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BACHELOR'S THESIS

Influence of temperature on development and virulence of entomopathogenic *Metarhizium anisopliae*

Vliv teploty na vývoj a virulenci entomopatogenní houby *Metarhizium anisopliae*

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ABSTRACT

Entomopathogenic fungus *M. brunneum* has a high potential in integrated pest management against targeted pests. The commercial bio-product Met52 is based on the strain F52 of entomopathogenic fungus *M. brunneum*. Temperature is the key factor in the inoculums production of strain F52. The optimal temperature for the cultivation of *M. brunneum* strain F52 is 25 °C. However, the influence of the temperature of inoculum production plays an important role on the growth and spore production during the subsequent cultivation in different temperature conditions. The optimal temperature for radial grows of strain F52 of *M. brunneum* from different inoculums is 25 °C and subsequently 30 °C. The cultivation of inoculums at 10 °C appears to be negative because at this temperature the strain grows very slow, and spore production is minimal. The morphology phenotype change of inoculums is influenced by 30 °C of cultivation and also this temperature affects the appearance of inoculum central colonies. The highest production of spores was determined from the colonies established from all inoculums cultivated at 25 °C. The highest mortality and the greatest fungus development was recorded at 25 °C and the most virulent to larvae T. molitor were inoculums produced at 15 °C and 20 °C.

Keywords: entomopathogenic fungi, *Metarhizium brunneum*, temperature, radial growth, spore production, virulence

ABSTRAKT

Entomopatogenní houba *M. brunneum* má vysoký potenciál v rámci Integrované ochrany rostlin proti cílovým škůdcům. Komerční biopreparát Met52 je koncipován na bázi kmene F52 entomopatogenní houby *M. brunneum*. Teplota je klíčovým faktorem pro produkci inokula kmene F52. Optimální teplota pro kultivaci kmene F52 houby *M. brunneum* je 25 °C. Vliv teploty na produkci inokula hraje důležitou roli při růstu, výtěžnosti spor během následné kultivace v různých teplotních podmínkách. Optimální teplota pro radiální růst kmene F52 houby *M. brunneum* získaného z různých inokul je 25 °C a následuje teplota 30 °C. Kultivace inokul v 10 °C není vhodná, protože při této teplotě kmen F52 roste velmi pomalu a výtěžnost spor je minimální. Morfologický fenotyp kultur narostlých z testovaných inokul je ovlivněn kultivací ve 30 °C a také tato teplota pro produkci inokula ovlivňuje vzhled středových kultur. Nejvyšší produkce spor byla zjištěna z kultur založených ze všech inokul kultivovaných ve 25 °C. Nejvyšší mortalita a nejlepší vývoj kmene byl zaznamenán opět při kultivaci ve 25 °C. Inokula produkované v 15 °C a 20 °C byla nejvíce virulentní k larvám *T. molitor*.

Klíčová slova: entomopatogenní houby, *Metarhizium brunneum*, teplota, radiální růst, produkce spor, virulence

CONTENT

1. PREFACE

Many entomopathogenic fungi were examined in the late 20th centuries for possible use against pests and pathogens. Of recent years people have reconsidered fungi for protection because of the increase in insecticide resistance and environmental problems caused by insecticide use. Because of this, there has been a process going on like identification of native fungi isolation from a variety of hosts. Awareness about problems caused by pesticide use has also helped in finding alternative solutions and control that are environmentally friendly. Such reasons have led to the consideration of entomopathogenic fungi as a component of IPM. Before the birth of "Green revolution," the farmers of 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. Variety of fungal species occur on pests and pathogens in different ecosystems, that means maintaining biotic tolerance to keep the pest or host population limited and below the economic damage level. 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 fungi are very and usually important in natural mortality factor in insect population. Some species such as *Metarhizium* and *Beauveria* belong to order Hypocreales are facultative generalist pathogen, but most of other species which belong to order Entomophthorales are obligate pathogens and they are quite specific. Facultative generalist fungi have frequently a wide range of host even-though, there is typical genetic diversity within species. Most of the entomopathogenic fungi are quite common and usually influence epizootics and are an important factor in regulating pest and insect population. Unlike like other biocontrol agents, fungi do not need to be ingested to infect their host, they can enter the body of the host directly through the cuticle and this shows how they can be effectively useful against all insects including sucking insects.

The aim of the thesis was focused on the influence of inoculums produced at different temperatures based on their characteristics of development and virulence consequently tested in various temperatures of cultivation.

2. INTRODUCTION

2.1. Integrated Pest Management (IPM)

IPM is an ecologically based system of pest management (Strand 2008). IPM is a combination of use of tactics to limit the pests or plant pathogen populations to a certain level that they cannot cause economic damage (Rapisarda and Cocuzza 2017). Concept of IPM excelled in the mid-70s when there was environmental and people health damage caused by synthetic pesticides on large scales (Dhaliwal *et al.* 2004). It has been found that the use of pesticides caused a lot of problems thus it reduced the abundance of world life, increased soil toxicity even caused deaths to some extents. Half of the people who have died by poisons related to pesticides are originated from developing countries (Uneke 2007).

According to Hall (1995), there are more than 10,000 species of pests, 30,000 species of weeds 100,000 plant diseases caused by fungi, bacteria, virus pathogens which decrease the yield of crops. The pesticide resistance is also slowly increasing given that some pest, plant pathogens and weeds are resistant to even more than one pesticide. According to statistics, it shows that there are at least 450 species of insects and mites, 100 species of plant pathogens and 48 species of weeds worldwide that have shown resistance to more than one pesticide active ingredient (Luckmann and Metcalf 1994). IPM use different methods to eliminate pests which caused crop damages. Some methods are traditional, like cultural, mechanical and physical methods, also biological control is used for many pests, and next methods like genetic and biorational methods are used. This alternative method can decrease the negative influence of pesticides using on the environment. Because of the resistance caused by pesticides, the development of new methods is important (Norris *et al.* 2003).

2.1.1. Aims of IPM

1) One of the aims is profit. This is aimed at maintaining and increasing profitability despite the presence of pests and this can be achieved by some factors, for example, preventing or avoid pest problems before an economic loss of crop production occurs.
2) Environmental quality. The aim is to improve the qualities of the environment, and this can be achieved by other factors for example choosing a selected method or insecticide with minimum risk on the non-targeted organisms (Uneke 2007).

2.1.2. Definition of IPM

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 minimise risks to human health and the environment. 'Integrated pest management' emphasises the growth of a healthy crop with the least possible disruption to agro-ecosystems and encourages natural pest control mechanisms (Anonymous 1).

2.1.3. Monitoring

Pest identification is the most important procedure in IPM. Correct identification of pests helps to choose proactive or reactive methods to eliminate pest populations. Positive identification of pests may be obtained from university personnel or private consultants, who are professionals in plant protection. 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 done routinely at chosen intervals or periods. The aim of the monitoring is to obtain information on the occurrence of pests, their developmental stages, population density, and the effectiveness of natural enemies. For monitoring, various tools such as yellow or blue glue plates can be used in the greenhouse, but the identification of pest "in situ" in the plant cover is very important. For small pest, 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 are helpful to select a suitable method of plant protection and it allows to predict the probability of the damage in the plants (Pultar 2003).

2.2. Biological control

A report from Hussey (1985) showed 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 did not give good control of the crops against the targeted pest. The first successful attempt happened in the year 1926,

when the *Ecarsia formosa* was introduced against whiteflies, which caused diseases on the tomato crops (Eilenberg 2006). Biological control methods are methods, which are used on living organisms to maintain the population of that organism. The agents which are used in biological controls are known as predators, parasitoids and pathogens. They are used for the population suppression of the 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 thereafter reduce the damage that would be caused on crops (Osborne *et al.* 2004).

2.2.1. 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 macroorganisms including parasites, parasitoids and predators. The second group includes different kinds of microorganisms, especially viruses, bacteria, nematodes and fungi (Hajek 2004).

Parasite

- ✓ An organism that develop inside or outside their hosts
- ✓ Immatures are closely associated with their hosts, they don't move from one host to another
- ✓ They are generally smaller than its hosts
- ✓ Free living are only adults
- ✓ They are specialists
- ✓ Order Hymenoptera

Predator

- ✓ An organism that kill and eat another organism (prey)
- ✓ adults and immature feeds on their prey
- ✓ Usually larger than their prey
- ✓ They need many preys to complete their development
- ✓ Adult and immatures are free-living
- ✓ They have wide range of prey from different orders
- ✓ Order Coleoptera, Hemiptera, Neuroptera, Diptera, predatory mites

Pathogens

- ✓ an obligatory or facultative pathogen that is capable of causing its host disease
- ✓ entomopathogenic microorganisms that are directly associated with insects (entomopathogenic viruses, entomopathogenic bacteria, entomopathogenic fungi, entomopathogenic or entomoparasitic nematodes)

Biological agents are introduced into the crops against pests or diseases by different strategies of biological control. There are different ways in which biological control can be applied, namely: Classical biological control, Inoculative biological control, Augmentative biological control (Inundative control, Sesonal inoculative control), Conservation control.

2.2.2. Strategies of biological control

Strategies of biological control are based on introduction of natural enemies into the agroecosystem of interest against harmful organisms (pests, plant pathogens, weeds). The natural enemies are defined as basic methodological equipments 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 the importation of an alien from a region where the pest species originated from. Once the alien is released, it is self-sustainable and is normally used at the regional or national level by government agencies working together with research institutions.

During this strategy of classical biological control, the natural enemies introduced against exotic pest which was unknowingly spread into the new agroecosystem. It is important to obtain the information about the exotic pests and observed if they have any natural enemies in the new area of distribution. If there are not any natural enemies observed. The government must cooperate with scientist from the area of exotic pest origin. The exotic natural enemies are imported and introduced into the agroecosystems for elimination of exotic pests before long-term occupation of those pests in the nature. The aim of this strategy is 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).

Before new natural enemy is imported into the new agroecosystem, the report for submission to the authority must be prepared. This document must include the information about exotic pests and about natural enemy. A summary of all available information on exotic pest origin, distribution, biology, natural enemies and impact in its area of distribution must be prepared. Also, the report with information on the natural enemy must be prepared. Accurate identification of natural enemy is required if necessary. Information should also include the biology and bionomy of natural enemy. What is important is to know the host specifications of the natural enemy. The potential hazards to non-targeted host must be analysed before the release of the natural enemy commences into the new area of interest (FAO 1996).

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 purchase* Mask. (Homoptera: Margarodidae), in California in the late 1800s (Caltagirone and Doutt 1989). The ladybird *R. cardinalis* were collected in Australia citrus orchards and consequently imported to California for elimination of the scale insect. The scale *Iceryapurchasi* originated from Australia and pest was spread into the areas where the citrus crops are grown (Normark 2009).

However, the first use of natural enemies to control insect pests was done by the Chinese. They sold near Canton nests of the ant *Oecophylla smaragdina* for use in control of citrus insect pests such as *Tessaratoma papillosa* (Lepidoptera) in the 3rd century (Orr and Lahiri 2014).

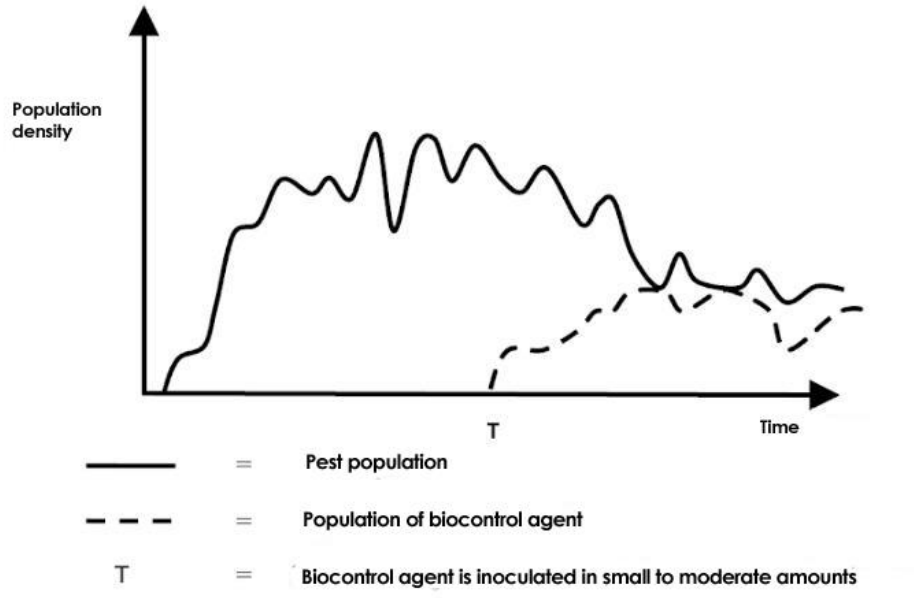
Inoculate biological control

In some publication, the Classical biological control is included as a part of 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 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.

The principle of inoculate biological control is using of the endemic or non-endemic species of natural enemies which are intentionally introduced into a new area of the spread of the harmful organism or reintroduced into the area in which these natural enemies

previously lived. The aim of intentional inoculum introduction is to ensure a long-term effect in agroecosystem against targeted pests. Positive effect can be achieved, to achieve in the case of successful attachment of the newly introduced or reintroduced of natural enemy, which is able to adapt, reproduce and spread in the new area and finally eliminate the population of the pests (Landa 2002, Sanda and Sunusi 2014).

Figure 1 The strategy of inoculate biological control/classical biological control (Eilenberg 2006)



The import and inoculum introduction of a new species of bioagents into the pest introduction area has been highly effective (e.g. the introduction of parasitoid *Aphelinus* had against *Eriosomala nigrum* or *Typhlodromus pyri* against *Tetranychus urticae* or *Tentranychus cinnebarinus*) (Landa 2002).

Augmentative biological control

The aim of this strategy is to periodically release natural enemies that do not multiply in nature in enough quantities to eliminate pests below the economic threshold. The practice of augmentation is based on the knowledge or assumption that in some crop systems there are not enough natural enemies to provide optimal biological control, but that the bigger number of individuals of the natural enemy can be increased by their introduction to agroecosystems (Landa 2002).

The main principle is mass-produce populations of endemic or non-endemic species, which are subsequently released into targeted agroecosystems to enhance their suppressive

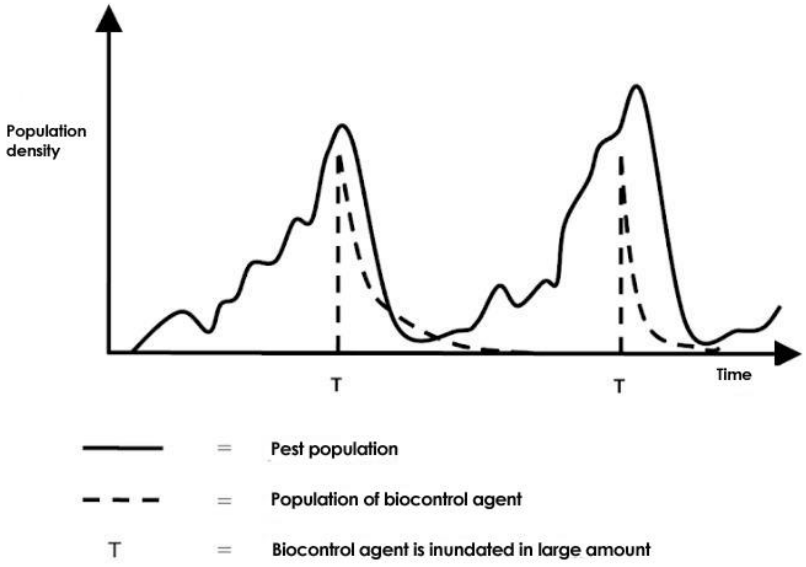
activity. The augmentative strategy is thus of a highly technological nature. A key condition for the practical implementation of this strategy is the existence of mass rears of parasites and predators, respectively large-scale production of microorganism. There are companies in the world that commercially breed and reproduce large numbers of different types of natural enemies and distribute them to growers in the form of standard biological agents (microorganisms) and bioproducts (microorganisms). 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 and Sunusi 2014). In the augmentative strategy, two basic methodological approaches are distinguished: Inundative biological control and Seasonal inoculative biological control.

