 2014 to 2016 globally to over $1.8 billion and PPT reported projected to reach USD 6.60 Billion by 2022 (*Anonymous 3*) of biopesticides market. One of the challenges is that they also have weaknesses that can be turned into strengths. An example is the narrower host spectrums that provide safe use. These features make it possible to be combined with natural enemies (Ravensberg, 2010). In testing single fungal species, it was found that *M. anisopliae* has a very good ability to persist in the soil environment (Greenfield et al., 2016). In the combination of fungi *I. fumosorosea* with *M. anisopliae*, the results obtained indicate that there was significant suppression of both growth and spore production. By *I. fumosorosea*. This suggests that *M. anisopliae* in combination with the fungus *I. fumosorosea* is unable to flourish in the same medium. In the compatibility test, the fungus *M. anisopliae* performed the worst of all the tested fungal species. On the other hand, the best was the combination of *I. fumosorosea* with *L. muscarium*, which were no mutual influence observed.

The other objective of this thesis was to assess the effectiveness of humidity on selected entomopathogenic fungi *L. muscarium* and *I. fumosorosea* and mycoparasitic fungus *T. Virens*. The application of these two entomopathogenic fungi is particularly beneficial against sucking pests particularly on leaves, with *L. muscarium* being able to infect not only moths, worms, but especially whiteflies and aphids (Kim et al., 2010) and *I. fumosorosea* can suppress, in addition to all greenhouse pests, populations of mealybugs (Kim et al., 2008; Fiedler et al., 2007) and powdery mildew (Kavkova et al., 2005). Ghule et al. (2019) isolate from the powdery mildew *Erysiphe necator* the entomopathogenic fungus *Lecanicillium antillanum*. Species *Lecanicillium longisporum* was determined as a mycoparasitic of the cucumber powdery mildew Sphaerotheca *fuliginea (*Kim et al., 2010). So, the application of entomopathogenic species can infect not only greenhouse pests but they have a side effect against different species of biotrophic plant pathogens such as powdery mildew. All the results obtained showed that the higher humidity is better for the growth and development of entomopathogenic fungi.

And the last objective was to find out when does the fungi produce better spores in the short or long term, and it was found that the best result came after 14 days and later they started decreasing if we didn´t add more suspension on the leaves the CFU would have significantly decreased. The experiment in the thesis showed that entomopathogenic fungi *B. bassiana, M. anisopliae, L. muscarium* and *I. fumosorosea* and mycoparasitic fungus *T. virens* can be isolated from the different environments, by using different methods of isolations. Entomopathogenic fungi can be isolated by two methods.

First is based on traps insects such as *Galleria mellonella, Tenebrio mollitor* or *Delia floralis* where the virulent strains of entomopathogenic fungi can cause diseases on the traping insects and the strains can be directly reisolated from the soils (Medo et al.,2011, Klingen et al., 2002, Zimmermann 2007).

The second method is based on selective mediums, the selective medium can be based on various nutrient media which are enriched with selective components such as the fungicidal active ingredient dodine (N-dodecylquanidine monoacetate) and antibiotics such as cycloheximide, chloramphenicol, streptomycin, and tetracycline (Chase et al. 1986; Shimazu et al., 1996). For the thesis experiments, the selective medium based

on dodine was used for the re-isolation of entomopathogenic fungi from the soil substrate and the cucumber leaves. All the strains were re-isolated not only on artificial medium PDA with antibiotics but also on the selective medium based on dodine. Mycoparasitic fungus *T. virens* was re-isolated using artificial medium PDA with antibiotics and *Trichoderma* selective medium (TSM). The *Trichoderma* selective medium is better for re-isolation of fast-growing fungus *T. virens* because selective medium inhibits the rapid growth of colonies compared to artificial medium PDA. Because of this property, the colony-forming units (CFU) of *T. virens* were objectively determined.

6 CONCLUSIONS

After all the experiments, the conclusion was made based on the results obtained or observed during the experiments, below is a list of conclusions that were made.

