Addis Ababa University
College of Natural and Computational Sciences
Department of Microbial, Cellular and Molecular Biology

Evaluation of *Beauveria bassiana* Isolates against *Varroa destructor* of *Apis mellifera* under Laboratory Condition.

By
Olyad Daniel

Advisors: Asnake Desalegn (PhD)
Yitbarek Woldehawariat (PhD)
Zewdu Ararso (PhD)

A Thesis Submitted to the School of Graduate Studies of Addis Ababa University in Partial Fulfilment of the Requirements for the Degree of Master of Science in Microbial, Cellular and Molecular Biology (Applied Microbiology).

April, 2024

Addis Ababa, Ethiopia

DECLARATION

I hereby declare that the thesis I have submitted for the Master of Science (MSc) degree in Microbial, Cellular and Molecular Biology (Applied Microbiology) to the School of Graduate Studies of Addis Ababa University, under the title "**Evaluation of *Beauveria bassiana* Isolates against *Varroa destructor* of *Apis mellifera* under Laboratory Condition.**" is my original work. Neither I nor anyone else has submitted it anywhere before. The thesis includes appropriate acknowledgments for any content used from outside sources.

ACKNOWLEDGEMENTS

I am deeply grateful to my main advisor, Dr. Asnake Desalegn, and my co-advisors, Dr. Yitbarek Woldehawariat and Dr. Zewdu Ararso, as well as Denberu Kebede, for their invaluable advice, guidance, and support throughout my study. I also extend my sincere thanks to the Oromia Agricultural Research Institute for allowing me to pursue my Master's degree. Additionally, I would like to thank Dr. Birru Yitaferu and Dr. Binyam Tesfaw for their financial support. Special thanks are also due to the National Agricultural Biotechnology Research Center, particularly to all members of the Microbiology Research Team, and the Holeta Bee Research Center for providing laboratory facilities and study samples. My sincere appreciation goes to Dawit Melese and Dr. Belay Habtegebriel for their facilitation and support throughout this study. Last but not least, I express my deepest gratitude to my family and friends for their love, encouragement, and unwavering support. Above all, I thank Almighty God for being alongside me throughout this journey.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

PDA	Potato Dextrose Agar
EPF	Entomopathogenic Fungi
DWV	Deformed Wing Virus
ABPV	Acute Bee Paralysis Virus
IPM	Integrated Pest Management
CRD	Completely Random Design
ANOVA	Analysis of Variance
LSD	Least Significant Difference
dpi	day post inoculation
CCD	Colony Collapse Disorder
GDP	Gross Domestic Product

ABSTRACT

Varroa, *Varroa destructor* (Acari: Varroidae), is an ectoparasitic mite of honeybees, *Apis mellifera*. This parasite poses a substantial threat to the health, welfare and production of *A. mellifera* globally, including Ethiopia. Without chemical treatment, colony losses worldwide are common. However, resistance to synthetic chemicals in beekeeping is increasingly concerning. Entomopathogenic organisms provide an eco-friendly alternative to pesticides and can prevent resistance development. Therefore, isolates of *Beauveria bassiana* were tested for their pathogenicity against *V. destructor* and their negative effect on the brood and adult stages of the central highland honeybees of Ethiopia, *A. mellifera*. Varroa mites were immersed in 5 millilitres of a conidial suspension containing 1 x 10^8 conidia/mL of three different fungal isolates (APPRC-44BC, APPRC-27, and S#10H), as well as control solutions (0.05% Tween 80 and distilled water). The inoculated mites were placed on honeybee brood inside capped cells. Then, the infected brood combs were kept in an incubator at 33 ℃ and 60% relative humidity for ten days. The fungal isolates and control treatments were also applied to young adult workers and healthy brood to observe the effects of the treatments. Fungal isolate APPRC-44BC displayed the highest (73%) potential for killing varroa mite. All three isolates (APPRC-44BC, APPRC-27, and S#10H) were found to be highly efficient between the sixth and eighth days post-application, accounting for 96.8% to 100% of the fungal-induced deaths. Interestingly, treatment with *B. bassiana* isolates did not show a significant effect on brood emergence and the weight of newly merged adults. However, the treatments had significant effect on adult honeybee survival. It is clearly observed from these results that isolates of *B. bassiana* are potential bio-control agents against *V. destructor*. However, further studies are needed to evaluate the efficacy of this promising fungal isolate (APPRC-44BC) in honeybee colonies under field conditions, as well as to develop application methods that have minimal impact on adult honeybee survival.

Keywords: *Apis mellifera*, *Beauveria bassiana*, Biocontrol, Honeybees, *Varroa destructor*

1. INTRODUCTION

1.1. Background

Bees play a significant role in the complex web of life and emerge as vital providers of ecosystem services. These ecosystem services may include provisioning services like honey, beeswax, pollen, and royal jelly; cultural services like beekeeping traditions' symbolism, educational value, and recreational prospects; and regulatory services like pollination and the preservation of genetic variety (Hung *et al*., 2018; Hepburn and Radloff, 2011). Because of their impact on ecosystems and human populations, honeybees are an integral part of our natural environment and cultural legacy (Aryal *et al.*, 2020).

Apis members, including honeybees, pollinate plants and provide valuable ecologic and economic services. Consequently, honeybees play a significant role in the global agricultural economy, as they pollinate 35% of the world's food production (Klein *et al*., 2007). The honeybee's social and instinctive behavior, as well as its physical structure, are responsible for the largest portion of crop pollination. Both managed and wild honeybees are essential to pollination, which benefits humans. The managed honeybees are the most commonly known pollinators, accounting for 90% of active agricultural pollination and being kept in large-scale commercial apiaries due to their honey and other bee products (Graystock *et al*., 2016). Keeping these insects also creates employment opportunities in rural regions of the world and produces useful products. Therefore, their protection is vitally important. Despite their ecological and economical values, a large number of honeybee colony losses have been observed worldwide in recent years. Beekeepers, agricultural crop growers, scientists and the general public are concerned about the mysterious deaths of honeybee (*A. mellifera*) colonies in many nations over the past decade. The phenomenon is known as colony collapse disorder (CCD) in the US. VanEngelsdorp *et al*. (2008) identify several possible causes for the losses, but no conclusive explanation has yet been found. Many scientists believe the cause is a combination of factors (Neumann and Carreck, 2010).

One of the important factors associated with colony death is a parasitic mite (*Varroa destructor*). Honeybee colonies infested with *V. destructor* often perish after 2 years of an initial varroa infestation. Many beekeepers employ synthetic pesticides to stop the death of their colonies. The chemical pesticides were preferred for their effectiveness and convenience of use. Nevertheless, the preparation of these pesticides can contaminate hive products and negatively affect honeybee health (Mullin *et al.*, 2010). Moreover, the continuous usage of chemicals have resulted in pesticide resistance, which is reported to be increasing in Europe, the USA and Canada. Despite the use of synthetic miticides in Canada, varroa mite infestation is responsible for the death of considerable honeybee colonies (Higes *et al.,* 2020). All these reasons, combined with environmental protection, consumer awareness and an ever evolving international eco-friendly legislation, have led to the development and adoption of low-impact management practices and chemicals.

The optimal varroa control method is environmentally friendly, varroa-selective, harmless to honeybees, and leaves minimal residues in bee products. In this regard recent scientific research has focused on alternative varroa mite control strategies. The use of entomopathogenic microorganisms, particularly entomopathogenic fungi (EPF), as a biological agent against *V. destructor* is among the alternative strategies. Entomopathogens can aid in mitigating miticide resistance within pest populations. Additionally, EPF have the ability to infect varroa, are non-toxic to humans, can be mass-produced, and are naturally present in the environment (Farenhorst *et al*., 2009). The entomopathogenic fungus *Beauveria bassiana* has been applied as bio-control agents against many insects, bovine ticks, as well as *V. destructor* in honeybees (Bava *et al*., 2022). Using *B. bassiana* in honeybee colonies could provide control against *V. destructor* (Meikle *et al*., 2008). However, it is unclear whether this fungus affects adult honeybees and their brood stages.

In Ethiopia, several studies carried out have reported the frequency and presence of varroa mites across different regions and seasons and indicated that varroa mites are becoming an important parasite of local honeybees (Desalegn Begna *et al.* 2016; Ebisa Mezgabu *et al.* 2016; Amsalu Arega 2020; Tesfu Shegaw *et al.* 2022; Alemayehu Gela *et al.* 2023;). These studies suggested that varroa mite could be one of the underplaying causes of colony absconding and associated diseases in honeybees. EPF, like *B. bassiana*, can lessen varroa mite damage to honeybee by infecting the parasite and reducing immune suppression (Hamiduzzaman *et al.,* 2012). However, there is no report thus far about whether the application of EPF can control varroa infestation in bee hives without causing significant effect in local honeybees. Hence, generating detailed and concrete information on the effectiveness of selected isolates of *B. bassiana* as potential varroa mite bio-control agent and whether these isolates could negatively impact adult workers and brood is crucial.

1.2. Statement of the problem

Beekeeping plays a crucial role in the agricultural economies of emerging countries, including Ethiopia, providing a significant source of income for smallholders (Haftey Sahle, 2018). However, honeybee colonies face various challenges, including the parasitic varroa mite, *V. destructor,* which poses a serious threat to bee health and honey production (Kerealem *et al*., 2009). Despite the widespread distribution of varroa mites in Ethiopia and their negative impact on honeybee populations, no effective control measures have been implemented. To protect the health and sustainability of beekeeping in Ethiopia, it is imperative to explore alternative, environmentally friendly pest control methods.

One promising approach is the use of entomopathogenic fungi, such as *B. bassiana,* which have shown effectiveness in controlling varroa mite infestations while posing minimal risk to honeybees. This biological control method offers a sustainable and eco-friendly alternative to chemical pesticides, which may leave residues in hive products and contribute to the development of pesticide resistance in mites.

Despite the growing interest in biological control methods for varroa mites, further research is needed to evaluate the efficacy of *B. bassiana* isolates under laboratory conditions. This study aims to assess the impact of these fungal isolates on varroa mite populations and their potential for integration into sustainable varroa mite management strategies in Ethiopia.

1.3. Significance of the study

This study holds significant implications for the beekeeping industry in Ethiopia. Varroa mites pose a serious threat to honeybee populations, leading to lower yields and colony losses. Current control methods, including synthetic pesticides, are not sustainable due to concerns about chemical residues and mite resistance. By exploring the use of entomopathogenic fungi, such as *B. bassiana,* as a biological control method, this study seeks to provide a sustainable and eco-friendly alternative for managing varroa mites.

The findings of this study will contribute to the body of knowledge on the effectiveness of *B. bassiana* isolates in controlling varroa mites under laboratory conditions. This research is particularly relevant to beekeepers, researchers, and policymakers in Ethiopia, where beekeeping plays a vital role in the economy and livelihoods of smallholders. By demonstrating the efficacy of biological control methods, this study has the potential to inform the development of more sustainable varroa mite management strategies, ultimately benefiting the health and sustainability of honeybee colonies in Ethiopia.

1.4. Objectives of the study

1.4.1. General objective

- To evaluate the efficacy of *B. bassiana* isolates as biological control agent against *V. destructor* and investigate whether this entomopathogenic fungus could detrimentally affect the brood and adult stages of worker bees in central highland of Ethiopia.

1.4.2. Specific objectives

- To assess the pathogenicity of three distinct *B. bassiana* isolates against *V. destructor*.
- To examine the detrimental impact of *B. bassiana* isolates on brood development and final emergence as adult worker bees.
- To investigate the effect of *B. bassiana* isolates on the survival of adult worker bees in a laboratory bioassay.

2. LITERATURE REVIEW

2.1. Beekeeping practices in Ethiopia

There is a long history of interaction between honeybees and humans. All around the world, beekeeping is a diverse and long-standing tradition. According to (Kritsky, 2017), the actual practice of beekeeping is thought to have started in antiquity, between 3000 BCE and 500 CE. This ancient tradition, which includes many different practices, crosses cultural boundaries. Beekeeping is a global industry with an established routine that plays a significant role in both the environment and human society. Beekeepers participate in a range of activities that reflect their diverse cultures, climates, and purposes (Gupta *et al.*, 2014).

Ethiopia is one of the African countries with the highest potential for honey production. Beekeeping has a long history and is important to the nation's environment, economy, and culture identity. Honey, with its delicious white, red, and yellow varieties used in cooking, medicine, and as a main ingredient in the local mead known as "tej," has long played a significant part in Ethiopian culture (Fenet Belay and Alemayehu Oljirra, 2016).

Though it is not as common among farmers as other agricultural industries, beekeeping nevertheless contributes significantly to the nation's economy, accounting for 1.3% of the agricultural GDP. It is significant to farmers in terms of revenue generation. Out of the harvested honey, only 10% is used for home consumption; the remaining 90% is sold, generating additional revenue (Sebsib Ababor and Yibrah Tekle, 2018). For many rural Ethiopian households, beekeeping is a significant source of revenue. In areas with limited agricultural options, it provides small-scale farmers and communities with an opportunity to supplement their income (Gupta *et al*., 2014).

In Ethiopia, beekeeping can be divided into four categories: improved, transitional, traditional backyard, and traditional forest. Traditional backyard beekeeping with comparatively superior management is prevalent throughout the majority of the country (Kenesa Teferi, 2018). In rural areas, traditional beekeeping methods are still practiced and are an essential component of the community's livelihoods (Sisay Fikru, 2015; Sebsib Ababor and Yibrah Tekle, 2018). In Ethiopia, traditional methods of beekeeping have a long history. Traditional beehive designs, such as hollowed-out logs or woven baskets, are used by a large number of rural populations for beekeeping. These customs, which have been passed down through the generations, indicate the close relationship between humans and bees (Kenesa Teferi, 2018). Ethiopia is well-known for its wide diversity of honey varieties, thanks to the abundance of flower varieties in the nation. Due to the fact that different regions produce diverse varieties of honey, each with unique flavors and attributes, Ethiopian honey is highly sought after in both domestic and international markets (Aman Dekebo *et al.*, 2019).

Up to 500,000 tons of honey and 50,000 tons of beeswax might be produced in the country annually. However, according to (Kenesa Teferi, 2018) report only 47,706 tons of honey and 5542 tons of beeswax were produced. Experts in beekeeping, or apiculture, believe the industry is still far from reaching its full potential in a country where 85 percent of employment is in agriculture. According to experts, thousands of impoverished farmers could be lifted out of poverty by producing honey using modern beekeeping technologies and equipment (Sisay Fikru, 2015).

2.2. Beekeeping constraints

Despite being deeply embedded in Ethiopian culture and the country's economy, beekeeping faces several challenges that prevent it from expanding and becoming more sustainable. Bee characteristics and environmental conditions, such as excessive use of pesticides and herbicides, a shortage of forages, honeybee pests and predators, and a lack of beekeeping equipment such as modern hives and accessories, are among the primary causes of beekeeping's problems. Improved beekeeping procedures cannot succeed without protective clothing, smokers, casting mold, and honey extractors—all of which are lacking for most beekeepers in the country, even with top bar and moveable frame hives. In addition, beekeepers face a knowledge gap and the expense of beekeeping equipment in comparison to their purchasing power (Kerealem *et al.*, 2009; Aman Dekebo *et al.*, 2019; Sebsib Ababor and Yibrah Tekle, 2018; Gratzer *et al.*, 2021). The challenges arising from the combination of environmental, social, and institutional elements mandate collaborative efforts to overcome and unleash the complete possibilities of the industry.

Moreover, habitat loss and degradation are caused by increased agricultural production, deforestation, and urbanization, all of which reduce honeybees' access to natural food available to. Bee colonies become weaker due to reduced floral diversity and quality, which affect their nutrition and make them more susceptible to illnesses and pests (Mustafa *et al.*, 2015).

Particularly, diseases and pests pose serious risks to bee health. Lack of knowledge and access to suitable treatments worsens the problems, leading to colony losses and decreased honey production (Haftey Sahle, 2018). Despite extensive studies on the host-parasite relationship between *A. mellifera* and *V.destructors*, varroa continues to pose a significant threat to beekeeping worldwide. Future advancements in basic and applied research are crucial to creating long-term control strategies for these dangerous pests (Noël *et al.*, 2020).