Inundative biological control

The aim of this release is single or repeated introductions of usually large amounts of natural enemies to achieve immediate regulation of pest population or suppression of development of plant pathogens. Inundative introductions are predominantly used in the biological control of annual crops against univoltine pests (1 generation per year) or bivoltinepests (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 the immediate, i.e. bio-insecticidal effect.

The classic models of successful implementation of the initiative introduction method include the use of parasitic wasps of the genus *Trichogramma* (eg. *T. evanescens* against the European corn borer *Ostrinia nubilalis*) or the single application of bioproducts based on entomopathogenic microorganisms (eg. *Bacillius thuringiensis* or *Beauveria bassiana* against the Colorado potato beetle *Leptinotarsa decemlineata, Metarhizium anisopliae against* against grasshoppers, wireworm beetles), of application of bioproduct based on mycoparasitic fungi (eg. *Coniothyrium minitans* against white mold *Sclerotinia sclerotiorum*). Also, indundative biological control is based on commercially rearing of predators, parasitoids or microorganisms to successfully establish the programs (Landa 2002, Eilenberg *et al.* 2001).

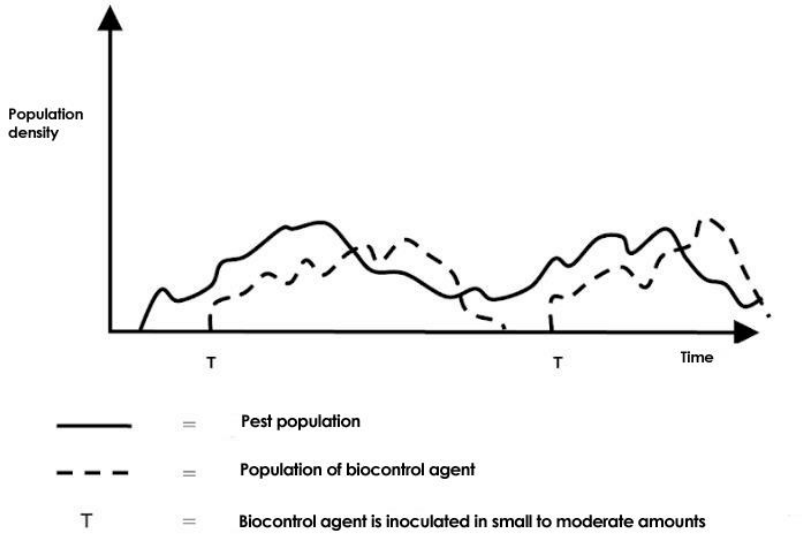
Figure 2 The strategy of inundative biological control (Eilenberg 2006)



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 already 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 the targeted pests.

Figure 3 The strategy of seasonal inoculate biological control (Eilenberg 2006)



The method of introducing seasonal inoculations is used in the biological control of greenhouse crops against multivoltine pests (many generations per vegetation season). In comparison with the inundation method, the main difference in seasonal inoculation is that

the structure of a biological control program is not focused on single applications to suppress one generation of pests or the developmental cycle of plant pathogenic microorganisms, but to induce a state in which even many generations of pests do not to exceed the tolerable level (critical number, economic threshold). During the growing season, monitoring of targeted pest populations and natural enemies is important. If it is necessary, the introduction of the next adequate amounts of bioagents must be considered (Minkenberg 1990, Landa 2002).

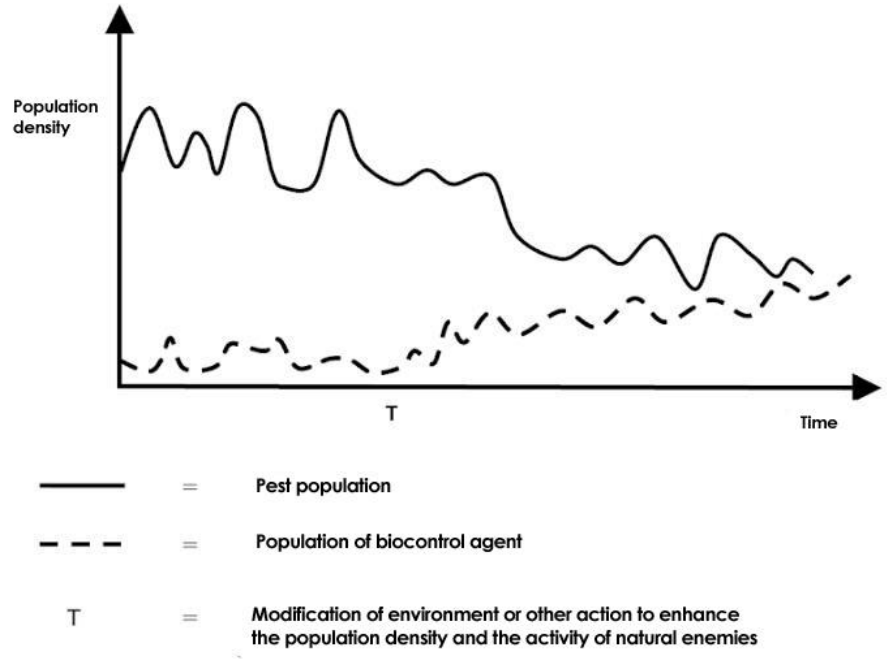
During the introduction of natural enemies, the alternative food for these beneficial species can be added, because if the pest occurs on the crops in small populations, natural enemies are able to feed on these alternative food sources. This method implements a very effective complex of biological control programs for various kinds of vegetables (tomato, cucumbers, peppers, etc.) grown in greenhouses. Successful programs are based on the introduction of *Encarsia formosa* Gahan (Hymenoptera: Aphelinidae) against whiteflies, predator of *Phytoseiulus persimilis* (Acarina: Phytoseidae) against two-spotted spider mite *Tetranychus urticae* and other natural enemies, nowadays established in greenhouses (Eilenberg *et al.* 2000, van Lenteren 2000, Minkenberg and van Lenteren 1986). At the end of the growing cycle, the crops are removed from the greenhouse and in the next round of growing cycle; the natural enemies must be reintroduced into the same greenhouse. This introduction to control a pest, it strongly depends on the density of populations and regulation of pest populations (Sanda and Sunusi 2014).

Conservative biological control

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

An example is the establishment of various agro-ecological and agronomic operations (e.g. intentional establishment of stable biocorridors, diversification of crops grown in the field, minimization of agro-technical interventions, establish of "beetle banks" and sowing of flowering plants near the cultivated field crops) (Eilenberg *et al.* 2001, Eilenberg 2006, Landa 2002).

Figure 4 The strategy of conservative biological control (Eilenberg 2006)



2.3. 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)

Entomopathogenic fungi are potentially the most adaptable biological control agents, because of their wide range of hosts. They usually result in natural epizootics. Fungi often cause spectacular epizootics in the population of many species of insects and they grow on the surface of cadavers (Hall and Papierok 1982). It is easy to recognize the infection because fungi are visible out-grown. The feature that makes these fungi attractive is the contact way with infected cadaver (Nadeau *et al.* 1996).

Entomopathogenic fungi are heterogenous and belong to many orders in a different division of Fungi kingdoms. Approximately 750 species, reported from different insect families. And a lot of fungi from order Hypocreales have great potentials 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).

Beauveria bassiana and *Metarhizium anisopliae* are known to be pathogenic to all stage of different insect and these fungi are the most known commercialized in the biological

control industry because bioproducts are produced around the world (Faria and Wright 2007). In the year 1883, Metchinikoff initiated mass culturing of fungus and carried out the first experiment with two beetle pests. *Metarhizium spp*. are known for attacking more than 200 species of insect pests belonging to different orders (Inglis *et al.* 2001).

2.3.1. Mode of infection

Pathogenesis is the sequence of processes in disease development from the time of infection to the final reaction in the host (Kůdela and Polák 2007). Differing to 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, production of blastospores, proliferation within the body of the host, utilization 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 2007, Nadeau *et al.* 1996; Thomas *et al.* 1996).

The first step in fungus development is quite 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 is ensured by 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 hemagglutinin, N-acetyl glucosamine, glycoproteins, sterols, polar lipids) (Sosa-Gomez *et al.* 1997, Altre *et al.* 1999, Vestergaard *et al.* 1999, Malsam *et al.* 2002).

The interaction between the spores and the body surface of a targeted host with the subsequent development of the pathogen is illustrated by the model of the interactional system - the parasitic part of the developmental cycle of the entomopathogenic fungi *Metarhizium anisopliae* (Hajek and St. Leger 1994).

The germinating spore may form a variety of penetration structures at the end of the sprout (e.g., the swollen tip of the spleen, the appressorium or the extracellular capsule). On the surface of the body, the conjugated and sprouted spores penetrate the cuticle with hyphae either directly or firstly spreading in all directions along the surface of the host body

and after they go inside of the body by natural openings (Vilcinskas and Götz 1999). Before direct penetration, fungi further form a penetration hypha that can penetrate into the body of the host. There are several factors involved in the penetration process. The environmental factors and the presence of inhibitory substances, such as fatty acids, contained in insect kits, are involved in the penetration. During penetration, the pathogen employs a combination of biochemical (enzymes) and physic-mechanical (pressure-penetrating hyphae) mechanisms. The penetration is mechanical which is aided by the production of enzymes such as endoproteases, esterases, lipases, chitinases, and chitobiose, with the most important role being exoproteases (Sosa-Gomez *et al.* 1997, Butt *et al.* 1998; Gillespie *et al.* 2000). Even if the conidia are germinated, the fungus may not be able to penetrate the insect's cuticle. This fungus or strain grows into the body of the host through natural openings, intermediate segments, or active penetration of the conidial key into the host body (Osborne and Landa 1992).

After the invasion of hyphae into the body of the host, the pathogen usually rapidly colonizes 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 hemolymph, it must overcome 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 and Götz 1999). The killing of the host by a pathogen is caused by a combination of several processes, including invasion of the pathogen into the host body tissues and their destruction (Inglis *et al.* 2001).

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 and death. Fungi from both orders differ in the way how they disperse the spores. Spore of Entomophthorales fungi are actively discharged from the cuticle by hydrostatic pressure, while later are spread by wind. If primary spores do not land on the host, it germinates and forms secondary spores. Resting spores allow Entomophthorales species to temporarily survive during unfavourable periods or the temporal

lack of new hosts. Conidia of Hypocreales fungi are passively spread by e.g. wind, water or by another insect (zoochory). Hypocreales fungi can survive in thenature without a new host as preserved cadavers or as conidia in the soil or generally saprotrophically on organic matter (Hajek and St. Leger 1994, Hajek and Shimazu 1996).

2.3.2. Factors affecting the effectiveness of entomopathogenic fungi

Insect infections caused by entomopathogenic fungi are conditioned by biotic factors (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 and Hoover 2006; Cory and Ericsson 2010; Jaronski 2010; Vega *et al.* 2012). Significant differences were noted not only among the different species of entomopathogenic fungi but also among their individual strains. Within one species of entomopathogenic fungi, there is a wide variety of strains that are differently tolerant to different environmental conditions. The strains can have differences in germination rates, vitality, growth, virulence to individual host species, and sporulation ability on the surface of the cadaver and spore production. Based on these characteristics, the strains can be compared, and the best strains can be good candidates for the production of bioproducts potentially use for practical biological control of plants against pests (Butt and Goettel 2000). One of the critical factors in the effective use of microbial agents as insecticides is their relatively short persistence on leaf surfaces and on soil surface (Gindin *et al.* 2000).

Abiotic factors

Abiotic or environmental factors like temperature, humidity and sunlight play an important role on the field persistence of entomopathogenic fungi. Abiotic factors which influence the virulence of entomopathogens must be considered for successive development of the fungus as a biocontrol agent (Inglis *et al.* 2001). These factors play a very significant role in the effectiveness of entomopathogenic fungi and hence on the course of infection in pest populations. In the order of decreasing relevance, humidity and temperature are most noticeable than other environmental factors. Factors as a composition and airflow, light and photoperiod are of less importance. Abiotic factors can influence predominantly the propagation of conidia, conidia germination, invasion hyphae penetration and finally fungus sporulation on the dead host (Drummond *et al.* 1987, Tanada and Kaya 1993, Inglis *et al.* 2001).

Temperature

The optimum temperature for the entomopathogenic fungi ranges from 20 °C to 25 °C (Osborne and Landa 1992). Hywel-Jones and Gillespie (1990) examined spore germination of *M. anisopliae* and *B. bassiana* at 20 - 30 °C and found higher germination levels in *M. anisopliae* as compared to *B. bassiana.* Most *Metarhizium* species, generally considered to be mesophilic fungi, have severely restricted growth below 10 °C and above 37 °C (Rangel 2006; Fernandes *et al.* 2008). Critical environmental factors can have a serious impact not only on growth and development, but also on pathogenicity of the fungal pathogen against the targeted pest (Faria and Wraight 2001). Many studies are focused on the determination of favourable temperature conditions of different strains, such as conidial germination (Fargues and Luz 1998, Thomas and Jenkins 1997, Bugeme *et al.* 2008a, Hywel-Jones and Gillespie 1990, Leemon and Jonsson 2008), or vegetative (hyphal) growth (Ekesi *et al.* 1999, Brooks *et al.* 2004) and production (Arthur and Thomas 2001, Tumuhaise *et al.* 2018).

The optimum temperature for the development of the fungus is not necessarily the same for the development of the disease. The rapidity of mycelial growth and development of infection depends on temperature. Infection and subsequent sporulation may range from 15 °C to 30 °C. In general, optimum values range between 20 °C and 30 °C (for example, 23 °C for *Beauveria brongiartii*, 24 °C for *Entomophthora obscura*, 25 °C for *Beauveria bassiana* and *Nomuraea rileyi* and 27 °C – 28 °C for *M. anisopliae*) with limits between 5° and 35°C. Temperatures lower than the optimum, distinctly retards the development of mycosis without necessarily affecting the total mortality. Temperatures above 30 °C commonly inhibit mycelial growth which is usually completely stopped at temperatures above 37 °C (Hywel-Jones and Gillespie 1990, Ferron 1978, Rangel *et al.* 2008).

However, the influence of temperature on the host insect must be taken into consideration, since very short periods between moults resulting from a high temperature may reduce, for example, the duration of the instar to an extent that penetration of the fungus through the integument is obstructed. The temperature tolerance can be affected by the existence of genotypes of fungus strains, which may also exhibit the stem-specific temperature differences. The geological origin of the strain matters,this means that a strain that has been isolated in a tropical or subtropical area may be more tolerant to higher temperatures, unlike a strain obtained from cooler areas, which, on the contrary, can develop better and faster even at low temperatures (Fargues and Luz 2000). Vidal *et al.* (1997) dealt with the influence of temperature on the rate of growth and the effectiveness of various isolates of *Isaria fumosorosea* against whitefly *Bemisia tabaci*. The strains used were

obtained from different insect hosts in different geographic regions (South America, Europe, Pakistan, Nepal and India). The temperature range for isolates obtained from the south of the USA (wet and dry subtropical regions) and from the region of West Asia (wet tropical region) was broad (8-35 °C) with optimal growth of isolates at 25 °C, 25-28 °C; 28 °C. Indian (dry tropical region) strains showed the highest tolerance to high temperatures (32 °C and 35 °C).