- Temperatures play an important role in the developments of colony-forming units of different genes of entomopathogenic fungi and mycoparasitic fungus.
- All entomopathogenic fungi can be well re-isolated from the soil using selective medium based on dodine and medium PDA with antibiotics.
- All entomopathogenic fungi had high colony forming units when applied in the soil individually as observed from the results.
- *M. brunneum* was the most suppressed entomopathogenic fungi in all combinations, having fewer colony-forming units.
- During isolation, *I. fumosorosea* had more colony-forming units in all combinations.
- Entomopathogenic fungi *L. muscarium* and *I. fumosorosea* can be well re-isolated from the cucumber leaves using selective media based on dodine
- Mycoparasitic fungus *T. virens* can also be well re-isolated from the cucumber leaves using *Trichoderma* selective medium (TSM).
- *L. muscarium* showed a continuous development, compared to another entomopathogenic fungus *I. fumosorosea* and mycoparasitic fungus *T. virens*.
- Re-application of suspension of entomopathogenic and mycoparasitic fungi on the cucumber leaves in the greenhouse, every 7 days increased the number of spores and an increase in colony-forming units was observed.
- Optimum humidity is a very important factor in the development of fungi.
- Low humidity reduces the development of fungi and sporulation.
- All used strains of entomopathogenic fungi and mycoparasitic fungus *T. virens* could be used in IPM, taking into consideration the effects of each entomopathogenic fungi on the other.

REFERENCE

Allen G. E., Greene G. L., Whitcomb W. H. (1971). An Epizootic of *Spicaria rileyi* on the Velvetbean Caterpillar *Anticarsia gemmatalis*, in Florida. *The Florida Entomologist* **54**, 189.

Altre J. A, Vandenberg J. D, Cantone F. A (1999). Pathogenicity of *Paecilomyces fumosoroseus* Isolates to Diamondback Moth,*Plutella xylostella*:Correlation with Spore Size Germination Speed, and Attachment to Cuticle. *Journal of Invertebrate Pathology* **73**, 332–338.

Anonymous1. Https://eurlex.europa.eu/lexuriserv/lexuriserv.do?Uri=OJ:L:2009:309:0071:0086:EN:PDF

Anonymous2. Https://bio.libretexts.org/Bookshelves/Microbiology/Book%3A_Microbiology_(Boundless)/6%3A_Culturing_Microorganisms/6.3%3A_Culturing_Bacteria/6.3C%3A_Selective_and_Differential_Media

Anonymous3. Https://www.valagro.com/media/filer_public/cb/9f/cb9f2118-783e-4a74-ab4e-55bd1ed2b1e6/ppt7.pdf

Ansari M. A., Butt T. M. (2011). Effects of successive subculturing on stability virulence, conidial yield, germination and shelf-life of entomopathogenic fungi. *Journal of Applied Microbiology* **110**, 1460–1469.

Arthurs S., Thomas M. B. (2001). Effects of temperature and relative humidity on sporulation of Metarhizium *anisopliae* var. *acridum* in mycosed cadavers of *Schistocerca gregaria*. *Journal of Invertebrate Pathology* **78**, 59–65.

Askary H., Yarmand H. (2007). Development of the entomopathogenic hyphomycete *Lecanicillium muscarium* (Hyphomycetes: *Moniliales*) on various hosts. *European Journal of Entomology*, **104**(1), 67.

Beilharz V. C., Parbery D. G., Swart, H. J. (1982). Dodine: a selective agent for certain soil fungi. *Transactions of the British Mycological Society*, 79(**3**), 507-511.

Bischoff JF, Rehner SA, Humber RA. A multilocus phylogeny of the Metarhizium anisopliae lineage. Mycologia. 2009 Jul-Aug;**101**(4):512-30

Boman H. G. (1981). Insect responses to microbial infections. *Microbial control of pests and plant diseases, 1970-1980*.

Boucias D. G., Pendland J. C. (1991). Attachment of Mycopathogens to Cuticle. 101–127 In The Fungal Spore and Disease Initiation in Plants and Animals.

Boucias D. G., Pendland J. C., Latge J. P. (1988). Nonspecific factors involved in the attachment of entomopathogenic deuteromycetes to host insect cuticle. *Applied and environmental microbiology* **54**, 1795–1805.

Butt T. M. (2002). Use of Entomogenous Fungi for the Control of Insect Pests. 111–134 In *Agricultural Applications*. Springer Berlin Heidelberg.

Caltagirone L. E., Doutt R. L. (1989). The history of the vedalia beetle importation to California and its impact on the development of biological control. *Annual review of entomology*, **34**, 1-16.

Carruthers R. I., Feng Z., Ramos M. E., Soper R. S. (1988). The effect of solar radiation on the survival of *Entomophaga grylli* (Entomophthorales: *Entomophthoraceae*) conidia. *Journal of Invertebrate Pathology* **52**, 154-162.

Cory J. S., Hoover K. (2006). Plant-mediated effects in insect pathogen interactions. *Trends in Ecology & Evolution* **21**, 278–286.

Cory J., Jerry S., Ericsson D. (2009). Fungal entomopathogens in a tritrophic context. 75–88 In *The Ecology of Fungal Entomopathogens*. Springer Netherlands.