Finally, but just as importantly, limited access to technology and a lack of technical knowledge can make it difficult for beekeepers to resolve problems successfully. Traditional, low-input practices are still widely used by Ethiopian beekeepers, which limits the effectiveness of hive management and the quality of honey produced. Access to modern beekeeping technologies, such as improved hive designs and equipment, is restricted for small-scale beekeepers (Haftey Sahle, 2018; Aman Dekebo *et al.*, 2019).

2.2.1. Varroa mites (*Varroa destructor*)

The genus *Varroa*, belongs to the family *Varroidae* in the order *Mesostigmata*, encompasses the species *V. destructor*. Parasitic mesostigmatan mites closely associated with honeybees are classified within the order *Mesostigmata*, a member of the class *Arachnida*. *Arachinida* comprises animals with jointed appendages, which are not vertebrates and are commonly referred to as arthropods.

There are several different kinds of mites in the genus *Varroa*; two prominent species are *Varroa destructor* and *Varroa jacobsoni*. It is known that these species parasitize honeybee adults as well as their young. *V. jacobsoni* mostly attacks Asian honeybees, *A. cerana*. In contrast, *V. destructor* has become a well-known pest that affects the western honeybee, *A. mellifera*, worldwide, as well as *A. cerana* in mainland Asia (Anderson and Trueman, 2000).

2.3. Varroasis

Honeybee colonies become infected with *V. destructor* commonly referred to as varroasis, also known as varroa infestation or varroa mite infection. Varroasis is a serious problem in beekeeping because of the detrimental effects these mites have on the health of honeybees and the survival of their colonies. It also causes a number of diseases that impact honeybees. In addition to being external parasites that damage bee colonies, varroa mites also serve as carriers of viruses that affect bee population. According to Traynor *et al.* (2020), these viruses are frequently cause of illnesses that have a significant impact on longevity and well-being of honeybee colonies. These mites are thought to be the most significant danger facing honeybees. Thus they represent a severe risk to honeybee populations worldwide. These tiny organisms are accountable for over 85% of colony deaths in the fall and approximately 27.2% of colony mortality in the winter (Guzmán-Novoa *et al.*, 2010).

These ectoparasitic pests do not have a free-living stage and are dependent on their honeybee hosts. The life cycle of female varroa mites consists of two separate stages: the reproductive phase, which takes place within the sealed drone and worker brood cells, and the phoretic phase, during which they attach to adult bees (Figure 1). Different chemicals regulate almost every stage of this cycle. The mite's males and nymphal stages can only found in sealed brood chambers and have a short life span. Female varroa mites use adult bees, particularly nurse bees, for dispersal within honey bee colonies, often hitching rides on them to move between cells (Nazzi and Le Conte, 2016). For many years, it was considered that varroa mites feed honeybee hemolymph and that varroa primarily infects honeybees by transmitting viruses. Recent research reveals that instead of hemolymph, they digest and devour the fat body tissue of the bee (Ramsey *et al.*, 2019).

Bees' adipose tissue is distributed throughout their bodies and performs several vital tasks. One important function is serving as a storehouse for energy reserves, holding onto lipids or fats, which is especially important in times of scarcity or cold (Strachecka *et al.*, 2021). During these times, this stored energy becomes an essential resource. Furthermore, the fat body plays a crucial role in some metabolic activities, including the transformation of nectar carbohydrates into lipids that are stored for later use. In addition to storing energy, it actively creates molecules required for energy-intensive tasks like flying (Li *et al.*, 2019; Strachecka *et al.*, 2021).

Notably, the generation of hormones and other regulatory factors is significantly dependent on the fat body, and this influences a variety of physiological processes in bees, such as growth, development, reproduction, and behavior control (Li *et al.*, 2019; Strachecka *et al.*, 2021).

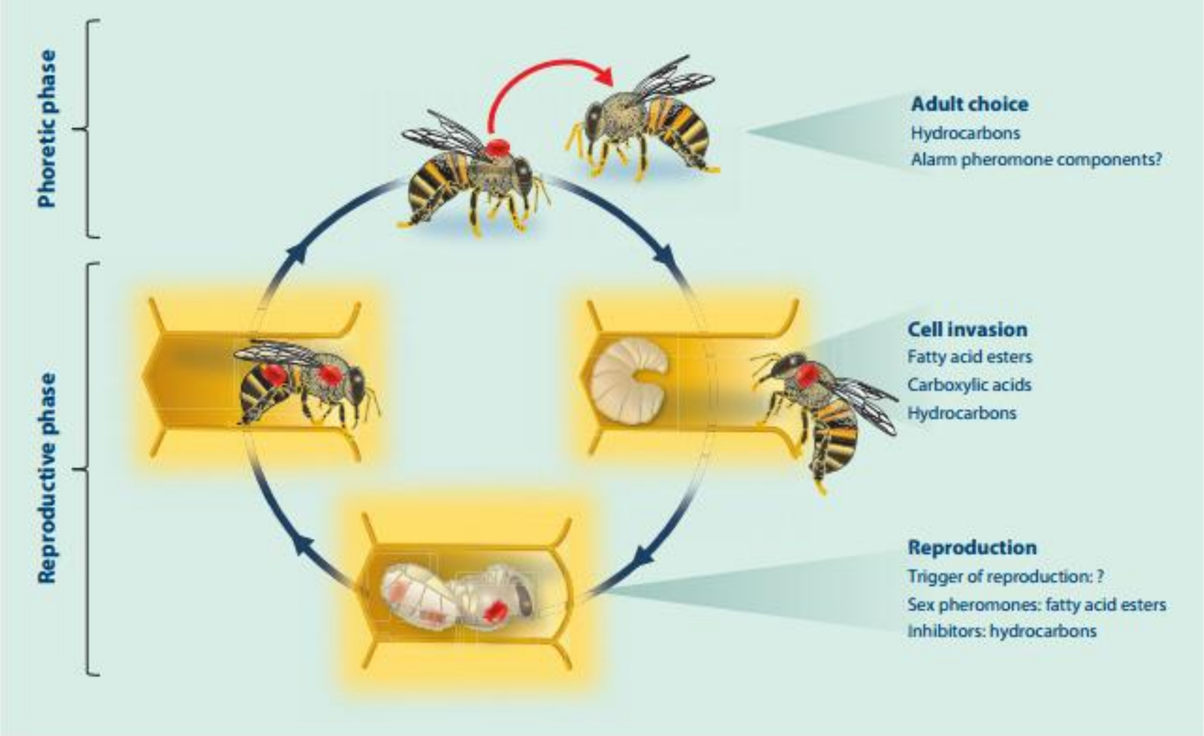

Figure 1. The life cycle of *V. destructor* and the various chemicals that play a crucial role in regulating distinct stages of this cycle. (Source: Nazzi and Le Conte, 2016)

Moreover, the bee's ability to fight off diseases is greatly enhanced by its fat content. By producing immune-related compounds and antimicrobial peptides, it protects bees from infections and diseases. Last but not least, the fat body's numerous functions include aiding in the detoxification of harmful substances such as toxins and pesticides that are present in the environment (Li *et al.*, 2019).

There are several detrimental effects on a bee's overall health and survival that might arise from the reduction or breakdown of its body fat. The fat body is a vital source of energy and a metabolic hub, thus any malfunctions can lead to a variety of issues. A reduced fat body significantly reduced bees' energy reserves, making it more difficult for them to perform essential tasks such as flying, foraging, and thermoregulation. Moreover, disrupting the metabolic processes of the fat body can result in nutritional imbalances in the bee. According to Li *et al.* (2019), Bataglia *et al*. (2022), and Deeter *et al.* (2023), this shortcoming reduces their overall strength.

The importance of the fat body extends beyond metabolic control to hormone regulation, which includes processes that control growth, reproduction, and behavior. Abnormalities in these processes may occur when these hormones are depleted from the bee's fat body, affecting the bee's overall fitness and ability to reproduce. Additionally, they reduce drone size, lowering reproductive fitness by reducing sperm production (Arrese and Soulages, 2010; *Li et al.,* 2019).

In addition, a bee's fat body is essential for its immune system and for detoxifying harmful substances in the environment. When a bee's fat reserves are depleted, its immune system weakens, making it more vulnerable to diseases and infections. Moreover, bees with an unhealthy fat body may struggle to absorb and eliminate pesticides and poisons, increasing their susceptibility to harmful effects. To put it simply, a bee's loss of body fat has a negative impact on its energy reserves, ability to fly, metabolic functions, hormonal balance, immune system, and defense against environmental pollutants. This cumulative effect jeopardizes the bees' overall health, survival, and reproductive ability. (Li *et al.,* 2019). Varroa may also influence bee behavior because of its ability to alter brain functions that impair the non-associative host bee's capacity for learning. Guzmán-Novoa *et al.* (2010), found that parasitized bees developed an olfactory stimulus habituation faster and responded less to sugar.

Abbo *et al.* (2016) found that honeybees exposed to varroa mites had a higher abundance of immune transcript than control bees. There were strong correlations ($R^2 \geq 0.90$ for Defensin, ≥ 0.85 for Hymenoptaecin, and ≥ 0.70 for Eater) between immune-related proteins and varroa mite parasitism. Honeybees respond to varroa infestation by producing host defense immune peptides, as evidenced by increased levels of Defensin (small antimicrobial proteins required for the innate immune system), Eater (a gene involved in bacterial recognition and phagocytosis), and Hymenoptaecin (an antimicrobial peptide found in insects).

On the other hand, a varroa mite infestation may result in proteomic alterations in the immune response of honeybees as well as down regulation of some immune gene expression in newly infected adults. The immune response of the bee is impacted by its disruption of the cascade immune response. For example, varroa reduces the amount of melanin produced by prophenol oxidase, which in turn reduces the quantity of hemoglobin circulating in the hemolymph. Insect immune responses and healing mechanisms are influenced by pathogens, which can be encapsulated by melanin and hemoglobin after infection or injury (Guzmán-Novoa *et al.,* 2010).

Moreover, these mites act as a carrier of certain viruses. Practically speaking, the only viruses with a clear varroa-vector link are Deformed Wing Virus (DWV) and Acute Bee Paralysis Virus (ABPV) Traynor *et al.* (2020). DWV is the most common and extensively studied virus connected to infestations of varroa mites. Bees lose their ability to fly as a result of DWV-induced wing distortion. The wings of infected bees are often curled, shriveled, or malformed. DWV is carried by varroa mites, which feed on bees and transfer the virus from individual to individual. Beekeepers are extremely concerned about DWV since varroa mites are involved in its transmission (Martin and Brettell, 2019; Traynor *et al.*, 2020). Adult bees are the main target of ABPV, which results in abrupt paralysis and death. Infected bees experience symptoms include shaking wings, lack of coordination, and paralysis. Additionally, "shivering"—a distinctive shaking of the wings—may be displayed by bees (de Miranda *et al.,* 2010).

2.4. Varroa mite prevalence in Ethiopia

Various studies conducted in Ethiopia have continuously demonstrated a notable prevalence of *V. destructor* in diverse locations of the nation. Varroa mites were widely distributed during the study seasons in all three geographic regions—Oromia, Amhara, and SNNPR—according to a recent study by Alemayehu Gela *et al.* (2023). With an overall prevalence of 89.7% in apiaries and 89.4% in colonies, the mite was remarkably common. The three most remarkable regional prevalence rates at the apiary level were 96.3% in Oromia, 85.7% in Amhara, and 60.0% in the SNNP region. The predominance of the mite also showed a similar trend at the colony level, with rates of 95.8% in Oromia, 85.2% in Amhara, and 71.9% in SNNPR.

A different study conducted by Tesfu Shegaw *et al.* (2022) found that the prevalence of 48.44% in colonies and 73.21% in apiaries. It's interesting to note that they also reported a different viewpoint which was greater incidence rate in the rainy season than in the dry. In contrast, Desalegn Begna *et al.* (2016) found that varroa prevalence was marginally greater during dry seasons. Across the study months, there were only slight variations in the mites' population through the entire season. In an identical manner, Amsalu Arega (2020) discovered that adult bee infestation was higher in the dry season (69.6%) than in the wet season (60.9%). A similar pattern could be seen in the predominance of sealed brood, which was 52.2% in the rainy season and 56.5% in the dry.

Comparatively lower prevalence of varroa mite was reported by Dereje Tulu *et al.* (2023), with an overall prevalence of 39.3%. On the other hand, the terrifying 100% overall prevalence of varroa mites was found in Ebisa Mezgabu *et al.* (2016) study which caused concerns due to the unusually high infestation rate. A study conducted in 2014 by Desalegn Begna added to the range of results by reporting an overall prevalence of 82% in adult bees and 64% in sealed brood. The wide range of findings emphasizes the complex nature of varroa mite prevalence in Ethiopia, stressing the necessity of ongoing study and observation to comprehend and manage this important problem in beekeeping.

2.5. Control of *V. destructor*

There are several methods for managing varroa mite infestations, including chemical, biological, and cultural approaches. Natural enemies are used in biological control to lower pest populations (Lacey *et al.,* 2015). The life cycle of mites is purposefully disrupted by cultural techniques such brood removal, queen caging, and trapping (de Guzman *et al.,* 2015; Giacobino *et al.,* 2016, Giacomelli *et al.,* 2016; and Liu *et al.,* 2020). Chemical control is the effective management and suppression of mite populations through the use of acaricides, either synthetic or organic (Haber *et al.,* 2019).

Generally speaking, an efficient varroa mite management strategy may combine many of these methods, depending on the particular requirements and conditions of beekeeping operations. A comprehensive method to efficiently control and minimize the impact of varroa mites on bee colonies is provided by the combination of biological, cultural, and chemical approaches (Devi *et al.,* 2019).

2.5.1. Cultural control

Techniques such as queen caging (Giacomelli *et al.,* 2016), brood removal (de Guzman *et al.,* 2015), and the trapping and removal of mites from brood cells (Liu *et al.,* 2020) are examples of cultural approaches. Integrated Pest Management (IPM) strategies, which try to lower varroa mite numbers while minimizing harm to the honeybee colony and the environment, frequently include cultural procedures as a key component.

Beekeepers use a variety of cultural techniques to control infestations of *V. destructors*. One method involves using tools like screened bottom boards, which have mesh screens installed to allow varroa mites to fall through and out of the hive, thus catching and removing mites from the hive. The fallen mites can then be readily collected and disposed of by beekeepers

(Liu *et al.,* 2020). Another method used in beekeeping to manage and control infestations of varroa is caging the queen. In case the infection of varroa mites becomes too much to handle, beekeepers could decide to temporarily confine the queen bee. The queen must be captured and placed in a different area of the hive as part of this procedure. For this, a variety of techniques can be used, such as excluders or specific queen cages (Giacomelli *et al.,* 2016).

The elimination of brood, particularly drone brood, is another successful strategy. Drone (male) bee brood is the preferred host for varroa mite infestations. To lower the mite population without endangering worker bees, beekeepers can remove frames containing drone brood that are affected with mites selectively (de Guzman *et al.*, 2015).

2.5.2. Chemical control

Chemical control techniques, such as acaricides or pesticides, are essential for pest management and control. Synthetic acaricides were previously the preferred method of controlling varroa mites. However, due to residues found in honeybee products and the emergence of chemical tolerance by varroa mites, alternative approaches are now required. These chemical control agents are sold by different producers under different product names. CheckMite+, Apiguard, Bayvarol, Thymovar, and ApiLife Var are a few of the most often found alternatives (Tlak Gajger *et al.,* 2020).

These products contain a range of ingredients, each of which is the exclusive active ingredient. Coumaphos is an active ingredient in CheckMite+, thymol is the active ingredient in Apiguard, Thymovar is a cellulose wafer containing thymol, and ApiLife Var is a blend of active ingredients that includes thymol, eucalyptus oil, levomenthol, and camphor. On the other hand, the active ingredient in bayvarol is flumethrin. These compounds all cause high mite mortality rates, although their efficiency in managing varroa varies greatly.

