ENTOMOPATHOGENIC FUNGI: AN ALTERNATIVE FOR THE BIOLOGICAL CONTROL OF APHIDS (*Phorodon cannabis*) IN CANNABIS (*Cannabis sativa*) PLANTS

DANIEL ANDRES LOPEZ RESTREPO
Bachelor of Biotechnology, Colegio mayor de Antioquia, 2018

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Department of Biological Sciences
University of Lethbridge
LETHBRIDGE, ALBERTA, CANADA

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DANIEL ANDRES LOPEZ RESTREPO

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Dr. Igor Kovalchuk Thesis Supervisor	Professor	MD/ Ph.D
Dr. Dmytro Yevtushenko Thesis Examination Committee Member	Associate Professor	Ph.D
Dr. Andriy Bilichak Agriculture and Agri-Food Canada Thesis Examination Committee Member	Research Scientist	Ph.D
Dr. Theresa Burg Chair, Thesis Examination Committee	Professor	Ph.D

ABSTRACT

The rapid expansion of the cannabis industry in Canada post-legalization has heightened the prevalence of pests, particularly the cannabis aphid *Phorodon cannabis,*which poses significant threats to crop health. This study investigates the immediate effects of *P. cannabis* on *Cannabis sativa* plants and explores biological control strategies utilizing entomopathogenic fungi. The research aims to test the antagonistic activity of various fungal isolates against aphids, analyze the immune responses of cannabis plants to infection, assess the impact on metabolite production and yield, and develop effective application strategies for these biocontrol agents.

Fungal isolates of *Beauveria* and *Metarhizium* were isolated and characterized. Infection tests on aphids demonstrated the potential of these fungi to control aphid populations without the environmental drawbacks associated with chemical insecticides. Bioassays revealed that both fungi achieved 100% aphid mortality at high conidial concentrations (1×10^7 conidia/mL), with *Beauveria bassiana* demonstrating faster efficacy. In greenhouse trials, *Beauveria bassiana* maintained aphid populations below 20 aphids throughout the experiment across all varieties and maintaining cannabis growth parameters comparable to the chemical insecticide. Untreated aphid infections substantially reduced plant height and biomass across three cannabis varieties tested, reaching heights of 40-48 cm and under 4 g of dry biomass.

Cannabinoid and terpene analyses revealed that *Beauveria bassiana*-treated plants exhibited higher concentrations of key metabolites, including THCa, CBDa, and total terpenes, compared to chemically treated plants. The findings highlight *Beauveria bassiana* as an eco-friendly alternative for pest management that not only effectively controls aphids but also supports the biochemical quality of cannabis plants.

Findings suggest that entomopathogenic microorganisms can significantly mitigate the impact of *P. cannabis* on cannabis seedlings, offering a sustainable alternative to chemical controls. This research contributes to the understanding aphid interactions with cannabis plants and promotes eco-friendly pest management practices within the burgeoning cannabis industry.

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LIST OF ABBREVIATIONS

EPF	Entomopathogenic fungi
IPM	Integrated pest management
Bb	*Beauveria bassiana*
CEA	Controlled environment agriculture
Bt	*Bacillus thuringiensis*
THCa	Tetrahydrocannabinol
CBD	Cannabidiol
Δ9-THC	Delta-9- Tetrahydrocannabinol
CBDa	Cannabidiolic acid
CBGa	Cannabiderolic acid
CBC	Cannabigerol
UV	Ultraviolet
DAT	Days after treatment
PVY	Potato Virus Y
HPLC	High-performance liquid chromatography
USP	United States Pharmacopeia classification of HPLC columns
HPLC-DAD	High-performance liquid chromatography with diode-array detection

CHAPTER 1: INTRODUCTION

1.1. Background on Cannabis cultivation in Canada

Cannabis sativa (*C. sativa* or cannabis) cultivation in Canada has undergone a transformative journey, evolving from a historical use primarily for industrial applications to becoming a major agricultural sector following its legalization (Pusiak et al., 2021). Historically, cannabis was cultivated for its fiber, particularly during the late 19th and early 20th centuries. However, with increasing regulation and stigma, its use became largely restricted by the mid-1900s. The landscape shifted dramatically with the passage of the Cannabis Act in October 2018, which legalized recreational cannabis and established a regulated framework for its production and sale (Hall et al., 2023). This landmark legislation positioned Canada as a global leader in cannabis reform, influencing both domestic cultivation practices and international policies (Drohan, 2021).

The Cannabis Act splits cannabis into two main categories: industrial hemp, which is cultivated for its low THC content and various industrial applications, and marijuana, which includes higher THC strains for both medicinal and recreational use (Cox, 2021). Under this framework, licensed producers must comply with stringent regulations that cover cultivation practices, product safety, and distribution protocols. The legislation also permits individuals to cultivate a limited number of cannabis plants for personal use, further promoting a culture of cannabis cultivation across the country (Imtiaz et al., 2023).

In terms of cultivation practices, Canadian cannabis production predominantly occurs indoors and in greenhouses (Drohan, 2021), enabling growers to optimize environmental conditions and ensure consistent quality. Advanced techniques such as hydroponics, aeroponics, and controlled

environment agriculture (CEA) have become commonplace, allowing producers to maximize yield while minimizing resource inputs (Vernon et al., 2023). The increasing consumer demand for organic and sustainably produced cannabis has also prompted many growers to adopt environmentally friendly practices, reflecting a broader trend toward sustainable agriculture (Valleriani, 2020). Economically, the legalization of cannabis has generated significant benefits for Canada. The cannabis industry has created thousands of jobs, generated substantial tax revenues, and attracted investment in the agricultural sector (Amlung & MacKillop, 2019). In addition to direct economic impacts, the industry has spurred research and development initiatives, particularly in agricultural biotechnology and pest management.

However, the burgeoning cannabis sector also faces challenges. Market saturation, competition from illicit sources, and evolving consumer preferences present ongoing hurdles for producers (Myran et al., 2023). Moreover, compliance with regulatory standards can be complex, requiring continual adaptation to new guidelines and practices. Environmental concerns, particularly regarding water use and waste management, are critical as the industry seeks to establish sustainable practices (Valleriani, 2020). The increase in cannabis crops has brought the appearance of new pests and diseases. *Phorodon cannabis*, commonly known as the cannabis aphid, has only recently been reported in North America and the impacts as well as many aspects of its interaction with the host plant are still largely unknown (Cranshaw et al., 2018).

1.2. Background on the importance of cannabis aphids as pests

The cannabis aphid is a small, light-colored insect recently found in the northern United States and Canada (Cranshaw et al., 2018; Lagos-Kutz et al., 2018). It usually lives on the stems and undersides of the leaves of plants and feeds by inserting its mouthpieces to extract from the phloem of the plant. These aphids affect the plant both directly and indirectly. The direct effects are related to their way of feeding since they weaken the plant causing slower growth, loss of vigor and yellowing of the leaves. On the other hand, they indirectly transmit diseases (Eastop, 1977; Pitt et al., 2022). Additionally, when feeding, they excrete a sugary substance known as honeydew which accumulates in small droplets on the leaves and is the ideal substrate for the appearance of phytopathogenic fungi and other pest such as ants (R. Singh & Singh, 2016). As aphids grow they need to change their exoskeleton to continue their life cycle. As the colony grows, these exoskeletons accumulate on the leaves of the plant and in cases of extreme infection, they can interfere with the photosynthetic processes of the host plant (R. Singh & Singh, 2016). There is very little information about the short and long-term impacts of the cannabis aphid and viable ways to control its population in cannabis crops, so it is necessary to carry out research to find alternatives to deal with this pest. It is important to facilitate the understanding of the mechanisms involved and find a better-suited biocontrol alternative for this pest.

Given the economic importance of cannabis as a crop, effective management of aphid populations is essential. Traditional pest control methods, including chemical insecticides, have been widely used; however, concerns about chemical residues, environmental impacts, and the development of pest resistance highlight the need for alternative approaches (Punja, 2021). Integrated pest management (IPM) strategies incorporating biological control methods are increasingly being explored to mitigate the impacts of aphids while promoting sustainable practices (Cranshaw et al.,

2019). In this context, exploring of entomopathogenic fungi as a biological control agent for *P. cannabis* presents a promising avenue for sustainable pest management. By leveraging natural enemies to suppress aphid populations, growers can reduce their reliance on chemical pesticides and promote a healthier growing environment for cannabis plants (Mantzoukas et al., 2022).

1.3. Background on the role of entomopathogenic fungi in biological control

Integrated pest management practices are normally recommended to solve aphid infections, which consists of the use of insecticides, repellent plants or insects, resistant plants and good crop management techniques (Mweke et al., 2018). However, chemically manufactured insecticides remain widely used. It's a common understanding that using these compounds not only develops resistance in the targeted insects but also has adverse effects on the environment., leads to health issues for humans, and is non-specific and affects beneficial organisms (Dhakal et al., 2019).

Entomopathogenic fungi are a viable alternative for insect control (Aker & Hasan Abacı, 2016). Fungi have been reported as plant protective agents since they affect insect populations under natural conditions, do not generate associated resistance and take advantage of the pest's feeding conditions to easily come into contact with them (Juliya, 2020). Generally, entomopathogenic fungi are opportunistic pathogens being capable of infecting a wide variety of species, and infectious responses can vary greatly depending on both biotic and abiotic factors, as well as the insect host and the type of crop associated with the insect (Shah & Pell, 2003). Commercial formulations of the fungi *Beauveria Bassiana*, *Metarhizium anisopliae* and *Lecanicillium lecanii* are available in Canada, but their application and studies of their effectiveness against *Phorodon cannabis* are scarce (Cranshaw et al., 2019).

1.3.1. Mechanisms of action

Unlike viruses, nematodes, and many bacteria, which often need specific entry points to infect insect hosts, entomopathogenic fungi (EPF) can penetrate nearly any part of the insect's cuticle. Although certain insects may have more susceptible entry sites, infection generally begins when single-celled spores, such as conidia or blastospores, attach to the insect's outer cuticle (Ortiz-Urquiza & Keyhani, 2013). This attachment triggers the production of various hydrolytic enzymes, such as proteases, chitinases, and lipases, which facilitate the fungal spores' germination, growth on the host's surface, and subsequent penetration through its cuticular layers.

During this invasion process, the fungus forms specialized structures like penetration pegs and appressoria, which enable the hyphae to breach the host's outer layers and advance into the integument. At this stage, the pathogen begins interacting with the host's immune defenses. The insect cuticle is highly complex and varies significantly in composition, even across the insect's life stages. The outermost epicuticle is a lipid-rich, hydrophobic barrier, while beneath it lies the procuticle, containing chitin and sclerotized proteins consisting of exo-, meso-, and endo-cuticular layers. The procuticle is followed by the epidermis, which encases the insect's internal structures (Afifah et al., 2022).

1.3.2. Advantages of using EPF

The use of EPF as insecticides is advantageous largely due to their high specificity in targeting only pest insects. Unlike broad-spectrum chemical insecticides that can inadvertently harm a wide range of insects, including beneficial predators and pollinators, entomopathogenic fungi selectively infect and kill specific insect pests (Trinh et al., 2020). This precision minimizes

the impact on non-target species, such as beneficial insects that help control other pest populations or those that contribute to pollination. Beneficial insect predators—like ladybugs, lacewings, and certain types of wasps—naturally regulate pest populations, and sparing them enhances the ecosystem's ability to maintain balanced pest control (Mantzoukas et al., 2022) . Additionally, this specificity helps maintain biodiversity within the ecosystem. Non-harmful parasites that do not damage crops or spread disease are also left unaffected, preserving their ecological role and further supporting a balanced natural environment (Shah & Pell, 2003). By using fungi that target only the pests, there's less risk of disrupting the food web and the complex relationships between different species in the ecosystem. Consequently, fungi-based insecticides can offer effective pest control without disturbing beneficial insects, promoting a healthier, more resilient ecosystem.

Entomopathogenic fungi-based insecticides are environmentally friendly and pose minimal risks to mammals, offering a safer alternative to traditional chemical insecticides. Unlike chemical pesticides, which can accumulate in soil, water, and non-target organisms, fungi-based biopesticides decompose more readily in natural environments. This biodegradability reduces the risk of environmental contamination and minimizes the threat to soil and aquatic health (Jaronski, 2010).