Danielson R. M., Davey C. B. (1973). Non nutritional factors affecting the growth of *Trichoderma* in culture. *Soil Biology and Biochemistry* **5**, 495–504.

Dirlbeková O. (1991). Biologické zdroje pro nechemickou ochranu rostlin (I. Deuteromycetes, *Beauveria bassiana* [Bals.] *Vuill.). Ústav vědeckotechnických informací pro zemědělství.*

Eilenberg J. (2006). Concepts and visions of biological control. 1–11 In An ecological and societal approach to biological control.

Eilenberg J., Hajek A., Lomer C. (2001). Suggestions for unifying the terminology in biological control. *BioControl* **46**, 387–400.

Ekesi S., Dimbi S., Maniania N. K. (2007). The role of entomopathogenic fungi in the integrated management of fruit flies (Diptera: *Tephritidae)* with emphasis on species occurring in Africa. *Use of entomopathogenic fungi in biological pest management* 239–274.

Elad Y., Chet I., Henis Y. (1981). A selective medium for improving quantitative isolation of *Trichoderma* spp. from soil. *Phytoparasitica* **9**, 59–67.

Faria M., Wraight S. P. (2001). Biological control of Bemisia tabaci with fungi. *Crop Protection* **20**, 767–778.

Faria M., Wraight S. P. (2007): Mycoinsecticides and mycoacaricides: A comprehensive list with worldwide coverage and international classification of formulation types. *Biological Control*, **43**(3): 237–256.

Fernandes É.K.K., Keyser C.A., Rangel D.E.N., Foster R.N., Roberts D. (2010). CTC medium: A novel dodine-free selective medium for isolating entomopathogenic fungi, especially *Metarhizium acridum*, from soil. *Biological control* **54**, 197–205.

Ferron P. (1978). Biological Control of Insect Pests by Entomogenous Fungi. *Annual Review of Entomology* **23**, 409–442.

Fiedler Ż., Sosnowska D. (2007). Nematophagous fungus *Paecilomyces lilacinus* (Thom) Samson is also a biological agent for control of greenhouse insects and mite pests. *BioControl* **52**, 547–558.

Fornallaz C. (1992). Demökologische Grundlagen und praktische Durchführung der mikrobiellen Bekämpfung des Maikäfers Melolontha melolontha L. Mit dem Pilz Beauveria brongniartii (Sacc.) Petch.

Gams W., & Zare, R. (2001). A revision of Verticillium sect. Prostrata. III. Generic classification. Nova Hedwigia, 329-337.

Ghule S.B., Sawant I.S., Sawant S.D., Saha S., Devarumath R.M. (2019): Isolation and identification of three new mycoparasites of *Erysiphe necator* for biological control of grapevine powdery mildew. *Australasian Plant Pathology*, **48**:351–367.

Gindin G., Geschtovt N. U., Raccah B., Barash I. (2000). Pathogenicity of *Verticillium lecanii* to different developmental stages of the silverleaf whitefly,*Bemisia argentifolii*. *Phytoparasitica* **28**, 229–239.

Greenfield M., Gómez-Jiménez M. I., Ortiz V., Vega F. E., Kramer M., Parsa S. (2016). *Beauveria bassiana* and *Metarhizium anisopliae* endophytically colonize cassava roots following soil drench inoculation. *Biological Control* **95**, 40–48.

Hajek A. E., St. Leger R.J. (1994). Interactions Between Fungal Pathogens and Insect Hosts. *Annual Review of Entomology* **39**, 293–322.

Hajek A., Eilenberg J. (2018). Introduction to Biological Control. In Natural Enemies: An Introduction to Biological Control (22-38).

Hall R.A. (1981). The fungus *Verticillium lecanii* as a microbial insecticide against aphids and scales. *Microbial control of pests and plant disease*.

Harman G.E. (2000). Myths and Dogmas of Biocontrol Changes in Perceptions Derived from Research on *Trichoderma harzinum* T-22. *Plant Disease* **84**, 377–393.

Hussey N.W., Scopes N.E.A. (1977). The Introduction of Natural Enemies for Pest Control in Glasshouses: Ecological Considerations. 349–377 In *Biological Control by Augmentation of Natural Enemies*.

Hywel-Jones N.L., Gillespie A.T. (1990). Effect of temperature on spore germination in *Metarhizium anisopliae* and *Beauveria bassiana*. *Mycological Research* **94**, 389–392.

Chase A.R., Osborne L.S., Ferguson V.M. (1986): Selective isolation of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* from an artificial potting medium. *Florida Entomologist*, **69**: 285-292.