Tlak Gajger *et al.* (2020) found that acaricides based on coumaphos, like CheckMite+, produced the best effects, while those based on thymol, like Apiguard, produced the least effect with a statistically significant difference. Beekeepers must be mindful of these differences in efficacy when choosing and using chemical control techniques to manage varroa mites.

2.5.3. Biological control

Biological control refers to the utilization of living creatures to decrease the number of pests and minimize their harm. Numerous pests, such as insects, weeds, plant infections, and animals, can be managed with its help. However, depending on the pest, different techniques and agents are used. Natural enemies of insects have a crucial role in limiting the population of possible pests (Stenberg *et al.,* 2021; Barratt *et al.,* 2018).

Biological control is a better option to chemical pesticides, which may have negative effects on the environment and human health, by reducing the target pest or species without endangering non-target creatures, the environment, or human health. Biological control falls into two categories: augmentative biological control, which releases natural enemies in large quantities into the environment to suppress pest populations and is frequently employed in agricultural contexts, and classical biological control, which involves introducing natural enemies into a new environment where the pest is problematic (Stenberg *et al.,* 2021).

In honeybee colonies, biological management methods for varroa mites include a range of agents, including as helpful arachnids as chelifer and entomopathogenic fungi (EPF). In particular, it has been shown that EPF such as *Beauveria bassiana* and *Metarhizium anisopliae* are effective at both infecting and suppressing varroa mite populations. It is noteworthy that these fungi accomplish this without endangering honeybee populations in a major way (Ferrari *et al.,* 2020; Hamiduzzaman *et al.,* 2012). Furthermore, because they are varroa mites' natural predators, chelifer plays a critical part in this biological control strategy. Without endangering their honeybee hosts, they feed on the mites (Fagan *et al.,* 2012; van Toor *et al.,* 2015).

Generally speaking, biological control is a highly advantageous and sustainable method of managing pests. It addresses problems like pollution from chemicals and harm to non-target organisms by lowering dependency on chemical pesticides. Because biological control agents are highly adaptable and precisely target pests, they reduce the disturbance of ecosystems. Biological controls, as opposed to chemical alternatives, enable ongoing pest management, reducing the need for frequent reapplication and preventing the emergence of resistance. Additionally, by protecting beneficial organisms, this strategy promotes the general health of the environment and ultimately proves to be cost-effective. Notably, biological agents provide no health hazards to people, making it a financially and environmentally sound option.

2.6. Chelifers as biological control agents of varroa mites

Chelifers, *Chelifer cancroides,* also known as pseudoscorpions or chelipedes (*Arachnida: Pseudoscorpionida*), are becoming more and more recognized as useful generalist predators of pests in honeybee colonies. They are little in size, but they have a lot of energy and live a long time in the hive. Chelifers are known to prey on varroa mites as part of their predatory habit, making them their natural enemies. Specifically, during the mite breeding season, their efficient searching abilities and an adult stage with early predatory tendencies allow them to eat a significant number of mites, which in turn affects the mite population as a whole (Fagan *et al.*, 2012; van Toor *et al.*, 2015).

In 2012, Fagan *et al*. published video surveillance results showing that chelifers were seen eating one to nine mites every day. These discoveries have important significance because the varroa model indicates that as few as 25 chelifers per hive may be able to stop the exponential increase in mite numbers. The projection was specifically constructed with a 10,000-bee hive that was up against a 1,000-mite infestation. This finding raises the prospect of using chelifers as biological control agents in beehives to deal with varroa mite-related problems.

2.7. Entomopathogenic fungi (EPF)

A class of microorganisms known as EPF is notable for being a pioneer in the field of pest biocontrol. These fungi are remarkably effective in infecting and eliminating a wide variety of insect pests. They are considered as natural enemies of insects because of their important function in controlling insect populations across a range of settings. Because of this, EPF have become important players in the continuous endeavor to eradicate pests via biological methods (Pell *et al.,* 2010; Sinha *et al.,* 2016). They are worldwide and can be found in many different types of natural environments. The spread and prevalence of these fungi are influenced by a number of factors, including climate, habitat features, host insect demography, and local environmental variables (Sinha *et al.,* 2016).

In both agricultural and natural environments, a wide variety of EPF flourish in soil habitats and integrate themselves into the soil's microbial population. According to Sinha *et al.* (2016), soil protects them from solar radiation damage and serves as a barrier against temperature fluctuations and water scarcity. According to Behie *et al.* (2015), fallen leaves, plant surfaces, and plant remains also provide a home for these microorganisms.

2.8. Entomopathogenic fungi as biocontrol agents

EPF have revolutionized the management of agricultural pests by offering an effective barrier against a wide range of agricultural pests. *B. bassiana* and *M. anisopliae* are two notable candidates in this field of biopesticides, both of which demonstrate their adaptability and efficacy (Singh *et al.,* 2017; Sinha *et al.,* 2016).

Different strains of these fungi can effectively manage varroa mites, with each strain exhibiting a different level of activity against this problematic mite. Ferrari *et al.* (2020) investigated *M. anisopliae* var. BIPESCO 5's efficacy against *V. destructor*. The effect of this particular isolate on the nucleus and parental bee colonies was assessed in this study. The weekly mite fall in the nucleus colonies treated with *M. anisopliae* var. BIPESCO 5 was significantly lower than in the control group, with a decrease of around 50%, according to the results. The difference between the treated and control groups was, however, hardly noticeable in the parental colonies. The isolate's dry conidia were applied by the researchers as a wet table powder, with each gram containing roughly $1x10^{10}$ conidia.

More importantly, in 2012, Hamiduzzaman *et al.* treated honeybee colonies with three distinct strains of *M. anisopliae* and found amazing results in the management of *V. destructor*. According to their investigation, using these isolates resulted in mite mortality rates ranging from 50% to as high as 90%. Two isolates in particular, *M. anisopliae* UAMH 9197 and *M. anisopliae* UAMH 9198, stood out for their remarkable 90% mite mortality rate, suggesting that they could be useful in controlling this honeybee pest.

On the other hand, in a two-round experiment, Romo-chacón *et al.* (2016) manually dusted *M. anisopliae* on honeycomb plates. Remarkably, their results showed that in both experiment rounds, there were much fewer mite deaths. The complexity of the relationship between *M. anisopliae* and *V. destructor* is demonstrated by this variation in findings, which also raises the possibility that a number of variables could affect how effective this strategy is.

A great deal of research has been done on *B. bassiana*'s potential use as a bio-control agent against varroa mites, a major pest in beekeeping. In a study by Sewify *et al.* (2015), an intriguing experiment was done where dry *B. bassiana* conidiospores were applied to beehives. Some important discoveries were made by this investigation. When compared to control groups, the study showed that *B. bassiana* had a significant effect on the number of varroa mites infected with fungi in treated colonies. The average number of dead mites in the colonies treated with *B. bassiana* and the untreated colonies differed significantly. These findings showed that, in comparison to other concentrations and the control group, a fungal concentration of 5x10^6 spores/g resulted in greater mortality rates in varroa mites. Furthermore, it was found that using *B. bassiana* for two rounds of dusting was successful in managing *V. destructor*, indicating a possible solution for this beekeeping problem.

In an important study carried out in 2012 by Hamiduzzaman *et al.,* treatment of honeybee colonies with three distinct isolates of *B. bassiana* produced remarkable outcomes in the control of *V. destructor*. In this investigation, a conidial concentration of 1x10^8 conidia/mL was used. Because of the use of these isolates, mite mortality rates in this study ranged from a significant 50% to an extreme 90%. Among these isolates, *B. bassiana* GHA in particular, stands out for showing an impressive 90% mite mortality rate by itself. This discovery emphasizes how potent these *B. bassiana* isolates could be as agent against the *V. destructor*.

The same fungal isolate, *B. bassiana* GHA, was tested in a related investigation by Sinia in 2013 at five different concentrations (1x10^2, 1x10^4, 1x10^5, 1x10^6, and 1x10^8 conidia/mL). The results of this experiment were significant especially showing how varying concentrations affected the rates of mite mortality.

The study's findings showed that at a fungal concentration of 1x10^8 conidia/mL, the maximum mite deaths were seen. This finding emphasizes how crucial concentration levels are to *B. bassiana* GHA's efficiency as a mite-control agent against *V. destructor*. More concentrations of the fungal isolate may have more noticeable effects on mite populations, according to this suggestion, which offers important information for maximizing the use of this bio-control technique in beekeeping.

2.9. Mechanism of action of entomopathogenic fungi (EPF)

EPF infect insects through a number of different processes. First, the process begins with adhesion or attachment. The creation of mucilage, a gelatinous or sticky substance, aids in the attachment or adhesion of fungal spores onto the host cell, a necessary step in the process. In addition to mucilage, this infection technique is also greatly aided by a variety of enzymes, lectins, and different types of interactions, such as hydrophobic and electrostatic forces (Singh *et al.,* 2017).

The fungal spores are activated once they are securely attached to the mite's body, at which point they start to produce enzymes and filamentous structures called hyphae. These enzymes—which include chitinases, lipases, and proteases—are essential for hydrolyzing the insect's epidermis. The creation of an appressorium (Figure 2), a specialized structure which links to the cuticle layer and forms a restricted bridge, or the germ tube itself are the two ways that an organism can enter the insect cuticle layer (Singh *et al.*, 2017).

The fungus enters the insect's hemocoel and proceeds to colonize and proliferate after making a successful entrance. After successful penetration, the fungus either forms discrete yeast-like structures or blastospores that are released into the hemolymph. After that, it enters the insect's respiratory system to get the most nourishment possible. A number of things contribute to the insect's mortality, such as nutrients depletion, mechanical damage from tissue invasion, and the development of toxins in the body (toxicosis). The other thing is that the process adhesion, germination, growth, and penetration on the insect cuticle and its constituent parts are likely to be influenced by environmental conditions such temperature, humidity, and sunlight (Sinha *et al.*, 2016; Singh *et al.*, 2017).

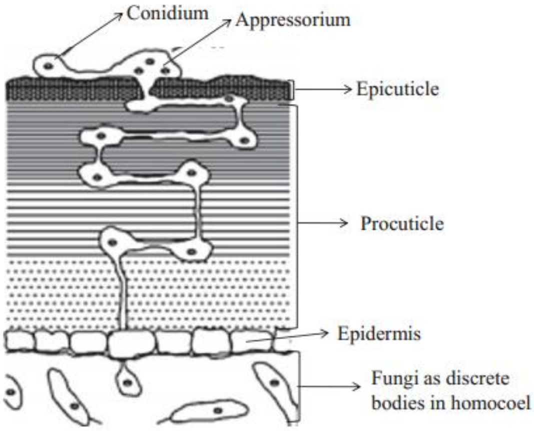

Figure 2. Invasion process of *B. bassiana*. (Source: Sinha *et al.*, 2016)

3. MATERIALS AND METHODS

3.1. Study area

In this investigation, honeybee colonies from the apiary of Holeta Bee Research Center were used. The laboratory operations were conducted at the National Agricultural Biotechnology Research Center of the Ethiopian Institute of Agricultural Research, located in Holeta. Holeta town is found in the Oromia Regional State of Ethiopia. It is located at 2391 meters above sea level and has a latitude and longitude of 9°3′N 38°30′E.

3.2. Experimental design

This study employed a completely random design (CRD). Each experimental unit was randomly assigned to a treatment group. A total of five treatment groups, incorporating three distinct fungal isolates and two control groups—one treated with distilled water and the other with a Tween 80 solution were used. Within each treatment group, one replication involved the examination of 20 varroa mites, 60 sealed honeybee brood, and 20 adult worker bees. Each treatment was performed in triplicate.

3.3. Fungal cultures and production of conidial suspension

In this experiment, three *Beauveria bassiana* isolates, namely APPRC-44BC, S#10H, and APPRC-27, were used. These isolates, obtained from Ambo Agricultural Research Center, were originally isolated from soil. Each isolate was grown on Potato Dextrose Agar (PDA) under controlled conditions to ensure their optimal growth and performance. A consistent temperature of 26±1℃ and a relative humidity of 70±10% were maintained during the culture procedure. These regulated environmental conditions created an ideal condition for the fungi, facilitating their growth.

Conidia, which were essential to this investigation, were carefully extracted from fungal cultures after two weeks of fungal growth according to the approach described by Hamiduzzaman *et al.* (2012) and Oliveira *et al.* (2015). Ten mL of sterile 0.05% Tween 80 solution was gently poured over culture plates to start the conidia extraction process. To help conidia release the culture, the surface of the fungal culture was then gently scraped with a sterile spatula. After that, the resulting conidial suspensions were put into 15 mL test tubes and vigorously vortexed for three minutes to ensure consistent conidia dispersion throughout the suspension. The conidial suspensions were further refined using double layer cheesecloth.

Serial dilutions were performed in 10 mL of sterile distilled water enriched with 0.05% Tween 80. To ensure uniform conidia distribution, each dilution underwent 30 seconds of gentle vortexing (Hamiduzzaman *et al.,* 2012; Oliveira *et al.,* 2015). Numerical density was measured using a Hemocytometer, and the conidial suspension was adjusted to attain a final concentration of 1×10^8 conidia/mL. To prevent contamination and counting errors, the cover glass and Hemocytometer were cleaned with 70% ethanol and dried with sterile lens paper. Distilled water moistened the Hemocytometer's shoulders before gently pressing on a cover-slip. The spore suspension was mixed by shaking test tubes, then filled into the chamber via capillary action, ensuring it was not overfilled or underfilled. Grid localization was achieved by observing the Hemocytometer setup under a microscope with a 10x objective lens. After a two-minute settling period, spores were counted in selected squares at 40x magnification power. Inclusions were made for spores on the upper and left boundary lines. The exhaustive counting procedure included each of the five large squares in the center and at the corners. On both sides of the Hemocytometer, the number of conidia in the large squares (i, ii, iii, iv, and v) were counted, and the average of one large square was determined (Avin, 2019).

Average number of conidia in one large square (A):

$$A = \frac{(i + ii + iii + iv + v) + (i + ii + iii + iv + v)}{5 + 5}$$

Using unitary method average number of spores per mL of the suspension was calculated.

$$\text{Spores per mL of suspension} = \text{A x Dilution factor x } 10^4$$

3.4. Vegetative growth rate and spore production evaluation

The sporulation and vegetative growth rate evaluation of the isolates were carried out following the techniques described by Amha Gebremariam *et al.* (2021) and Awan *et al.* (2021). One millilitre suspension containing 1×10^8 conidia/mL from each isolate was evenly spread onto separate plates containing PDA. The plates were covered with Parafilm and allowed to sit complete in darkness at 26 ± 1℃ for 72 hours to grow. Using a 5-mm cork borer, the unsporulated mycelial mats were carefully removed from the culture plates following the 72 hour incubation period. After that, each excised agar plug was moved independently to the center of a fresh PDA plate. For every isolate, this procedure was repeated three times. The plates were once more sealed with Parafilm and kept at 26 ± 1℃ in total darkness for duration of 15 days. The colonies' radial growth was recorded every day to track the growth process. The measurement was carried out using a basic plastic ruler. A simple linear regression analysis was then used to determine the mycelial growth rate (mm/day).

After the 15-day growth phase, conidia output was counted. This was accomplished by cutting out a 5 mm diameter circular disc of mycelial mat from the sporulated fungal culture plates using cork borer. The circular disc of mycelial mat encompassed an area of approximately 19.63mm². This circular disc was placed in to 10 milliliters of 0.05% Tween 80 solution. To ensure full mixing, the suspension was vortexed vigorously for five minutes. After that, the suspension was filtered through double layer of cheesecloth and the number of conidia in the suspensions was counted using Hemocytometer.

3.5. Characterization of *B. bassiana* isolates

Isolates underwent thorough cultural and morphological characterizations for confirmation purpose.

3.5.1. Cultural characterization

Beauveria bassiana isolates were subjected to a culture examination, with particular focus given to colony properties such as color, shape, texture, and growth pattern on PDA. According to Amha Gebremariam *et al.* (2021) the following are descriptions of the observed cultural traits of *B. bassiana* isolates on growing media. The isolates had a round colony shape and a scattered growth pattern. The color of the colony varied from yellow-white to white. The colonies are said to have a smooth texture that might be either powdery or cottony. These macroscopic features were helpful in identifying and distinguishing *B. bassiana* isolates from other fungal species during the culture evaluation of the isolates.