Additionally, because fungi-based insecticides target insect physiology specifically, they are generally safe for humans and other mammals. Chemical insecticides, on the other hand, have been shown to impact mammalian health, potentially causing adverse effects ranging from endocrine disruption to carcinogenicity (Aktar et al., 2009). Fungi-based insecticides avoid these risks as they do not contain the harmful active ingredients typically found in synthetic pesticides, which can be inhaled, ingested, or absorbed by humans and animals, potentially causing both acute and chronic health issues. Moreover, entomopathogenic fungi have a selective mode of action that infects and

kills insects without harming vertebrates or other non-target species (Shahid et al., 2012). By using insect-specific pathways for infection and reproduction, these fungi minimize collateral damage, unlike chemical insecticides that often harm beneficial insects, birds, and other wildlife. This selective action of fungal insecticides not only lowers toxicity risks to humans but also prevents unintended harm to ecosystems, making them a promising, sustainable pest control solution. They carry genes that produce insect-specific toxins, offering potential for further enhancement through biotechnology (Juliya, 2020). Some fungi can live endophytically within plants, potentially boosting the host's immune response (Clifton et al., 2018). Their high persistence in the environment provides sustained pest suppression.

1.3.3. Applications in pest management

The application of entomopathogenic fungi in agriculture has gained traction as growers seek sustainable pest management strategies. In the context of cannabis cultivation, the use of EPF to target pests like *P. cannabis* could be particularly beneficial. Studies have shown that certain strains of EPF can effectively reduce aphid populations, improving plant health and yield without the adverse effects associated with chemical insecticides (Saranya et al., 2010; Ullah et al., 2022; Vu et al., 2007).

The effectiveness of EPF varies by fungal strain, environmental conditions, and host susceptibility factors, such as population density and nutritional status. EPF also have potential in post-harvest pest management, reducing reliance on chemical fumigants by controlling pests in stored products like grain (Mantzoukas et al., 2022) . Studies show that combined treatments with EPF and natural insecticidal agents, such as diatomaceous earth and plant extracts, often produce synergistic effects that enhance insect mortality, such as combinations of *B. bassiana* with neem extracts or

diatomaceous earth against storage pests like rice and corn weevils. Further research on mixed entomopathogen applications could optimize these synergies and reduce the need for chemical interventions.(Cranshaw et al., 2019).

1.3.4. Challenges and considerations

Despite their potential, the implementation of EPF in pest management faces challenges. Factors such as environmental conditions, host plant resistance, and the formulation of fungal products can influence the efficacy of these biological agents. Research into optimizing application methods, understanding the ecological interactions of EPF, and developing robust formulations is essential for enhancing their effectiveness in the field (Khan et al., 2012).

This research is relevant because due to the increase of *Cannabis sativa* crops in Canada, the report of new pests is also increasing. This project will allow us to understand not only the negative impacts *of Phorodon cannabi*s on cannabis but also propose new alternatives for the biological control of these insects, thus reducing the use of chemical pesticides and their environmental impact.

CHAPTER 2: HYPOTHESIS AND OBJECTIVES

We hypothesized that entomopathogenic microorganisms are efficient biocontrol agents for *Phorodon cannabis* in *Cannabis sativa* seedlings under greenhouse conditions. To test this hypothesis, we have proposed the following objectives:

2.1. Objectives

1. To propose additional strategies for controlling this pest, entomopathogenic microorganisms will be tested for their antagonistic activity against *Phorodon cannabis* in cannabis plants (*Cannabis sativa*) under greenhouse conditions.
2. Analyze the immune response generated by *Phorodon cannabis* infection in cannabis plants, to improve understanding of the mechanisms involved and the insect's biology.
3. Test the effects of *Phorodon cannabis* infection on cannabis metabolite production.
4. Develop a strategy for effectively applying entomopathogenic microorganisms for the biological control of *Phorodon cannabis* in cannabis plants under greenhouse conditions.

CHAPTER 3: LITERATURE REVIEW

3.1. Overview of aphid biology and damage

Aphids, small, soft-bodied insects, belong to the family *Aphididae* and exhibit a wide range of host-plant specializations. *P. cannnabis*, commonly known as the cannabis aphid, is a specific pest of *Cannabis sativa* plants. It has a piercing-sucking mouthpart that enables it to extract plant sap, particularly phloem sap rich in sugars aphid and other nutrients, which serves as their primary food source (Cranshaw et al., 2018).

Aphids reproduce rapidly via parthenogenesis (asexual reproduction) under favorable conditions, allowing them to colonize host plants quickly (Nielsen & Hajek, 2005). In general, aphids can produce multiple generations in a single growing season, and populations can explode if unchecked. Many species, including *P. cannabis*, have complex life cycles, often involving sexual reproduction towards the end of the growing season or under stress, as well as overwintering in egg form on host plants. This ability to reproduce rapidly makes aphids a significant agricultural pest.

Aphids also excrete a sugary substance called honeydew, which promotes the growth of sooty mold fungi on plant surfaces. This mold, in turn, impedes photosynthesis, reducing the plant's ability to grow and produce flowers. Moreover, aphids are known vectors for plant viruses, and *P. cannabis* may potentially contribute to the spread of plant diseases in *Cannabis sativa*, though its specific role as a vector in cannabis systems requires further study.

3.2. Damage caused by aphids on *Cannabis sativa*

Aphid infection damage initially leads to leaf drop, with aphid injury further reducing flower and resin production, which can ultimately kill the plants (Pulkoski & Burrack, 2023). Growers report that aphids can cause substantial damage in greenhouses, making it essential to develop sustainable pest management strategies to protect mother plants, seedlings, and clones before transplanting (Cranshaw et al., 2018). Currently, there are few pesticides approved for hemp, none with confirmed effectiveness, and research-based recommendations for managing insect or aphid pests in hemp remain limited (Visković et al., 2023). This is why aphid infestations can have devastating effects on *Cannabis sativa* plants. Infection with *P. cannabis* can cause direct and indirect damage to cannabis plants.

3.2.1. Direct damage

Aphids can directly damage Cannabis plants by sucking their nutrients, leading to curling and twisting of tender shoots, general plant devitalization, and in severe cases, the death of young seedlings. Flowers may develop abnormally, showing malformations such as twisted pods and impaired seed development. Aphid infestations can also cause yellowing of foliage, stunted growth, and even form galls on leaves and stems, which serve as temporary shelters for the aphids (R. Singh & Singh, 2016). This type of injury is especially common on older mother cannabis plants.

Beyond these direct effects, aphid infestations have indirect impacts on plant physiology that vary with the growth stage at the time of infestation. Aphid feeding on flowers often results in stunted twig growth and reduced flower yield. Many injuries remain asymptomatic, with aphids reducing root growth, plant size, and overall yield without visible symptoms. Large aphid populations

excrete honeydew, which coats the leaf cuticle, attracting sooty molds that block photosynthesis and decrease the plant's marketability and aesthetic appeal. Additionally, dust, dirt, and shed skins adhere to the sticky honeydew, further affecting the plant's appearance (Dedryver et al., 2010). The extent of aphid injury typically depends on (i) the aphid population level at each developmental stage and (ii) the plant's sensitivity to aphid feeding.

3.2.2. Indirect damage

Aphids are primary vectors of plant viruses, which, significantly impact global crop health and yield. These viruses can directly or indirectly manipulate aphid behavior, physiology, and host plant health to enhance virus spread. For instance, non-persistent viruses are quickly acquired from plant epidermal layers and influence vector feeding behaviors, while persistent viruses, acquired from phloem, affect feeding over the insect's lifespan (Pitt et al., 2022).

Aphids, often highly host-specific, can transmit viruses to non-host plants during brief feeding probes, increasing virus spread potential. For example, the potato virus Y (PVY) severely impacts potato and other *Solanaceae* crops like peppers and tomatoes, causing yield loss and symptoms like leaf chlorosis and tuber necrosis (Pitt et al., 2022). Spread by over 65 aphid species, including the green peach aphid, PVY transmission is challenging to control with insecticides, as short virus acquisition times can actually increase transmission by inducing vector movement (Ullah et al., 2022).

The recent legalization of industrial hemp in Canada has introduced this crop to a new range of pests, including the cannabis aphid, which can spread viruses such as alfalfa mosaic and cucumber mosaic virus (Pitt et al., 2022). Though cannabis aphids have yet to be confirmed as PVY vectors, their presence in Canadian hemp greenhouses warrants concern for virus transmission.

3.3. Relevance to biological control via entomopathogenic fungi

IPM for controlling insect pests includes the use of resistant crop varieties, intercropping, optimal planting dates, and reduced chemical pesticides, which help protect crops while minimizing environmental and health risks. Despite these benefits, synthetic chemical controls are still commonly used, leading to concerns over pesticide residues, resistance, and harm to beneficial organisms. Growing consumer demand for reduced pesticide residues has spurred interest in alternatives such as IPM strategies and microbial insecticides. Microbial insecticides, though slower-acting, appeal to horticultural farmers due to their minimal residue, shorter pre-harvest intervals, and lower environmental impact (Mweke et al., 2018).

Among microbial insecticides, EPF is particularly effective against sap-feeding insects like aphids, as they infect through the cuticle rather than ingestion. Aphid species, including *P. cannabis*, are vulnerable to EPF, and several fungi have been developed into mycoinsecticides. Public awareness of synthetic pesticide risks is also driving demand for safer pest control options and biopesticides (Mweke et al., 2018; H. Singh & Kaur, 2020).

Developing EPF-based mycoinsecticides involves selecting potent strains, assessing their efficacy across various environmental conditions, and optimizing production and formulation. Key EPF species in pest control include *Metarhizium anisopliae*, *Beauveria bassiana*, and *Lecanicillium* species. Studies show the effectiveness of *M. anisopliae* isolates, against various aphid species (Aker & Hasan Abacı, 2016; Juliya, 2020). *B. bassiana* has also been reported as an EPF species to control aphid species (Juliya, 2020; Mweke et al., 2018). These previously mentioned reports lead us to believe that EPFs could be an important alternative for aphid pest control in cannabis crops.

3.4. Aphid biology

Aphids, belonging to the family *Aphididae*, are notorious pests in agriculture due to their rapid reproduction, sap-feeding behavior, and ability to transmit plant diseases. Among the many species that impact crops, *P. cannabis* is a specialized pest that primarily infests *Cannabis sativa* (Cranshaw et al., 2018). Understanding the biology of *P. cannabis* is essential for developing effective biological control strategies, such as using entomopathogenic fungi, which provide an eco-friendly alternative to chemical pesticides.

3.4.1. Morphology and life cycle of *P. cannabis*

The cannabis aphid, is a small, soft-bodied insect commonly found on the leaves and stems of cannabis plants. It is typically light-colored, with forms indoors and early-season outdoor forms ranging from cream to pale yellow. As the season progresses and daylight hours decrease, the aphids' coloration changes to shades of light green, pale pink, and light brown. This aphid species was recently identified in North America and is widely distributed in Canada. It exists in both winged and wingless forms, with winged aphids occasionally showing dark spots. Wingless aphids lack this spotting but may exhibit pale stripes along the body. The adult aphids are typically pale green to yellow and can measure about 1.7 to 2.0 mm in length. Their body is pear-shaped, and they possess characteristic cornicles (small tube-like structures) on the dorsal side of their abdomen, a typical feature of aphids. The nymphs resemble the adults but are smaller and without wings (Cranshaw, 2018).

Cannabis aphids reproduce primarily through parthenogenesis, meaning only females are involved, and they give birth to live, genetically identical offspring. As the aphids mature, they molt several

times, each time shedding their old exoskeleton. Most adult aphids are wingless, but in late summer, some may develop wings. This mixed stage of wingless and winged aphids is particularly noticeable at the end of the growing season (Cranshaw, 2018).

The biological cycle of cannabis aphid has not been well studied, but it is likely similar to that of other aphids. Aphid development is strongly influenced by temperature, and adults may live for a few weeks. During their lifetime, female aphids can produce 1-5 offspring daily. When natural predators are present, the aphids' lifespan is typically shorter (Cranshaw et al., 2018). In late summer or early fall, as day length decreases, cannabis aphids begin to produce sexual forms, including winged males and egg-laying females. This is the only time of year when aphid eggs are laid externally on plant surfaces, such as leaves, flowers, and stems. At other times, aphids reproduce asexually by giving birth to live young (Lagos-Kutz et al., 2018).