Humidity

The key factor in disease development in the pest population is also humidity, especially relative air humidity (RH %) (Luz and Fargues 1999). Of all the important factors, that influences the epizootics of mycopathogen, none is more critical for sporulation, germination, and invasion of the host than high humidity (Allen et al. 1971). Pathogenesis occurs at much lower surrounding values of temperature and humidity (Ferron 1978, Ramoska 1982). Relative atmospheric humidity is necessary for germination since most conidia of entomopathogenic fungi germinate at a humidity of more than 90% (Hall 1981). If the humidity is high on the insect cuticle, this microclimate allows the parasitic phase of its development. If the humidity is low on the insect cuticle, the fungus never causes disease of insect. The external sporulation never occurs on the killed insect, if the relative humidity is too low. In the natural environment, these humidity conditions occur during the day several times, with decreases and rises in temperature, whereby each living organism produces a water-saturated layer on the surface of the cuticle (Weiser 1966).

As a rule, the relative air humidity requirements increase considerably even in the sporulation period. Most entomopathogenic fungi need for sporulation relative humidity above 95% (Hall 1981). For example, it has been shown that entomopathogenic fungus *B. bassiana* sporulated on the surface of the mummified host (*Rhodnius prolixus*) at a relative humidity of 97 % (Fargues and Luz 2000).

In the case of inappropriate conditions, most entomopathogenic fungi produce only hyphae (or mycelium) inside the body of the infected host, and the saprotrophic phase develops under suitable conditions (Drummond *et al.* 1987). Under favourable conditions, the mycelium is more easily penetrated from the body cavity to the surface of the dead host and, with enough moisture, forms typical morphological structures related to sporulation (Arthurs and Thomas 2001). The disintegration of the infected individual can take place under less favourable humidity ratios. Because of the production of chitinolytic enzymes, the cuticle can be disintegrated (Milner *et al.* 1997).

Conditions affecting sporulation are key factors also for horizontal transmission

of fungi. Lower moisture is particularly suitable in the conidia spreading phase, especially in those types of fungi that produce conidia with a hydrophobic surface without mucilagenic cover (Hall 1981, Gottwald and Tedders 1982). Secondary infection again is limited by sufficiently high humidity (Arthurs and Thomas 2001). The study of the conditions necessary for sporulation allows understanding of the dynamics of the course of fungal epizootics as well as the optimization of the conditions of the environment to allow the development of secondary infection after application of myco-insecticides (Inglis *et al.* 2001, Arthurs and Thomas 2001).

The influence of moisture on the development of entomopathogenic fungi can be influenced to a limited extent. The addition of oily substances to the conidia suspension helps to bridge the critical period of germination of conidia of entomopathogenic fungi. The oil not only allows the conidia to adhere to the surface of the host but also creates suitable moisture conditions for germination. Oil is likely to reduce the need for high air humidity that is normally needed to induce infection. At the same time, it prevents the rapid destruction of conidia and protects it against UV rays (Prior et al. 1988, David-Henriet *et al.* 1998, Malsam *et al.* 2002).

Solar radiation

An important obstacle to field use of fungal pathogens against insect-pest control is their susceptibility to ultraviolet (UV) radiation which has been shown to reduce viability after only a short period (usually a few hours) of direct exposure to sunlight (Zimmermann 2007, Fargues *et al.* 1997a, Rangel *et al.* 2008). The fraction of the ultraviolet spectrum that is not filtered out by earth's atmosphere is composed of UV-A (wavelength 320-400nm) and UV-B (280-320nm). Of the two fractions, UV-B is considered more biologically damaging because of its direct interaction with DNA. UV-A, which can still cause damage, does so through oxidation (Fargues *et al.* 1997a, Braga *et al.* 2001, Fernandes *et al.* 2007).

Metarhizium is susceptible to both UV-A and UV-B which can cause delayed germination after only 1 hour of exposure and significant reduction in viability after only 2 hours. Some isolates of *M. acridum*, like ARSEF 324, are more tolerant to UV radiation and have greater than 50% germination after 4 hours of exposure (Braga *et al.* 2001, Rangel *et al.* 2008). Visible and infrared radiation is less harmful than UV radiation (Fargues *et al.* 1997a). Significant differences in sensitivity to radiation were also observed between entomopathogenic fungi. *M. flaviviridae* conidia were more resistant to artificial radiation than *B. bassiana, M. anisopliae, I. fumosorosea* (Fargues *et al.* 1996).

Soil

The soil is an extremely complex environment in which many factors are being applied to entomopathogenic fungi. The most important factors determining the occurrence and effects of entomopathogenic fungi include soil type (soil texture, organic matter content, and pH), moisture (water capacity) and soil microflora. These factors can affect the lifespan, persistence, and effectiveness of fungi that are used in biological plant protection as regulating microbiological agents (Inglis et al. 2001). Various forms of the formulation (WP, WDG, lyophilized mycelium, mycelial granules) are intentionally developed for the application of entomopathogenic fungi to the soil, and various forms of application (spraying, grouting, pickling, granulation and pellet injection) are used. Soil for many species of entomopathogenic fungi is a natural habitat, and the presence and natural occurrence of many species of fungi, such as *B. bassiana, M. anisopliae* and *I. farinosa* (Landa *et al.* 2002).

Fungi conidia persist in the soil of mild climate longer than blastospores (Butt 2002). Many entomopathogenic fungi can withstand different temperatures and conditions of high humidity and dryness (Inglis *et al.* 2001). Many studies have shown that conidia applied to the surface of soil or conidia introduced into the soil exhibit considerable stability in mild climate conditions (Zimmermann 2007). In the soil, fungi can survive on both organic matter and saprotrophic parts (Tanada and Kaya 1993).

2.3.3. Biotic factors affecting the effectiveness of entomopathogenic fungi

Pathogen

Pathogenicity is the ability of the pathogen to induce disease in the insect population and as such is dependent on a variety of factors such as the physiology of a targeted host (e.g., defence mechanisms), the physiology of entomopathogenic fungi (e.g., the production of enzymes and secondary metabolites) and environmental conditions. Fungi, as opposed to other entomopathogenic microorganisms, usually have a relatively wide range of hosts (McCoy *et al.* 1988, Inglis *et al.* 2001). The host spectrum of entomopathogenic fungi varies significantly depending on the species of fungus. For example, *Aschersonia aleyrodis* is strictly selective. Fungus *A. aleyrodis* infects only species of family Aleyrodidae and *Nomuraea rileyi* almost exclusively infecting butterfly caterpillars of the Noctuidae family. By contrast, the species belonging to the genera *Beauveria*, *Lecanicillium*, *Metarhizium*, *Isaria*, *Paecilomyces*, and *Tolypocladium* are generally broad polyphagous and can parasite

on insect reptiles (Inglis *et al.* 2001).

In one species of fungus, there are many strains belonging to one species of fungus which may have a narrow or wide host specialization due to their virulence. Strains with wide host specialization are more polyphagous than with narrow host specialization (McCoy *et al.* 1988). For the successful biological control of the insect population, it is necessary to select a suitable strain that is capable of penetrating and infecting individuals. The importance of the selected strain consists mainly of: a) easy cultivation and high and rapid sporulation on artificial area; b) high virulence of the selected strain against the targeted pest; c) the ability of the strain to withstand the environment in which the target pest is located (Meekes *et al.* 2000, Ravensberg 2010). In many strains of entomopathogenic fungi, their cultivation on artificial media or long-term storage may reduce the virulence to the target pest, or in a worse case, the virulence may be completely lost (Brownbridge *et al.* 2001, Goettel 1992). The rate of reduction to complete loss of virulence depends more on the individual strain than on the genus or species of entomopathogenic fungi. Some strains are able to maintain their virulence even after long-term repeated in vitro cultivation, e.g., *B. bassiana* (Inglis *et al.* 2001), *L. Lecanii* (Hall 1980), *I. farinose* (Hayden *et al.* 1992) they can completely lose virulence after few cultivations on artificial broth (Butt 2002). Nagaich (1973) states that the strain of *Lecanicillium lecanii*, which is virulent to aphid has lost its virulence already after the second passage via the artificial medium. To protect or increase the virulence of a strain used, therefore, it is often stressed that the need to maintain the virulence of strains by passage through a natural insect host (Hirte *et al.* 1989).

Host

Significant physiological and morphological factors affecting the development of diseases caused by entomopathogenic fungi in the insect host population include population density, behaviour and host bionomy, developmental stage, availability of food, genetic basis, possible mechanical or chemical injury or injury caused by parasites or predators. One of the most important aspects of fungal efficacy is stress. The stressed organism is more susceptible to disease caused by entomopathogenic fungus. Insect stress (individuals, populations) can be caused by one or more factors (population overcrowding, lack of food, exposure to chemical stressors, adverse environmental conditions or loss of immunity) (Inglis *et al.* 2001).

The development stage of the insects is a significant element of the predetermination of the course of the fungal disease. Not all stages of development of insects can be infected

with entomopathogenic fungi. In many cases, juvenile stages of insects (larvae, nymphs) are more susceptible to infection than adults (Butt and Goettel 2000, Inglis *et al.* 2001). Juvenile stages of insects are more susceptible to Hypocreales fungi because they have a weaker cuticle and the exterior cuticle is not as important a defensive barrier as in the case of adults (Weiser 1966). For example, young larvae of *Ostrinia nubilalis* are more susceptible to infection caused by *B. bassiana* spore than in adults (Feng *et al.* 1985).

Older instars nymphs of *Trialeurodes vaporariorum* are less susceptible to infection caused by *A. aleyrodis* than young instars, and adults are rarely infected with this fungus (Osborne and Landa 1992). On the other hand, the adult of *Frankliniella occidentalis* showed a higher susceptibility to infection caused by *L. lecanii* spores than was the case with nymphal stages (Vestergaard *et al.* 1995). Entomophthoraceae often infect older instars that are weakened after molting and are less resistant to younger individuals (Weiser 1966).

The density of the insect population is particularly important at the beginning and during the disease epizootics as it increases the likelihood of contact of healthy individuals with infected individuals. Process of transfers the spores from infected cadaver to healthy individual calls horizontal transmission. The process of transfers the spores from an infected cadaver to a healthy individual is called horizontal transmission. In addition, at a higher population density, a specific microclimate (temperature and RH %) and possible acceleration of the pathogen development occur within the pest population (Gindin *et al.* 2000). Introduction of fungal pathogens into the host population initiates epizootics and prevents or reduces the damage caused by the pest. Artificial epizootics have been accomplished for long-term use especially in areas with high humidity (Sandhu *et al.* 2012).

Host plant

Many characteristics (production of chemicals, growth, and morphology) of host plants can have a direct or indirect effect on the survival, development and efficacy of entomopathogenic fungi (Elliot *et al.* 2000). Plants produce a wide range of chemicals which depending on their nature, concentration and biological activity and it can affect the life of spores or pest susceptibility to entomopathogenic fungi (Vega *et al.* 1997, Butt 2002).

Experimentally, nymphs of the 3rd instar whitefly *T. vaporariorum* have been shown to be highly susceptible to infections caused by *B. bassiana* and *I. fumosorosea* fungi in cucumbers, whereas the same stage on tomato leaves was significantly less susceptible to infection. It has been shown that tomatoes has antimicrobial (including antifungal) effects (Poprawski *et al.* 2000). Poprawski and Jones (2001) found that whitefly *Bemisia tabaci* was

less susceptible to *B. bassiana* and *I. fumosorosea* infection than whitefly *T. vaporariorum* on cotton plants. The probable cause is that cotton plants produce gossypol, which has been shown to inhibit fungi growth.

2.4. Fungi of genus *Metarhizium*

Fungi of the genus *Metarhizium spp*. (Hypocreales: Clavicipitaceae) are cosmopolitan widespread, and the species naturally occur in agricultural and non-agricultural soils all over the world (Meyling and Eilenberg 2007, Sung *et al.* 2007). Bioagents of this genus are significant pathogens of insects and are widely used in biological control against significant soil pests (Zimmermann 2007).

Tulloch (1976) classified the species of genus *Metarhizium spp*. according to their morphological characteristics. The difference among the species is based on the length of the conidia, the presence of the sub-hymenial zone, formation of conidiophores with conidia, the color of conidia and fungal colony growth on artificial medium (Humber 1997). There are a number of different *Metarhizium* species that include for example *M. anisopliae, M. brunneum, M. flavoviride var. minus, M. pingshaese, M. cylindrosporae, M. guizhouensis, M. taii, M. pinsahensis* and *M. biformisporae* (Bidochka and Small 2005). Samson *et al.* (1988) recognized only three species of the genus of *Metarhizium*, which are pathogenic to insects, namely *M. anisopliae, M. flavoviride* and *M. album*. The fungus *M.anisopliae* is the most common species for its wide host range. *M. anisopliae* is pathogenic to over 200 host species and more can be discovered with time. Fungus *M. anisopliae* is a cosmopolitan fungus and its strains are widely isolated from different places around the World. The dense and dark green growth of mycelium of *Metarhizium* species often seen on the infected host is called „green muscardins“ disease (Zimmermann 2007).

Although some species of *Metarhizium* may not be formally recognized based on morphological characteristics, the genetic and molecular methods were used to assess the taxonomy of *Metarhizium*. The current taxonomy of *Metarhizium* is based on multilocus phylogenetic DNA sequence analyses. *M. anisopliae* and *M. flavoviride*, which are normally common species in the genus, are currently known species complexes. *M. pingshaense, M. anisopliae, M. robertsii*, and *M. brunneum* comprise the *M. anisopliae* species complex, which are called the PARB clade, and have particularly variety host ranges and global distributions (Bischoff *et al.* 2006, Bischoff *et al.* 2009). Multilocus phylogenetic analyses reveal pervasive congruent hierarchical structure among the genomic regions analyzed, which

suggest that current PARB species delimitations likely encompass additional cryptic complexes. Further, the interpolation of isolates from different continents throughout each species lineage indicates periodic inter-continental dispersals. As one example of the utility of IGS markers, the commercially registered *M. anisopliae* strain F52, which is widely used for pest control in North America, Canada and Europe, is shown to be a member of the *M. brunneum* complex (Rehner and Kepler 2017).

The morphology of the colonies can differ depending on the species or on the kind of artificial media. The edges of these colonies may be white with different thickness. Some *Metarhizium* species produce compact powdery colonies which are described as predominantly dark green, light green, white or brownish. Other species can produce filamentous white colonies that later sporulate and they are bicolour. These species very often sporulate in the centre of the colonies. The underside of the colony may be white, yellow, yellowish or brownish. The genus *Metarhizium* does not produce synnemata. Conidiophores are branched with apices of branches bearing one to several phialides that may be truncated or elongated. Conidia are hyaline, unicellular, green or brown and form chains (Brunner-Mendoza et al. 2019). The shape of conidia is ellipsoidal, and size depends on the species but generally from 4.0−14.5 × 2.0−5.0 μm. *M. majus* is the species with the largest conidia (8.5−14.5 × 2.5−2.0 μm) and *M. acridum* is the species with the smallest conidia (4.0−5.5 × 2.0−3.0 μm). Fungus *M. bruneum* produces conidia in size 4.5-8.0 x 6.0-13.5 μm (Bischoff *et al.* 2009). Conidia size of *M. flavoviride* is from 4.7–5.9 x 2.8-3.4 μm (Xavier-Santos *et al.* 1999). Conidia morphology is used for identification of species, but conidia morphology is indistinguishable between closely related species, such as *M. anisopliae*, *M. brunneum*, *M. pingshaense* and *M. robertsii* (Brunner-Mendoza *et al.* 2019).