Chaverri P., Castlebury L. A., Overton B. E., Samuels G. J. (2003). *Hypocrea/Trichoderma*: species with conidiophore elongations and green conidia. *Mycologia* **95**, 1100–1140.

Ignoffo C.M., Hostetter D.L., Sikorowski P.P., Sutter G., Brooks W.M. (1977). Inactivation of representative species of entomopathogenic viruses, a bacterium, fungus, and protozoan by an ultraviolet light source. *Environmental Entomology* **6**, 411–415¨

Inglis G. D., Johnson D. L., Goettel M. S. (1997). Effects of temperature and sunlight on mycosis (*Beauveria bassiana*) (Hyphomycetes: *Sympodulosporae*) of grasshoppers under field conditions. *Environmental Entomology* **26**, 400–409.

Inglis G.D., Goettel M.S., Butt T.M., Strasser H. (2001). Use of hyphomycetous fungi for managing insect pests. 23–69 In *Fungi as biocontrol agents: progress problems and potential*.

Jaronski S. T. (2007). Soil ecology of the entomopathogenic ascomycetes: a critical examination of what we (think) we know. *Use of entomopathogenic fungi in biological pest management* 91–143.

Jaronski S. T. (2009). Ecological factors in the inundative use of fungal entomopathogens. 159–185 In *The Ecology of Fungal Entomopathogens*.

Kant P., Reinprecht Y., Martin J., Islam R., Pauls K. (2011). Disease Resistance/Pathology/*Fusarium*. *Comprehensive Biotechnology* **4**, 729-743.

Kavková M., Čurn V. (2005): *Paecilomyces fumosoroseus* (Deuteromycotina: Hyphomycetes) as a potential mycoparasite on *Sphaerotheca fuliginea* (Ascomycotina*: Erysiphales*). *Mycopathologia*, **159**(1): 53–63.

Keller S., Zimmermann G., Wilding N., Collins N.M., Hammond P. M., Webber J. F. (1989). Mycopathogens of soil insects. Insect-fungus interactions, 239-270.

Khun K.K., Ash G.J., Stevens M.M., Huwer R.K., Wilson B.A.L. (2020): Compatibility of *Metarhizium anisopliae* and *Beauveria bassiana* with insecticides and fungicides used in macadamia production in Australia. *Pest Management Science*, **77**(2): 709-718.

Kim J., Roh J., Choi J., Shin S., Jeon M., Je Y. (2008). Insecticidal activity of *Paecilomyces fumosoroseus* SFP-198 as a multi-targeting biological control agent against the greenhouse whitefly and the two-spotted spider mite. *International Journal of Industrial Entomology* **17**, 181–187.

Kim J.J., Goettel M.S, Gillespie D.R. (2010). Evaluation of *Lecanicillium longisporum*, Vertalec against the cotton aphid, Aphis gossypii, and cucumber powdery mildew, *Sphaerotheca fuliginea* in a greenhouse environment. *Crop protection* **29**, 540–544.

King A.D., Hocking A.D., Pitt J.I. (1979). Dichloran-rose bengal medium for enumeration and isolation of moulds from foods. *Applied and environmental microbiology* **37**, 959–964.

Klingen I., Eilenberg J., Meadow R. (2002): Effects of farming system, field margins and bait insect on the occurrence of insect pathogenic fungi in soils. *Agriculture, Ecosystems and Environment*, **91**(1-3), 191–198.

Kolesidis D., Boddy L., Eastwood D., Yuan C., Fowler M. (2019). Predicting fungal community dynamics driven by competition for space. *Fungal Ecology* **41**, 13-22.

Krauss U., Bidwell R., Ince J. (1998). Isolation and preliminary evaluation of mycoparasites as biocontrol agents of crown rot of banana. *Biological control* **13**, 111–119.

Krauss U., Hidalgo E., Arroyo C., Piper S. R. (2004). Interaction between the entomopathogens *Beauveria bassiana*, *Metarhizium anisopliae* and *Paecilomyces fumosoroseus* and the mycoparasites Clonostachys spp., *Trichoderma harzianum* and *Lecanicillium lecanii*. *Biocontrol Science and Technology* **14**, 331–346.

Kudela V. (1989). Obecná fytopatologie. Academia.

Kundu J.K., Stará J., Kocourek F., Pultar O. (2003). Polymerase chain reaction assay for *Cydia pomonella* granulovirus detection in *Cydia pomonella* population. *Acta Virol* **47**, 153-7.

Landa Z. (1998). Biopreparáty na bázi entomopatogenních hub. Agro-ochrana a vyživa rostlin 1998; 10: 7–12. Samson RA. *Paecilomyces* and some allied hyphomycetes. *Studies in Mycology. Baarn: Centralbureau voor Schimmelcultures* **6**, 58.