Outdoors, the aphid eggs survive through the winter, lying dormant until the following spring. The eggs hatch when temperatures rise and day length increases, and they may colonize nearby cannabis plants. However, survival rates are higher indoors, where aphids can persist on living plants throughout the winter and spread to outdoor plants through infested transplants. In Canada, cannabis aphid populations are highest in late summer and early fall. However, natural predators such as lady beetles, flower flies, lacewings, and parasitoid wasps often help control aphid outbreaks (R. Singh & Singh, 2016). In indoor environments, these natural predators are usually absent unless intentionally introduced (Cranshaw, 2018).

Cannabis aphids share a similar appearance with hop aphids (*Phorodon humuli*), a species found primarily on hops (Cranshaw et al., 2018; Lagos-Kutz et al., 2018). However, the two can be differentiated through careful examination under a microscope, particularly at the front of the head. Other aphid species, like the green peach aphid (*Myzus persicae*), cotton aphid (*Aphis gossypii*),

and bean aphid (*Aphis fabae*), have been reported to feed on cannabis (Margaritopoulos et al., 2002; Nazir et al., 2019).

3.4.2. Feeding behavior and damage to *Cannabis sativa*

Cannabis aphids feed by piercing plant tissue with their needle-like mouthparts to extract phloem sap. Although their feeding does not damage plant cells directly, it can reduce plant vitality over time. High aphid populations can lead to symptoms such as stunted growth, wilting, and yellowing of leaves (Dedryver et al., 2010).

As they feed, cannabis aphids produce honeydew, a sticky substance that drips onto nearby leaf surfaces. This shiny residue is a clear indicator of aphid activity and can be useful for detection. Additionally, aphids molt as they grow, shedding their exoskeletons. These discarded exoskeletons, or "cast skins," accumulate near aphid colonies and can also help identify infestations (Singh & Cunningham, 1981).

3.4.3. Ecological adaptations

The cannabis aphid has several ecological adaptations that allow it to thrive in indoor cultivation environments, where cannabis is increasingly being grown for medicinal and recreational use. The aphid's ability to repq |roduce asexually, combined with its rapid life cycle, means that even small populations can quickly reach damaging levels. In protected environments like greenhouses, the lack of natural predators can exacerbate aphid outbreaks. Furthermore, environmental factors such as consistent humidity and temperature control within cannabis cultivation facilities provide optimal conditions for aphid survival and reproduction (Cranshaw, 2018).

P. cannabis exhibits a high degree of host specificity to *C. sativa*, making it a particularly concerning pest in cannabis production. This host specialization allows the aphid to optimize its feeding behavior and reproductive success on cannabis plants, unlike more generalist aphid species that infest multiple plant species (Schreiner & Cranshaw, 2020).

The close interaction between *Phorodon cannabis* and *Cannabis sativa* highlights the importance of tailored pest management strategies in cannabis cultivation systems. Unlike many traditional crops, cannabis is subject to strict pesticide regulations, making the use of biological controls, such as entomopathogenic fungi, even more critical (Schreiner & Cranshaw, 2020).

3.5. Current methods of pest management in cannabis

Pest management in cannabis cultivation, particularly targeting aphids like *P. cannabis*, presents unique challenges. Cannabis is often grown for medicinal and recreational purposes, meaning that chemical residues on the plants can directly affect consumers. As a result, the use of synthetic chemical pesticides is heavily regulated or even prohibited in many regions. This has prompted the cannabis industry to adopt a range of pest management strategies, combining traditional, chemical-free approaches with biological controls. This section outlines the current pest management methods employed in cannabis cultivation, with a focus on their application in controlling *P. cannabis*.

3.5.1. Cultural practices

Cultural control methods aim to create conditions that promote plant health and either reduce pest pressure or enhance the presence of beneficial organisms. These strategies can help make the environment less inviting for pests and more conducive to natural predators. Cultural

practices are often the first line of defense against pests and can prevent their entry into production facilities or prevent infestations from becoming established.

Key elements of cultural control include biosecurity and sanitation, which are crucial for preventing pest outbreaks. For instance, incoming plant material should be thoroughly inspected under a microscope for pests and pathogens, including eggs. New plants should be isolated for a period of 24 to 72 hours before being introduced into the growing area. This isolation helps to detect any pests or diseases that may be present. Additionally, vents should be equipped with screens of appropriate mesh size to prevent pest entry. While these measures are effective in minimizing pest risks, they do not guarantee the complete elimination (Lemay & Scott-Dupree, 2022).

Human activity is another vector for pest introduction, so it is important to implement clean practices, such as workplace-only clothing and restricted access to growing areas. These precautions help reduce the likelihood of pest transfer from outside sources (Lemay & Scott-Dupree, 2022). Workflow is another cultural control consideration: always move from clean, pest-free areas toward more infested zones to avoid spreading pests and diseases through workers and equipment. Effective sanitation also plays a pivotal role in controlling pest movement between crop cycles (Ilikj et al., 2020). For example, it is advisable not to place new plants near older crops, as pests can easily spread to the new plants and continue their life cycle. To avoid this, batches should be isolated from one another, and crop-free periods should be scheduled. These intervals help to break pest cycles by depriving them of host plants. For many insect pests, a week without a host can be sufficient to reduce their population. During crop-free periods, the growing environment should be maintained at temperatures above 40°C and humidity below 50% for a few days to help further reduce pest populations (Vernon et al., 2023).

In addition to isolation and environmental control, regular disinfection of the growing space is essential. This includes cleaning benches, plant supports, irrigation systems, and any equipment used during crop production. Sanitizing tools such as pruners, shears, and sprayers between uses is also critical in preventing pest transmission between different crop areas. While cultivar selection plays a role in pest management, there are currently no known cannabis cultivars that are resistant to pests (Ilikj et al., 2020). Some cultivars may be more susceptible, so maintaining detailed records of pest occurrences across different cultivars can help identify which varieties are more prone to pest problems.

Physical control methods involve manually eliminating or deterring pests. These methods can be preventive or reactive and typically include actions such as using screens on vents, vacuuming or aspirating pests, hand-removal, de-leafing, and mass trapping (using sticky cards, pheromone traps, UV lights, or electric bug zappers). When using de-leafing as a technique, removed leaves should be immediately bagged to prevent pests from re-entering the growing area (Ilikj et al., 2020). While physical controls can be labor-intensive and less efficient compared to other methods, screening vents is an effective and low-cost measure for preventing pest ingress. These physical controls can also provide an immediate reduction in pest numbers, complementing other pest management practices (Lemay & Scott-Dupree, 2022).

3.5.2. Chemical control

When cultural and physical controls fail to maintain pest populations at acceptable levels, chemical control is often considered the next step. However, reliance on chemical pesticides should be minimized. There are few chemical pesticides approved for use on cannabis, and their registration can vary depending on jurisdiction. In some regions, insecticidal soaps, horticultural

oils, and microbial biopesticides are among the most common products available (Craven et al., 2019).

Before using any chemical pesticide, it is critical to check local regulations, ensure the product is legal for use on cannabis, and carefully read the label for application guidelines, safety instructions, and compatibility with other pest management measures (Atapattu & Johnson, 2020). When applying pesticides, thorough coverage is essential, especially on the underside of leaves, which may require additional equipment, such as higher-pressure sprayers or de-leafing to improve spray penetration. Water-sensitive paper can be used to check the spray coverage in various parts of the canopy (Lemay & Scott-Dupree, 2022). Though chemical control methods are generally discouraged in cannabis, organic options such as soaps, oils, and biopesticides are used in combination with other methods to manage pest populations without leaving harmful residues.

The use of synthetic chemical pesticides is highly restricted in cannabis cultivation due to health concerns associated with pesticide residues on the final product. However, certain organic and less toxic chemical products are permitted for use in some regions, depending on local regulations. These include:

Organic insecticidal soaps, derived from natural sources, can be used to break down the outer coating of aphids, causing them to dehydrate and die. Horticultural oils, such as neem oil, are also used to smother aphids and disrupt their ability to feed (Shannag et al., 2014). These products are commonly applied as a foliar spray and are generally considered safe for cannabis plants (Tremblay et al., 2009).

Plant-based insecticides, such as pyrethrins (extracted from chrysanthemum flowers), are sometimes used in cannabis pest management. Pyrethrins work by disrupting the nervous system

of insects, causing paralysis and death. However, they must be used with caution, as some formulations can be toxic to beneficial insects and predators (Hanson et al., 2017).

Neonicotinoids, such as imidacloprid, are chemically similar to nicotine and affect the nervous system of insects. While they are highly effective against sucking insects like aphids and whiteflies, neonicotinoids are notorious for their persistence in the environment and their potential to harm non-target organisms, including pollinators and other beneficial insects (Bass & Field, 2018).

Systemic insecticides are absorbed by the plant and circulate through its tissues, making the plant toxic to feeding pests. This method ensures prolonged protection but raises concerns about the pesticide residues that may remain in the plant tissue, especially in flowers and leaves that are harvested for human consumption. Systemic insecticides can persist in cannabis plants for weeks or months, posing a significant risk to consumers (Ricupero et al., 2020).

The use of chemical insecticides in cannabis cultivation presents several significant risks, particularly due to the unique nature of cannabis as a consumable product. One of the primary concerns is the potential for pesticide residues to remain on the plant material after harvest. These residues, which can persist on cannabis flowers, pose serious health risks when the product is smoked, vaped, or processed into edibles and concentrates (Craven et al., 2019). Systemic insecticides, in particular, can be toxic or carcinogenic if consumed over time. Moreover, pesticides can alter the plant's natural chemical profile, including the levels of cannabinoids like THC and CBD, as well as terpenes, which are responsible for the flavor and aroma of cannabis (Lemay & Scott-Dupree, 2022). This can not only affect the consumer experience but also reduce the marketability and overall value of the crop. In addition to chemical residues, insecticides can cause direct damage to the plants, leading to symptoms like leaf curl, chlorosis (yellowing), and stunted growth, which ultimately lower crop yield and quality. The widespread use of chemical insecticides

can also harm beneficial insects, such as pollinators and natural pest predators, disrupting the ecological balance of the growing environment (Grammenos et al., 2021). This may lead to secondary pest outbreaks and a dependency on continued pesticide use, creating a harmful cycle that becomes difficult to break.

Another issue is the development of pest resistance, as over-reliance on insecticides can cause pests to evolve resistance, making them harder to control over time. This may result in the need for higher doses or more toxic chemical treatments, further exacerbating the environmental and plant health impacts (Erdos et al., 2021; Hanson et al., 2017). Additionally, pesticides that are not absorbed by the plant can leach into the soil or runoff into nearby water sources, contaminating ecosystems and harming wildlife. The long-term persistence of certain pesticides can disrupt local biodiversity and soil health, further complicating the sustainability of cannabis cultivation (Lemay & Scott-Dupree, 2022).

3.5.3. Biological control

Biological control is a key component of sustainable pest management in cannabis cultivation. This method relies on the use of natural predators, parasitoids, and entomopathogens microorganisms to reduce pest populations. Given the restrictions on pesticide use in cannabis and all the associated problems related to the use of chemical insecticides, biological control is particularly valuable for managing aphids such as *P. cannabis*.

3.5.3.1. Natural predators

Natural predators of aphids, such as lady beetles (*Coccinellidae*), lacewing larvae (*Chrysopidae*), and predatory mites (*Phytoseiulus persimilis*), are commonly introduced into

cannabis crops to reduce aphid populations. These predators feed on aphids at various stages of their life cycle and are highly effective in IPM programs. Ladybird beetles, in particular, are the most common aphid predators encountered throughout the world. Some of the common genera predaceous on aphids are: *Adalia*, *Adonia*, *Brumoides*, *Coccinella*, *Cheilomenes*, *Exochomus*, *Hippodamia*, *Oenopia*, *Micraspis*, *Scymnus*, etc (R. Singh & Singh, 2016).

One of the challenges of using natural predators is that they may also impact non-target species. These predators, especially generalists like lady beetles or lacewings, can consume other beneficial insects or even harm the existing ecological balance. Previous reports have shown that introduced predators can disrupt native species and sometimes lead to unintended ecological consequences (Hemptinne, 2012).