3.5.2. Morphological characterization

The study employed slide culture techniques and observation of sporulated fungal culture under microscope for morphological characterisation of fungal isolates, adhering to the procedure found on Amha Gebremariam *et al.* (2021). The process started with the standard methods for preparing PDA medium. After being put onto plates, the media were left to solidify. Then a piece of sterile filter paper and a sterile glass slide were placed inside another sterile Petri dish together with sterile straw tubes. A 1-by-1-cm block of solidified PDA was cut from a plate with a sterile blade and placed onto the glass slide. Using a sterile needle, the two weeks old fungal culture conidia were injected onto each side of the agar block. After that, a coverslip was put over the block and pressed to ensure adherence and produce a tight seal. To provide ideal growth conditions, about 2 millilitres of sterile distilled water was added to the Petri dish's bottom. After that, the plates were incubated for three days at a controlled temperature of 26 ± 1 ℃. Following the observation of fungal development, the coverslip was carefully taken off using forceps and put on a clean glass slide with a drop of lactophenol cotton blue solution. After preparation, this slide was closely examined using a compound microscope with a 40x magnification power. A piece of sporulated fungal cultures, which were used as conidia source during slide culture technique, were also stained with lactophenol cotton blue and examined using a compound microscope with a 40x magnification power.

3.6. Conidia viability test

A germination test was done to evaluate the viability of the conidia from the isolates in accordance with the protocols that was described elsewhere (Belay Habtegebriel *et al.*, 2016; Denberu Kebede *et al.*, 2022). Ninety millimetre Petri dishes with 10 mL of PDA culture medium were used to start the germination tests. After that, a Hemocytometer was used to adjust the conidial suspension's concentration to 3×10^6 conidia/mL. Next, 100µl of the conidial suspension was distributed equally among the PDA media in every Petri dish. When the free water evaporated, the plates were sealed with parafilm to create a controlled environment during 16 hours incubation period. A consistent temperature of 26 ± 1 ℃ was upheld in a growth chamber throughout the entire incubation period. After 16 hours of incubation, one millilitre of 70% alcohol was added to each Petri dish to prevent over-germination. Each Petri plate was pseudo-triplicated by placing three sterile coverslips on it in order to increase the assessment's accuracy. With a minimum of 300 conidia counted on each plate, this pseudo-triplication allowed for extensive examinations under a light microscope at 40x magnification. A conidium was considered to be germinated for the purposes of the experiment if its germ tube growth was at least as big as itself. In order to ensure the production of solid and data that is reliable, this procedure was carried out precisely and repeated three times for each isolate. Calculating the viability percentage for each isolate was the last stage of the assessment. To do this, the number of conidia that germinated was divided by the total number of conidia that were inspected, and then the result was multiplied by 100. The percentage of germination for each isolate was calculated using the following formula:

$$\text{\% of spore germination} = \frac{\text{Number of spores germinated}}{\text{Total spore count}} \text{x } 100$$

3.7. Collection of *V. destructor*

Frames containing sealed drone brood were brought to the lab for the varroa mite collection. Using tiny forceps to carefully open sealed brood cells, mites were carefully removed off the body surface of honeybee brood. Three hundred female mites were collected and placed into several 20 mL glass scintillation vials. Two late-stage honeybee larvae were supplied as a food source in each vial to keep the mites alive until the experiments. These mites were used in bioassays very quickly; all of the experiments were carried out in two hours after the mites were collected, according to the protocol described by Hamiduzzaman *et al.* (2012).

3.8. *V. destructor* inoculation with fungal isolates

This test was performed following the protocol stated in Hamiduzzaman *et al.* (2012). For each treatment twenty varroa mites were individually submerged in 5 milliliters of fungal suspension containing conidia at a concentration of 1×10^8 conidia/mL for ten seconds. The same procedure was done for the control groups (0.05% Tween 80 and distilled water). The treated mites were then arranged on sterilized filter paper with a 90 mm diameter inside a 90 mm diameter sterilized Petri dish to facilitate drying. For each replication of a single treatment, a brood comb containing more than 50 freshly-capped brood cells was prepared from a healthy colony in order to infest honeybee brood with treated varroa mites. Using non-toxic, water-based paint markers, the rims of these brood cells were uniquely colored; each color denoted a different treatment. Each capped cell was carefully loaded with two varroa mites that had been exposed to the same treatments using a fine paintbrush. For one replication of a single treatment a total of ten capped cells were used. The capped cells were opened by making a small, 2mm long slit in the cap using a sterile blade and then resealed by gently touching it with liquid beeswax. After that, the infested combs were kept in an incubator at 33°C and 60% relative humidity for ten days. At 48-hour intervals, the mite mortality within each infested cell was checked by cutting off the cell cap, removing the brood with fine forceps, and looking for dead mites. If a mite showed no signs of life after being pierced with a pin, it was considered dead. Any alive mites were transferred to other cells containing live brood, followed by sealing and labeling of the new cell. After being extracted from the cells, the dead mites were surface sterilized with 90% ethanol and placed on PDA that contained chloramphenicol. Once this was done, plates were incubated for 36 hours at 26℃ under controlled conditions with a relative humidity of 70±10%. Indeed, the primary sign of fungal-induced death was the development of mycelia from a varroa mite cadaver. For each of the five different treatments, this extensive experiment was conducted three times.

3.9. Honeybee brood collection and inoculation with fungal isolates

The inoculation of honeybee brood with fungal isolates was conducted according to the protocol described in Hamiduzzaman *et al.* (2012). The brood combs were taken from a honeybee colony and kept overnight in an incubator in the lab, which was maintained at 33℃ and 60% relative humidity. The brood cells within each comb were systematically arranged into rows and color-coded after the overnight incubation. Every distinct color was associated with a particular fungal isolate or control groups. Following this, 5µL of conidial suspension, distilled water, and 0.05% Tween 80 solution were randomly given to each row of brood cells. For each treatment, sixty freshly capped brood were used. Twenty of the sixty brood were set aside to record bee emergence, twenty more were set aside to track fungal colonization the day following inoculation (1dpi), and twenty more were set aside to evaluate fungal colonization seven days later (7dpi). Every treatment group was given a separate set of screen cages that were made to fit the exact shape of the brood cells that were getting the same treatment. The combs were then put back into the incubator, and the bees' emergence were monitored carefully. Manually inserting wire mesh screened cages into the comb allowed for the confinement of the treated brood cells and allowed for the observation of bee emergence. The number of emerging bees from the treated broods as well as cases of brood mortality (brood not hatching successfully) were recorded. Then, the freshly hatched bees were carefully taken out of their cages with wire mesh screens and placed in feeding cages so that their body weight could be determined. Then, after being exposed to low-temperature narcosis for anaesthesia as described by Tutun *et al.,* (2020), the body weights of emerging adult bees were measured. The brood cells were also opened 1dpi and 7dpi. Using tiny forceps, infected broods were extracted in order to evaluate the extent and timing of fungal colonization in honeybee brood. After being surface sterilized, the broods were placed on microscope slides. On these slides, the broods were crushed and stained with lactophenol cotton blue, enabling examination of the spores and hyphae in different tissues at 100x magnification power.

3.10. Collection of adult honeybees

From healthy colonies, combs containing emerging worker bee pupae were collected and brought to the lab. The pupae's maturity was assessed by randomly opening a few cells and examining the color of the thorax and eyes. The combs were carefully covered with cheesecloth and kept at 33℃ after being taken out of the hive. After being emerged, twenty bees were moved to each of 10 x 8 x 6 cm cage. The bees had access to food and water within their cages. The bees in the cages were then maintained for one week prior to the start of the treatment at a constant temperature of 30℃ (Shaw *et al.,* 2002).

3.11. Adult honeybee inoculation with fungal isolates

Fungal isolates were used to inoculate honeybee brood following the procedures described in Shaw *et al.* (2002), García-Fernández *et al.* (2008), and Omuse *et al.* (2022). The adult worker honeybees received distinct treatments once they were matured, one week old. After being briefly subjected to low-temperature narcosis for anaesthesia, groups of twenty matured adult worker honeybees were moved to specific spray cages. A 12 x 12 cm piece of 6 mm thick polyurethane foam (PU foam) with a 9 cm-diameter central hole was used to build each spray cage. A 12 x 12 cm square of 0.7 mm mesh with an aperture size of 2.46 mm (offering 60% open area) was firmly secured on one side of the PU foam, and another piece of mesh was pinned to the other side. Then, using a handheld sprayer, 1 mL of a watery suspension containing 1×10^8 conidia/mL was sprayed on them. The bees in the control group were sprayed with 1 mL of distilled water and 1 mL of a 0.05% Tween 80 solution. Treated bees were then immediately returned, under no further anaesthesia, to their respective bioassay cages. Afterwards, for the entire period of the experiment, the cages were kept at 30℃ and 60±10% humidity. Over the period of two weeks, daily counting was done to record the number of live and dead honeybees in each cage. Dead bees were taken out, surface sterilized, and placed on PDA containing chloramphenicol at a temperature of 26±1 ℃ and a relative humidity of 70 ± 10%. After incubation the presence of sporulating mycelia on honeybee cadavers, served as a marker for fungal-induced mortality. This experiment was repeated three times for each of the three different fungal isolates and control groups.

3.12. Data analysis

The experimental data were subjected to statistical analysis employing one way analysis of variance (One way ANOVA), and means were compared at the 5% significance level using Least Significant Difference (LSD) test. The calculations for mean values, standard deviation, and standard error of the mean were performed using R-Studio (version 4.3.1).

4. RESULTS

4.1. Conidial viability

After 16 hours of incubation, remarkably high conidia viability percentages were observed in all fungal isolates. None of the isolates registered an average viability value lower than 89.88%. In particular, APPRC-27 demonstrated the highest average conidia viability percentage of 91.89 ± 0.64, while lowest viability percentage of 89.88 ± 0.64 was observed for S#10H (Table 1). However, there was no statistically significant difference ($P > 0.05$) in conidia viability among the isolates.

Table 1. Conidia viability percentage of fungal isolates.

Fungal isolates	Viability (%)
APPRC-27	91.89 ± 0.64^a
APPRC-44BC	90.89 ± 0.64^a
S#10H	89.88 ± 0.64^a

Means followed with the same letters in a column are not significantly different from each other ($P > 0.05$).

4.2. Vegetative growth rate

After monitoring growth trend for a duration of fifteen days (Figure 4), the analysis of growth patterns among three distinct isolates revealed a highly significant difference ($P < 0.001$) among the radial growths of the isolates. Notably, isolate APPRC-44BC displayed highest daily radial growth compared to the isolates S#10H and APPRC-27. However, there was no significant ($P > 0.05$) growth difference between S#10H and APPRC-27 (Table 2).

Table 2. Mean ± SE radial growth rate of fungal isolates per day

Fungal Isolates	Growth rate (mm) /day
APPRC-44BC	3.38±0.18^a
APPRC-27	2.18±0.18^b
S#10H	1.96±0.18^b

Means followed with the same letter in a column are not significantly different from each other ($P > 0.05$).

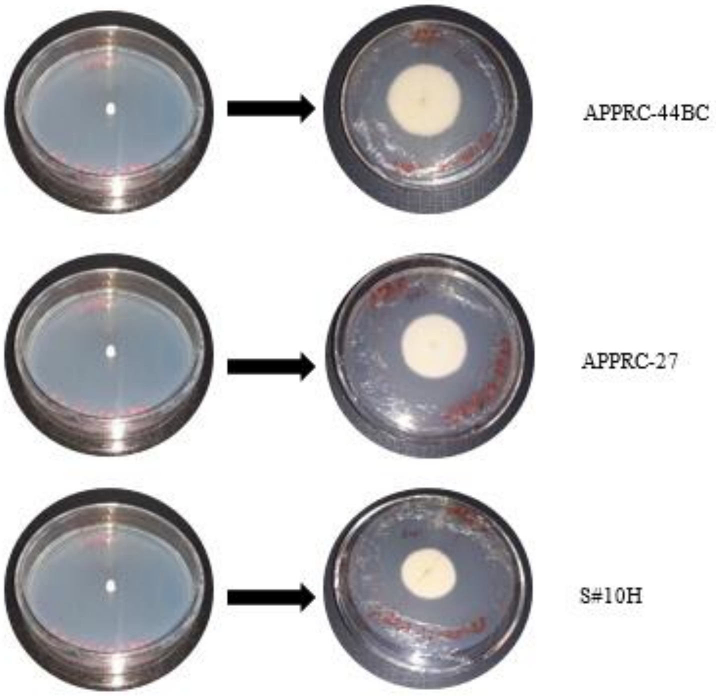

Figure 3. Radial growth of *B. bassiana* isolates on PDA. The pictures on the left side of the arrows indicate inoculation day, while those on the right show growth after two weeks.

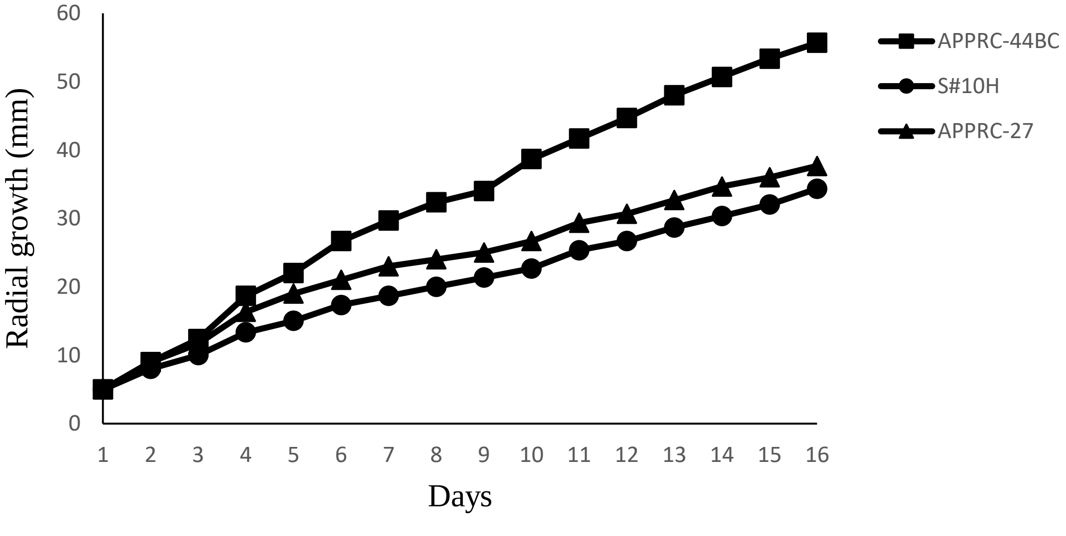

Figure 4. Mycelial growth of three *B. bassiana* isolates on PDA.

The correlation analysis of number of days and the radial growth showed a strong positive correlation for all three isolates with the correlation coefficients of 0.9954, 0.9959 and 0.9861 for APPRC-44BC, S#10H, and APPRC-27, respectively. These coefficients indicated that there was almost a perfect positive linear relationship between the two variables (days and radial growth). The Pearson's correlation coefficient was calculated between radial growth and the duration isolates spent on growth media. The p-value for the slope coefficient was found to be 1.013×10^{-14} for APPRC-44BC, 6.563×10^{-16} for S#10H and 2.567×10^{-12} for APPRC-27.

In regression analysis, the relationship between radial growth and the duration (days) isolates spent on growth media for three distinct isolates: APPRC-44BC, S#10H, and APPRC-27 were examined. The regression equations were found to be $Y = 8.172 + 3.289X$, $Y = 7.291 + 1.786X$, and $Y = 9.758 + 1.919X$ for isolates APPRC-44BC, S#10H and APPRC-27 respectively. A strong model fit was demonstrated with an R^2 value of 0.99, 0.99 and 0.97 for isolates APPRC-44BC, S#10H and APPRC-27, respectively.