Landa Z. (2002). Biologická ochrana zahradních rostlin proti chorobám a škdcm v polních podmínkách, ve sklenících a fóliovnících. *Demo M., Hričovsky I.(Eds.): Trvalo udržatel'né technológie v záhradnictve. Slovenská pol'nohospodárska univerzita v Nitre, 225* **280**.

Landa Z., Bohatá A. (2008). Záměrné využívání autochtonních kmenů vybraných druhů entomopatogenních hub. *Jihočeská univerzita v Českých Budějovicích, Zemědělská fakulta*.

Lisansky S. (1997). Microbial biopesticides. 3–10 In *BCPC Symposium Proceedings*.

Litwin A., Nowak M., Różalska S. (2020): Entomopathogenic fungi: unconventional applications. Reviews in *Environmental Science and Bio-Technology*, 19(1): 23-42.

Liu Z.Y., Milner, R.J., McRae, C.F., Lutton G.G. (1993). The use of dodine in selective media for the isolation of *Metarhizium* spp. from soil. Journal of Invertebrate Pathology, **62**(3), 248-251.

Medo J., Cagáň L. (2011). Factors affecting the occurrence of entomopathogenic fungi in soils of Slovakia as revealed using two methods. *Biological Control* **59**, 200–208.

Medo J., Michalko J., Medová J., Cagáň L. (2016). Phylogenetic structure and habitat associations of *Beauveria* species isolated from soils in Slovakia. *Journal of invertebrate pathology* **140**, 46–50.

Milner R.J., Lutton G.G. (1976). *Metarrhizium anisopliae*: survival of conidia in the soil. In *Proceedings of the International Colloquium on Invertebrate Pathology*.

Mora M.A.E., Castilho A.M.C., Fraga M.E. (2017). Classification and infection mechanism of entomopathogenic fungi. *Arquivos do Instituto Biológico* **84**.

Müller-Kögler E., Zimmermann G. (1986). Zur Lebensdauer von Beauveria bassiana in Kontaminiertem Bonden unter Freilandund Laboratoriumsbedingungen. *Entomophaga* **31**, 285–292.

Nadeau M.P., Dunphy G.B., Boisvert J. L. (1996). Development ofErynia conica(Zygomycetes: Entomophthorales) on the Cuticle of the Adult Black Flies *Simulium rostratumandSimulium decorum*(Diptera: *Simuliidae). Journal of Invertebrate Pathology* **68**, 50–58.

Osborne L.S., Landa Z. (1992). Biological Control of Whiteflies with Entomopathogenic Fungi. *The Florida Entomologist* **75**, 456.

Pal K.K., Gardener BM. (2006). Biological control of plant pathogens. *The plant health instructor* **2**, 1117–1142.

Park Y.H., Stack, J.P., Kenerley, C.M. (1992). Selective isolation and enumeration of *Gliocladium virens* and *G. roseum* from soil. *Plant disease*, 76(3), 230-235.

Persoon C.H. (1794). Disposita methodical fungorum. Romers Neues Mag Bot, **1**, 81-128.

Quintela E. D., McCoy C. W. (1998). Synergistic effect of imidacloprid and two entomopathogenic fungi on the behavior and survival of larvae of *Diaprepes abbreviatus* (Coleoptera: Curculionidae) in soil. *Journal of economic entomology* **91**, 110–122.

Ram R., Keswani C., Bisen K., Tripathi R., Singh H. (2018). Biocontrol Technology. *Omics Technologies and Bio-Engineering* 177-190.

Ramanujam B., Rangeshwaran R., Sivakumar G., Mohan M., M(2014). Yandigeri management of Insect Pests by Microorganisms.

Ramoska W. A. (1982). An examination of the long-term epizootic potential of various artificially introduced mosquito larval pathogens. *Mosquito News* **42**, 603–607

Rangel D.E., Anderson A.J., Roberts D.W. (2008). Evaluating physical and nutritional stress during mycelial growth as inducers of tolerance to heat and UV-B radiation in *Metarhizium anisopliae conidia*. *Mycological research*, **112**(11), 1362-1372.

Ravensberg, W. J. (2010). The development of microbial pest control products for control of arthropods: a critical evaluation and a roadmap to success.

Roberts D.W., Campbell A. S. (1976). Stability of entomopathogenic fungi. *Misc. Publ. Entomol. Soc. Amer.* **10**, 19.

Samson R.A. ((1974). *Paecilomyces* and some allied hyphomycetes. *Studies in mycology* **6**, 1–119.