In Colombia and Brazil, the phylogenic classes were distributed. Some *Metarhizium* species such as *M. anisopliae var. majus*, *M. flavoviride*, and *M. album* were reported to be specific to insects that include order Coleoptera, Orthoptera, and Hemiptera. While *M. flavoviride var. flavoviride* and *M. flavoviride var. minus* were also reported to show specificity to Coleoptera and Homoptera, respectively. This data research was obtained based on Allozyme polymorphisms. Generally, strains of *Metarhizium* that show host specificity have a tropical–subtropical distribution (St. Leger *et al.* 1992). Nashi and Sato (2017) analysed Japanese insect-derived isolates of *M. anisopliae* and *M. flavoviride* from species complexes to reveal their species diversity using phylogenetic analysesand the results showed that 57 isolates were identified as nine species. Fungus *M. pingshaense* was the most

frequently isolated species its and the 29 isolates came from six orders and 14 families of insects.

Around 58 registered and unregistered fungi of the genus *Metarhizium* are commercially produced in the world. The most important registered bioproducts are Green Muscle® and Met52. Bioproduct Muscle® was developed under the LUBILOSA research program. This product started to be important bioproduct mainly in Africa. Bio-product is based on strain IMI 330189 of fungus *M. anisopliae var. acridum* and is used against locusts (Acridoidea: Pyrgomorphoidea), grasshoppers and cicadas. Under favourable conditions, it causes epizootics in populations of locust populations in Africa. Bioproducts Met52 G and Met52 EC are manufactured and distributed by Novozymes Biologicals BioAg Group. Both bio-products contain the strain F52 entomopathogenic fungus *M. brunneum*. Granulated Met52 G is used against larvae and eggs of the *Otiorhynchus sulcatus* and *Otiorhynchus ovatus* while Met52 EC is used against greenhouse pests (thrips, mites, whiteflies) that seriously damage vegetables and ornamental plants (Faria and Wraight 2007).

3. MATERIAL AND METHODS

Fungal strain F52

Strain F52 of entomopathogenic fungus *M. brunneum* was re-isolated from MET52 on artificial medium PDA (Potato-dextrose agar, Himedia laboratories Pvt. Ltd., India). The isolate is stored in the alginate pellets in mycological collection in the Department of Genetic and Special Plant Protection, Faculty of Agriculture, University of South Bohemia, Czech Republic. For experiments, the strain F52 was activated on Petri dishes with PDA. The mother culture of strain F52 was maintained on PDA by regular sub-culturing on the same medium. The strain was incubated in thermostats (without light) for 14 days at different temperatures 15, 20, 25, 30 °C and 25 days at 10 °C before experiments. The inoculum produced at different temperature was consequently evaluated at different temperature (10, 15, 20, 25, 30 °C) to find out the optimal condition for its germination, growth and spore production. The efficacy of different inoculums on larvae of *T. molitor* was tested after incubation at 15, 20, 25, 30 °C. Below is a sample of the diagram that simply shows the wall process but in a simplified way.

Figure 5 Strain inoculum produced at each temperature was consequently tested for its quality using "in vitro" (germination, radial growth and spore production) and "in vivo" (virulence) test.

Inoculum produced at different temperature (10, 15, 20, 25, 30°C)	*Temperature of cultivation*		
	10°C	Radial growth and spore production	Germination (GI, %)
	15 °C	Radial growth, spore production and bioassay (mortality %, FDI)	Germination (GI, %)
	20 °C	Radial growth, spore production and bioassay (mortality %, FDI)	Germination (GI, %)
	25 °C	Radial growth, spore production and bioassay (mortality %, FDI)	Germination (GI, %)
	30 °C	Radial growth, spore production and bioassay (mortality %, FDI)	Germination (GI, %)

Insect

The mealworm, *Tenebrio molitor* (Coleoptera: Tenebrionidae) was obtained from rear at the Department of Plant Production, University of South Bohemia in Ceske Budejovice. Individuals they fed on mixtures such as groats, bran, flour and dog food. All stages were kept separately to avoid cannibalism and the rear was maintained at 25 ± 1°C and 16L:8D photoperiod.

Bioassays were conducted with synchronized population of *T. molitor* larvae. The larvae of the same size were collected and the surface was sterilized. The larvae were dipped for 3 seconds into the 3% solution of sodium hypochlorite (NaClO) and immediately they were rinsed three times in sterile water. Afterwards, the larvae were placed on filter paper for drying.

Germination and Growth Index

The full sporulated culture of *M. brunneum* was poured by 0.05% Tween 80 and the spores were scraped off from the surface using inoculation loop. The concentration of spores in the suspension was estimated in counting chambers, the spore suspension was adjusted to $1.0x10^7$ spores ml^{-1}. Before germination test, the sterile glass slide was covered with a thin layer of PDA. The drops of adjusted spore suspension were placed in two raw on the surface of medium. After soak of drops, the glass slide was placed into the water chamber (90mm Petri dish lined with wetted filter paper) and incubated at 10, 15, 20, 25 and 30 °C. The percentage of germination and growth index (GI) was determined after 24 hours under light microscope.

Table 1: Index scale used for evaluation of spore germination (Growth index, GI)

GI	Specification
0.0	spore has uniform shape, spore is not germinated
0.5	spore is elongated shape, the germ is observed in rate 1:0.5 (spore:germ)
1.0	the germ size is 1:1 (spore:germ)
1.5	the germ is 2–3 times longer than spore
2.0	the germ is more than 3 times longer than spore, branches of hyfae can be observed
2.5	sporulation begin, 1 or 2 spores in the chain are formed on the conidiophore
3.0	full sporulation, more than 2 spores are in the chains

Radial growth and spore production

For radial growth, one drop of adjusted suspension was inoculated with the inoculation loop in the middle of the Petri dish containing the PDA medium. The Petri dishes were incubated at different temperatures (10, 15, 20, 25 and 30 °C). For each temperature, 8 replications were prepared. The radial growth was evaluated after 7, 14 and 21 days. The

radial growth of colonies was measured in two perpendicular directions at right angles to each other. The colonies area in 1 mm^2 was calculated.

Spore production was determined separately from two central colonies after 21 days of incubation. Each central culture was cut from PDA plate and placed into the blender and adequate amount of solution with 0.05% detergent Tween 80 and the contents was properly mixed. The spore concentration was estimated in counting chamber. For each temperature, the spore production was assessed on 1mm2 of the culture.

Virulence against T. molitor larvae

For bioassay, the collected and sterilized larvae of *Tenebrio molitor* were used for bioassay. For each tested inoculums temperature, the 36 larvae were dipped singly into the strain suspension containing $1.0x10^7$spores ml^{-1} for 3 s. and another 36 larvae were dipped into 0.05% solution of Tween 80 for control. The surplus suspension or water was drained off quickly by suction with the filter paper (Goettel and Inglis 1997). The treated larvae were placed individually into the wet chambers (12 Multiwell culture plates, each containing well moistened filter paper) in three repetitions for each inoculums temperature. The multiwall plates were enclosed in polyethylene bags to prevent water loss during incubation and put into different tested temperatures (15 °C, 20 °C, 25 °C and 30 °C). Bioassay was not effective at 10 °C. The mortality and fungal development index (FDI) was assessed after 4 days for the first time and routinely daily until the 11^{th} day or till fully sporulation of fungus on cadaver (value 3.0 according to the Fungal Development Index – FDI). The FDI evaluation shown in figure 1; by a modified scale published in Landa *et al.* (1994).

Statistics

The differences in radial growth of colonies, spore production and mortality were compared using one-way analysis of variance (ANOVA, $p<0.05$ was considered significant). Colony size and spore production data were log10 (x+1) - transformed and mortality data were Arcsine square root-transformed before the analysis. Data are expressed as the mean ± SE. Statistical analyses were performed using StatSoft software StatSoft Inc. (2013).

Figure 6 (Part A): The scale used to evaluate the development of entomopathogenic fungus *M. brunneum* on a host *T. molitor* larva; Fungus development index - FDI (visual scale of the infection development on the larva body)

FDI 0.0	Larva is alive; no symptoms of infection are presents on the body surface	
FDI 0.5	Beginning of infection; the melanise spots are seen on the larva surface, but the larva is still alive; the larva movement can be restricted.	
FDI 1.0	Larva is dead; killed after the defence mechanisms of the larva inside the body have been colonized by fungus; more the melanise spots can be seen.	
FDI 1.5	**Fungus proliferation; the hyphae are present on the body surface; the visual infection of fungus is observed on the body.**	

Figure 6 (Part B): The scale used to evaluate the development of entomopathogenic fungus *M. brunneum* on a host *T. molitor* larva; Fungus development index - FDI (visual scale of the infection development on the larva body)

FDI 2.0	**The compact mycelium on the body surface; larva is overgrown by the fungus.**	
FDI 2.5	**Beginning of sporulation; the light green colour of sporulation is observed on the larva body surface.**	
FDI 3.0	**Full sporulation; fungus complete its life cycle; the green muscardina is observed on the larva body surface.**	

4. RESULTS

The temperature profile of the entomopathogenic fungi of the strain F52, shows how the strain produced at different temperatures can subsequently evolve under favourable or unfavourable temperature conditions. Growth, development, and virulence of the strain were tested. The parameters of central cultures were evaluated, the radial growth of the central cultures was evaluated and the area of the central culture in mm^2 was calculated. Furthermore, spore production from the central culture was set and consequently, the spore production per 1 mm^2 was counted.

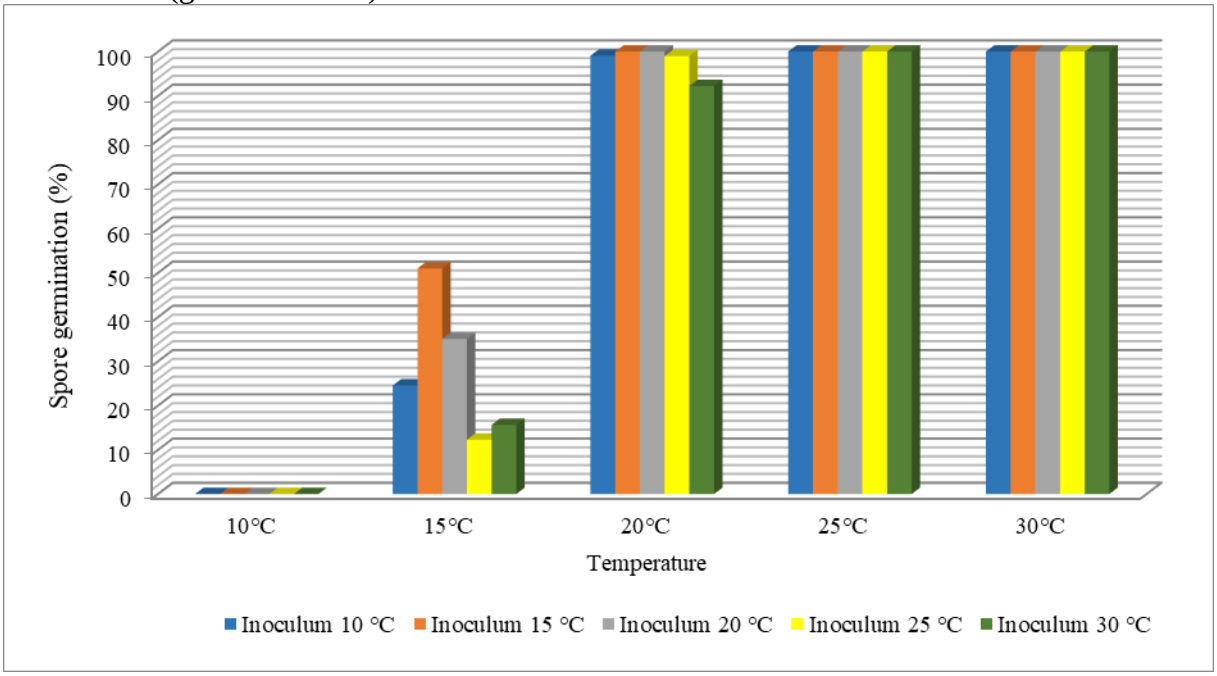
Virulence of the strain was in the interaction among pathogen, susceptible host and favourable abiotic conditions. The larvae of *T. molitor* were used as a susceptible host. Virulence of the strain was tested under the favourable conditions for strain development, i.e. RH 95 – 100 %, at 20 °C. The bioassay was established for all the inoculums. However, the virulence bioassay was not established at 10°C because of screening time and this temperature is unfavourable not only for strain development but also for infection of larvae *T. molitor*.

The radial growth and colony area are presented in Table 2 – 6. Inoculum temperature plays a very important role in strain development. The production of inoculums at 10°C was produced for a long time approximately 30 days in comparison with other inoculums production in other temperatures. In higher temperature profiles, the results of the inoculum were obtained after 10-15 days.

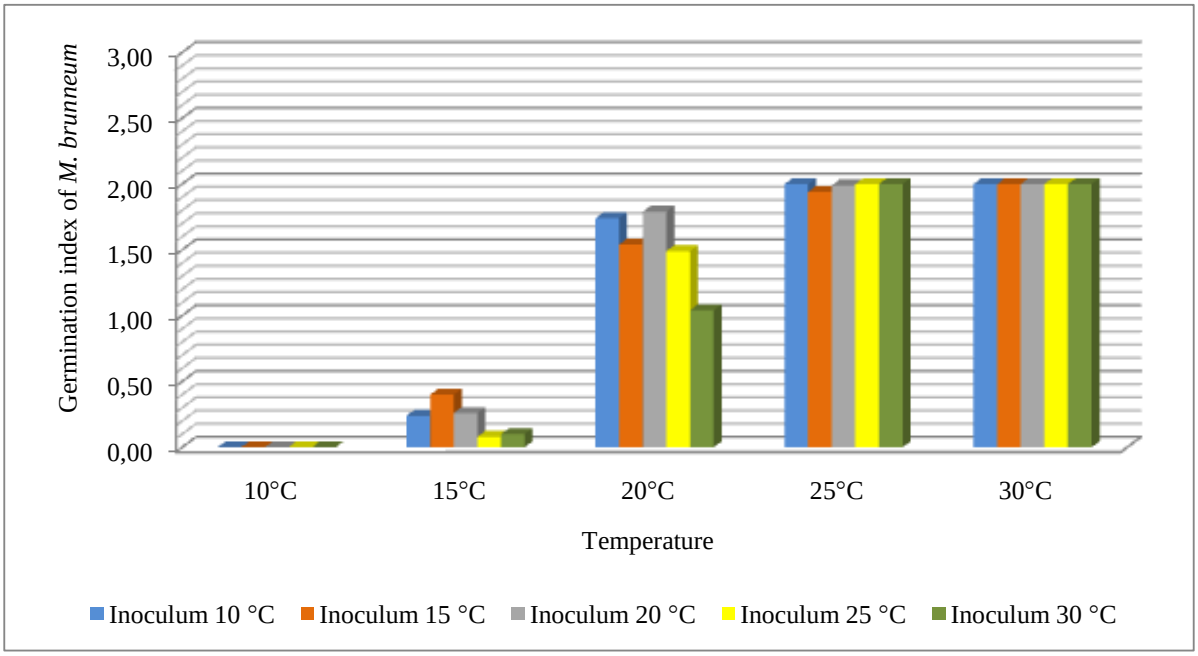
The germination test

All spores from all the produced inoculums at temperatures 25 and 30 °C germinated after cultivation. While at 20 °C incubation, the germination among the inoculums ranged between 92.21 % (inoculum from 30 °C) to 100 % (inoculum from 15 and 20 °C). Considerable lower germination of spores was recorded after incubation at 15 °C. The inoculum produced at 15 °C was the most germinated variant (50.98 %) after incubation, as compared to other inoculums incubated at the same temperature. According to the results, this inoculum was adapted to the same temperature where it was originally produced. The lowest germination at 15 °C was observed in inoculums obtained from 25 °C (12.28 %) and 30 °C (15.63 %). But from all the inoculums incubated and cultivated at 10°C did not germinate.