4.3. Conidia production potential

During the quantification process using a haemocytometer, the APPRC-44BC isolate exhibited conidia yield of about $1.93 \pm 0.582 \times 10^6$ conidia/mL (9.83×10^4 conidia/mm²). Additionally, APPRC-27 and S#10H demonstrated conidia counts of around $1.66 \times \pm 0.582 \times 10^6$ conidia/mL (8.44×10^4 conidia/mm²) and $1.61 \pm 0.582 \times 10^6$ conidia/mL (8.20×10^4 conidia/mm²), respectively. These results revealed that there was no statistically significant difference ($P > 0.05$) among isolates in conidia production potential. However, APPRC-44BC was noted for having a marginally superior conidia production.

4.4. Characterization of *B. bassiana* isolates

4.4.1. Cultural characterization

At the colony level, the three *B. bassiana* isolates exhibited interesting visual characteristics. Their growth on solid media was characterized by a white color, showcasing distinctive textures that range from a fluffy, cotton-like appearance to a powdery texture, raised to flat elevation, and circular shape (Figure 5A and 5B). APPRC-27 isolate exhibited cotton-like texture which then transformed into a powdery surface after two to three weeks, attributed to the prolific growth of conidia. In contrast, the APPRC-44BC and S#10H isolates consistently displayed a smooth texture which was later transformed in to powdery texture as a result of conidia formation. Notably, the reverse side (underside) of the colonies exhibited a yellowish-white color (Figure 5C), forming distinct patterns that contributed to the identification process. All three isolates exhibited colonies with a circular shape on PDA

(Figure 5D). As the colonies expanded, an obvious radial growth pattern emerged, with the mycelium extending outward from the center. The filamentous growth of *B. bassiana* colonies were characterized by hyaline hyphae extending radially, playing an essential role in the colony's expansion and coverage of the growth medium.

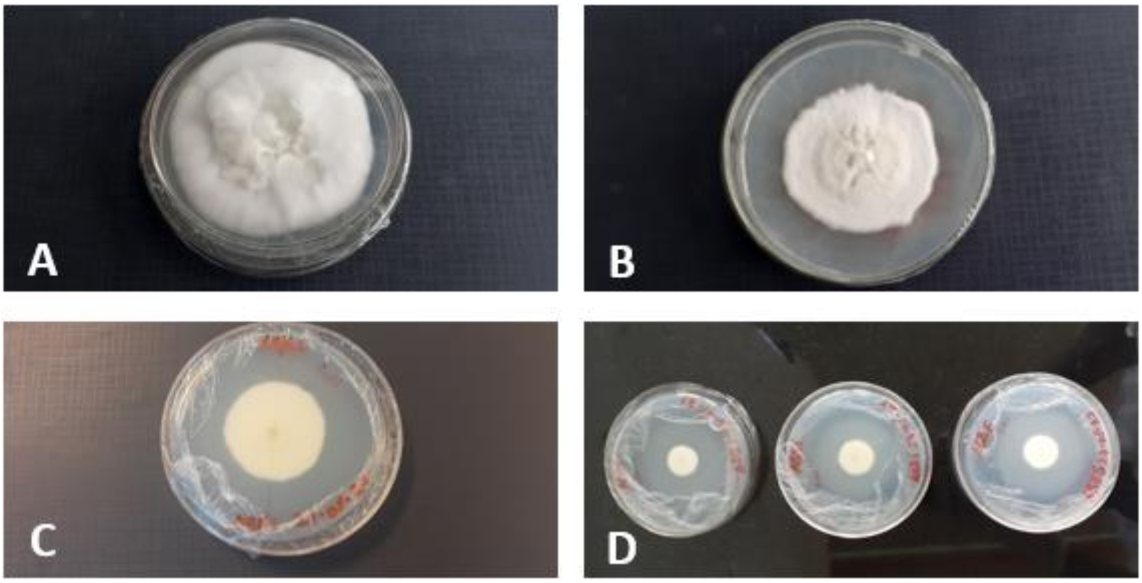

Figure 5. The cultural characteristics of *B. bassiana* isolates on PDA. The cotton-like appearance (A), smooth texture (B), reverse side (C), and circular shape (D) of *B. bassiana* isolates.

4.4.2. Morphological characterization

The *B. bassiana* fungus' hyphal network, or mycelium, was examined under a compound microscope. A complex network of filaments that extended from the colony's center was observed. These filament structures started to form conidiophores—specialized hyphal extensions—during their development on PDA. The conidia were arranged in columns or chains at the tips of these conidiophores, producing a spectacle. The conidia, the single-celled structures were observed to be oval in shape and white in color under a 40x magnifications of compound microscope (Figures 6). After a period of development on the growth media, the color of the conidia changed to gray.

Figure 6. Stained fungal structures (conidia and hyphae) of *B. bassiana* isolate.

4.5. Varroa mite inoculation

In the test conducted to examine the effect of three distinct fungal isolates on *V. destructor,* the treatment involved fungal isolates demonstrated a highly significant ($P < 0.001$) mortality of varroa mite compared to the control groups. Among the five treatments, varroa mite cadavers collected from the three treatments with *B. bassiana* fungal isolates (APPRC-44BC, APPRC-27, and S#10H) exhibited a significantly high level of fungal mycelia development on the varroa cadaver (Figure 7). Fungal mycelia development was not observed in any of the varroa cadavers collected from the control groups.

Among the three distinct fungal isolates, isolate APPRC-44BC was found on average on 14.67 ± 0.77 cadavers of varroa mites out of 20 varroa mites that received the treatment. Likewise, isolate APPRC-27 was found on average on 13.67 ± 0.77 cadavers of varroa mites out of 20 varroa mites that received the treatment. The other isolate, S#10H, was found on average on 10.33 ± 0.77 cadavers of varroa mites out of 20 varroa mites that received the treatment (Figure 8).

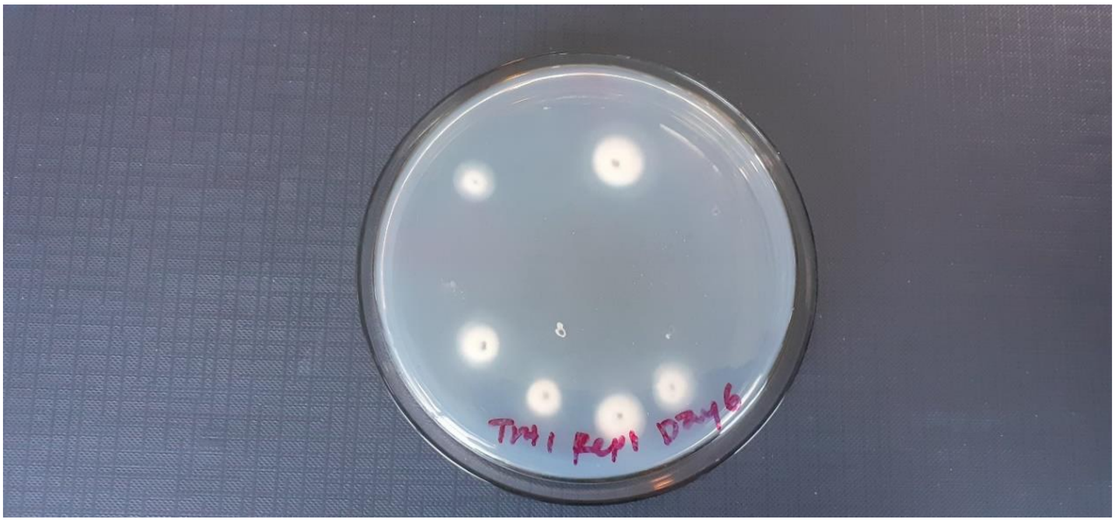

Figure 7. The growth of fungal isolates on the cadaver of varroa mites.

Of the three fungal isolates, isolate APPRC-44BC was found to have the most effective potential in controlling of varroa mites with 73%, followed by isolate APPRC-27 with a 68% controlling effect. However, S#10H was the least effective isolate (52%).

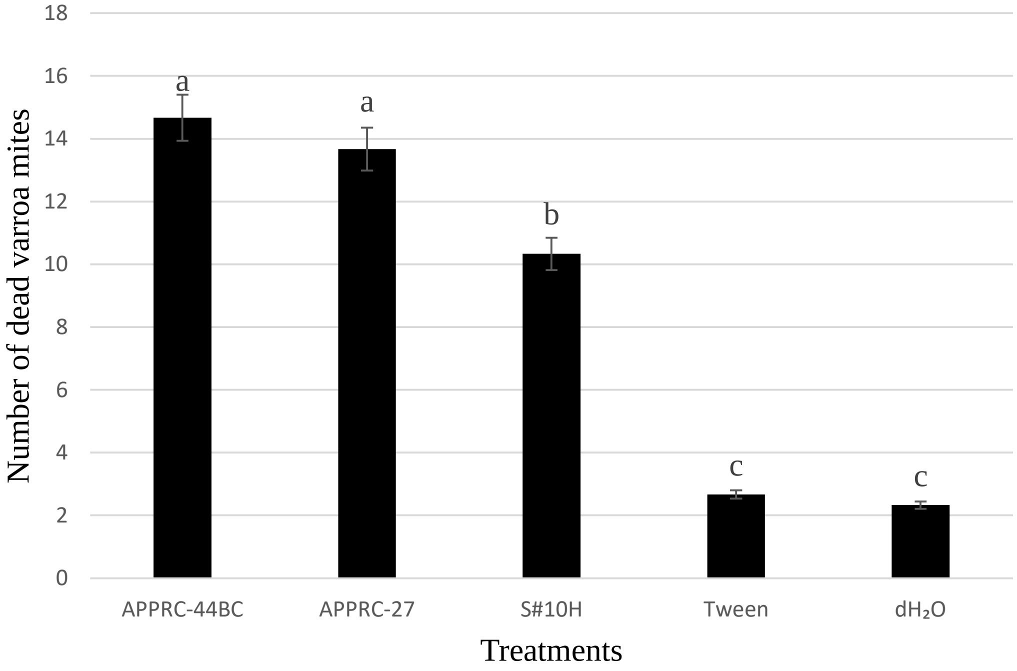

Figure 8. Mean mortality of varroa mites inoculated with different isolates of *B. bassiana*.

Regarding the timing of varroa mite death, significant differences in the timing of varroa mite death were observed among treatments. The fungal isolates APPRC-44BC and S#10H showed highly significant differences ($P < 0.001$) in mortality timing, while APPRC-27 exhibited a very significant difference ($P < 0.01$). APPRC-44BC induced the fastest varroa mite mortality, with about 98% occurring between the sixth and eighth days post-inoculation, with 55% on the sixth day and 43% on the eighth, and only 2% on the tenth; no deaths were noted on the second and fourth days (Figure 9). However, significant difference was not observed ($P > 0.05$) between day 6 and day 8 (Table 3). Similarly, S#10H caused significant mortality on the sixth and eighth days ($P < 0.001$), with about 96.8% of deaths occurring between these days, 61.3% on the eighth day, 35.5% on the sixth, and only 3.2% on the tenth, with no deaths on the second and fourth days (Figure 9). However, there was significant difference ($P < 0.01$) between sixth and eighth days (Table 3). APPRC-27 caused 100% mortality on the sixth and eighth days, with a very significant difference ($P < 0.01$) between these days, around 68% of deaths on the eighth day, 32% on the sixth, and no deaths on days 2, 4, and 10. Overall, the APPRC-44BC isolate was the fastest killer, with a mortality rate of 55% on the sixth day post-application (Figure 9).

Table 3. Mean ± SE of varroa mite mortality within 10 days post-application of fungal isolates.

APPRC-44BC		**S#10H**		**APPRC-27**	
Days	Mean	Days	Mean	Days	Mean
6	8.00 ± 0.61^{a}	8	6.33 ± 0.68^{a}	8	9.33 ± 1.33^{a}
8	6.33 ± 0.61^{a}	6	3.66 ± 0.68^{b}	6	4.33 ± 1.33^{b}
10	0.33 ± 0.61^{b}	10	0.33 ± 0.68^{c}	10	0^{c}
2	0^{b}	2	0^{c}	2	0^{c}
4	0^{b}	4	0^{c}	4	0^{c}

Mean followed with the same letter within a column are not significantly different from each other ($P > 0.05$).

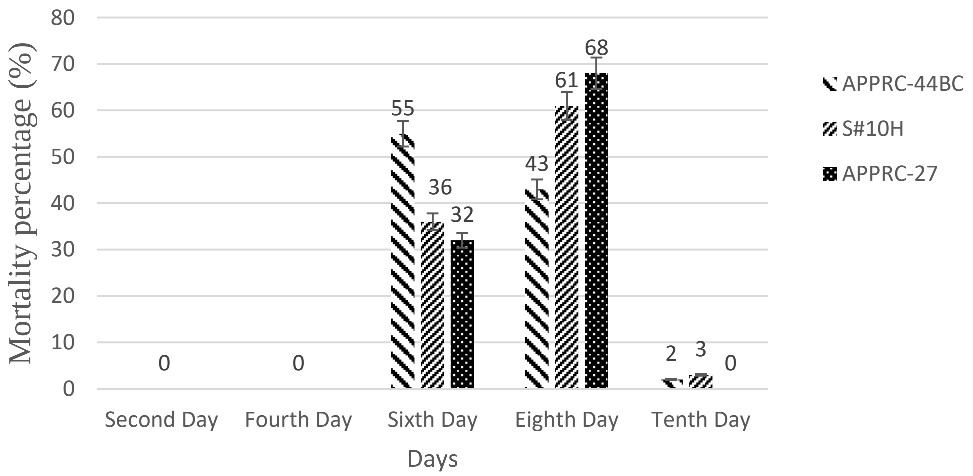

Figure 9. Mean mortality rate (%) caused by each isolate throughout the entire experiment.

4.6.The effect of fungal isolates on the emergence of brood to adult bee

During daily monitoring, a number of emerged bees were found trapped in wire mesh screened cages (Figure 11A). This indicated that fungal suspension had no significant (P > 0.05) effect on the emergence of honeybees from brood cells compared to the control groups. Likewise, significant brood emergence difference was not found among the isolates used to kill varroa mites. However, a relatively higher mean value was recorded for the isolate APPRC-27 (16.00 ± 2.61), followed by APPRC-44BC (15.33 ± 2.61). Relatively, the lowest mean brood emerging value was recorded for S#10H (12 ± 2.61). The control groups had brood emergence mean value of 19.67 ± 2.61. In terms of percentage, the APPRC-27 isolate had relatively the highest brood emergence rate (80%) (Figure 10).

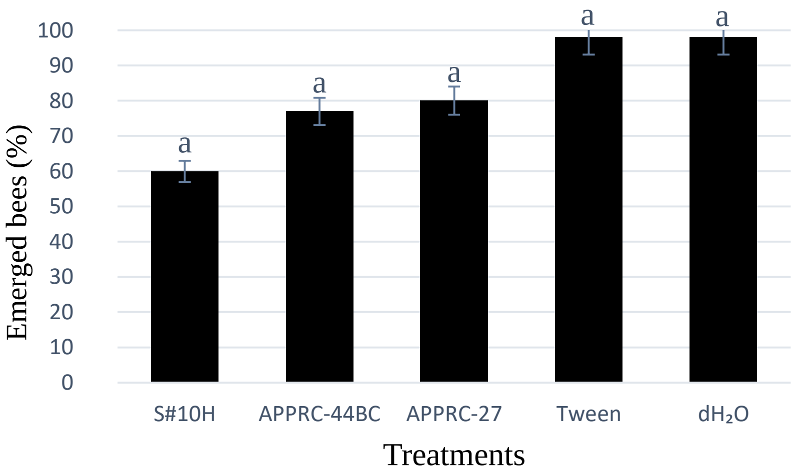

Figure 10. Mean percentage of bee emerged across the treatments.