Samuels R., Paula A., Carolino A., Gomes S., Paula C., Cypriano M., Silva L., Ribeiro A., Bastos J., Peres C. (2016). Entomopathogenic organisms: conceptual advances and real-world applications for mosquito biological control. *Open Access Insect Physiology* 25.

Sanda N. B., Sunusi M. (2014). FUNDAMENTALS OF BIOLOGICAL CONTROL OF PESTS.

Shah M. M., Afiya H. (2019). Introductory Chapter: Identification and Isolation of *Trichoderma* spp.-Their Significance in Agriculture, Human Health, Industrial and Environmental Application. In *Trichoderma-The Most Widely Used Fungicide*.

Shimazu M., Sato H. (1996): Media for selective isolation of an entomogenous fungus, *Beauveria bassiana* (Deuteromycotina: *Hyphomycetes*). *Applied Entomology and Zoology*, 31(**2**): 291-298.

Shin T.Y., Choi J.B., Bae, S.M., Koo H.N., Woo S. D. (2010). Study on selective media for isolation of entomopathogenic fungi. *International Journal of Industrial Entomology*, **20**(1), 7-12.

Schwartz, W. (1967). Erwin Müller-Kögler, Pilzkrankheiten bei Insekten. XVI und 444 S., 40 Abb., 12 Tab. *Berlin 1965: Paul Parey*. DM **98**.

Sosa-Gomez D. R., Boucias D. G., Nation J. L. (1997). Attachment of *Metarhizium anisopliae* to the Southern Green Stink Bug Nezara viridula Cuticle and Fungistatic Effect of Cuticular Lipids and Aldehydes. *Journal of Invertebrate Pathology* **69**, 31–39.

Steinwender B. M., Enkerli J., Widmer F., Eilenberg J., Thorup-Kristensen K., Meyling N. V. (2014). Molecular diversity of the entomopathogenic fungal *Metarhizium* community within an agroecosystem. *Journal of invertebrate pathology* **123**, 6–12.

Storey G. K., Gardner W. A. (1987). Vertical movement of commercially formulated *Beauveria bassiana* conidia through four Georgia soil types. *Environmental Entomology* **16**, 178–181.

Storey G. K., Gardner W. A. (1988). Movement of an aqueous spray of *Beauveria bassiana* into the profile of four Georgia soils. *Environmental Entomology* **17**, 135–139.

Strasser H., Forer A., Schinner F. (1996). Development of media for the selective isolation and maintenance of viruIence of *Beauveria brongniartü*.

Stukenberg N.M. (2018). LED based trapping of whiteflies and fungus gnats: From visual ecology to application (Doctoral dissertation, Hannover: *Institutionelles Repositorium der Leibniz Universität Hannover).*

Subramaniam M. S. R., Babu A., Deka B. (2021). *Lecanicillium lecanii* (Zimmermann) Zare & Gams, as an efficient biocontrol agent of tea thrips, Scirtothrips bispinosus Bagnall (Thysanoptera: Thripidae). *Egyptian Journal of Biological Pest Control* **31**, 1–14.

Sung G.H., Hywel-Jones N.L., Sung J.M., Luangsa-Ard J.J., Shrestha B., Spatafora J.W. (2007). Phylogenetic classification of *Cordyceps* and the clavicipitaceous fungi. *Studies in mycologi, 57: 5-59.*

Tumuhaise V., Ekesi S., Maniania N.K., Tonnang H.E.Z, Tanga C.M., Ndegwa P.N., Irungu L.W., Srinivasan R., Mohamed S.A. (2018). Temperature-Dependent Growth and Virulence and Mass Production Potential of Two Candidate Isolates of *Metarhizium anisopliae*(Metschnikoff) Sorokin for ManagingMaruca vitrataFabricius (Lepidoptera: Crambidae) on Cowpea. *African Entomology* **26**, 73–83.

Usta C. (2013). Microorganisms in biological pest control a review (bacterial toxin application and effect of environmental factors). 287–317 In *Current progress in biological research*. Intech Rijeka.

Vänninen I. (1995): Distribution and occurrence of four entomopathogenic fungi in Finland: effect of geographical location, habitat type and soil type. Mycological Research, **100**: 93-101.

Veen KH, Ferron P. (1966). A selective medium for the isolation of *Beauveria tenella* and of *Metarrhizium anisopliae*. *Journal of Invertebrate Pathology* **8**, 268–269.