Graph 1 the comparison of spore germination of inoculums cultivated in various temperatures for 24 hours (germination %)



Graph 2 the comparison of germination index of inoculums cultivated in various temperatures for 24 hours (germination %)



At the same time, the germination index (GI) was evaluated with the germination rate of all evaluated inoculums. The germination index describes in detail the phase of growth of entomopathogenic fungus spore. The GI scale ranges from 0 to 3 and is described in the material and methods chapter. The GI was evaluated after 24 hours. All of the *M. brunneum* inoculums reached a similar germination index under different temperatures of cultivation. After cultivation, all the inoculum at 25 and 30 °C reached index 2 except for the inoculum

produced at 15 °C. A lower GI (1.94) was observed here. The GI for inoculum at 20 °C was more varied. The range was from 1.04 (inoculum from 30 °C) to 1.79 (inoculum from 20 °C). A similar phenomenon was also observed for inoculum at the culture temperature of 15 °C, slight differences among the inoculums variants in the GI were observed. When the inoculums were cultivated at 10 °C, spores of F52 strain did not germinate (GI = 0) after 24 hours (GI=0).

Radial growth of inoculums of strain F52 produced at different temperatures

The spores of *M. brunneum* produced at 10 °C showed the largest central colonies at 25 °C during cultivation. The area of colonies cultivated at 20 °C and 30 °C were almost similar after 7 and 14 days of measurement. After 21 days, higher differences between colonies were observed. The length of radial colony was longer, about 6 cm from the colony obtained from 30 °C as compared to the colony produced at 15 °C which was shorter. The measurements of radial growth correlate with the area in 1 mm^2 of central culture. The smallest cultures were observed during incubation at 10°C. After 7 days, the inoculums were not growing at this temperature. The cultures kept the trend of their size and area in all observed days during incubation from all the tested inoculum from different temperatures. (see Table 2 and 7).

Table 2 Influence of inoculum production temperature of entomopathogenic fungus *M. brunneum* strain F52 on radial growth and area (mm^2) of central cultures cultivated at 10 °C during 21 days (mean ± smodch)

Parametr /Day		10°C	15°C	20°C	25°C	30°C
Ø culture	Day 7	0.00±0.00	10.88±0.34	15.13±0.34	24.00±0.00	16.19±0.40
	Day 14	4.69±0.48	22.81±0.40	30.81±0.54	46.38±0.81	32.94±0.44
	Day 21	10.00±0.00	35.00±0.00	43.38±0.50	67.94±1.29	49.50±1.03
Parametr /Day		10°C	15°C	20°C	25°C	30°C
area (mm2)	Day 7	0.00±0.00 e	92.89±5.63 d	179.67±8.32 c	452.39±0.00 a	205.80±10.45 b
	Day 14	17.26±3.38 e	408.73±14.25 d	745.66±26.29 c	1689.11±58.75 a	852.06±22.84 b
	Day 21	78.54±0.00 e	962.10±0.00 d	1477.60±34.16 c	3625.00±136.78 a	1924.42±78.79 b

a,b,c Means within a row followed by the same letter are not significantly different (ANOVA procedure; α=0.05; Tukey HSD test)

For the inoculum of strain F52 produced at 15°C, the most suitable temperature for development and showed better results was 25 °C. After 21 days of incubation, the culture of *M. brunneum* produced colonies in diameter 63.81 mm in diameter (3198.17 mm^2).

The colonies of *M. brunneum* increased the growth from the lowest cultivation temperature 10 °C to a higher temperature of 25 °C. On the contrary, the highest temperature of 30°C, the colonies were slightly suppressed in comparison to 25°C. The inoculum of strain obtained from 15°C, formed the average area of the colony only 2078.02 mm2 cultivated at 30 °C after 21 days, so it is around 35% area, the colony was smaller than that observed from the 25 °C temperature. The lowest cultivation temperature is not optimal for the growth of *M. brunneum.* After 7 days, the inoculum produced at 15 °C was not able to grow at 10 °C. The *M. brunneum* inoculum produced at 15 °C showed statistically significant differences in growth at all cultivated temperatures during 21 days (see Table 3 and Table 7).

Table 3 Influence of inoculum production temperature of entomopathogenic fungus *M. brunneum* strain F52 on radial growth and area (mm^2) of central cultures cultivated at 15 °C during 21 days (mean ± smodch)

Parametr /Day		10°C	15°C	20°C	25°C	30°C
Ø culture	Day 7	0.00±0.00	8.69±0.48	13.88±0.72	21.94±0.77	17.69±0.60
	Day 14	4.13±0.34	19.13±1.15	27.25±0.58	42.69±0.60	34.81±0.75
	Day 21	8.44±0.81	29.69±1.35	40.38±0.71	63.81±0.83	51.44±1.55
Parametr /Day		10°C	15°C	20°C	25°C	30°C
area (mm2)	Day 7	0.00±0.00 e	59.28±6.39 d	151.20±15.74 c	377.98±26.65 a	245.71±16.78 b
	Day 14	13.36±2.41 e	287.27±34.78 d	583.21±24.72 c	1431.17±39.90 a	951.83±40.73 b
	Day 21	55.91±10.27 e	692.21±62.75 d	1280.31±50.76 c	3198.17±83.43 a	2078.02±127.73 b

a,b,c Means within a row followed by the same letter are not significantly different (ANOVA procedure; α=0.05; Tukey HSD test)

Inoculum of *M. brunneum* produced at 20 °C showed similar size and areas of colonies as in the previous variants. The culture temperature for the inoculum of 25 °C, was again optimal for growth and development of the strain colonies. The strain produced at 25 °C, were the largest colonies from all the tested inoculums cultivated at the same temperature after 21 days of incubation. At 25 °C, the area of colony was 3786.85 mm^2. It is evident that for the production of inoculum the most optimal temperature is just 20 °C, which provides the strain F52 of *M. brunneum* with optimal growth conditions during incubation at 25 °C. The cultivation temperature of 30 °C again slightly inhibits fungal growth in comparison with cultivation temperature of 25 °C. The growth of the strain F52 was not observed at 10 °C after 7 days of incubation. Cultivated temperature 15 °C and 20 °C are not the best conditions for colonies growth. At these temperature colonies were produced of area 842.39 mm^2 (15 °C) and 1473.38 mm^2 (20 °C), respectively. However, the statistical

differences were observed in colonization of Petri dishes from inoculum produced at 20 °C over the time (see Table 4 and Table 7).

Table 4 Influence of inoculum production temperature of entomopathogenic fungus *M. brunneum* strain F52 on radial growth and area (mm^2) of central cultures cultivated at 20 °C during 21 days (mean ± smodch)

Parametr /Day		10ºC	15ºC	20ºC	25ºC	30ºC
Ø culture	Day 7	0.00±0.00	10.00±0.00	14.75±0.45	25.50±0.73	16.31±0.70
	Day 14	4.75±0.45	21.94±0.44	28.81±0.54	48.56±0.51	30.50±0.63
	Day 21	10.50±0.52	32.75±0.45	43.31±0.60	69.38±1.26	46.00±0.00
Parametr /Day		10ºC	15ºC	20ºC	25ºC	30ºC
area (mm2)	Day 7	0.00±0.00 a	78.54±0.00 b	170.87±10.19 c	510.70±28.82 e	208.99±17.86 d
	Day 14	17.72±3.16 a	377.98±15.20 b	652.01±24.58 c	1852.22±39.03 e	730.62±30.55 d
	Day 21	86.59±8.52 a	842.39±22.83 b	1473.38±41.43 c	3786.85±137.23 e	1661.90±37.31 d

a,b,c Means within a row followed by the same letter are not significantly different (ANOVA procedure; α=0.05; Tukey HSD test)

The only inoculum produced at 25 °C was able to grow at 10 °C after 7 days of incubation. The colony reached 3.5 mm in diameter (9.62 mm^2). After 14 and 21 days, the area of colonies was 19.63 mm^2 and 78.54 mm^2, respectively.

Table 5 Influence of inoculum production temperature of entomopathogenic fungus *M. brunneum* strain F52 on radial growth and area (mm^2) of central cultures cultivated at 25 °C during 21 days (mean ± smodch)

Parametr /Day		10ºC	15ºC	20ºC	25ºC	30ºC
Ø culture	Day 7	3.50±0.52	9.63±0.50	14.81±0.40	22.56±0.51	16.19±0.40
	Day 14	5.00±0.00	21.00±0.00	29.81±0.40	43.94±0.57	31.88±0.54
	Day 21	10.00±0.00	33.00±0.00	43.19±0.66	65.00±0.00	45.88±0.81
Parametr /Day		10ºC	15ºC	20ºC	25ºC	30ºC
area (mm2)	Day 7	9.62±2.84 a	72.76±7.46 b	172.32±9.18 c	399.82±18.11 e	205.80±10.45 d
	Day 14	19.63±0.00 a	346.36±0.00 b	698.05±18.68 c	1516.21±39.59 e	797.98±16.90 d
	Day 21	78.54±0.00 a	855.30±0.00 b	1464.89±44.87 c	3318.30±0.00 e	1652.88±58.21 d

a,b,c Means within a row followed by the same letter are not significantly different (ANOVA procedure; α=0.05; Tukey HSD test)

It is possible that the inoculum produced at 25 °C is able to increase the adaptation to lower temperature. The similar size of colonies was again observed at the other cultivated temperatures in comparison to previously evaluated inoculums. The inoculum produced

at 25 °C produced significantly different colonies at all tested temperature during all evaluated days (see Table 5 and Table 7). After 21 days, the colony area produced at 25 °C was 3318.30 mm^2.

An assumption for inoculum produced at 30 °C was that the strain F52 increases the adaptation to higher temperatures, specifically for consequently cultivation of strain at 30 °C. The idea was not confirmed. The temperature 30 °C for cultivation was not optimal for the growth of strain F52 of *M. brunneum*. In comparing to temperature 25 °C for cultivation, colonies were smaller in size and area, in all evaluated days. The 25 °C was again optimal for growth of strain. The colonies area was at this temperature of cultivation 3585.10 mm^2 after 21 days of incubation. The area is almost two times larger than the area of colony produced at 30 °C. The colonization of the Petri dishes by an inoculum of strain F52 produced at 30 °C was statistically significant at all temperatures of cultivation (see Table 6 and Table 7). After 7 days, the strain produced at 30 °C did not grow again after incubation at 10 °C. The growth was observed after 14 days when the average of the colony was 7.31 mm in diameter.

Table 6 Influence of inoculum production temperature of entomopathogenic fungus *M. brunneum* strain F52 on radial growth and area (mm^2) of central cultures cultivated at 30 °C during 21 days (mean ± smodch)

Parametr /Day		10°C	15°C	20°C	25°C	30°C
Ø culture	Day 7	0.00±0.00	10.06±0.25	15.00±0.00	24.00±0.37	16.69±0.48
	Day 14	7.31±0.70	23.00±0.00	30.00±0.00	46.44±0.63	32.81±0.40
	Day 21	13.50±0.52	35.13±0.81	44.00±0.52	67.56±0.63	47.94±0.57
Parametr /Day		10°C	15°C	20°C	25°C	30°C
area (mm2)	Day 7	0.00±0.00 a	79.52±4.12 b	176.71±0.00 c	452.39±13.77 e	218.71±12.41 d
	Day 14	42.00±8.83 a	415.48±0.00 b	706.86±0.00 c	1693.66±46.22 e	845.61±20.58 d
	Day 21	143.14±10.95 a	969.00±44.37 b	1520.53±35.69 c	3585.10±66.45 e	1804.85±43.20 d

a,b,c Means within a row followed by the same letter are not significantly different (ANOVA procedure; α=0.05; Tukey HSD test)

Table 7 Statistical evaluation of the effect of different temperatures of *M. brunneum* on colony area colony production (mm^2) from inoculum produced at different temperatures

Inoculum temp. (°C)	Day 7	Day 14	Day 21
10°C	F=56199,4; df=4,75;p<0.0000	F=5633,3; df=4,75;p<0.0000	F=45529,0; df=4,75;p<0.0000
15°C	F=14241,4; df=4,75;p<0.0000	F=6887,4; df=4,75;p<0.0000	F=3940,5; df=4,75;p<0.0000
20°C	F=32717,3; df=4,75;p<0.0000	F=6107,5; df=4,75;p<0.0000	F=11324; df=4,75;p<0.0000
25°C	F=1759,61; df=4,75;p<0.0000	F=119431; df=4,75;p<0.0000	F=76896; df=4,75;p<0.0000
30°C	F=73231; df=4,75;p<0.0000	F=4984,8; df=4,75;p<0.0000	F=12587; df=4,75; p<0.0000

From all inoculums of strain F52 produced at different temperatures, the largest area of colonies in all variants was produced after cultivation at 25 °C for 21 days. The area among all inoculum variants was statistically significant (F=70.0; df=4,75; p<0.0000). The largest colony area was recorded at this temperature from the inoculum produced at 20 °C (3787 mm^2) and subsequently from the inoculum at 10 °C (3625 mm^2). On the contrary, the smallest colony was observed from inoculum produced at 15 °C (3198 mm^2). At cultivated temperature 30 °C, the largest colony produced a strain F52 from the inoculum produced at 15 °C and a slightly smaller colony was recorded from the inoculum produced at 10 °C. The size of colonies were also statistically significant (F=100.2; df = 4,75; p<0.0000).

Table 8 Influence of inoculum production temperature of *M. brunneum* on area size (mm^2) of central culture after 21 days of cultivation (mean±SE)

Cultiv temp.	Temperature of inoculum °C				
	10°C	15°C	20°C	25°C	30°C
10°C	78.54±0.00 Eb	55.91±10.72 Ec	86.59±8.52 Eb	78.54±0.00 Eb	143.14±10.95 Ea
15°C	962.11±0.00 Da	692.21±62.75 Dc	842.39±22.83 Db	855.30±0.00 Db	969.00±44.37 Da
20°C	1477.64±34.16 Cab	1280.31±50.76 Cc	1473.38±41.43 Cb	1464.89±44.87 Cb	1520.53±35.69 Ca
25°C	3625.01±136.78 Ab	3198.17±83.43 Ad	3786.85±203.41 Aa	3318.30±0.00 Ac	3585.10±66.45 Ab
30°C	1924.42±78.79 Bb	2078.02±127.73 Ba	1661.90±37.31 Bd	1652.88±58.21 Bd	1804.85±43.20 Bc
	*F=5326.8;df=4,75; p<0.0000	*F=4010.7;df=4,75; p<0.0000	*F=3404.2;df=4,75; p<0.0000	*F=21343.0;df=4,75 ; p<0.0000	*F=13498.8;df=4,75 ; p<0.0000

*A.B.C – Means within a column followed by the same letter are not significantly different (ANOVA procedure; α=0.05; Tukey HSD test) (Anova for column)

⋆ a,b,c Means within a row followed by the same letter are not significantly different (ANOVA procedure; α=0.05; Tukey HSD test)

The inoculums produced at all temperatures formed almost balanced colonies after their incubation at 20 °C. However, the colony area produced from different inoculums was again statistically significant (F=84.0; df=4,75; p<0.0000). After cultivation of strain F52 at 15 °C, the largest colonies produced strain from inoculum obtained from extreme

temperatures of 10 °C (962.11 mm^2) and 30 °C (969.00 mm2). The smallest colony was recorded from the inoculum produced at 15 °C (F=133.0; df=4,75; p<0.0000). The cultivation temperature 10 °C is not optimal for growth of entomopathogenic fungus *M. brunneum*. However, the big differences were observed among the inoculums variants. The largest colony was obtained from inoculum produced at 30 °C (143.14 mm^2). On the contrary, the smallest colony was produced from inoculum obtained from temperature 15 °C (55.91 mm^2) (F=173.7; df = 4,75; p<0.0000).