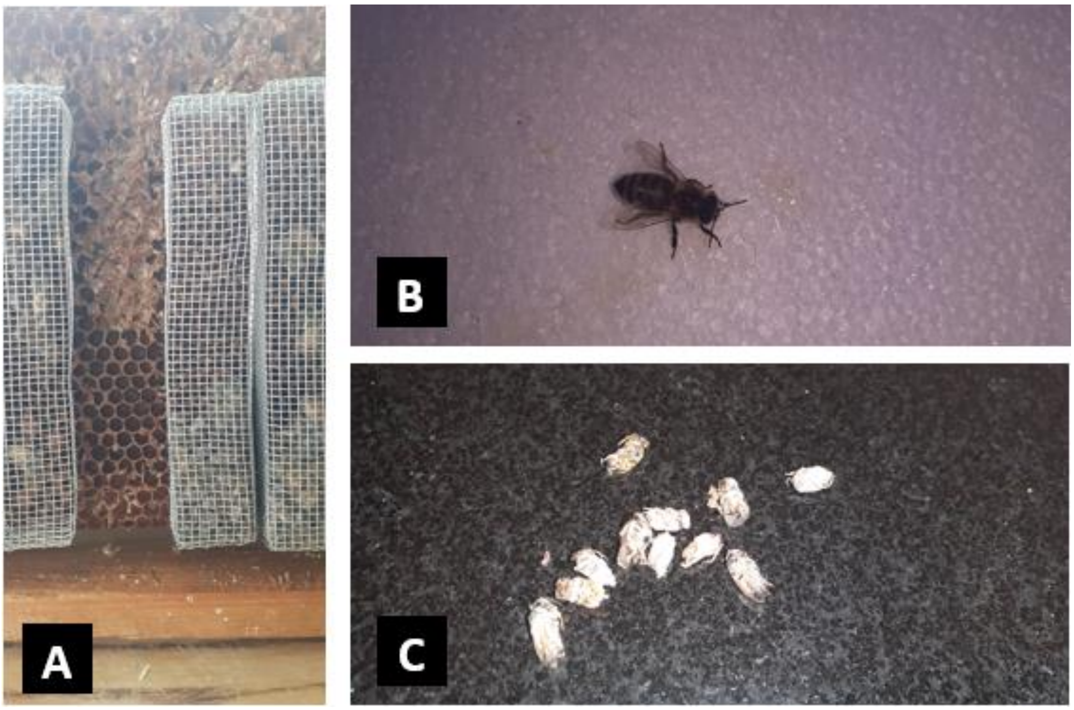

Figure 11. Outcomes of brood inoculation. (A) Emerged honeybees within the mesh wire enclosure. (B) Emerged honeybee. (C) Unmerged honeybees extracted from brood cells.

4.7. The effect of fungal isolates on the body weight of emerged bees

This experiment assessed the impact of fungal isolates on the body weight of emerging honeybees. The results indicated that inoculating bee brood with fungal isolate suspensions had no significant effect (P > 0.05) on the body weight of emerged bees. Nevertheless, a slightly highest average body weight of 0.107 ± 0.01 grams was observed for the isolate APPRC-44BC (Figure 12).

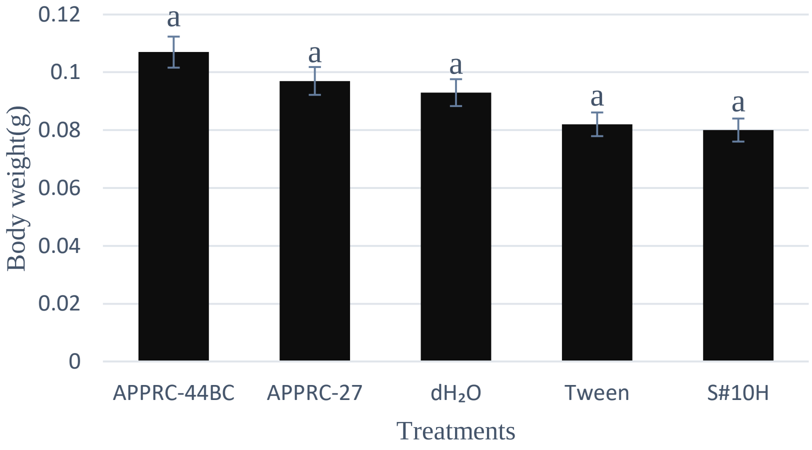

Figure 12. Mean body weight of newly emerged bees treated with five treatments.

4.8. Fungal colonization on brood

All treatments did not show any signs of fungal colonization in honeybee broods on the first day post-inoculation (1 dpi). No fungal spores or hyphae were found when the broods from these treatments were crushed and stained with lactophenol cotton blue for microscopic examination. Likewise, by the seventh day post-inoculation (7 dpi) there was no significant ($P > 0.05$) fungal colonization of honeybee broods. However, some fungal colonization became apparent in honeybee broods that had received treatment with the three fungal isolates with mean values of 9.00 ± 2.31, 7.33 ± 2.31 and 6.33 ± 2.31 infected broods with isolates APPRC-44BC, APPRC-27 and S#10H respectively. This suggests that, while not quite reaching the level of statistical significance, there is an obvious trend in fungal colonization over time among the treatment groups with fungal isolates.

4.9. Effect of fungal isolates on the survival of adult honeybees

Over the course of the 14-day period of daily monitoring, mycelial development was observed on the cadavers of honeybees collected from cages in which the fungal isolate suspensions were applied. According to the observed result, adult honeybees' survival was significantly ($P < 0.05$) lower in the treatment groups that received the application of three fungal isolates than in the control groups. However, no significant differences ($P > 0.05$) were found when comparing the impacts of these three fungal isolates with one another. In particular, the treatment group which received the application of the isolate APPRC-44BC had slightly higher daily mortality rate of adult bee compared to other fungal isolates, with a mean of 1.52 ± 0.26 adult bee death per day. After all, with a mean of 1.43 ± 0.26 adult bee death per day, the S#10H isolate showed a significant effect on adult bee survival. Comparatively, out of the three isolates, the APPRC-27 isolate had the least impact on adult bee survival, with a mean of 1.36 ± 0.26 adult bee death per day. Conversely, the group treated with distilled water had a mean adult honeybee death rate of 0.40 ± 0.26 adult bee death per day, while the control group treated with 0.05% Tween 80 solution had a mean mortality rate of 0.52 ± 0.26 adult bee death per day (Figure 13).

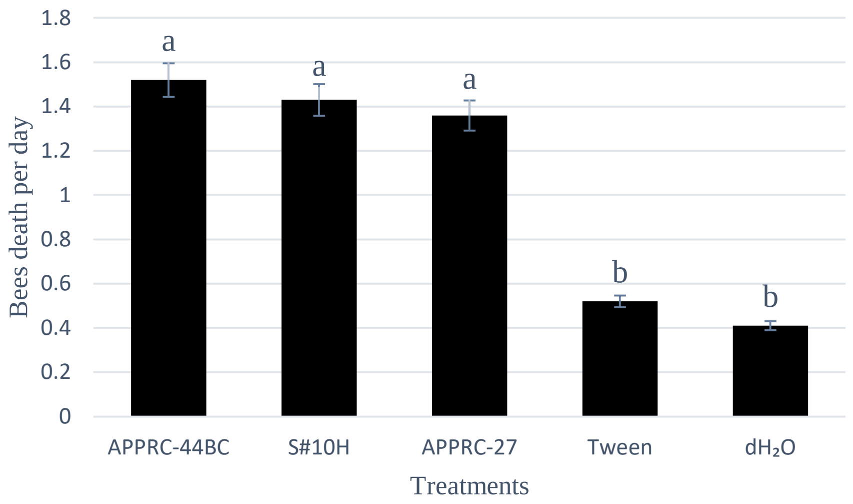

Figure 13. The mean value of adult bees’ death per day.

5. DISCUSSION

Conidial viability is commonly used to assess the quality of the fungal inoculum used in biological control studies and applications. Organisms treated with conidia from low-quality batches containing a large proportion of debilitated conidia, lived significantly longer than those treated with comparable quantities of conidia from high-quality batches. Thus, the evaluation of conidial viability before application proved to be a crucial factor affecting the final reported results. To this end, all three isolates of *B. bassiana* used in this investigation showed mean conidial viability values higher than 89%. During the test, conidial viability percentages for APPRC-27, APPRC-44BC, and S#10H were 91.89 ± 0.64, 90.89 ± 0.64, and 89.88 ± 0.64, respectively. Despite showing slightly lower percentages than Oliveira *et al.* (2015), who reported a 93.4 ± 1.2 percentage of conidial viability of pure *B. bassiana* conidia on PDA, our results fell within the range of *B. bassiana* isolate conidial viability percentages reported by Denberu Kebede *et al.* (2022), which ranged from 87.63 ± 5.09 to 96.35 ± 0.87. The results of this study are also comparable with those reported by Sinia (2013), who reported more than 90% of the *B. bassiana* isolates' conidia germination.

Good conidia viability percentage usually falls between 80% and 100% (Hsia *et al.,* 2014). This means that the majority of the fungal spores are viable and capable of germinating to infect the target organism. With this regard, the three isolates used in this study demonstrated good conidia viability. Furthermore, these isolates were among those proven to have good conidia viability in the previous study conducted by Denberu Kebede *et al.* (2022). The viability of conidia can vary depending on the age of the spores, storage conditions, and the specific strain of *B. bassiana*. The good conidia viability found in this study could be attributed to various factors, such as the freshness of the conidia used and the specific strain of *B. bassiana*. Additionally, the surfactants used may have diverse effects on the viability of conidia (Mwamburi *et al.*, 2015). The effects of Tween 80 on *B. bassiana* conidia germination vary depending on the concentration and isolates. According to Mwamburi *et al.* (2015), it may have stimulatory effects on certain isolates while having inhibitory effects for others.

The effectiveness of EPF as a biological control agent can be significantly influenced by their vegetative growth rate. This is because a faster growth rate serves as a pathogenicity determinant, positively correlating with fungal virulence. In addition, factors such as the geographical origin of isolates and the specific fungal strain can exert notable influences on the growth rates (Amha Gebremariam *et al*., 2021). The daily growth rate observed in this study ranges between 0.89 to 3.43 mm per day, which is consistent with the result published by Amha Gebremariam *et al.* (2021). On average, the highest radial growth rate (3.38 mm day^{-1}) was achieved with isolate APPRC-44BC, while the lowest radial growth rate (1.96 mm day^{-1}) was observed with isolate S#H10. This variation could be attributed to genetic differences among isolates of *B. bassiana* and the geographical origin of the isolates, which may affect their growth rates. Genetic variation can result in differences in the types and quantities of metabolites produced, consequently influencing growth rates (Kubicek *et al.*, 2003). Based on the observations and findings, it is evident that the vegetative growth rate of EPF plays a crucial role in their effectiveness as biological control agents.

The growth and development of fungi increase proportionally with the length of the incubation time until reaching a point of stationary growth, which influences the conidial density (Afifah and Saputro, 2020). This study used regression analysis to evaluate the association between the radial growth of isolates and the number of days spent growing on growth media. The regression equations $Y = 8.172 + 3.289X$, $Y = 7.291 + 1.786X$, and $Y = 9.758 + 1.919X$ for the isolates APPRC-44BC, S#10H, and APPRC-27, respectively, with R-squared values of 0.99, 0.99, and 0.97 for radial growth, demonstrated strong model fits for the tested relationship of the factors. Moreover, the slope coefficients and small p-values of the Pearson's correlation results provide evidence for a strong relationship between the number of days the isolates spent on growth media and their radial growth. This indicates that an increase in the day length of one resulted in an increase in radial growth of 3.289 mm for APPRC-44BC, 1.786 mm for S#10H, and 1.919 mm for APPRC-27. The high R-squared values of the isolates, especially isolates APPRC-44BC and S#10H, imply that the days spent on growth media account for 99% of the variability in radial growth. Therefore, it is important to keep the *B. bassiana* culture on the growth medium until it reaches a point of stationary growth to obtain optimum conidial density, which is crucial in the battle against pests.

The density of conidia directly affects EPF's ability to suppress target pests. As insects develop resistance to pathogen infections, a higher concentration of conidia becomes necessary (Afifah and Saputro, 2020). Upon conducting the quantification process, the conidial yield values for APPRC-44BC, APPRC-27, and S#10H were determined to be $1.93 \pm 0.582 \times 10^6$ conidia per mL (9.83×10^4 conidia per mm²), $1.66 \times \pm 0.582 \times 10^6$ conidia per mL (8.43 x 10^4 conidia per mm²), and 1.61 ± 0.582 x 10^6 conidia per mL (8.20 x 10^4 conidia per mm²), respectively. These results exceeded the result reported by Awan *et al.* (2021), for which a maximum conidial production of 2.89 ± 0.28 × 10^3 conidia/mm^2 was recorded. However, the results of this study were marginally less than those reported in Amha Gebremariam *et al.* (2021), with spore production ranging from 3 x 10^6 to 4.60 x 10^8 spores/mL. Some factors, such as the isolate's characteristics, growth conditions, and environmental effects, could be responsible for these observed variations (Afifah and Saputro, 2020). Therefore, care should be taken while culturing the isolates to have a maximum conidial yield for the intended purpose.

Beauveria bassiana is known for its wide distribution and ecological importance in the natural ecosystem. Most commonly, the morphological and cultural characteristics of these fungi are used to isolate them from their natural habitats and to distinguish them as well (Amha Gebremariam *et al.,* 2021). The isolates used in this study exhibited cultural characteristics such as dispersed growth patterns, white to yellowish-white colony color, a smooth, cottony to powdery texture, a raised to flat elevation, and a circular shape. On the other hand, the microscopic observation of *B. bassiana* isolates revealed oval conidia with hyaline hyphae. These morphological and cultural characteristics are distinct from those of other fungal species. Thus, these characteristics were crucial for confirming that the isolates used in this study were *B. bassiana*. Furthermore, the observed color changes on the conidia as they grew older on the growth medium indicated that they were ready for their eventual interaction with their insect hosts.

In the biological control of varroa mites, *B. bassiana* is a notable contender whose adaptability and effectiveness have been shown in a number of studies. In this study, the goal was to test the effectiveness of *B. bassiana* isolate against *V. destructor*. This was accomplished by analyzing the overall and daily mortality rates of varroa mites treated with the isolates, as well as determining the mortality caused by each fungal isolate. Based on this analysis, *B. bassiana* isolate APPRC-44BC exhibited the highest mortality rate (73%). This may have been due to its stronger varroa pathogenic features, such as vegetative growth rate, conidial density, and enzymes synthesized by the isolate. This demonstrated the potential for APPRC-44BC of *B. bassiana* to manage *V. destructor* infestations in the future. This finding is in consistent with the varroa mite mortality data published by Sinia (2013), who found that the use of *B. bassiana* fungal isolates was responsible for 50% to 90% of the varroa mite mortality. Furthermore, the findings of our study are in line with those of Hamiduzzaman *et al.* (2012), who found that the use of *B. bassiana* fungal isolates resulted in varroa mite mortality ranging from 50% to more than 90%. The variation in virulence among *B. bassiana* isolates could be attributed to differences in vegetative growth rate, conidial density, conidia viability percentage, or their biochemistry, all of which require further investigation.

The temporal dynamics of fungal infection are critical in the biological control of pests. Understanding these dynamics allows us to track how the fungal infection progresses over time within the varroa mite population, how long it takes for infected mites to die, and how easily the fungus to spread from mite to mite within a colony. In this study, the mortality rate of varroa mites varied over time, with highly significant differences observed between the days on which deaths were reported for various isolates. In addition, the percentage distribution of varroa mite deaths on different days revealed the temporal dynamics of fungal infection. Six days after application, all three fungal isolates started to kill varroa mites. The sixth and eighth days following the application of fungal isolates were particularly important, accounting for 96.8% up to 100% of all fungal-induced varroa mites' deaths. APPRC-44BC in particular, was the fastest varroa mites' killer with a 55% mortality rate on the sixth day after application. Similar to our finding, Hamiduzzaman *et al.* (2012) reported that *B. bassiana* isolates caused significant mortality in *V. destructor* following 7 days after inoculation. According to Hamiduzzaman *et al.* (2012) and Sinia (2013), isolates that killed more than 50% of the mites within a 7 days were considered pathogenic, giving credibility to this finding. These results, which reveal the effectiveness and temporal patterns of each isolate, have implications for developing targeted varroa mite management plans. Optimizing treatment protocols based on the observed patterns could help reduce varroa mite infestations and improve honeybee health. Thus, future research may focus on application methods, dosage, and assessing long-term effects on honeybee colonies to establish sustainable and effective varroa mite management strategies before using the isolated in integrated pest management.