Vestergaard S., Butt T.M., Bresciani J., Gillespie A.T., Eilenberg J. (1999). Light and Electron Microscopy Studies of the Infection of the Western Flower Thrips *Frankliniella occidentalis* (Thysanoptera: *Thripidae*) by the Entomopathogenic Fungus *Metarhizium anisopliae*. *Journal of Invertebrate Pathology* **73**, 25–33.

Vidal C., Fargues J., Lacey L.A. (1997). Intraspecific variability of *Paecilomyces fumosoroseus*: effect of temperature on vegetative growth. *Journal of Invertebrate Pathology* **70**, 18–26.

Vilcinskas A., Götz P. (1999). Parasitic Fungi and their Interactions with the Insect Immune System. 267–313 In *Advances in Parasitology Volume **43***.

Watanabe, T. (2010). Pictorial atlas of soil and seed fungi: morphologies of cultured fungi and key to species.

Woo S.L., Ruocco M., Vinale F., Nigro M., Marra R., Lombardi N., Pascale A., Lanzuise S., Manganiello G., Lorito M. (2014). *Trichoderma*-based products and their widespread use in agriculture. *The Open Mycology Journal* **8**.

Wraight S.P., Butt T.M., Galaini-Wraight S., Allee L.L., Soper R.S., Roberts D. W. (1990). Germination and infection processes of the entomophthoralean fungus *Erynia radicans* on the potato leafhopper Empoasca fabae. *Journal of Invertebrate Pathology* **56**, 157–174.

Zhou Z., Luo X., Zhang Y., Li,Y., Zang Y. (2010). Machine-based technologies for detecting and monitoring insect pests of crops: a review. Acta Entomologica Sinica, **53**(1), 98-109.

Zimmermann G. (1986). The 'Galleria bait method'for detection of entomopathogenic fungi in soil. *Journal of applied Entomology* **102**, 213–215 Wiley Online Library.

Zimmermann G. (1993). The entomopathogenic fungus *Metarhizium anisopliae* and its potential as a biocontrol agent. *Pesticide Science* **37**, 375–379.

Zimmermann G. (2007a). Review on safety of the entomopathogenic fungus *Metarhizium anisopliae*. *Biocontrol Science and Technology* **17**, 879–920.

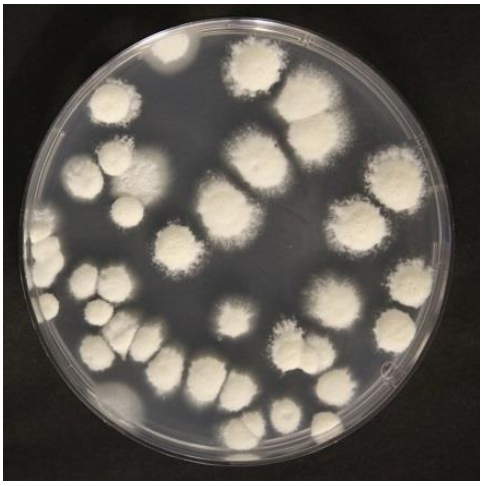
Zimmermann G. (2007b). Review on safety of the entomopathogenic fungi *Beauveria bassiana* and *Beauveria brongniartii*. *Biocontrol Science and Technology* **17**, 553–596.

Zimmermann G. (2008). The entomopathogenic fungi *Isaria farinosus* (formerly *Paecilomyces farinosus*) and the *Isaria fumosorosea* species complex (formerlyPaecilomyces fumosoroseus): biology ecology and use in biological control. *Biocontrol Science and Technology* **18**, 865–901.

Zimmermann G., Papierok B., Glare T. (1995). Elias Metschnikoff, Elie Metchnikoff or Ilya Ilich Mechnikov (1845-1916): a pioneer in insect pathology, the first describer of the entomopathogenic fungus *Metarhizium anisopliae* and how to translate a Russian name. *Biocontrol Science and technology*, **5**(4), 527-530

List of Appendix

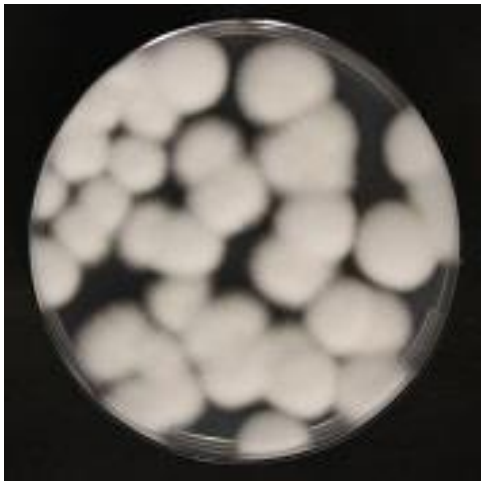
Fig. 1a Re-isolation of entomopathogenic fungi from the soil substrate after 14 day of incubation at 25±1 °C