Predators can also be affected by fluctuations in aphid populations. If aphid populations decrease, predators may not be sustained and can diminish, leading to ineffective long-term control. Also, some predators may not specialize in aphids and may not be effective at controlling aphid populations consistently (Heimpel, 2000). Aphids can also reproduce rapidly and may outpace predator populations, making it difficult for the predators to keep up. Some reports suggest that natural enemies alone may not be able to reduce aphid populations to economically insignificant levels, especially in high-density aphid infestations (Hemptinne, 2012).

3.5.3.2. Parasitoids

The use of parasitoids, such as *Aphidius colemani* and *Lysiphlebus testaceipes*, for aphid control is widely regarded as an effective and environmentally sustainable biological control strategy due to its target specificity and minimal environmental impact. Parasitoids lay their eggs inside aphids, and the developing larvae consume and ultimately kill the host, directly reducing

aphid populations (Van Lenteren, 2008). Advantages of using parasitoids include their ability to establish populations in fields, thus providing long-term control, and their reduced likelihood of impacting non-target species compared to generalist predators (Harmon, 2009). However, there are limitations to this strategy, parasitoids may have slow response times in high-density aphid outbreaks, and their effectiveness can be constrained by environmental conditions such as temperature and humidity, which influence their survival and reproductive rates (Sigsgaard, 2010) this is particularly important in Canada. Moreover, some aphids may develop resistance mechanisms, such as behavioral defenses or symbiotic relationships with bacteria that help ward off parasitoid attacks, thus limiting their efficacy (Oliver, 2003). Additionally, the need for mass-rearing facilities and timely release programs can increase operational costs, posing challenges for large-scale implementation (Harmon, 2009). Despite these challenges, parasitoids remain a valuable tool in IPM programs for aphid control, particularly when combined with other biological or cultural control methods to enhance overall pest management.

3.5.3.3. Entomopathogenic fungi

Entomopathogenic fungi, such as *Beauveria bassiana* and *Metarhizium anisopliae*, are being increasingly explored as biological control agents for aphid management in multiple crops due to their ability to infect and kill aphids by penetrating their exoskeleton and proliferating inside the host (Aker & Hasan Abacı, 2016; Lee et al., 2015; Nazir et al., 2019). These fungi offer advantages as they are generally target-specific, environmentally friendly, and pose minimal risk to non-target organisms, beneficial insects and human health, making them ideal for IPM programs (Mweke et al., 2018). However, the effectiveness of entomopathogenic fungi can be limited by environmental conditions: high humidity is typically required for spore germination and infection, and extremes of temperature may reduce fungal efficacy and survival in field conditions; this

highlights the necessity of doing experiments to prove their effectiveness under specific conditions (Khan et al., 2012; Mantzoukas et al., 2022).

Additionally, application and persistence challenges arise because fungal spores degrade quickly under ultraviolet (UV) light, limiting their efficacy in outdoor environments and requiring repeated applications, which can increase labor and costs (Shah & Pell, 2003). The development of resistant aphid strains and variations in fungal virulence also pose challenges to achieving consistent control (Shah & Pell, 2003). Despite these limitations, entomopathogenic fungi remain a promising biocontrol option for the control of the cannabis aphid. When combined with other control methods, they may enhance the efficacy of aphid management.

3.6. Mechanisms of action of entomopathogenic fungi

3.6.1. Attachment and adhesion to the insect cuticle

The initial step in the infection process is the attachment of fungal spores (conidia) to the external surface of the aphid's body. Entomopathogenic fungi such as *B. bassiana* and *M. anisopliae* produce hydrophobic conidia, which adhere to the insect's cuticle through hydrophobic interactions. This attachment is facilitated by various fungal surface proteins and enzymes that promote adhesion, even under adverse environmental conditions (Mora et al., 2018). The sticky nature of the fungal spores allows them to remain on the host despite insect movement or environmental fluctuations. Specialized infection structures, such as appressoria and penetration pegs, assist in overcoming the host’s outer defenses (Islam et al., 2021).

These fungi face various host defenses, particularly in response to enzyme-mediated degradation, which triggers immune reactions. The primary mechanism of infection relies on enzymes that target

major cuticular components, suggesting a broad-spectrum virulence across insect species (Ortiz-Urquiza & Keyhani, 2013). Genetic studies show variations in the virulence mechanisms of fungi, highlighting their evolutionary adaptation to insect infection. Engineering these fungi to overexpress specific enzymes has enhanced virulence, though excessive enzymatic activity can cause host sclerotization, reducing fungal sporulation and transmission potential. Other factors like mechanical pressure from hyphae growth and the production of organic acids (e.g., oxalate) further degrade insect cuticle integrity, facilitating fungal invasion (Ortiz-Urquiza & Keyhani, 2013).

3.6.2. Penetration of the cuticle

To infect insects, entomopathogenic fungi must adhere to and penetrate the host's outer epicuticle, a layer rich in waxes and lipids that vary between insect species and life stages. This attachment process involves two steps: a passive binding and a tighter adhesion (Ortiz-Urquiza & Keyhani, 2013). For the previously mentioned entomopathogenic fungi *B. bassiana* and *M. anisopliae*, surface proteins known as hydrophobins contribute to the initial non-specific binding, while specific adhesion proteins (e.g., Mad1 in *M. anisopliae*) facilitate stronger attachment (Islam et al., 2021). These fungi also degrade epicuticular hydrocarbons as nutrients, relying on enzymes, including cytochrome P450 proteins, to break down these lipids into metabolites used for fungal growth (Islam et al., 2021). Fungal adaptations to metabolize these hydrocarbons are crucial for virulence, as shown in studies where pre-induction on hydrocarbon-rich media enhanced fungal infectivity. Temperature, humidity, and UV exposure further affect fungal adhesion, growth, and virulence on the insect surface (Ma et al., 2023).

3.6.3. Invasion of the hemocoel and growth

Entomopathogenic fungi infect insects by first entering their hemocoel and then switching from mycelial to yeast-like forms, allowing them to avoid detection by the insect's immune system and multiply. This evasion is due to the lack of a cell wall in these fungal cells, making them harder for immune cells to recognize (Ma et al., 2023). Some species, like *Nomuraea rileyi*, remain unrecognized by hemocytes due to the absence of recognizable surface markers. As nutrients are depleted, the fungi revert to a mycelial form, causing physical and toxic effects on the host, including seizures and paralysis, which ultimately lead to death. The fungi produce toxins, such as destruxins, that disrupt cellular processes and weaken the insect's defenses (Ortiz-Urquiza & Keyhani, 2013). Under favorable conditions, fungal hyphae break through the insect's exoskeleton, producing spores that spread to other insects.

3.7. Previous research on fungal control of aphids

A study (Javed et al., 2019) investigated the virulence of *B. bassiana* and *Verticillium lecanii* against an aphid under both laboratory and field conditions. The research demonstrated that *B. bassiana* caused mortality rates of over 80% in aphid populations within 6 days of application. The study also explores some other factors like the viability of conidia, germination speed, the growth rate for hyphae, and the effect of environmental factors (temperature, humidity, UV light) on spore production also influence the virulence of fungal isolates and their mode of action on different insects. These findings suggest that *B. bassiana* can serve as a robust biocontrol agent in environments such as greenhouses, where humidity can be controlled an important factor in cannabis cultivation.

In another recent study, Homayoonzadeh et al. (2022) examined the effectiveness of *B. bassiana* to enhance resistance to cotton aphids (*Aphis gossypii*) in cucumber plants. This research found that inoculation of cucumber plants with *B. bassiana* elevates levels of secondary metabolites, which alter the physiology of cotton aphids that feed on them and, therefore, endophytic *B. bassiana* has the potential to alter herbivore-plant interactions in favor of cucumber plants, and at the expense of *A. gossypii* fitness and population growth. Not only that, but this study also says that it is likely that plants inoculated with *B. bassiana* had elevated levels of alkaloids, flavonoids, and phenols, in and this was at least partially responsible for the reduced activity of detoxifying enzymes in *A. gossypii* that fed on these plants (Homayoonzadeh et al., 2022). These results are interesting because if entomopathogenic fungi are able to induce metabolite production in plants to help them enhance resistance or immune response to aphid infection, cannabis metabolites may also be altered when treated with EPF.

Another 2023 study by Akrich et al. evaluated an isolate of *B. bassiana* against *Aphis craccivora*, the cowpea aphid a polyphagous aphid species that causes various damage on different crops. The study identified a highly virulent fungal strain, isolated from dead insects' samples. some of which caused aphid mortality rates of 74% within seven days of exposure with concentrations of 1×10^6 and 1×10^8 conidia/ml. In the greenhouse experiments the same concentration caused a death rate of 70% after seven days post-treatment (Akrich et al., 2023). These results revealed the rapid development and highly insecticidal potential of the *B. bassiana* isolate against adults of *A. craccivora* and also reaffirmed the necessity of exploring and exploiting EPF to develop safer and more effective strategies for controlling pests and protecting crops.

M. anisopliae has continued to demonstrate strong potential as a biological control agent against aphids. Latiff et al (2022) conducted a study on the effectiveness of isolates of *M. anisopliae* in

controlling *Aphis gossypii* populations in *Capsicum annum* and *Solanum melongena* crops. The researchers found that *M. anisopliae* reached a 97% mortality rate at the concentration 1×10^7 conidia/ml seven days after treatment (DAT). They also noted that *A. gossypii* with various life stages were susceptible to fungal treatment irrespective of the host plant the aphid growth (Latiff et al., 2022).

3.8. Gaps in existing knowledge

Despite the significant advances in the use of entomopathogenic fungi for the biological control of aphids, several gaps in the current body of research remain, particularly in the context of *P. cannabis* infestations in *C. sativa* cultivation. As cannabis production becomes more widespread, the unique aspects of this crop's cultivation and pest pressures highlight the need for targeted research as the effects of ETF and the cannabis aphid over the cannabinoid and terpene concentration of the cannabis plants are mostly unknown.

3.8.1. Limited research on *P. cannabis* and ETF

One of the most significant gaps in existing knowledge is the lack of specific research focusing on the efficacy of entomopathogenic fungi against *P. cannabis*, the primary aphid pest in cannabis. While many studies have explored the effectiveness of fungi like *B. bassiana* and *M. anisopliae* against other aphid species, there is limited data on how well these fungi perform specifically against *P. cannabis*. The biology, behavior, and environmental preferences of this aphid species may differ from more commonly studied aphids, potentially affecting the outcomes of fungal applications.

Lagos-Kutz et al (2018) were among the first reports to identify two aphid species, *P. cannabis* and *Rhopalosiphum rufiabdominale* (rice root aphid), as potential pests on industrial hemp in the U.S. Midwest. They collected aphid samples from field-grown hemp, hydroponic systems, and suction traps, using both morphological and molecular methods to differentiate *P. cannabis* from the similar *P. humuli.* Findings confirmed *P. cannabis* on hemp in Minnesota and identified *P. cannabis* migrations across six Midwestern states, providing critical insight into the spread and potential pest impact of these species on hemp cultivation.

Other studies, like Pitt (2022), investigated *P. cannabis* as a vector of Potato Virus Y to both hemp (host) and potato (non-host). Researchers conducted transmission assays and used Electrical Penetration Graph (EPG) analysis to observe aphid feeding behaviors. Results showed high PVY transmission rates (96% for hemp and 91% for potato in group assays) and revealed differences in feeding behavior between viruliferous and non-viruliferous aphids, impacting virus transmission. Findings suggest PVY infection could alter aphid behavior to increase virus spread across crops.

Research by MacWilliams (2023) investigates cannabidiol's (CBD) role in cannabis defense against cannabis aphids. CBD, a primary cannabinoid in hemp, was hypothesized to contribute to plant defense. Aphid reproduction and longevity were reduced on high-CBD plants compared to low-CBD ones, though adding CBD to aphids' artificial diet unexpectedly increased their reproduction rate. Aphid feeding did not significantly alter CBD levels in the plant but increased certain phytohormones like salicylic acid, jasmonic acid, and abscisic acid, which are known to activate plant defenses. This research highlights the complex interactions between cannabinoids and phytohormone signaling in *C. sativa*, suggesting that cannabinoids and terpenes may work together in defense. Further studies are encouraged to understand the mechanisms better.