One of the requirements for an organism to function as a biological control agent is its specificity in targeting the pest species while avoiding harming non-target organisms. This specificity is critical in ensuring that the control method does not disrupt non-target communities. In this investigation, honeybee brood were inoculated with fungal suspensions from three different fungal isolates across three treatments, which provided valuable insights into the potential effects of fungal isolates on brood emergence to adult bees. There were no statistically significant differences in brood emergence between the fungal suspensions treated and control groups. This suggests that the fungal isolates used had non-significant impact on the honeybee brood's ability to hatch from its cells. However, this finding contradicts the Hamiduzzaman *et al.* (2012) study, which found that applying *B. bassiana* fungal isolate significantly reduced brood emergence by up to 60%. Furthermore, Al Mazra'awi (2007) also reported relatively less emerging brood in the fungal treated group. There could be several possible reasons for the non-significant effect of *B. bassiana* isolates on the emergence of brood to adult bees in this study. One possible explanation is that honeybee's resistance varies depending on the strain. The honeybee strain used in our study was claimed to be *Apis mellifera bandasii*, whereas Hamiduzzaman *et al.* (2012) and Al Mazra'awi (2007) were *Apis mellifera Linnaeus* and *Apis mellifera carnica*, respectively. Furthermore, the variation in the fungal isolates used may contribute to this effect. Additional research may be required to investigate this idea further.

Similarly, when honeybees come into contact with *B. bassiana* spores, the fungus can penetrate through their exoskeleton and infect them. The infection may or may not cause various effects on the honeybees, including physiological changes. This study showed that the fungal isolates had no significant effect ($P > 0.05$) on the body weight of emerged bees. This implies that fungal isolate treatments have non-significant effect on pupal development or body weights at emergence. Furthermore, Meikle *et al.* (2007) found no evidence that *B. bassiana* influenced adult bee or colony weight, which is consistent with our findings. These findings indicate that fungal isolates may not have a significant impact on honeybee physiology, at least in terms of body weight regulation.

The progression of fungal colonization on the honeybee brood could be used to predict the fungal ability to affect the brood emergence and the body weight of emerged bees (Hamiduzzaman *et al.*, 2012). In this research, there was no indication of fungal colonization on the first day post-inoculation (1 dpi). However, signs of fungal colonization were observed on the seventh day post-inoculation (7 dpi), but they were not statistically significant ($P > 0.05$) compared to the control. This indicates that, like varroa, the brood develops signs of fungal colonization over time. A similar finding indicating slow colonization of inoculated broods 7 dpi was reported (Hamiduzzaman *et al.*, 2012). Overall, the gradual emergence of fungal colonization on honeybee brood over time observed in this study could be one explanation for the slight decreased brood emergence to adult bees, even though both results were non-significant. More research is needed to investigate this concept further.

This study demonstrated a significant effect that isolates of *B. bassiana* had on adult honeybee survival. The presence of mycelia on the fungal-inoculated, surface-sterilized, deceased honeybees following incubation on PDA containing chloramphenicol established the demonstrated relationship between the applied fungal isolates and the observed mycelial growth on honeybee cadavers. All three isolates had comparatively similar overall means of death regardless of their variations. This implies the observed increased adult honeybee mortality was largely caused by the experimental fungal isolates. However, the reduction in the longevity of honeybees observed in this direct spray bioassay may be attributed to the method of applying the fungal suspension to the honeybees. This suggests that the fungus's conidia could be present in higher concentrations, potentially penetrating the exoskeleton of the bees through the intersegmental membranes of the abdomen. This is the most common adhesion site for fungi, leading to colonization and eventual death of the bees. In this regard, Omuse *et al*. (2022) exposed bees indirectly to *B. bassiana* ICIPE 284 isolate by spraying the fungal isolate suspension on to filter papers and introducing it in the bottom of the cages. Thus, all fungus-exposed bees were able to acquire conidia; however, bees were not significantly affected by the bioassay. Moreover, Potrich *et al.* (2018) found that the spraying technique reduced the longevity of bees to 62.6 hours, while contact with soy leaves and smooth surfaces containing *B. bassiana* reduced the longevity of bees to 89.1 hours and 97.3 hours, respectively, which was much longer than when it was sprayed. Similarly, Colombo *et al*. (2020) revealed that worker honeybee survival significantly reduced when *B. bassiana* was sprayed. However, exposing honeybees to *B. bassiana*-sprayed eucalyptus leaves did not significantly reduce honeybee survival, unlike exposure to *M. anisopliae*. On the other hand, Soni and Thakur (2011) reported that both the strip and spray methods were effective and did not cause significant mortality in bees. However, the dose to which the bees were exposed (10^8 spores/mL) was probably much higher than they would encounter in the field. Generally, the method of application of *B. bassiana* during biological control could be one of the factors influencing honeybee survival, highlighting the importance of considering application methods in efforts to mitigate potential effects on bee populations.

6. CONCLUSION AND RECOMMENDATIONS

6.1. Conclusion

In this study, all three fungal isolates demonstrated a varroa mite killing potential exceeding 50%, with APPRC-44BC showing the highest efficacy at 73%, followed by APPRC-27 at 68%, and S#10H at 52%. The most efficient control occurred between the sixth and eighth days' post-application, with fungal-induced death rates ranging from 96.8% to 100% within this period. APPRC-44BC was particularly effective, causing a 55% mortality rate on the sixth day post-application. Interestingly, the application of these isolates did not significantly impact the rate of emergence or body weight of the emerged bees. However, there was a significant effect on adult honeybee survival, likely due to the method of application exposing bees to higher concentrations of fungi. These findings suggest promising possibilities for more targeted and effective varroa mite control, offering valuable insights for beekeeping and agricultural practices.

6.2. Recommendations

- Consider the use of fungal isolates, especially APPRC-44BC, for the development of an alternative commercial biological control antagonist for varroa mite, while carefully considering the application method.
- Further research is needed to identify additional varroa pathogenic isolates and develop application methods that have a minimal impact on bee populations.
- Investigate the long-term effects of fungal isolate application on varroa mite populations and honeybee colonies to assess sustainability and efficacy over time.
- Collaborate with beekeepers and agricultural experts to implement and evaluate the effectiveness of fungal-based varroa mite control strategies in real-world settings.

7. REFERENCES

Abbo, P.M., Kawasaki, J.K., Hamilton, M., Cook, S.C., DeGrandi-Hoffman, G., Li, W.F., Liu, J., and Chen, Y.P. (2016) “Effects of Imidacloprid and *Varroa destructor* on survival and health of European honeybees, *Apis mellifera*.” *Insect Science*, 24(3): 467-477.

Afifah, L., and Saputro, N. W. (2020) “Growth and viability of entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin in different alternative media.” *IOP Conference Series: Earth and Environmental Science,* 468(1): 012037.

Alemayehu Gela, Anagaw Atickem, Amssalu Bezabeh, Yitbarek Woldehawariat and Araya Gebresilassie (2023) “Insights into varroa mite (*Varroa destructor*) infestation levels in local honeybee (*Apis mellifera*) colonies of Ethiopia.” *Journal of Applied Entomology*, 147(9): 798-808.

Al Mazra’awi, M. (2007) “Impact of the entomopathogenic fungus *Beauveria bassiana* on the honeybees, *Apis mellifera* (Hymenoptera: Apidae).” *World Journal of Agricultural Sciences*, 3(1): 7–11.

Aman Dekebo, Daniel Bisrat and Chuleui Jung (2019) “Opportunities and Constraints of Beekeeping Practices in Ethiopia.” *Journal of Apiculture*, 34(2): 169–180.

Amha Gebremariam, Yonas Chekol, and Fassil Assefa (2021) “Phenotypic, molecular, and virulence characterization of entomopathogenic fungi, *Beauveria bassiana* (Balsam) Vuillemin, and *Metarhizium anisopliae* (Metschn.) Sorokin from soil samples of Ethiopia for the development of mycoinsecticide.” *Heliyon*, 7(2021): e07091.

Anderson, D. L., and Trueman, J. W. H. (2000) “Varroa jacobsoni (Acari: Varroidae) is more than one species.” *Experimental and Applied Acarology,* 24: 165–189.

Amsalu Arega (2020) “The Prevalence of Honeybee Diseases and varroa mite in Selected Districts of East Wollega Zone , Oromia National Regional State, Ethiopia.” *International Journal of Advanced Research in Biological Sciences,* 7(4): 46-59.

Arrese, E. L. and Soulages, J. L. (2010) “Insect fat body: Energy, metabolism, and regulation.” *Annual Review of Entomology,* 55: 207–225.

Aryal, S., Ghosh, S., and Jung, C. (2020) “Ecosystem Services of Honeybees ; Regulating , Provisioning and Cultural Functions.” *Journal of Apiculture*, 35(2): 119–128.

Avin, F. A. (2019) “Easy way to count spores and prepare spore suspension by Hemocytometer” Available at: https://www.researchgate.net/publication/337772212

Awan, U. A., Xia, S., Meng, L., Raza, M. F., Zhang, Z., and Zhang, H. (2021) “Isolation, characterization, culturing, and formulation of a new *Beauveria bassiana* fungus against Diaphorina citri.” *Biological Control*, 158: 104586.

Barratt, B. I. P., Moran, V. C., Bigler, F., and van Lenteren, J. C. (2018) “The status of biological control and recommendations for improving uptake for the future.” *BioControl*, 63: 155–167.

Bataglia, L., Simões, Z. L. P. and Nunes, F. M. F. (2022) “Transcriptional expression of m6A and m5C RNA methyltransferase genes in the brain and fat body of honeybee adult workers.” *Frontiers in Cell and Developmental Biology*, 10: 1–9.

Bava, R., Castagna, F., Piras, C., Musolino, V., Lupia, C., Palma, E., Britti, D., and Musella, V. (2022) “Entomopathogenic Fungi for Pests and Predators Control in Beekeeping.” *Veterinary Science*, 9 (2): 95

Behie, S. W., Jones, S. J. and Bidochka, M. J. (2015) “Plant tissue localization of the endophytic insect pathogenic fungi Metarhizium and Beauveria.” *Fungal Ecology*, 13: 112–119.

Belay Habtegebriel, Emana Getu, Mohamed Dawd, Emiru Seyoum, Getnet Atnafu, Fathiya Khamis, Ylva Hilbur, Sunday Ekesi, and Mattias C. Larsson (2016) “Molecular characterization and evaluation of indigenous entomopathogenic fungal isolates against Sorghum Chafer, Pachnoda interrupta (Olivier) in Ethiopia” *Journal of Entomology and Nematology*, 8(5): 34–45.

Colombo, F. C., Maciel, R. M. A., Abati, R., Raulino-Domanski, F., Longhi, S. J., Costa-Maia, F. M., Vismara, E. S., Lozano, E. R., and Potrich, M. (2020) “Do *Beauveria bassiana* and *Metarhizium anisopliae* affect worker survival and the production of Africanized *Apis mellifera* queens?” *Journal of Apicultural Research*, 60(2): 260–269.

Deeter, M. E., Snyder, L. A., Meador, C., and Corby-Harris, V. (2023) “Accelerated abdominal lipid depletion from pesticide treatment alters honeybee pollen foraging strategy, but not onset, in worker honeybees.” *Journal of Experimental Biology*, 226(7): jeb245404.

de Guzman, L. I., Rinderer, T. E., Frake, A. M., and Kirrane, M. J. (2015) “Brood removal influences fall of *Varroa destructor* in honeybee colonies.” *Journal of Apicultural Research*, 54(3): 216–225.

de Miranda, J. R., Cordoni, G., and Budge, G. (2010) “The Acute bee paralysis virus-Kashmir bee virus-Israeli acute paralysis virus complex.” *Journal of Invertebrate Pathology,* 103: S30–S47.

Denberu Kebede, Tesfaye Alemu, and Tadele Tefera (2022) “Endophytic Potential and Larvicidal Efficacy of Entomopathogenic Fungi against the Spotted Stem Borer, *Chilo partellus*.” *A Journal of Entomology*, 2022.

Dereje Tulu, Shiferaw Temteme, Melkam Aleme, Ararsa Bogale, Awraris Getachew, Esayas Mendesil (2023) “Epidemiology, factors influencing prevalence and level of varroosis infestation (*Varroa destructor*) in honeybee (*Apis mellifera*) colonies in different agroecologies of Southwest Ethiopia.” *Parasite Epidemiology and Control*, 23: e00325.

Desalegn Begna (2014) “Occurrences and Distributions of Honeybee (*Apis mellifera* Jemenetica)Varroa Mite (*Varroa destructor*) in Tigray Region, Ethiopia.” *Journal of Fisheries and Livestock Production*, 2(3): 10-4172.

Desalegn Begna, Alemayehu Gela, Taye Negera, and Amssalu Bezabeh (2016) “Identifying The Species, Effects And Seasonal Dynamics of Honeybee Varroa Mites: A Newly Emerging Parasite To Ethiopian Honeybee.” *International Journal of Toxicology and Environmental Sciences*, 1(1): 4.

Devi, S., Devi, M. and Chitralekha (2019) “Different methods for the management of Varroa mite (*Varroa destructor*) in honeybee colony.” *Journal of Entomology and Zoology Studies*, 7(4): 178–182.

Ebisa Mezgabu, Eyob Hirpa, Dasselegn Begna, Lama Yimer, Abdisa Bayan and Misganu Chali (2016) "Occurrence and Distribution of Varroa Mite and Antivarroa Effect of Propolis in Walmara District of Oromia Special Zone Around Finfine, Ethiopia." *Journal of Veterinary Science and Technology,* 7(5): 10-4172.

Fagan, L. L., Nelson, W. R., Meenken, E. D., Howlett, B. G., Walker, M. K., and Donovan, B. J. (2012) "Varroa management in small bites." *Journal of Applied Entomology,* 136(6): 473–475.

Farenhorst, M.; Mouatcho, J.C.; Kikankie, C.K.; Brooke, B.D.; Hunt, R.H.; Thomas, M.B.; Koekemoer, L.L.; Knols, B.G.J.; Coetzee, M. (2009) "Fungal infection counters insecticide resistance in African malaria mosquitoes" *Proceedings of the National Academy of Sciences,* 106(41): 17443–17447.

Fenet Belay and Alemayehu Oljirra (2016) "The Significance of Honey Production for Livelihood In Ethiopia" *Journal of Environmental and Earth Sciences,* 6(4): 46–53.

Ferrari, M. C. F., Favaro, R., Mair, S., Zanotelli, L., Malagnini, V., Fontana, P., and Angeli, S. (2020) "Application of *Metarhizium anisopliae* as a potential biological control of *Varroa destructor* in Italy." *Journal of Environmental and Earth Sciences*, 59(4): 528–538.

García-Fernández, P., Santiago-Álvarez, C. and Quesada-Moraga, E. (2008) "Pathogenicity and thermal biology of mitosporic fungi as potential microbial control agents of *Varroa destructor* (Acari: Mesostigmata), an ectoparasitic mite of honeybee, *Apis mellifera* (Hymenoptera: Apidae)." *Apidologie,* 39(6): 662–673.

Giacobino, A., Molineri, A., Bulacio Cagnolo, N., Merke, J., Orellano, E., Bertozzi, E., Masciangelo, G., Pietronave, H., Pacini, A., Salto, C., and Signorini, M. (2016) "Key management practices to prevent high infestation levels of *Varroa destructor* in honeybee colonies at the beginning of the honey yield season." *Preventive Veterinary Medicine,* 131: 95–102.

Giacomelli, A., Pietropaoli, M., Carvelli, A., Iacoponi, F., and Formato, G. (2016) "Combination of thymol treatment (Apiguard®) and caging the queen technique to fight *Varroa destructor*." *Apidologie,* 47(4): 606–616.

Gratzer, K., Kibebew Wakjira, Fiedler, S., and Brodschneider, R. (2021) “Challenges and perspectives for beekeeping in Ethiopia. A review.” *Agronomy for Sustainable Development*, 41*: 46.*

Graystock, P., Blane, E. J., McFrederick, Q. S., Goulson, D., Hughes, W. O. H. (2016) “Do Managed Bees Drive Parasite Spread and Emergence in Wild Bees?” *International Journal for Parasitology: Parasites and Wildlife*, 5(1): 64− 75.