For all the tested inoculums of strain F52, the colony area increased depending on the increasing temperature of cultivation from 10 °C to 25 °C. After 21 days, strain inoculums cultivated at 30 °C produced smaller colonies in comparison to colonies growth at 25 °C. Increase in colonies growth from inoculums was not detected at 30 °C. The strain from all inoculums produced almost identical area of colonies after cultivation at 30 °C and cultivation at 20 °C. Influence of inoculum temperature is statistically significant on consequent growth of strain F52 at different temperature in all variants after 21 days of incubation (see Table 8).

Spore production of strain F52 inoculums

The same phenomenon in spore production appears as in the experiment where the growth properties of inoculums of strain F52 cultivated at different temperature after 21 days of incubation. The optimum temperature for spore production of *M. brunneum* from all inoculums was 25 °C (F=365.0; df=4,5; p=0.0000).

Table 9 Influence of inoculum production temperature of *M. brunneum* on spore production per culture after 21 days of cultivation (mean±SE)

Cultiv temp.	Temperature of inoculum °C				
	10°C	15°C	20°C	25°C	30°C
10°C	$3.28\pm0.18x10^6$	$5.43\pm0.38x10^6$	$4.60\pm0.20x10^6$	$4.93\pm0.23x10^6$	$4.88\pm1.38x10^6$
15°C	$2.70\pm0.00x10^8$	$2.94\pm0.06x10^8$	$3.71\pm0.04x10^8$	$2.49\pm0.24x10^8$	$1.44\pm0.06x10^8$
20°C	$5.95\pm0.25x10^8$	$7.98\pm0.52x10^8$	$1.08\pm0.01x10^9$	$5.98\pm0.03x10^8$	$3.58\pm0.51x10^8$
25°C	$2.53\pm0.03x10^{10}$	$2.72\pm0.05x10^{10}$	$1.80\pm0.04x10^{10}$	$3.05\pm0.00x10^{10}$	$1.97\pm0.00x10^{10}$
30°C	$9.84\pm0.34x10^8$	$1.19\pm0.00x10^9$	$6.18\pm0.18x10^8$	$5.58\pm0.48x10^8$	$6.77\pm0.02x10^8$

Table 10 Influence of inoculum production temperature of *M. brunneum* on spore production per 1 mm^2 of colony after 21 days of cultivation (mean±SE)

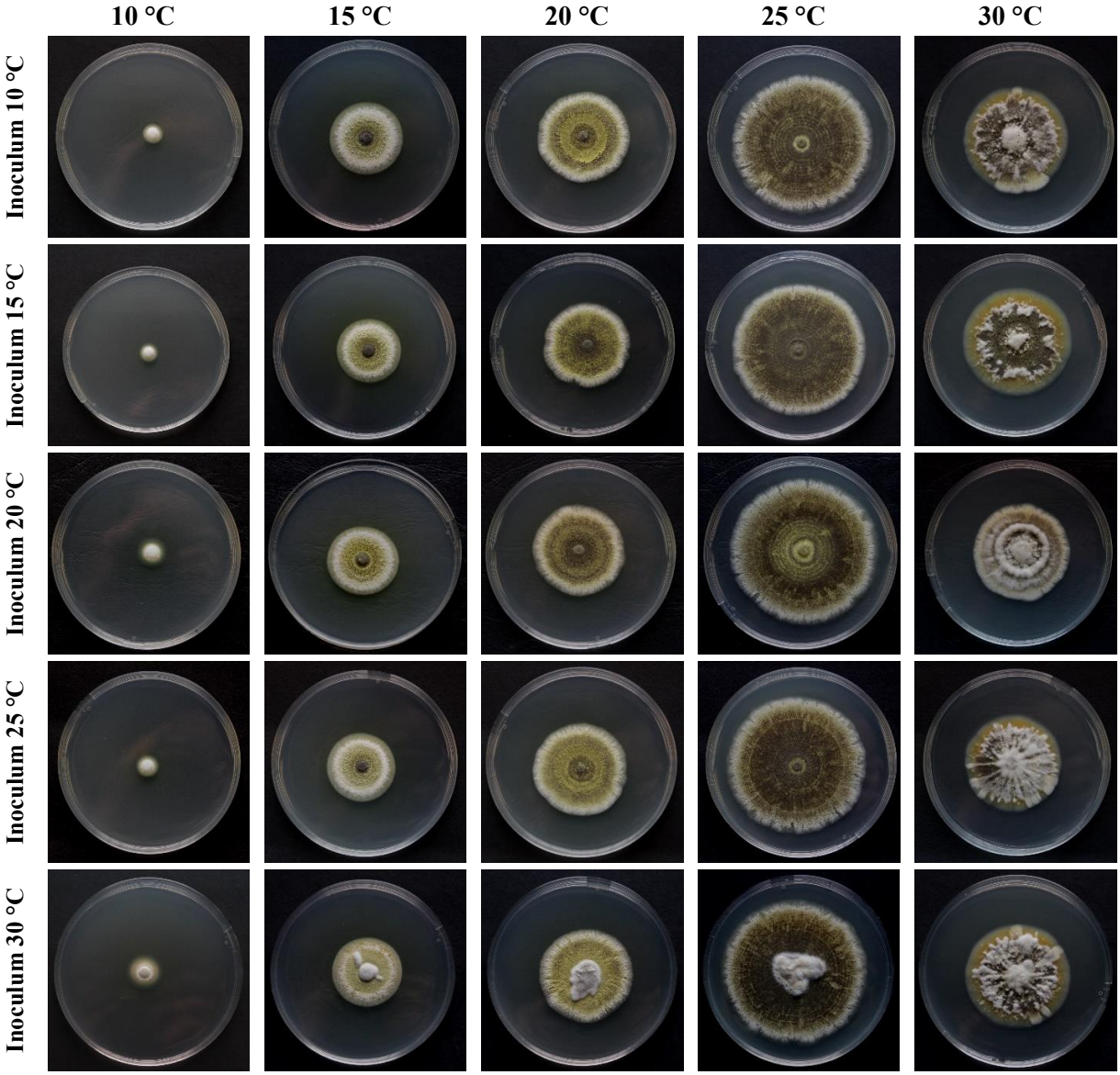
Cultiv temp.	Temperature of inoculum °C				
	10°C	15°C	20°C	25°C	30°C
10°C	$4.17\pm0.22x10^4$ Eb	$9.70\pm0.67x10^4$Da	$5.31\pm0.23x10^4$ Eab	$6.27\pm0.29x10^4$ Cab	$3.41\pm0.96x10^4$ Db
15°C	$2.81\pm0.00x10^5$ Db	$4.25\pm0.08x10^5$Ca	$4.41\pm0.04x10^5$Ca	$2.92\pm0.29x10^5$ Bb	$1.48\pm0.06x10^5$ Cc
20°C	$4.03\pm0.17x10^5$ Cb	$6.23\pm0.41x10^5$Ba	$7.33\pm0.07x10^5$ Ba	$4.08\pm0.02x10^5$ Bb	$2.36\pm0.33x10^5$ BCc
25°C	$6.98\pm0.07x10^6$ Ab	$8.50\pm0.16x10^6$ Aa	$4.97\pm0.12x10^6$ Ad	$9.19\pm0.02x10^6$ Aa	$5.48\pm0.00x10^6$ Ac
30°C	$5.12\pm0.18x10^5$Ba	$5.71\pm0.01x10^5$Ba	$3.72\pm0.11x10^5$ Db	$3.37\pm0.29x10^5$ Bb	$3.75\pm0.01x10^5$ Bb
	F=2824.0;df=4,5; p<0.0000	F=1324.4;df=4,5; p<0.0000	F=3851.0;df=4,5; p<0.0000	F=871.8;df=4,5; p<0.0000	F=164.4;df=4,5; p=0.0000

*A.B.C – Means within a column followed by the same letter are not significantly different (ANOVA procedure; α=0.05; Tukey HSD test) (Anova for column)

* a,b,c Means within a row followed by the same letter are not significantly different (ANOVA procedure; α=0.05; Tukey HSD test)

The production of spores per mm^2 from all inoculums was increased from the lowest temperature of cultivation up to temperature 25 °C. The optimum temperature for spore production of *M. brunneum* from all inoculums was 25 °C. The spore production of all variant cultivated at 25 °C ranged from $4.97x10^6$ per mm^2 (inoculum from 20 °C) to $9.19x10^6$ per mm^2 (inoculum from 25 °C). The strain F52 from all inoculums produced the lowest amount of spores At 10°C of cultivation in comparison to the other cultivated temperatures. 10 °C is not suitable for spore production from all inoculums of the strains F52 of fungus *M. brunneum*. The lowest spore production at this temperature was observed from inoculum produced at 30 °C, the spore yield was $3.41x10^4$. However, the spore production was in this lowest temperature of incubation statistically significant (F=8.92; df=4,5; p=0.0169). The spore production per mm^2 obtained from inoculums produced at 15, 20 a 30 °C was ranged in the order of 10^5. The lowest production was determined at variant where the inoculum from 30 °C was cultivated at 15 °C (1.48×10^5 per mm^2) and the highest production was obtained from variant where the inoculum from 20°C was cultivated at 20 °C ($7,33 \times 10^5$ per mm^2). These temperatures are not suitable for inoculum production of strain F52 in comparison with the productive temperature 25 °C. Differences among spore production at 15 °C (F=80.5; df=4,5; p=0.0001), 20 °C (F=37.6; df=4,5; p=0.0006) and 30 °C (F=28.2; df=4,5; p=0.0013) were also statistically significant.

Figure 7 Morphotypes of entomopathogenic fungus *M. brunneum* strain F52 (inoculum temperature/cultivation temperatures)



All inoculums obtained from various temperatures and consequently cultivated in different temperature conditions had the same course in the production of spores. The lowest production is determined after cultivation of strain F52 at 10 °C and the highest at 25 °C. The statistical evaluation is included in Table 10.

Efficacy of strain F52 inoculums

Efficacy did not set up at 10 °C of incubation because it takes long to be effective. But all inoculums produced in different temperature on larvae of *T. molitor* were evaluated on 4th day after incubation at 15, 20, 25 and 30 °C and then continuously evaluated until the 8th day

of incubation. The last day of the bioassay observation was the 11th day. Statistical analysis was performed on day 5 ($F=3442,81$; $df=1,16$; $p<0.0000$) and 6 ($F=15083,0$; $df=1,16$; $p<0.0000$) of the bioassay. The individual inoculums tested varied significantly among each other in the larvae mortality of *T. molitor* and the course of infection on larvae.

Table 11 Influence of inoculum production temperature of *M. brunneum* on larvae mortality of *Tenebrio molitor* and Fungus Development Index (mean±SE)