Other aphids have been reported and their impact studied. For example, Cranshaw (2020) describes the rice root aphid (*Rhopalosiphum rufiabdominale*) as an emerging pest for indoor-grown Cannabis sativa in North America, especially in marijuana production. These aphids primarily inhabit plant roots and reproduce through asexual cycles, with winged forms colonizing new plants as they mature. Infestations cause generalized plant decline and can damage growth. The paper also explores control strategies, including biological agents like fungi and predatory ants, highlighting the need for more pest management research in indoor cannabis cultivation.

Pulkoski (2023) examined the effects of two pests, the two-spotted spider mite (*Tetranychus urticae*) and the green peach aphid (*Myzus persicae*), on cannabinoid concentrations in greenhouse-grown industrial hemp. Researchers conducted greenhouse experiments, comparing cannabinoid levels in individual versus pooled plant samples and then analyzing THC and CBD changes in plants exposed to low and high mite infestations. Results showed that spider mite feeding could reduce THC and CBD concentrations, with higher infestation levels generally causing slower increases in these cannabinoids, although impacts varied by pest density and plant variety. Aphids, however, failed to survive on whole hemp plants despite reproducing on excised leaf discs, suggesting that aphid pest pressure on hemp would be minimal. Overall, the study underscores the need for early pest management to protect cannabinoid yields and encourages further research to understand long-term infestation effects and refine integrated pest management practices for hemp.

However, despite the fact that cannabis aphid infestations have been reported in several states in the northwestern United States and Canada, there are still scarce reports on the effects of these infestations on the production of metabolites, terpenes, or the impact on plant growth and flower yield. Likewise, there are no reports attempting to provide a solution to this issue using ETF or other biocontrol alternatives.

CHAPTER 4: MATERIALS AND METHODS

4.1. Isolation of entomopathogenic fungi

Infected insect samples were collected and stored in hermetically sealed plastic bags. For the isolation of entomopathogenic fungi, each sample was enriched in sterile distilled water supplemented with 0.1% peptone, 0.5% Tween 80 and 0.05 mg/ml chloramphenicol, these were incubated at room temperature and 100 rpm agitation on a rotatory shaker. Serial dilutions were prepared to cultivate and isolate fungi and then plated on potato dextrose agar (PDA: 200 g/L potato extract, 20 g/L dextrose, and 15 g/L agar in distilled water pH 3.5). After seven days of incubation, pure cultures were obtained and stored for future research. Subsequently, lactophenol blue staining was performed to enhance the visibility and contrast of fungal structures under a microscope, making key morphological details easier to observe. The stain binds to chitin in the fungal cell walls, highlighting hyphae, spores, and conidiophores with a blue tint against a clear background., as well as morphological keys of the colonies; fungal samples were first observed for colony color texture, and growth patterns on Potato Dextrose Agar (PDA) plates. Colors, such as white, green, or cream, and textures, such as cottony, powdery, or granular, were recorded as preliminary identifiers. to select isolates consistent with entomopathogenic fungi of the genera *Beauveria*, *Metarhizium* and *Lecanicillium*.

4.2. Molecular characterization of the entomopathogenic fungi isolates

The isolates were classified by molecular analysis, for which DNA extraction was performed using the NORGEN Plant/Fungi DNA isolation kit (Cat. 27300). At the end of the extraction process, DNA quantification was performed using the 260 nm light absorption method

(Nanodrop). For PCR, the oligos ITS1: 5'TCCGTAGGTGAACCTGCGG 3' and ITS4: 5'TCCTCCGCTTATTGATATGC 3' were used, which amplify a fragment around 750 base pairs (bp). The PCR products were sent to Eurofins Genomics, for sequencing using the Sanger method. Forward and reverse sequences were obtained, refined, edited and aligned. The consensus sequences were compared with the available sequences in the GeneBank database using nucleotide BLAST to determine the identity of the fungal isolates. Finally, the phylogenetic tree of the isolates was constructed using the Neighbor-Joining method integrated in MEGA 11.

4.3. Preparation of the inoculum solution of entomopathogenic microorganisms

The isolates were grown on PDA at 25±1 °C for 15 days. Conidia were harvested with sterile distilled water containing 0.05% Tween 80. Mycelia was removed by filtering conidia suspensions through 4 layers of sterile cheesecloth. Conidia was counted under a microscope using a Neubauer hemocytometer to adjust the suspension concentration to 1×10^7 cells/mL for each isolate.

4.4. Cannabis aphids

Phorodon cannabis insects were obtained from infected plants in Hepler Hall at the University of Lethbridge during the spring of 2023. Insects were isolated and cultivated in another greenhouse, and later some wingless females were transferred to cannabis plants of the variety “Congo diesel” to establish colonies. The plants were fed regularly with FloraGro, rich in nitrogen, during the vegetative stage to promote healthy leaf and stem development. As the plants transitioned to flowering, FloraBloom, high in phosphorus and potassium; was introduced, to support bud growth and root strength. Throughout both stages, FloraMicro was applied to provide essential trace elements. and defoliation was carried out periodically to control the population and

infection of aphids. Old plants were replaced with new ones every time that was necessary. The insects were observed using a stereo microscope at 20X magnification to determine the morphological characters to discriminate *Phorodon cannabis* from other aphid species (Lagos-Kutz et al., 2018).

4.5. Entomopathogenicity bioassay

The pathogenicity test of the isolates against *P. cannabis* was carried out using the leaf-dip method with some modifications (Nazir et al., 2019). For each microorganism, Cannabis leaf discs of 50 mm diameter were obtained from healthy plants and immersed for 10 seconds in 5 mL of fungal suspensions obtained as described above. Then, to reduce the amount of excessive fungal suspension, the leaves were placed on sterile filter paper for 15 minutes and taken to sterile Petri dishes containing 1% agar solution. As a control, leaf discs immersed in 0.05% Tween 80 were used. Subsequently, non-winged adults were collected from infected plants and inoculated into the leaf discs. The Petri dishes were incubated at room temperature (25°C) in the dark for 10 days. Each treatment was repeated ten times and data was taken on the mortality of the aphids on days 3, 5, 7 and 10 of incubation, during which time the dead aphids were removed and analyzed in the microscope to observe if the growth of the mycelium of the fungus was the cause of death.

4.6. Greenhouse bioassay

Cuttings from mother plants, approximately 6 months old, were rooted for 10 days after which the plants were transferred to pots with a mixture of peat moss and perlite 70:30 ratio. Plants were grown at 22°C with an 18 h light 6 h dark cycle for 4 weeks and then transferred to chambers with 12 h light/12 h dark regime to promote flowering. Five treatments were applied to the cannabis plants: (1) uninfected, treated with distilled water, (2) uninfected, treated with entomopathogenic

microorganism, (3) aphid-infected, treated with commercial chemical insecticide according to the manufacturer's specifications, (4) aphid-infected treated with distilled water, and (5) aphid-infected, treated with entomopathogenic microorganism. Wingless adult aphids were inoculated on each plant according to the treatment. One week after the inoculation of the aphids and using an atomizer, the plants were foliar sprayed with the fungal solution (1×10^7 cells/mL) as well as the chemical insecticide or the control with distilled water only according to each treatment. Spraying was repeated once a week until the flowering process was completed (Saranya et al., 2010). By the end of the flowering period, the flowers were harvested, dried, weighed, measured and later sent for subsequent analysis by HPLC and HPLC -DAD at Canvas Labs (Vancouver, BC), to determine the concentrations of important cannabinoids and terpenes respectively.

4.7. Data collection

To evaluate how effective entomopathogenic microorganisms are in controlling *P. cannabis* within greenhouse conditions, the aphid population was determined for each treatment described above.

Samples of three leaves were taken from each plant, one from the top, the middle and the base of each plant, before and after each treatment. Data was taken on the number of live aphids in each sample and the population was calculated each week for each treatment (Dhakal et al., 2019).

4.7.1. Measurement of growth parameters

To assess the efficacy of pest control measures by comparing growth parameters between plants subjected to treatment and those left untreated, the weight and height of the plants (base of the pylon to the highest point of the crown of the leaves), was measured. Dry biomass was

determined after flowering; for this, each of the plant was taken to an oven for 72 hours at 60°C, after which each plant was weighed on a top-loading laboratory balance (Islam et al., 2021).

Monitoring changes in plant size and weight will help assess the severity of pest infestations or the effectiveness of pest control methods. It will also offer tangible metrics to evaluate pests' impact on plant development.

4.8. Statistical analysis

The response variables were initially described through descriptive statistics and later on analyzed using parametric tests. In all cases the variables were expressed as the mean ± standard deviation. To determine the effects of the different treatments on the response variables, a one-way ANOVA was implemented, followed by a Tukey test. Before any statistical analysis, Brown and Forsythe tests was performed to determine the homogeneity of variance and Kolmogorov-Smirnov test to test Normality of the obtained data, with the aim of verifying compliance with these two assumptions without which the analysis did not proceed. In case of non-compliance with the above assumptions, the data in percentages was subjected to arc-sine transformations to be normalized, or ultimately, they were analyzed using the non-parametric Kruskal Wallis test. In all cases, $p<0.05$ was used as a statistical criterion to reveal significant differences between treatments, considering a 95% test confidence interval. All the data was analyzed using the IBS SPSS Statistics version 25 statistical program.

CHAPTER 5: RESULTS

5.1. Isolation of entomopathogenic fungi

After obtaining samples of dead or infected insects from Hepler Hall greenhouse, fungal isolates were obtained following a seven-day incubation period of serial dilutions of enriched samples, which were then plated onto Potato Dextrose Agar (PDA). A total of 20 fungal isolates were recovered after purification and subculturing. Subsequently, lactophenol blue staining was applied to enhance the visibility and contrast of fungal structures under the microscope, making key morphological features easier to observe. The stain binds to chitin in the fungal cell walls, highlighting hyphae, spores, and conidiophores with a blue tint against a clear background.

Among the isolates obtained, colonies consistent with *Trichoderma* (F17, F18), *Penicillium* (F1), and *Aspergillus* (F10, F16) were identified (Table 1). Two isolates, F2 and F12, were consistent morphologically with the genera *Beauveria* and *Metarhizium*, respectively, according to the literature.

Isolate F2 exhibited a white to pale cream color with a cottony or powdery texture (Table 1). Under the microscope, after staining with lactophenol blue, F2 showed small, single-celled, oval or globose conidia produced on short, zig-zag conidiophores. The conidia were often arranged in dense clusters, giving the colony a globular appearance.

Isolate F12, on the other hand, appeared green to olive in color with a granular texture (Table 1). After two weeks of incubation on PDA, the colonies developed a powdery appearance due to

conidia formation. Microscopic examination revealed that the conidia of F12 were cylindrical to elliptical and were borne on distinct, branched conidiophores, typically arranged in dense, brush-like clusters.

Table 1. Morphological description of fungal isolates with entomopathogenic potential.

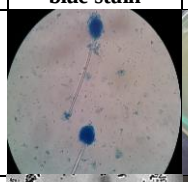
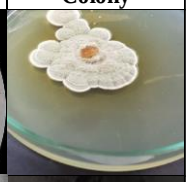
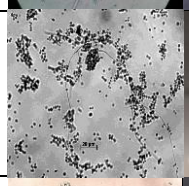
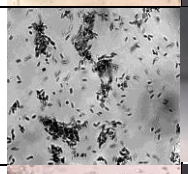
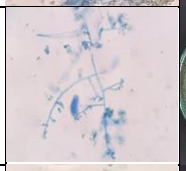
Isolate code	Elevation	Colony color	Reverse color	Appearance	Colony border	Lactophenol blue stain	Colony
F1	Raised	Grey	Dark Yellow	Rough	Circular		
F2	Raised	White	Yellow	Smooth	Irregular		
F10	Raised	Green	Yellow	Rough	Irregular		
F12	Raised	Dark green	Brown	Rough	Circular		
F15	Raised	Green	Brown	Rough	Irregular		
F17	Flat	Green	Yellow and Green	Rough	Circular		
F18	Flat	Yellow and Green	Yellow and Green	Rough	Circular		

Figure 1. Phylogenetic tree for fungal isolate F2 *Beauveria bassiana* based on the ITS sequences. Tree constructed using the Neighbor-Joining method integrated in MEGA 11, E value 5e-^{57}.