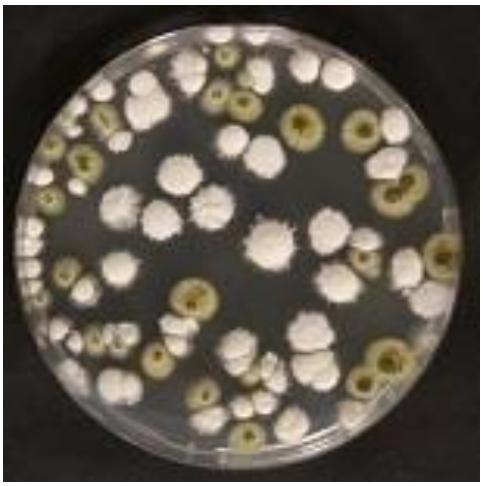
Re-isolation of *B. bassiana* from the soil using selective medium based on dodine

Re-isolation of *M. brunneum* from the soil using selective medium based on dodine

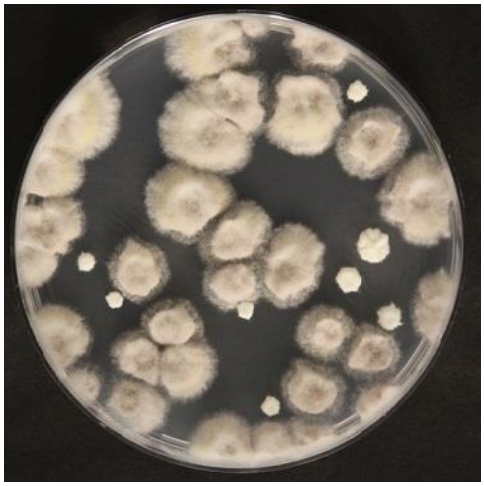
Re-isolation of *I. fumosorea* from the soil using selective medium based on dodine



Re-isolation of *L. muscarium* from the soil using selective medium based on dodine



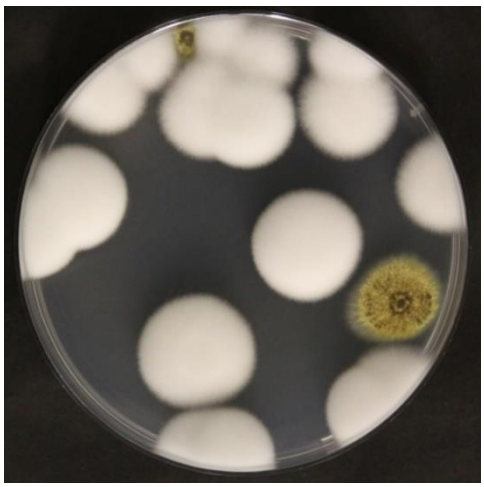
Re-isolation of combination of *B. bassiana* and *M. brunneum* from the soil



Re-isolation of combination of *B. bassiana* and *I. fomosorosea* from the soil

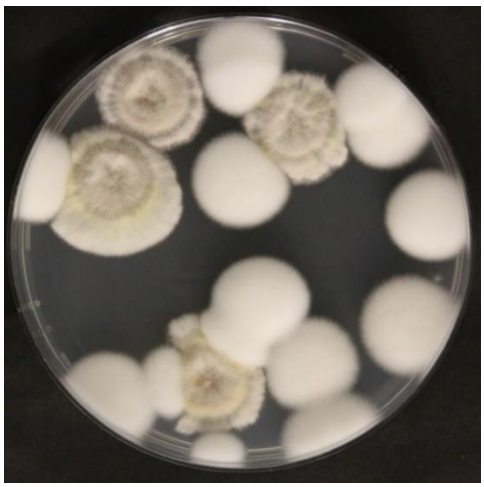
Fig. 1b Re-isolation of entomopathogenic fungi from the soil substrate after 14 day of incubation at 25±1 °C (continued)

Re-isolation of combination of *B. bassiana and L. muscarium* from the soil



Re-isolation of combination of *M. anisopliae and L. muscarium* from the soil

Re-isolation of combination of *M. anisopliae and I. fomosorosea* from the soil



Re-isolation of combination of *I. fomosorosea and L. muscarium* from the soil

List of abbreviations

CFU- colony-forming Units

IPM- Intergrated pest management

EPF- Entomopathogenic fungi

PDA – Potato dextrose agar

PDA + A - Potato dextrose agar with antibiotics

Dodine – selective medium based dodine