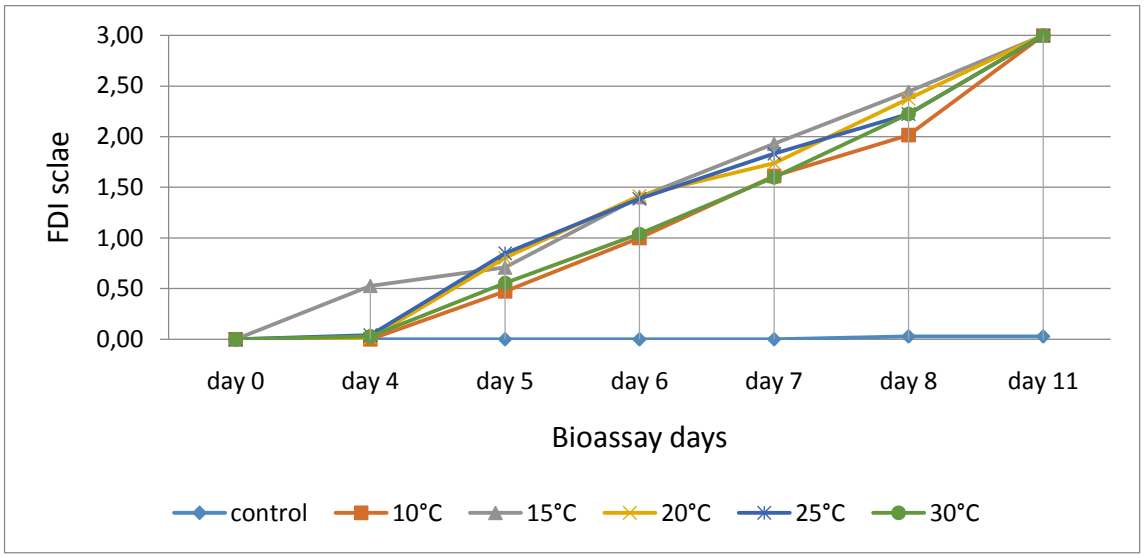
Incub. Temp.	Cultiv. temp.	Day 4	Day 5		Day 6		Day 7	Day 8	Day 11
		mean±SE	mean±SE	Tukey	mean±SE	Tukey	mean±SE	mean±SE	mean±SE
10°C	control	0.00±0.00* (0.00±0.0)**	0.00±0.00 (0.00±0.0)	g	0.00±0.00 (0.00±0.0)	c	0.00±0.00 (0.00±0.0)	0.00±0.00 (0.00±0.0)	0.00±0.00 (0.00±0.0)
	15°C	0.00±0.00 (0.0±0.0)	2.78±4.81 (0.47±0.13)	g	69.41±12.73 (1.00±0.08)	ab	97.21±4.83 (1.61±0.02)	97.21±4.83 (2.01±0.05	100.0±0.0 (3.00±0.00)
	20°C	0.00±0.00 (0.03±0.02)	13.87±4.80 (0.51±0.06)	cdefg	86.08±4.81 (1.14±0.05)	ab	100.0±0.0 (1.63±0.04)	100.0±0.0) (2.03±0.06	100. 0±0.0 (3.00±0.00)
	25°C	0.00±0.00 (0.00±0.0)	16.65±14.42 (0.44±0.09)	bcdefg	91.64±8.35 (1.22±0.19)	a	100.0±0.0 (1.58±0.23)	100.0±0.0) (2.04±0.23	100.0±0.0 (3.00±0.00)
	30°C	0.00±0.00 (0.03±0.02)	16.65±14.42 (0.50±0.07)	bcdefg	88.86±12.75 (1.15±0.15)	a	100.0±0.0 (1.51±0.02)	100.0±0.0) (2.03±0.10	100.0±0.0 (3.00±0.00)
15°C	control	0.00±0.00 (0.00±0.0)	0.00±0.00 (0.00±0.0)	g	0.00±0.00 (0.00±0.0)	c	0.00±0.00 (0.00±0.0)	2.78±4.81 (0.03±0.05)	2.78±4.81 (0.03±0.05)
	15°C	3.76±4.81 (0.53±0.05)	38.86±17.34 (0.71±0.14)	abcdef	100.0±0.0 (1.40±0.10)	a	100.0±0.0 (1.93±0.10)	100.0±0.0 (2.44±0.09)	100.0±0.0 (3.00±0.00)
	20°C	5.55±4.81 (0.53±0.06)	11.10±4.80 (0.61±0.05)	defg	74.96±8.34 (1.11±0.05)	ab	100.0±0.0 (1.64±0.13)	100.0±0.0 (1.90±0.21)	100.0±0.0 (3.00±0.00)
	25°C	0.00±0.00 (0.46±0.00)	49.96±8.33 (0.88±0.07)	abc	97.21±4.83 (1.47±0.05)	a	100.0±0.0 (2.06±0.02)	100.0±0.0 (2.43±0.05)	100.0±0.0 (3.00±0.00)
	30°C	0.00±0.00 (0.44±0.02)	49.96±8.33 (0.96±0.07)	abc	86.07±9.63 (1.35±0.17)	ab	100.0±0.0 (2.07±0.13)	100.0±0.0 (2.32±0.12)	100.0±0.0 (3.00±0.00)
20°C	control	0.00±0.00 (0.00±0.00)	0.00±0.00 (0.00±0.00)	g	0.00±0.00 (0.00±0.00)	c	0.00±0.00 (0.00±0.00)	0.95±3.93 (0.03±0.05)	0.95±3.93 (0.03±0.05)
	15°C	0.00±0.00 (0.01±0.02)	52.74±12.73 (0.81±0.13)	abc	94.62±4.66 (1.42±0.04)	a	100.0±0.0 (1.74±0.06)	100.0±0.0 (2.38±0.07)	100.0±0.0 (3.00±0.00)
	20°C	2.78±4.81 (0.03±0.05)	61.07±12.72 (0.83±0.15)	a	86.27±9.80 (1.35±0.10)	ab	97.31±4.66 (1.57±0.09)	100.0±0.0 (2.38±0.07)	100.0±0.0 (3.00±0.00)
	25°C	0.00±0.00 (0.00±0.00)	55.51±4.81 (0.85±0.06)	ab	97.31±4.66 (1.54±0.08)	a	100.0±0.0 (2.07±0.25)	100.0±0.0 (2.57±0.05)	100.0±0.0 (3.00±0.00)
	30°C	0.00±0.00 (0.00±0.00)	38.85±4.81 (0.68±0.06)	abcdef	77.75±21.00 (1.24±0.23)	ab	100.0±0.0 (1.58±0.04)	100.0±0.0 (2.19±0.02)	100.0±0.0 (3.00±0.00)
25°C	control	0.00±0.00 (0.04±0.04)	0.00±0.00 (0.04±0.04)	g	5.55±4.81 (0.06±0.05)	c	3.76±3.93 (0.06±0.10)	3.76±3.93 (0.06±0.10)	3.76±3.93 (0.06±0.10)
	15°C	0.95±4.81 (0.04±0.07)	61.07±12.72 (0.85±0.10)	a	94.42±4.83 (1.39±0.10)	a	100.0±0.0 (1.83±0.04)	100.0±0.0 (2.22±0.15)	100.0±0.0 (3.00±0.00)
	20°C	8.33±0.00 (0.10±0.02)	38.86±12.72 (0.71±0.07)	abcdef	97.21±4.83 (1.38±0.08)	a	100.0±0.0 (1.75±0.11)	100.0±0.0 (2.13±0.07)	100.0±0.0 (3.00±0.00)
	25°C	0.95±4.81 (0.03±0.05)	41.63±16.66 (0.69±0.10)	abcde	86.08±12.75 (1.13±0.18)	ab	100.0±0.0 (1.51±0.09)	100.0±0.0 (2.10±0.12)	100.0±0.0 (3.00±0.00)
	30°C	0.00±0.00 (0.00±0.00)	47.18±12.72 (0.79±0.07)	abcd	86.08±12.75 (1.31±0.24)	ab	100.0±0.0 (1.68±0.10)	100.0±0.0 (2.17±0.14)	100.0±0.0 (3.00±0.00)
30°C	control	0.00±0.00 (0.00±0.00)	0.00±0.00 (0.00±0.00)	g	0.00±0.00 (0.00±0.00)	c	0.00±0.00 (0.00±0.00)	0.00±0.00 (0.00±0.00)	0.00±0.00 (0.00±0.00)
	15°C	2.78±4.81 (0.03±0.05)	11.10±12.72 (0.56±0.06)	efg	69.40±19.25 (1.04±0.13)	ab	100.0±0.0 (1.60±0.10)	100.0±0.0 (2.22±0.21)	100.0±0.0 (3.00±0.00)
	20°C	0.00±0.00 (0.00±0.00)	8.32±8.32 (0.51±0.09)	fg	72.20±41.11 (1.19±0.42)	ab	100.0±0.0 (1.60±0.10)	100.0±0.0 (2.08±0.07)	100.0±0.0 (3.00±0.00)
	25°C	0.00±0.00 (0.00±0.00)	38.86±25.45 (0.74±0.16)	abcdef	100.0±0.0 (1.40±0.06)	a	100.0±0.0 (1.83±0.19)	100.0±0.0 (2.44±0.06)	100.0±0.0 (3.00±0.00)
	30°C	0.00±0.00 (0.00±0.00)	0.00±0.00 (0.42±0.04)	g	36.09±29.25 (0.71±0.15)	bc	72.19±29.28 (1.15±0.27)	94.42±4.83 (1.75±0.30)	100.0±0.0 (3.00±0.00)

a,b,c Means within a column followed by the same letter are not significantly different (ANOVA procedure; $\alpha=0.05$; Tukey HSD test); *the mortality is in first row and **value of FDI is in second row in the brackets

In control variants, the larvae were in good conditions, the index did not exceed the value 0.5 of FDI, and maximum mortality in control variant was 3.76 %. After 4 days of bioassay, the inoculum influences the mortality of larvae *T. molitor* incubated at 15 °C. Inoculum produced at 15 °C start to be virulent very fast in comparison to another inoculum. The mortality significantly increased one day later of incubation where the highest mortality was determined in inoculum produced at 25°C (61.07 %). The inoculum produced at 20 °C caused also good mortality (52.74 %). However, the inoculum produced at 10 °C and 30 °C was a week in virulence in the larvae population. The same results were obtained next days of the bioassay.

At extreme temperature was the mortality only 69.40 %, while inoculum obtained from 15 °C to 25 °C caused mortality between 94.42 % and 100 %. After 7 days, there was 100 % mortality in larvae in all the variants. Fungus development index (FDI) was assessed also every day of the bioassay. The index almost correlates the mortality results. However, differences between mortality and FDI are recorded after 7 days.

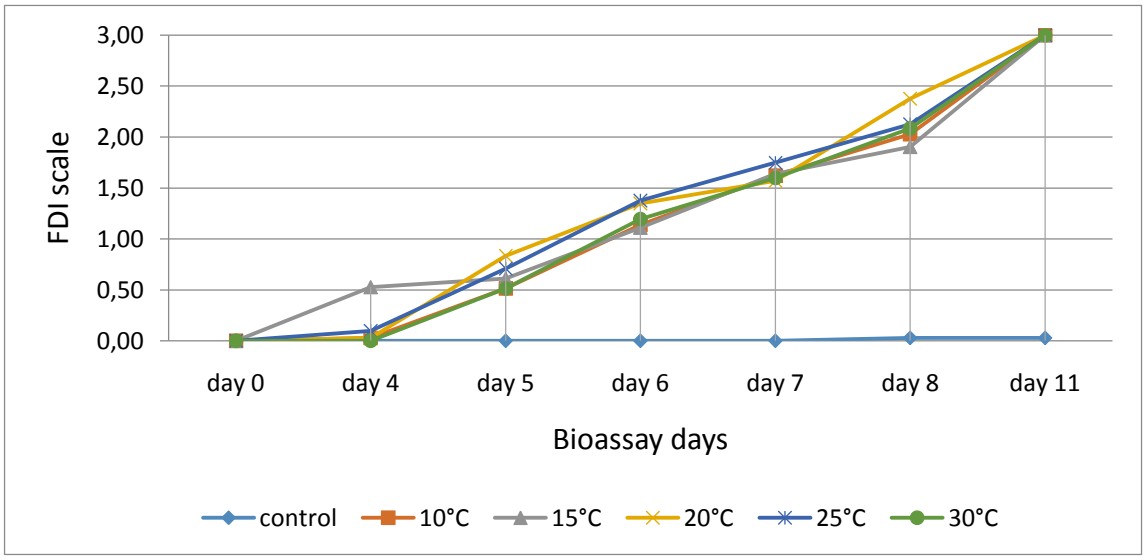
Graph 3 Comparison of *M. brunneum* development index (FDI) of different inoculum production temperature during bioassay on *T. molitor* cultivated at 15 °C



The inoculum of strain F52 produced at 15 – 25 °C caused mortality 100% but differences were recorded in FDI. The index of the inoculum ranged from 1.74 – 1.93. The fungus development was faster in the variant where the larvae were infected by inoculum produced at 15 °C, while inoculum produced at 20 °C was slower in development. At the end of bioassay, the mortality was in all variants 100 % and index reaches the value 3.0. In this

time, the strain F52 had completed its all live cycle of the larvae of *T. molitor* (see Table 11 and Graph 3).

Graph 4 Comparison of *M. brunneum* development index (FDI) of different inoculum production temperature during bioassay on *T. molitor* cultivated at 20 °C

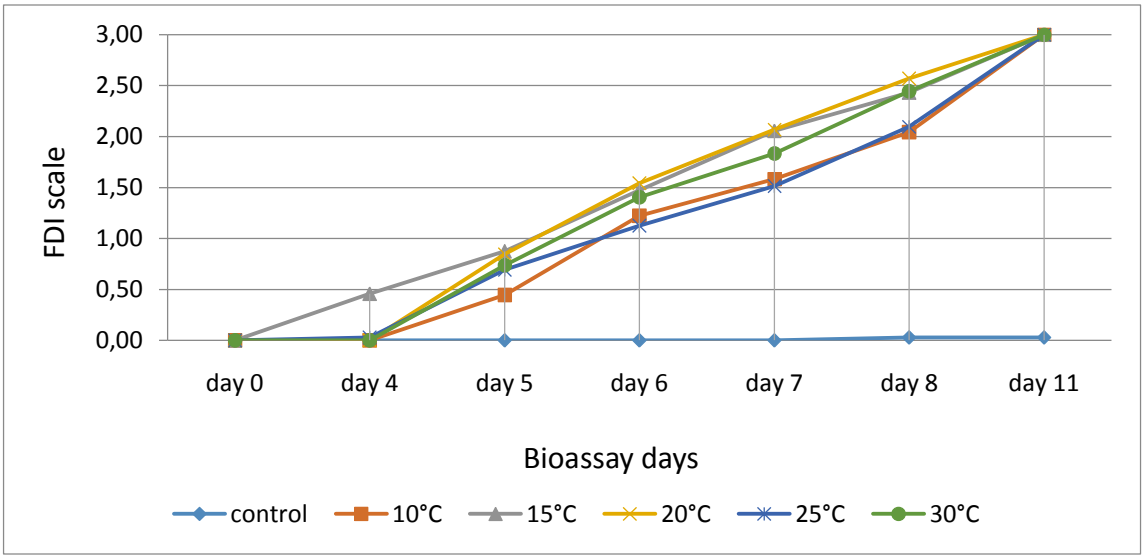


At 20 °C of incubation larvae treated by a different inoculum of strain F52 of *M. brunneum* were again differing in virulence on the host *T. molitor*. The invasion of infection into the larvae was faster by inoculum produced at 25 °C after 4 days of the bioassay. The inoculum produced at 15 °C was also more infective at the beginning of the bioassay. After 5 days, these inoculums did not continue in the growth of mortality. The highest mortality was observed at the inoculum, which was produced at 20 °C. After 6 days, the range of mortality in the larval population of *T. molitor* was between 72.20 (inoculum from 30 °C) to 97.21 (inoculum from 25 °C). The 100 % mortality was reached in all variants of inoculums except the inoculum produced at 20 °C.

The FDI was different in some days of incubation. The mortality 97.21 % was recorded in the variant where the inoculum was produced at 25 °C after 6 days, but the FDI (1.38) was similar as in the variant where the inoculum was produced at 20 °C (1.36). However, the morality in this form was about 11 % lower. These results show that the saprotrophic phase of the development in this variation is faster and don't correlate with the mortality. The same observation was recorded after 8 days of bioassay, where the mortality of larvae was 100 % in all variant but high differences in the FDI were recorded. FDI was ranged from 1.90 (15 °C) to 2.38 (20 °C). It means that in the alteration of 15 °C the stain F52 spores did not start to germinate yet in comparison with the second alteration of 20 °C where

the strain F52 fully sporulated on the parts of cadavers of larvae *T. molitor* (see Table 11 and Graph 4).

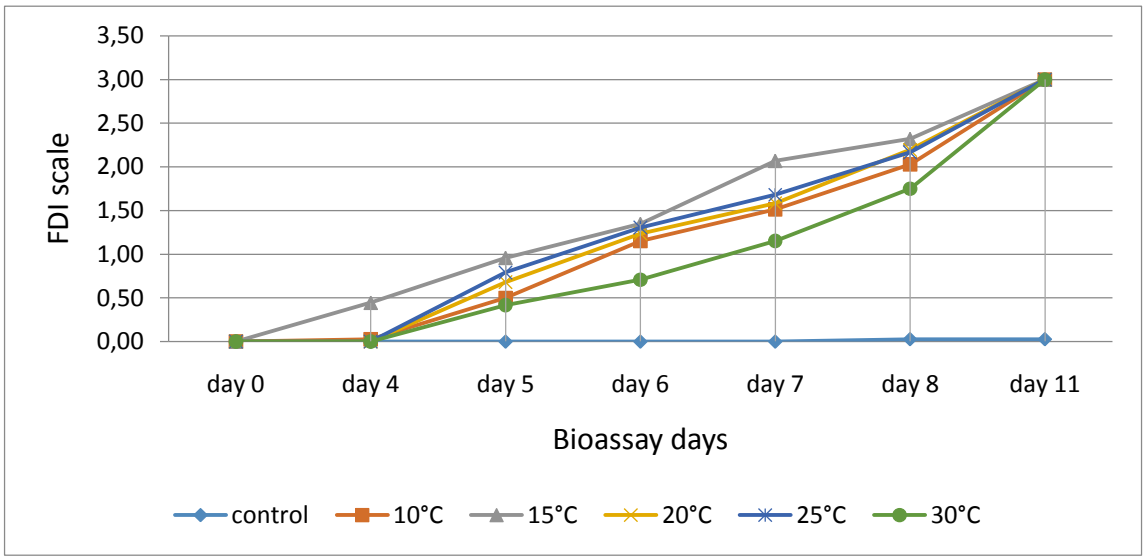
Graph 5 Comparison of *M. brunneum* development index (FDI) of different inoculum production temperature during bioassay on *T. molitor* cultivated at 25 °C



The cultivation of infected larvae at 25 °C seems to be the optimal temperature for bioassay because the invasion of mortality was high after 5 and 6 days of incubation. However, the beginning of fungus efficacy was the very slow recorded 4th day of bioassay in compare with other cultivated temperatures. The mortality considerable reached after 6 days of the bioassay. The mortality ranged from 86.08 % (inoculum from 25 °C) to 100 % except inoculum from 30 °C which had less.