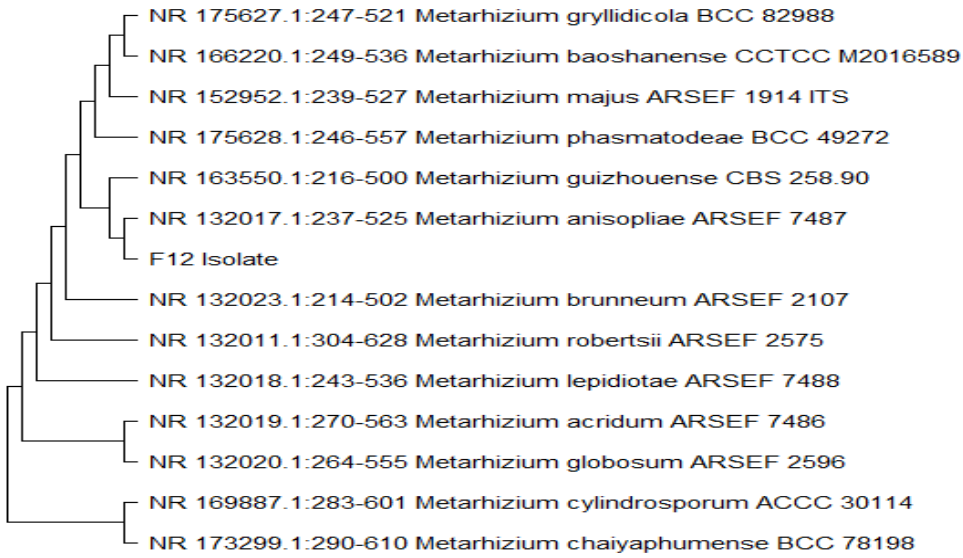


Figure 2. Phylogenetic tree for fungal isolate F12 *Metarhizium anisopliae* based on the ITS sequences. Tree constructed using the Neighbor-Joining method integrated in MEGA 11, E value 1e-^{149}.

Molecular analysis confirmed the morphological identification, revealing that the two EPF isolates (F2 and F12) obtained from insect cadavers were a *Beauveria bassiana* isolate and a *Metarhizium anisopliae*, isolate respectively. This identification was achieved by sequencing the internal transcribed spacer (ITS) region of the rDNA using the ITS1 and ITS4 primers. Phylogenetic analysis, conducted using the Neighbor-Joining method in MEGA 11 (Figure 1 and 2), produced a bootstrap consensus tree. The sequences of this isolate showed 99% homology with another *B. bassiana* and *Metarhizium anisopliae* isolates in the GenBank database.

5.2. Cannabis aphids

Aphid colonies were successfully established on cannabis plants of the Congo Durban variety. To maintain the colonies, new plants had to be infected approximately every two months. The aphid infection spread rapidly on the plant, and a new plant had to be infected about every month. Production of large amounts of honeydew was evident, and obvious damage to the plants (e.g., wilting, and yellowing) was observed (Figure 3).

Figure 3. Cannabis plants of the Congo Durban variety infected with aphids in the Hepler Hall greenhouse.

Using a stereo microscope at 20X magnification we determined the morphological characters of the aphids to discriminate *P. cannabis* from other aphid species (Figure 4).

It was possible to identify the aphid as *P. cannabis* and distinguish it from the most similar species *Phorodon humuli* (hop aphid) by key morphological features. *P. cannabis* has flabellate (spatulate) body setae, slightly swollen siphunculi, and shorter antennal segment III relative to the siphunculi. In contrast, *P. humuli* lacks flabellate setae, has straight siphunculi, and a longer antennal segment III (Cranshaw et al., 2018). Also *P. cannabis* has a slightly lighter color than *P. humuli*, especially evident in adults. These differences provide reliable markers for distinguishing between the two species on cannabis plants.

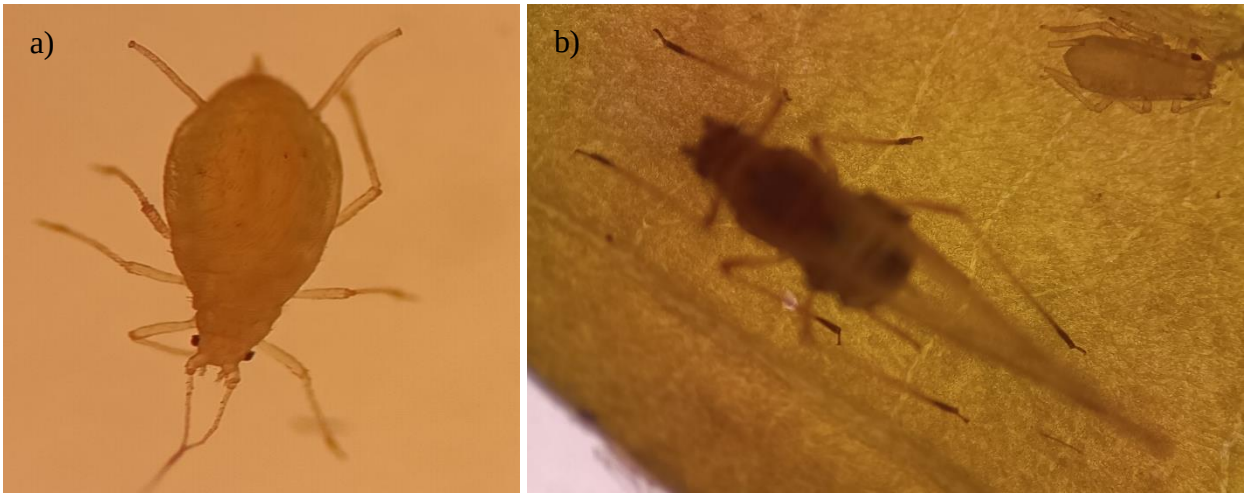


Figure 4. *P. cannabis*. a) Adult aphid. b) Winged adult. Under a stereo microscope at 20X magnification.

5.3. Entomopathogenicity bioassay

The pathogenicity test of the isolates against *P. cannabis* was carried out using the leaf-dip method with some modifications. Figure 5 shows the mean mortality rate of *P. cannabis* at different days after treatment (DAT) with the entomopathogenic fungi *B. bassiana* and *M. anisopliae* across two conidial concentrations (1×10^5 and 1×10^7 conidia mL^{-1}), plus a control group.

At the highest concentration (1×10^7), both fungi achieved a 100% mortality by the 10^{th} DAT, however there were no statistically significant differences between the two microorganisms ($P>0.05$). Lower concentrations and earlier DATs generally resulted in lower mortality, highlighting dose- and time-dependent effects on aphid mortality. However, with the concentration of 1×10^7 conidia mL^{-1} *B. bassiana* reached a higher mean mortality ($P<0.05$) by the 7th DAT, showing a higher infection rate compared to *M. anisopliae* at the same concentration and at the

same DAT. The average aphid mortality of the control treatment only increased starting on 7th DAT and only reached 22% on 10th DAT of treatment. Because of these results, it was decided to use the *B. bassiana* inoculum at a concentration of 1×10^7 conidia mL^{-1} for the Greenhouse bioassay.

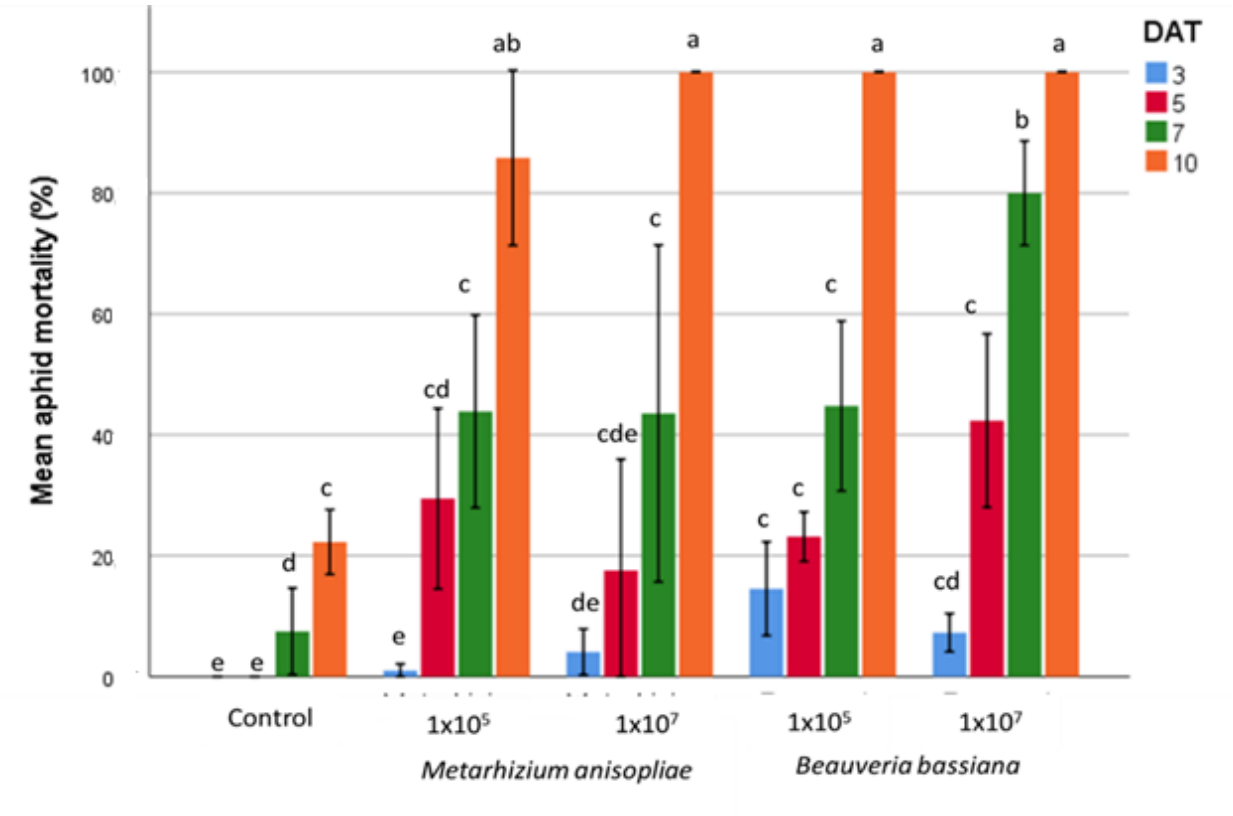


Figure 5. Mean mortality (%) of *P. cannabis* recorded at different time intervals (DAT: Days after treatment) for conidial bioassays performed with *B. bassiana* and *M. anisopliae*. Treatments included two conidial concentrations (i.e., 1×10^5 and 1×10^7 conidia mL^{-1} and one control. Columns represent mean percent mortality ± SD (n = 10). The means followed by the same letters within columns are not significantly different from each other according to Tukey's HSD ($P < 0.05$)

The dead aphids were removed and analyzed under the microscope to observe the growth of the fungal mycelium as a cause of death. On the 3th DAT of treatment, the first signs of infection were evident: fungal spores adhered and germinating in the aphid cuticle, with small germ tubes that began to penetrate the insect's exoskeleton.

Later, on the 5th DAT, it was seen that the infection was progressing, filiform hyphae were spreading throughout the aphid, consuming nutrients and altering its tissues. After this, on the 7th and 10th DAT, hyphae grew outward from the aphid's body, breaking the cuticle and covering the insect with a fuzzy white layer of fungal mycelium (Figure 6). This external growth produces new spores on the surface of the aphid, ready to infect other insects, demonstrating *B. bassiana*'s and *M. anisopliae*'s effectiveness as biological pest control agents.