Gupta, R. K., Reybroeck, W., van Veen, J. W., and Gupta, A. (Eds.). (2014). Beekeeping for Poverty Alleviation and Livelihood Security.

Guzmán-Novoa, E., Eccles, L., Calvete, Y., McGowan, J., Kelly, P. G., and Correa-Benítez, A. (2010) “*Varroa destructor* is the main culprit for the death and reduced populations of overwintered honeybee (*Apis mellifera*) colonies in Ontario, Canada” *Apidologie*, 41(4), 443–450.

Haber, A. I., Steinhauer, N. A. and Vanengelsdorp, D. (2019) “Use of Chemical and Nonchemical Methods for the Control of *Varroa destructor* (Acari: Varroidae) and Associated Winter Colony Losses in U.S. Beekeeping Operations” *Journal of Economic Entomology*, 112(4): 1509–1525.

Haftey Sahle, Gashaw Enbiyale, Ayalew Negash, and Tsehaye Neges (2018) “Assessment of Honey Production System, Constraints and Opportunities in Ethiopia” *Pharmacy and Pharmacology International Journal*, 6(1): 42–47.

Hamiduzzaman, M. M., Sinia, A., Guzman-Novoa, E., and Goodwin, P. H. (2012) “Entomopathogenic fungi as potential biocontrol agents of the ectoparasitic mite, *Varroa destructor*, and their effect on the immune response of honeybees (*Apis mellifera* L.)” *Journal of Invertebrate Pathology*, 111(3): 237–243.

Hepburn, H. R. and Radloff, S. E. (Eds.). (2011). Honeybees of Asia.

Higes, M., Martín-Hernández, R., Sara Hernández-Rodríguez, C., González-Cabrera, J. (2020) “Assessing the resistance to acaricides in *Varroa destructor* from several Spanish locations” *Parasitology Research*, 119: 3595–3601.

Hung, K.-L. J., Kingston, J. M., Albrecht, M., Holway, D. A., and Kohn, J. R. (2018) "The worldwide importance of honeybees as pollinators in natural habitats" *Proceedings of the Royal Society B: Biological Sciences*, 285(1870): 20172140.

Hsia, I. C. C., Islam, M. T., Ibrahim, Y., How, T. Y., and Omar, D. (2014). "Evaluation of conidial viability of entomopathogenic fungi as influenced by temperature and additive." *International Journal of Agriculture and Biology*, *16*(1): 146–152.

Kenesa Teferi (2018) "Status of Beekeeping in Ethiopia- A Review." *Dairy and Veterinary Science Journal*, 8(4): 555743.

Kerealem Ejigu, Tilahun Gebey and T R Preston (2009) "Constraints and Prospects for Apiculture Research and Development in Amhara region, Ethiopia." *Livestock Research for Rural Development,* 21(10).

Klein, A.-M.; Vaissiere, B. E.; Cane, J. H.; Steffan-Dewenter, I.; Cunningham, S. A.; Kremen, C.; Tscharntke, T. (2007) "Importance of Pollinators in Changing Landscapes for World Crops." *Proceedings of the Royal Society of London. Series B, Biological Sciences*, 274(1608): 303−313.

Kritsky, G. (2017) "Beekeeping from Antiquity Through the Middle Ages." *Annual Review of Entomology*, 62: 249–264.

Kubicek, C. P., Bissett, J., Druzhinina, I., Kullnig-Gradinger, C., and Szakacs, G. (2003). "Genetic and metabolic diversity of Trichoderma: A case study on South-East Asian isolates." *Fungal Genetics and Biology*, 38(3): 310–319.

Lacey, L. A., Grzywacz, D., Shapiro-Ilan, D. I., Frutos, R., Brownbridge, M., and Goettel, M. S. (2015) "Insect pathogens as biological control agents: Back to the future" *Journal of Invertebrate Pathology*, 132: 1–41.

Li, S., Yu, X. and Feng, Q. (2019) "Fat body biology in the last decade" *Annual Review of Entomology*, 64: 315–333.

Liu, F., Xu, X., Zhang, Y., Zhao, H., and Huang, Z. Y. (2020) "A meta-analysis shows that screen bottom boards can significantly reduce *Varroa destructor* population" *Insects*, 11(9): 1–8.

Martin, S. J. and Brettell, L. E. (2019) "Deformed Wing Virus in Honeybees and Other Insects." *Annual Review of Virology*, 6: 49–69.

Meikle, W. G., Mercadier, G., Holst, N., Nansen, C., and Girod, V. (2007) "Duration and spread of an entomopathogenic fungus, *Beauveria bassiana* (Deuteromycota: Hyphomycetes), used to treat varroa mites (Acari: Varroidae) in honeybee (Hymenoptera: Apidae) hives." *Journal of Economic Entomology*, 100(1): 1–10.

Meikle, W.G., Mercadier, G., Holst, N., Nansen, C., Girod, and V. (2008) "Impact of two treatments of a formulation of *Beauveria bassiana* (Deuteromycota: Hyphomycetes) conidia on varroa mites (Acari: Varroidae) and on honeybee (Hymenoptera: Apidae) colony health." *Experimental and Applied Acarology*, 46: 105–177.

Mullin, C.A.; Frazier, M.; Frazier, J.L.; Ashcraft, S.; Simonds, R.; VanEngelsdorp, D.; Pettis, J.S. (2010) "High levels of miticides and agrochemicals in north american apiaries: Implications for honeybee health." *PloS one*, 5(3): e9754.

Mustafa, M. O., Adeoye, O. T., Abdulalzeez, F. I., and Akinyemi, O. D. (2015) "Mitigating Effects of Climate Change and Deforestation on Bees With Respect to Their Ecology and Biology" *Journal of Environmental Ecology*, 6(2): 1-12.

Mwamburi, L. A., Laing, M. D., and Miller, R. M. (2015) "Effect of surfactants and temperature on germination and vegetative growth of *Beauveria bassiana*." *Brazilian Journal of Microbiology*, 46(1): 67–74.

Nazzi, F., and Le Conte, Y. (2016) "Ecology of *Varroa destructor*, the Major Ectoparasite of the Western Honeybee, *Apis mellifera*." *Annual Review of Entomology*, 61: 417–432.

Neumann, P and Carreck, N. L. (2010) "Honeybee colony losses." *Journal of Apicultural Research*, 49(1): 1- 6.

Noël, A., Le Conte, Y. and Mondet, F. (2020) "*Varroa destructor*: How does it harm *Apis mellifera* honeybees and what can be done about it?" *Emerging Topics in Life Sciences*, 4(1): 45–57.

Oliveira, D. G. P., Pauli, G., Mascarin, G. M., and Delalibera, I. (2015) “A protocol for determination of conidial viability of the fungal entomopathogens *Beauveria bassiana* and *Metarhizium anisopliae* from commercial products.” *Journal of Microbiological Methods*, 119: 44–52.

Omuse, E. R., Niassy, S., Wagacha, J. M., Ong’amo, G. O., Lattorff, H. M. G., Kiatoko, N., Mohamed, S. A., Subramanian, S., Akutse, K. S., and Dubois, T. (2022) “Susceptibility of the Western Honeybee *Apis mellifera* and the African Stingless Bee Meliponula ferruginea (Hymenoptera: Apidae) to the Entomopathogenic Fungi *Metarhizium anisopliae* and *Beauveria bassiana*.” *Journal of Economic Entomology*, 115(1): 46–55.

Pell, J. K., Hannam, J. J., and Steinkraus, D. C. (2010) “Conservation biological control using fungal entomopathogens.” *BioControl*, 55: 187–198.

Potrich, M., da Silva, R. T. L., Maia, F. M. C., da Silva, E. R. L., Rossi, R. M., Colombo, F. C., Tedesco, F. G., and de Gouvea, A. (2018) “Effect of entomopathogens on Africanized *Apis mellifera L.* (Hymenoptera: Apidae).” *Revista Brasileira de Entomologia*, 62(1): 23–28.

Ramsey, S. D., Ochoa, R., Bauchan, G., Gulbronson, C., Mowery, J. D., Cohen, A., Lima, D., Joklik, J., Cicero, J. M., Ellis, J. D., Hawthorne, D., and VanEngelsdorp, D. (2019) “*Varroa destructor* feeds primarily on honeybee fat body tissue and not hemolymph.” *Proceedings of the National Academy of Sciences*, 116(5): 1792-1801.

Romo-Chacón, A., Martínez-Contreras, L. J., Molina-Corral, F. J., Acosta-Muñiz, C. H., Ríos-Velasco, C., Ponce de León-Door, A., and Rivera, R. (2016) “Evaluation of Oregano (Lippia berlandieri) Essential Oil and Entomopathogenic Fungi for *Varroa destructor* Control in Colonies of Honeybee , *Apis mellifera*.” *Southwestern entomologist*, 41(4), 971-982.

Sebsib Ababor and Yibrah Tekle (2018) “Beekeeping Practice, Opportunities, Marketing and Challenges in Ethiopia.” *Dairy and Veterinary Science*, 5(3): 555662

Sewify, T., Ibrahim, Y. Y. and Salah El-Deen, M. (2015) "*Beauveria bassiana* (Balsamo), a Potential Mycopesticide for Efficient Control of the Honeybee Ectoparasitic Mite, *Varroa destructor* Anderson and Trueman." *Egyptian Journal of Biological Pest Control,* 25(2): 333–337.

Shaw, K. E., Davidson, G., Clark, S. J., Ball, B. V., Pell, J. K., Chandler, D., and Sunderland, K. D. (2002) "Laboratory bioassays to assess the pathogenicity of mitosporic fungi to *Varroa destructor* (Acari: Mesostigmata), an ectoparasitic mite of the honeybee, *Apis mellifera*." *Biological Control,* 24(3): 266–276.

Singh, D., Raina, T. K., and Singh, J. (2017) "Entomopathogenic fungi: An effective biocontrol agent for management of insect populations naturally." *Journal of Pharmaceutical Sciences and Research,* 9(6): 830–839.

Sinha, K. K., Choudhary, A. K., and Kumari, P. (2016). Entomopathogenic fungi. In Ecofriendly pest management for food security (475-505). Academic Press.

Sinia, A. (2013). Evaluation of the Fungi *Beauveria bassiana*, *Metarhizium anisopliae,* and *Clonostachys rosea* as Bio-control Agents against the Honey Bee Parasitic Mite, *Varroa destructor* (Doctoral dissertation). University of Guelph, Guelph, Ontario.

Sisay Fikru (2015) "Review of Honeybee and Honey Production in Ethiopia" *Journal of Animal Science Advances,* 5(10): 1413–1421.

Soni, J. and Thakur, M. (2011) "Effect of Biopathogens on Honeybees." *Pest Technology,* 5(1): 86–90.

Stenberg, J. A., Sundh, I., Becher, P. G., Björkman, C., Dubey, M., Egan, P. A., Friberg, H., Gil, J. F., Jensen, D. F., Jonsson, M., Karlsson, M., Khalil, S., Ninkovic, V., Rehermann, G., Vetukuri, R. R., and Viketoft, M. (2021) "When is it biological control? A framework of definitions, mechanisms, and classifications." *Journal of Pest Science,* 94(3): 665–676.

Strachecka, A., Olszewski, K., Kuszewska, K., Chobotow, J., Wójcik, Ł., Paleolog, J., and Woyciechowski, M. (2021) "Segmentation of the subcuticular fat body in *Apis mellifera* females with different reproductive potentials." *Scientific Reports,* 11(1).

Tesfu Shegaw, Asrat Arke, Nahom Belay and Dawit Habte Giorgis (2022) “Diagnostic survey on varroa mite (Varroa distractor) prevalence in South-Western Ethiopia.” *Cogent Food and Agriculture*, 8(1): 2143610.

Tlak Gajger, I., Svečnjak, L., Bubalo, D., and Žorat, T. (2020) “Control of *Varroa destructor* Mite Infestations at Experimental Apiaries Situated in Croatia.” *Diversity*, 12(1): 12.

Traynor, K. S., Mondet, F., de Miranda, J. R., Techer, M., Kowallik, V., Oddie, M. A. Y., Chantawannakul, P., and McAfee, A. (2020) “*Varroa destructor*: A Complex Parasite, Crippling Honeybees Worldwide.” *Trends in Parasitology*, 36(7): 592–606.

Tutun, H., Sevin, S. and Çetintav, B. (2020) “Effects of different chilling procedures on honeybees (*Apis mellifera*) for anesthesia.” *Ankara University Veterinary Faculty Journal*, 67(3): 289–294.

VanEngelsdorp, D., Hayes Jr, J., Underwood, R. M., and Pettis, J. (2008) “A survey of honey bee colony losses in the US, fall 2007 to spring 2008.” *PloS one*, 3(12): e4071.

van Toor, R. F., Thompson, S. E., Gibson, D. M., and Smith, G. R. (2015) “Ingestion of *Varroa destructor* by pseudoscorpions in honeybee hives confirmed by PCR analysis.” *Journal of Apicultural Research*, 54(5): 555-562.

8. APPENDICES

Appendix: One way ANOVA results

ANOVA result of conidial viability of the three fungal isolates

	Df	Sum sq	Mean sq	F value	Pr(>F)
Trts	2	6.02	3.01	2.47	0.165
Error	6	7.32	1.22		

CV = 2.45%

ANOVA result of radial growth of the isolates on PDA growth media

	Df	Sum sq	Mean sq	F value	Pr(>F)
Trts	2	52.68	26.34	17.47	1.86e-07***
Error	132	199.07	1.51		

CV = 1.98%   *** = Significant at 0.1% level

ANOVA result of spore production capability of fungal isolates

	Df	Sum sq	Mean sq	F value	Pr(>F)
Trts	2	1.793e+11	8.964e+10	0.0883	0.917
Error	6	6.089e+12	1015e+12		

CV = 2.45%

ANOVA result of all the five treatments applied on varroa mites

	Df	Sum sq	Mean sq	F value	Pr(>F)
Trts	4	628.9	157.2	87.35	9.77e^{-08} ***
Error	10	18	1.8		

CV = 2.23%;   *** = Significant at 0.1% level

ANOVA results for varroa mite death within 10 days post APPRC-44BC isolate application.

	Df	Sum sq	Mean sq	F value	Pr(>F)
Days	4	183.6	45.9	40.5	3.79e^{-06} ***

Error	10	11.33	1.13		

CV = 2.23%; *** = Significant at 0.1% level

ANOVA results for varroa mite death within 10 days post S#10H isolate application.

	Df	Sum sq	Mean sq	F value	Pr(>F)
Days	4	96.93	24.23	17.31	$1.72e^{-04}$ ***
Error	10	14.00	1.40		

CV = 2.23%; *** = Significant at 0.1% level

ANOVA results for varroa mite death within 10 days post APPRC-27 isolate application.

	Df	Sum sq	Mean sq	F value	Pr(>F)
Days	4	205.6	51.40	9.637	$1.84e^{-03}$ **
Error	10	53.33	5.33		

CV = 2.23%; ** = Significant at 1% level

ANOVA results for honeybee brood emergence post inoculation.

	Df	Sum sq	Mean sq	F value	Pr(>F)
Trt	4	125.7	31.43	1.541	0.263
Error	10	204.0	20.40		

CV = 2.23%

ANOVA results for body weight of emerged bees

	Df	Sum sq	Mean sq	F value	Pr(>F)
Days	4	0.0213	0.0053	0.974	0.423
Error	243	1.33	0.0054		

CV = 1.97%

ANOVA results for fungal colonization of honeybee brood

	Df	Sum sq	Mean sq	F value	Pr(>F)
Days	4	216.4	54.1	3.353	0.055
Error	10	161.3	16.13		

CV = 2.23%

ANOVA results for effect of fungal isolates on the survival of adult honeybee

	Df	Sum sq	Mean sq	F value	Pr(>F)
Days	4	54.4	13.112	4.382	0.026*
Error	205	613.4	2.992		

CV = 1.97%   * = Significant at 5% level