After 7 days, all larvae in each alteration were dead. However, the fungus development index was different. Inoculums produced at 10 °C and 25 °C reach the index around 1.50, which means that mortality is related to fungus. The hyphae of the fungus were observed on the cadavers. The highest indexes (2.06 and 2.07) were observed in the variant where inoculum was produced at 15 °C and 20 °C. The cadavers were covered by mycelium and it started sporulate. The beginning of sporulation was observed after 8 days of bioassay and full sporulation respectively completed development cycle of strain was observed in day 11 (see Table 11 and Graph 5).

Graph 6 Comparison of *M. brunneum* development index (FDI) of different inoculum production temperature during bioassay on *T. molitor* cultivated at 30 °C



The inoculums were not effective against the larval population of *T. molitor* during first four days of the bioassay during incubation at 30 °C. After 5 days of bioassay, the mortality was observed at all inoculums except inoculums produced at 30 °C. It seems that this inoculum was not adapted to the temperature of its production. The bioassays evaluated after 6 days showed higher mortality in the populations in all alterations, but again except inoculums produced at 30 °C. After 7 days of incubation, in the variants of inoculums from 10 °C to 25 °C the mortality 100 % was recorded. The inoculum produced at 30 °C was slower in the mortality of the bioassay. The 100 % of mortality was recorded on the last day of observation. The differences among the variants in the development of fungus were also observed and larval population was unbalanced after treatment with inoculums. The large differences were noted in the variant, where the inoculums were produced at 10 °C and 30 °C. In these variants, the alive, dead and infected larvae were observed. In the inoculum produced at 10 °C was population unbalanced after 5 days of bioassay and the strain F52 produced at 30 °C caused large difference also in the 6th day of the bioassay. In this temperature of cultivation, the inoculum produced at 15 °C was the most efficient of efficacy. The average fastest inoculum index (from 15 °C) on larvae was 2.32 after 8 days of incubation. The beginning of sporulation is marked by index 2.50, it means that inoculum from 15 °C already started to sporulate after 8 days of the bioassay. After 11 days, full sporulation was already visible on the surface of all larvae in all variants (see Table 11 and Graph 6).

5. DISCUSSION

The entomopathogenic fungus *M. brunneum* strain F52 is an incorporated commercial product (Novozymes Biological, Franklinton, NC, USA). Previously it was considered as species *M. anisopliae* var. *anisopliae*. But recently the scientific name was changed based on a multigene phylogenetic approach using near-complete sequences from nuclear DNA. This strain F52 of *M. brunneum* is still often described in commercial sources as *M. anisopliae* (Bischoff *et al.* 2009).

Strain F52 causes infection on the beetle larvae and originally was only developed for control of *Otiorhynchus sulcatus*. However, nowadays this product is used in nursery ornamentals in soft fruit crops against *O. sulcatus* (black wine weevil) and in greenhouse ornamentals against *Frankliniella occidentalis* (western flower thrip) (Ansari and Butt 2013, Klingen et al. 2015). Production is one of the critical points in the development of fungal bioproducts (Jackson et al. 2010). The status of facultative parasites allowed the production of biomass of infectious conidia by use of large-scale biotechnologies, which is a basic technological prerequisite for the development and market implementation of standard bioproduction (Ravensberg 2010). After application of entomopathogenic fungi on the targeted pest, it starts to control the population of thatpest. If the spores are attached to the body of a suitable host, they start to germinate if the environmental conditions are favourable. After germination, the hyphae penetrate into the host and growth within the pest starts and after few days the death of the insect occurs (Barra-Bucarei *et al.* 2016). The potential use of fungal strains in practical depends on their pathogenicity on the targeted pest. The virulence of the fungi is one of the key factors. For the development of the bioproduct, the strain/ inoculum which can easily be produced, high quality and high virulence obtained from its mother culture must be used (Skalicky *et al.* 2014).

After long-term storage, it is important to monitor the phenotypic and genotypic changes in the inoculum and the purity of the strain. If any changes or contamination occurs, the strain must be recovered from the culture collection or from the deposited product. If a new isolate is obtained, its properties must be carefully tested before being replaced by an older strain and used in the production of a new batch of bioproduct (Ravensberg 2010). Solid state fermentation is used for production of fungus *M. anisopliae* (Mendonca 1992, Cherry *et al.* 1999, Milner and Hunter 2001). However, Kleespies and Zimmermann (1992) studied the production of blastospores by submerged production.

In the thesis, the strain F52 re-isolated from bioproduct Met52 was used for the experiment focused on the temperature profile. The aim of this thesis focused on the influence of inoculums produced at different temperatures based on their characteristics of development and virulence consequently tested in various temperatures of cultivation. The radial growth was observed on a period of 21 days, of which all variants of each inoculum were measured radially every seven days. After measurement, the area of culture was calculated and the production of spores was determined per 1 mm^2. During the growth, there were significant changes in the size of the colonies and the number of spores produced varying from different temperatures of inoculums. Also, the temperature of cultivation played an important role in the inoculums characteristics of growth.

Temperature plays a very important role not only in the pathogenesis of entomopathogenic fungi, but also in their growth and development (Zimmermann 2007). *Metarhizium* strains have shown wide variation in their abilities to grow at low and high temperatures (Ouedraogo *et al.* 1997). Roddam and Rath (1997) isolated cold-active strains of *Metarhizium* from the subantarctic Macquarie Island. Rath *et al.* (1995) examined carbohydrate utilization and cold-active growth for 134 isolates of *Metarhizium* and distinguished several *Metarhizium* groups based on carbohydrate utilization, as well as a group of cold-active *M. anisopliae* isolates able to germinate at 5°C. Evidence of genetic groups associated with low or high-temperature growth was presented by Driver *et al.* (2000).

Significant variability in germination at different temperatures has been reported for inoculums of strain F52 produced at different temperatures. The different germination of spores was observed during incubation of all inoculums at 15 °C. The index germination of spores of all inoculums was not uniform not only at 15 °C but also at 20 °C of incubation. For example the resulted indicated or showed that, the inoculum produced at 15 °C adapted to the 15 °C cultivation temperature. These spores were most germinated in comparison with other inoculums. The same observation was determined when the inoculum produced at 20 °C as experimented. Fargues *et al.* (1997b) found that the strains of *B. bassiana* which are isolated from the various geographic origins can adapt to conditions with large thermal fluctuations. The spores of inoculums well germinated at 25 °C and 30 °C, but temperature beyond 30 °C negatively affects the germination of *M. anisopliae* spores (Walstad *et al.* 1970, Harman *et al.* 1991). The optimum temperature for spore germination is between 25 °C and 30 °C (Bugeme *et al.* 2008b, Niassy *et al.* 2012, Zimmermann 2007). Significant reduction in the germination of spores was also observed below 10 °C and above 40 ºC (Hallsworth and Magan 1999,

Chandra Teja and Rahman 2016). Fernandes *et al.* (2008) mentioned that the exposure the aqueous conidial suspensions to 43 or 45 °C for 1, 2, 3 or 4 h during germination was tested, and the results showed that the exposure to the temperature that was beyond the optimal temperature played a significant role in the decrease of spore production.

The inoculums produced in different temperatures produced non-identical colonies after their cultivation in various temperatures. After 7 days, significant differences were recorded after cultivation of inoculums at 15 °C and 25 °C. Inoculum from 15 °C produced the smallest colony at 20 °C temperature of incubation. The colony was more than 36 % smaller than the colony of the same inoculums cultivated at 10 °C. The results reviewed that inoculum produced at 10 °C was more adapted to higher temperatures than that of 15 °C. The 10 °C temperature of cultivation is the critical temperature for the development of strain F52 inoculums produced as in different temperatures. The only inoculum survived at this low temperature was the one from 25 °C. After 14 days, strain F52 was able to grow at low temperature of 10 °C from all inoculums variants. The inoculum produced at 30 °C was not growing after 7 days, but later it produced the largest colonies in comparison to other inoculums. De Croos and Bidochka (1999) found differences in cold activity among isolates, all isolates were grown at 22 °C, but only few of them grew at 8 °C. The inoculum produced at 30 °C produced also large colonies at 10 °C and 15 °C of incubation and second larger colonies at 20 °C and 25 °C. However, the morphology of colonies produced from inoculums obtained from 30 °C was different in comparison to the other inoculums. In the middle of the colonies, the white sterile mycelium was growing. And also the colonies formed from all the inoculums cultivated at 30 °C had different morphotypes, the sterile mycelium grew on all area of colonies in some colonies.

The optimum temperature for growth ranged between 25 ºC and 30 ºC which is consistent with reports of Ekesi *et al.* (1999) and Dimbi *et al.* (2004). Ouedrago *et al.* (1997) reported that the optimum temperature for the radial growth of four Ethiopian isolates of *M. anisopliae* ranged between 25 and 32 ºC. Similar results are presented by Milner *et al.* (1997). They found that *M. anisopliae* isolates, that there was no mycelial growth of the fungus at 35 °C, and that rapid growth occurred between 20 and 30°C, with the most growth at 30 °C. In the thesis, the best temperature for radial growth of strain F52 of *M. brunneum* is 25 °C. At this cultivation temperature, the colonies of strain F52 established from different inoculums produce the largest areas on each observation day.

The most important environmental factors that affect the mass production

of *M. anisopliae* are temperature and humidity, which are considered as critical factors during the incubation stage (Li and Feng 2009, Chen *et al.* 2014, Hallsworth and Magan 1999). The number of spores produced within one temperature of inoculum did not differ so much within one temperature of cultivation. However, some variance was noted among the variants after the spore yield was calculated per $1mm^2$. Almost in all variants, the area of colonies growth from different inoculums and cultivated at different temperatures was not correlated with spore production obtained after 21 days. The positive correlation was observed after yielding of spores after cultivation at 30 °C from inoculums produced at 10 °C and 15 °C. There was a correlation between the areas of the colony with the spore yield. The highest production of spores was determined from the colonies of all inoculum variants cultivated at 25 °C.

Ekesi *et al.* (1999) recorded that the tested strains of *M. anisopliae* and *B. bassiana* were most effective in terms of virulence to the bean flower thrip at 25 and30°C. Significant decrease in the development rate of fungal infections determined at 20 °C, but the mortality was not affected at this temperature. Ferron (1978) reported that temperatures lower than optimal reduce the speed of development of mycosis without necessarily affecting the total mortality. The same results were obtained in the bioassay, where the 100 % mortality was reached mostly after 7 days of virulence test, but the FDI was different. The faster development of strain F52 was determined from inoculums produced at 15 °C and 25 °C. One day later, the index was high in the variant where the inoculum was produced at 20 °C. The mortality of bean flower thrip caused by *B. bassiana* and *M. anisopliae* increased as temperature of incubation was raised (Ekesi *et al.* 1999). Vestergaard *et al.* (1995) reported that adult thrip *F. occidentalis* was susceptible to *M. anisopliae* isolate 275 at a temperature range from 18 ºC to 26ºC, but mortality was higher at upper temperatures of 23 ºC and 26 ºC compared to 18 ºC and 20ºC. Maniania *et al.* (2001) reported that application of *M. anisopliae* significantly reduced *F. occidentalis* in chrysanthemum crop, but the control of second instars was much lower than for adults. Similar observations were made in laboratory bioassays by Vestergard *et al.* (1995) and Ugine *et al.* (2005) with the efficacy of *M. anisopliae* and *B. bassiana* against *F. occidentalis*. Niassy *et al.* (2012) reported that the LT50 value for second instars were 8.2 days at 26 ºC as compared to 1.9 – 2.2 days for the adults as observed at a temperature range of 25 – 30 ºC. Such observations were also made by Ekesi *et al.* (1999) who reported that the LT50 values for the virulence of *M. anisopliae* on adult of bean flower thrip decreased as temperature increased and no significant difference was observed in the LT50 values at 25 °C and 30 ºC. The isolates of *M. anisopliae* used against *Agriotes lineatus* larvae in the laboratory were tested by Ansari *et al.* (2009). They found that

only two isolates of *M. anisopliae* caused significant mortality at the population of this soil-dwelling pest, the other tested isolated were less efficacy, they cause the mortality between 10 % and 70 %. The efficacy of entomopathogenic fungi is more effective in laboratory conditions than in the field conditions. The presence of fungi can be influenced by soil factors as a pH, contents of organic matter, salinity, etc. (Quesada-Moraga *et al.* 2007). High organic matter can have a positive effect on the occurrence and persistence of entomopathogenic fungi in the soil (Medo and Cagáň 2011). Fungus *M. anisopliae* and other species of the genus *Metarhizium spp*. are able to colonize the soil environment of plant roots and act as pathogens against pests (Liao *et al.* 2014). Klingen *et al.* (2015) revealed that indigenous Norwegian entomopathogenic fungal isolates decreased *O. sulcatus* survival in bioassays at 12 °C and 18 °C, but at 6 °C any isolates did not reduced larval survival. The larval stages of *O. sulcatus* are active in autumn and early spring, so the effect of entomopathogenic agents at a lower temperature as 12 °C is important for biocontrol of this pest in northern temperate regions (Klingen *et al.* 2015, Fitters *et al.* 2001). Persistence and even increase of fungal propagules in the rhizosphere are important for biocontrol agent applied against soil-dwelling or root feeding pests. Root inoculation used against *O. sulcatus* by strain F52 has proved effective biocontrol (Bruck 2005, Bruck and Donahue 2007, Ansari and Butt 2013).

Influence of temperature on the inoculums production play an important role in the germination, radial growth, spore production and efficacy on alternative host (*T. molitor*) after consequently cultivation of these inoculums in different temperatures. The result of this thesis can be used as knowledge in the potential production of mother cultures for mass production of bioproduct or results can be used for practical use of strain F52 for application in the field under the various conditions. The temperature plays an important role in the survival of entomopathogenic fungi in the soil or can have a positive effect on the production of spores. The ability of strain F52 to survive and be produced, the second generation of spores at low or high temperature can have great practical potential in the use of this strain or it can have a good basis for the next research.

6. CONCLUSIONS

- ✓ Temperature plays an important role in the inoculums production of strain F52 component for commercial bioproduct.
- ✓ The inoculum must be of good quality and must be tested and declared that the strain does not change its morphological phenotypes during spore production/cultivation.
- ✓ The optimum temperature for spore germination is 25 °C and 30 °C.
- ✓ The inoculum produced at 25 °C was the only one capable of growing at the lowest tested temperature of 10 °C after 7 days of incubation.
- ✓ After 7 days, the inoculums produced at 30 °C were retarded in the radial growth at 10 °C but the following two weeks the strain produced second largest colonies.
- ✓ The optimal temperature for radial growth of strain F52 of *M. brunneum* from different inoculums is 25 °C and subsequently 30 °C.
- ✓ Temperature at 30 °C influences the morphology phenotypes changes not only in inoculum production but also in consequently inoculums cultivation.
- ✓ The highest production of spores was determined from the colonies of all inoculums variants cultivated at 25 °C.
- ✓ The inoculums produced at 15 °C to 25 °C seemed to be of good quality for spore production.
- ✓ The best temperature for mortality test and fungus development is 25 °C and the most virulent were inoculums produced at 15 °C and 20 °C.
- ✓ Further research should be done on other climatic factors other than temperature that influence the efficacy of the entomopathogenic fungus.
- ✓ Entomopathogenic fungus *Metarhizium brunneum* has a high potential in pest management compared to chemical insecticides and thus should be used as one of the management options in the IPM.

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