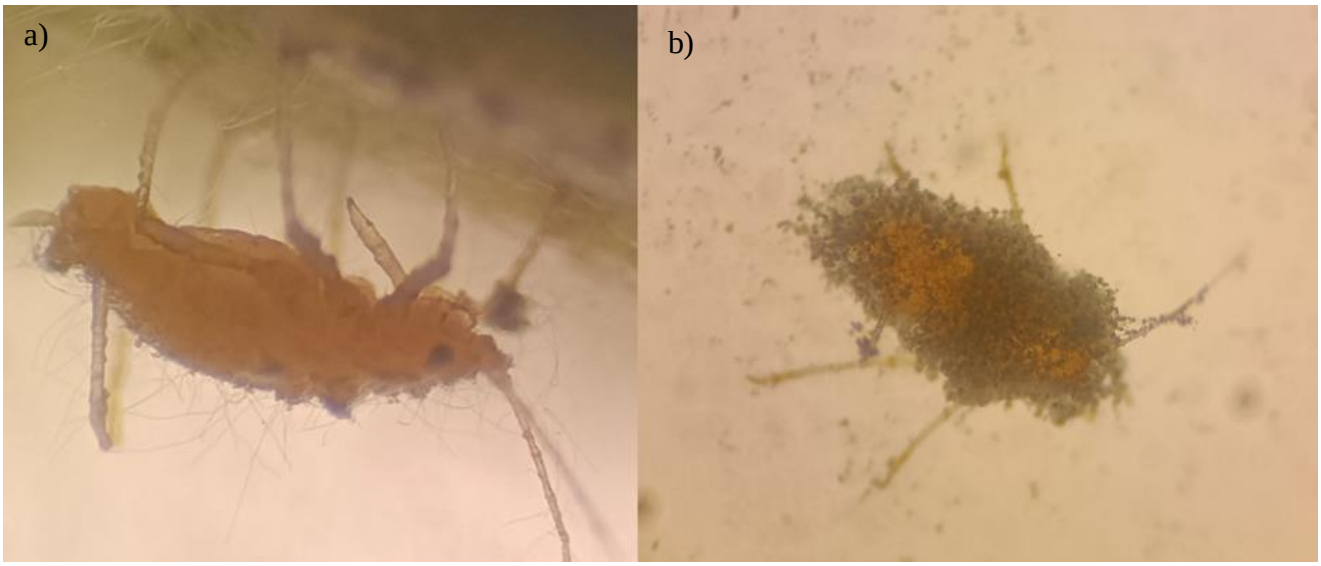


Figure 6. *Phorodon cannabis* infected with a) *Beauveria bassiana* and b) *Metarhizium anisopliae* on DAT 10 under a stereo microscope at 20X magnification.

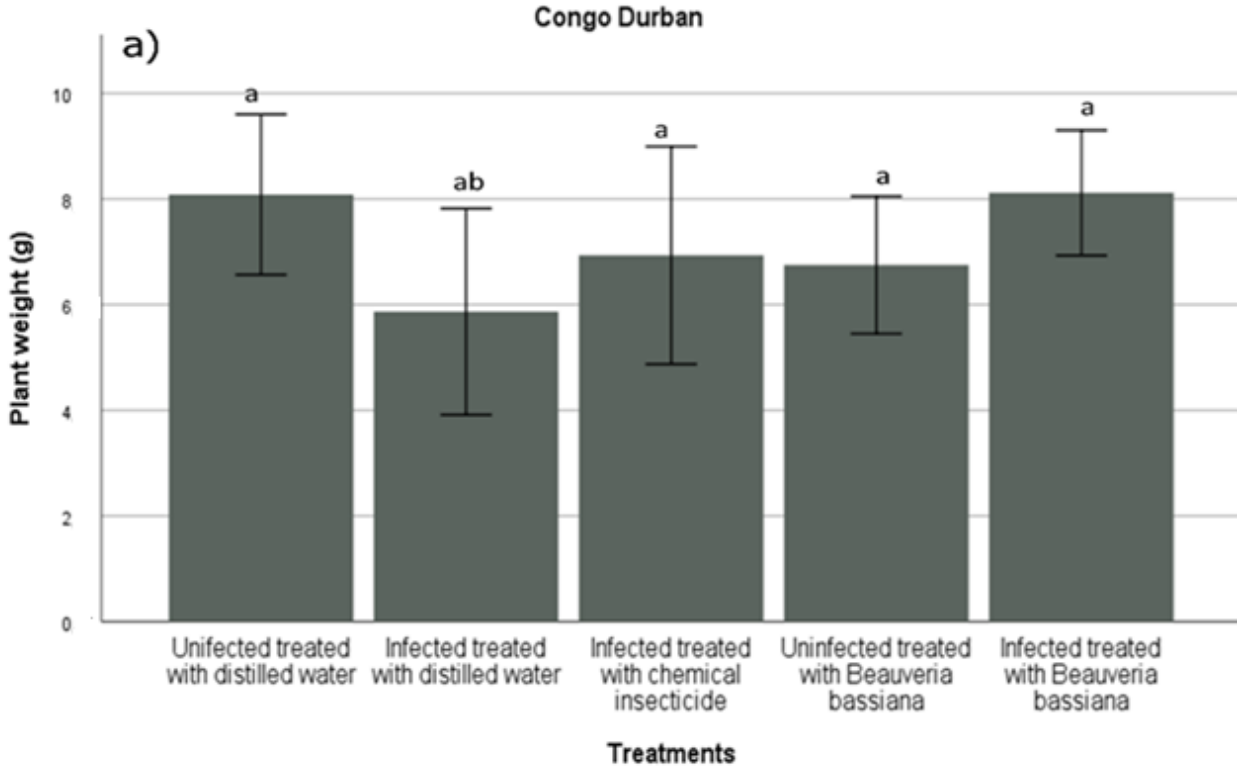
5.4. Green house bioassay

5.4.1. Effects of aphid infection on cannabis plants growth parameters

Approximately 9 weeks after the flowering process began, the three cannabis varieties were dried, weighed and measured to determine the growth parameters of the treatments. Figure 7 shows the growth parameters (dry biomass and height) of Congo Durban cannabis plants under different

treatments. The dry biomass (Figure 7a) of the plants that were uninfected and treated with distilled water showed the highest mean dry biomass, 8 g. Infected plants treated with distilled water had the lowest biomass, indicating that infection negatively impacted biomass production. There was no significant difference between treatments with *B. bassiana* and chemical insecticide, suggesting similar efficacy in mitigating biomass loss by aphid infection.

Uninfected and treated with distilled water plants were the tallest (around 55-60 cm). Infection reduced plant height across treatments, with infected plants treated with distilled water being the shortest. *B. bassiana* and the chemical insecticide helped maintain greater height compared to untreated infected plants, but both treatments resulted in slightly shorter plants than the uninfected control (Figure 7b).



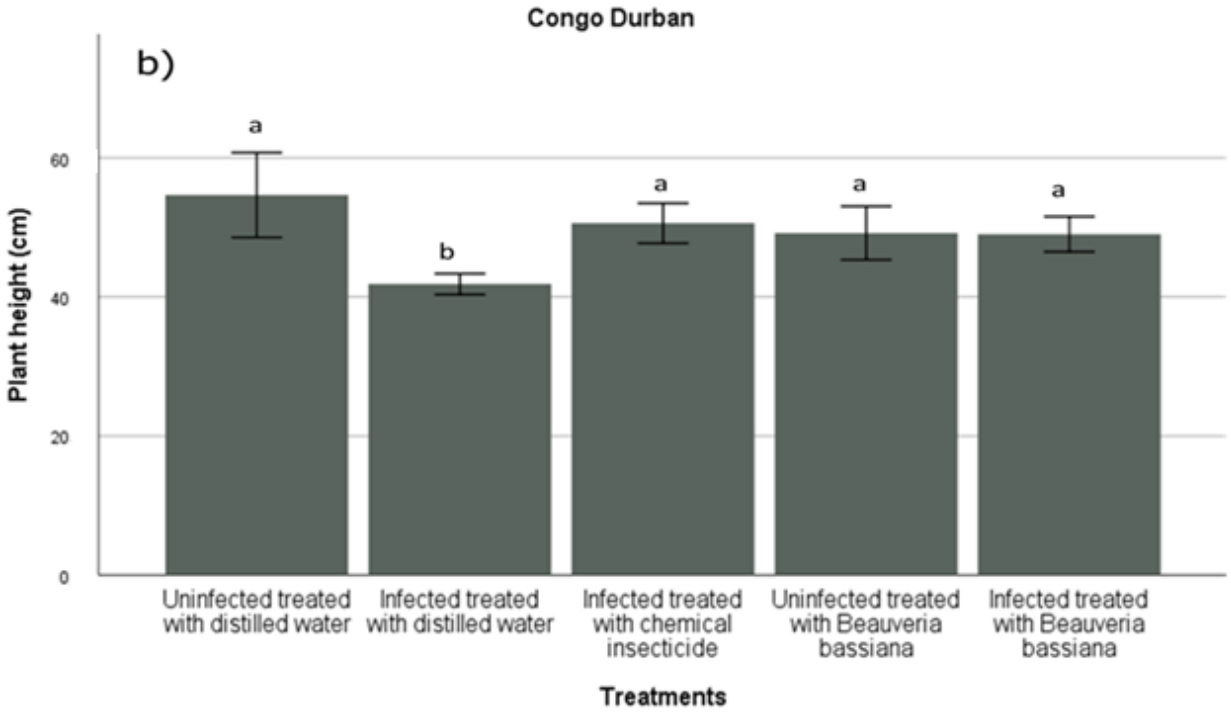
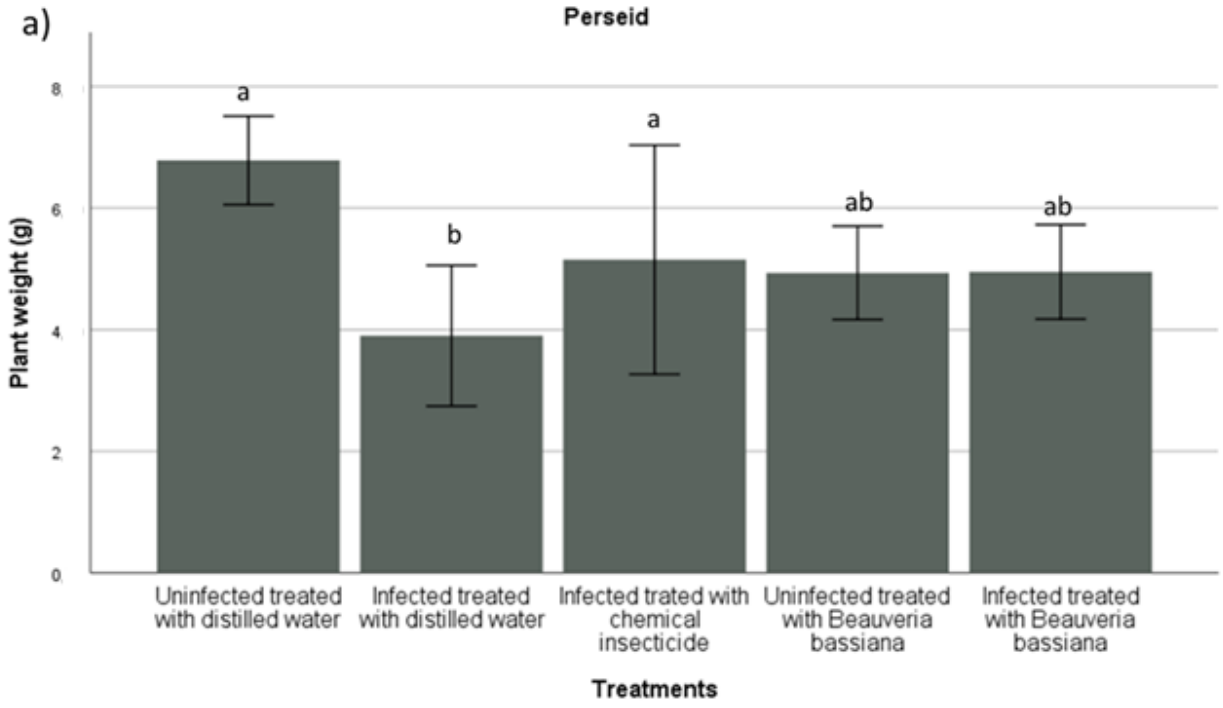


Figure 7. Growth parameters of Congo Durban (THC dominant) cannabis plants, a) Dry biomass after the flowering process and b) Height of the plants (base of the plant to the highest point of the crown of the leaves). Variables are expressed as the mean ± standard deviation. Means followed by same letter are not significantly different by Tukey's HSD multiple range test at $P < 0.05$.

Uninfected Perseid plants treated with distilled water had the highest average dry biomass (7 g), though there were no significant differences among the other treatments except for the infected plants treated with distilled water ($P < 0.05$). Both *B. bassiana* treatments—whether infected or uninfected by aphids—as well as the chemical control produced similar dry biomass levels, approximately 5 g (Figure 8a). In contrast, the infected plants treated with distilled water yielded the lowest biomass (under 4 g), highlighting that *P. cannabis* infection, if unmanaged, can significantly reduce the growth and productivity of host plants.

Again, uninfected Perseid plants treated with distilled water reached the greatest average height (48 cm), though there were no significant differences among the other treatments (P<0,05), except for the infected plants treated with water. Plants infected with *P. cannabis* and treated with chemical insecticide measured 42 cm in height, uninfected plants treated with *B. bassiana* reached 45 cm, and infected plants treated with *B. bassiana* were 41 cm tall. The infected plants treated with water showed the lowest height (40 cm), further indicating that untreated aphid infection can adversely impact plant growth.



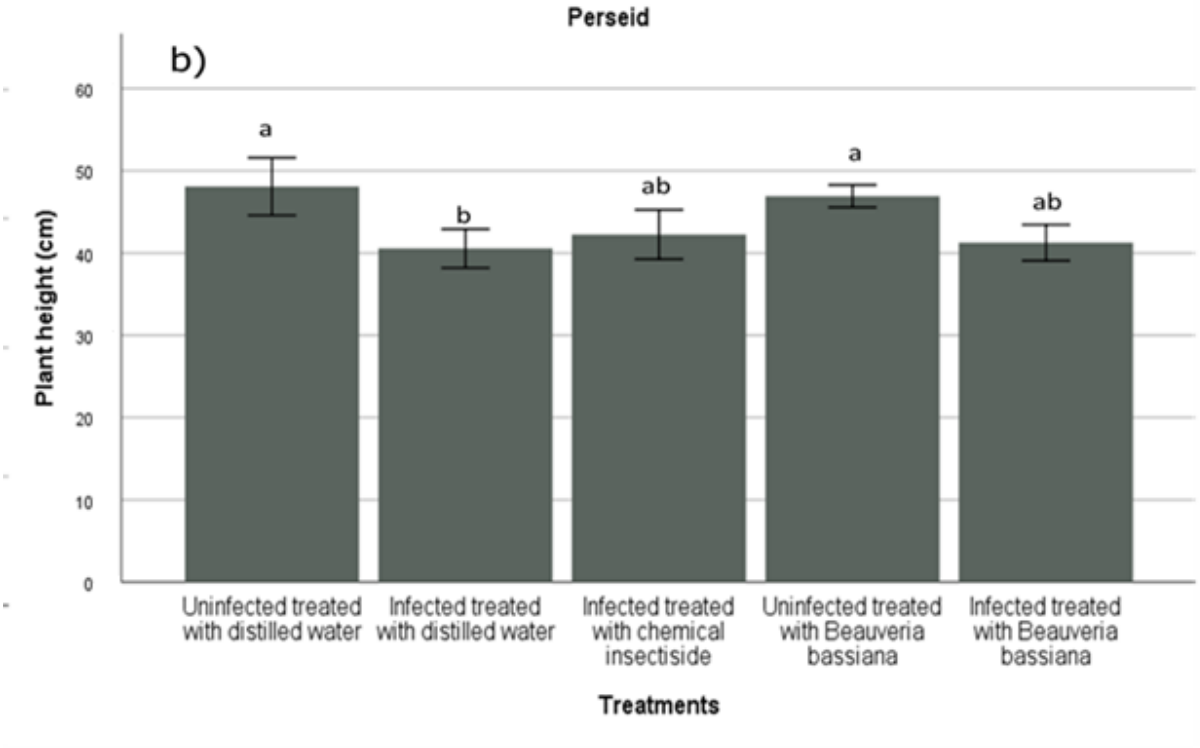


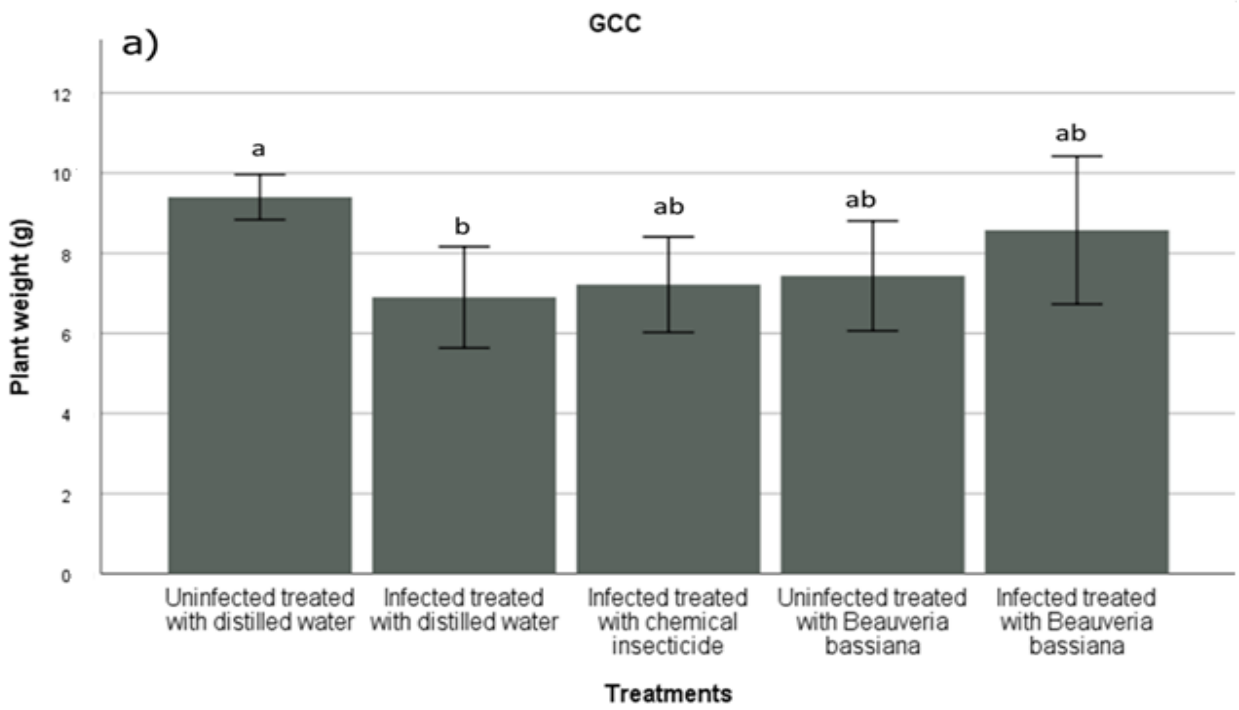
Figure 8. Growth parameters of Perseid (CBD dominant) cannabis plants, a) Dry biomass after the flowering process and b) Height of the plants (base of the plant to the highest point of the crown of the leaves). Variables are expressed as the mean ± standard deviation. Means followed by same letter are not significantly different by Tukey's HSD multiple range test at $P < 0.05$.

In summary, *P. cannabis* infection significantly reduced both height and dry biomass in Perseid plants, particularly when untreated. While plants treated with distilled water, chemical insecticide, or *B. bassiana* showed similar growth outcomes in height and biomass, infected plants treated only with distilled water exhibited the lowest values for both metrics. These findings highlight that *P. cannabis* infection can substantially impair plant growth and productivity if left unmanaged,

emphasizing the importance of appropriate pest control measures to maintain healthy plant development.

Uninfected GCC plants treated with distilled water exhibited the highest average dry biomass (9 g), although no significant differences were observed among the other treatments, with the exception of the infected plants treated with distilled water ($P < 0.05$) (Figure 9a). Notably, the infected plants treated with distilled water showed the lowest biomass, averaging 7 g.

In terms of plant height, the infected GCC plants treated with distilled water reached the shortest average height (45 cm). No significant differences were observed between the infected plants treated with the chemical insecticide (51 cm), the non-infected plants treated with water (51 cm), and those treated with *B. bassiana* (48 cm) ($P < 0.05$). However, the infected plants treated with water had the lowest height, measuring 43 cm (Figure 9b).



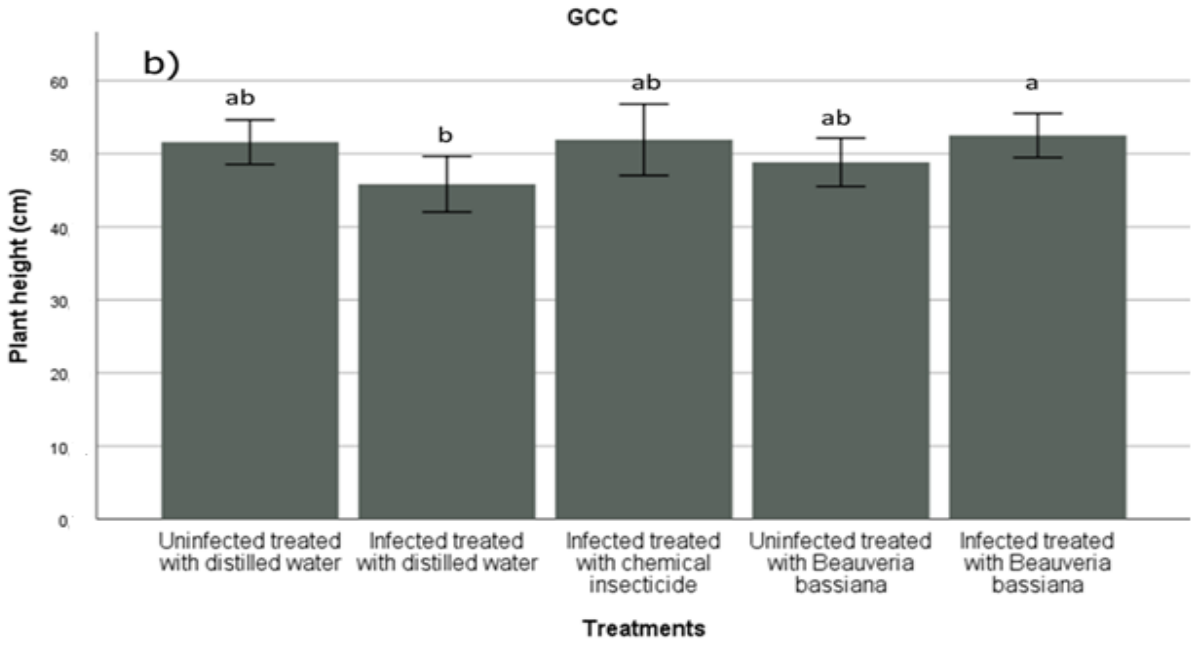


Figure 9. Growth parameters of GCC (CBD≈THC) Cannabis plants, a) Dry biomass after the flowering process and b) Height of the plants (base of the plant to the highest point of the crown of the leaves). Variables are expressed as the mean ± standard deviation. Means followed by same letter are not significantly different by Tukey's HSD multiple range test at $P < 0.05$.

The results suggest that infection with *P. cannabis* negatively impacted both the height and biomass of the GCC cannabis plants. Specifically, infected plants treated with distilled water showed the most pronounced reductions in both height and biomass compared to other treatments. While treatments with *B. bassiana* or a chemical insecticide did not significantly improve biomass or height compared with the uninfected plants treated with distilled water, they did provide some benefit over untreated, infected plants. These findings indicate that *P. cannabis* infection adversely affects the growth of GCC cannabis plants, reducing both their overall height and dry biomass.

5.4.2. Effects of *Beauveria bassiana* on aphid population

Figures 10, 11, and 12 show the effect of different treatments with *B. bassiana* on Congo Durban, Perseid and GCC infected with *P. cannabis* on aphid population, respectively. Based on the data, it can be seen that across all three varieties, the aphid populations remained low and stable in the plants treated with *B. bassiana* (Bb) and the chemical insecticide. The Bb and chemical insecticide treatments effectively controlled aphid population growth, maintaining it close to zero throughout the 9-week period after infection, with minimal variation.

Across all varieties, untreated plants (infected with aphids but given only water) experienced significant aphid population growth over the 9-week experimental period. In GCC, aphid populations increased steadily, reaching around 183 aphids (Figure 12) by the ninth week. Perseid showed even higher susceptibility, with populations soaring to over 350 aphids (Figure 11) by the end of the trial, indicating rapid aphid proliferation in the absence of treatment. Congo Durban, while also exhibiting unchecked growth, had a slightly slower rate of increase, peaking at around 210 aphids in week 9 (Figure10).

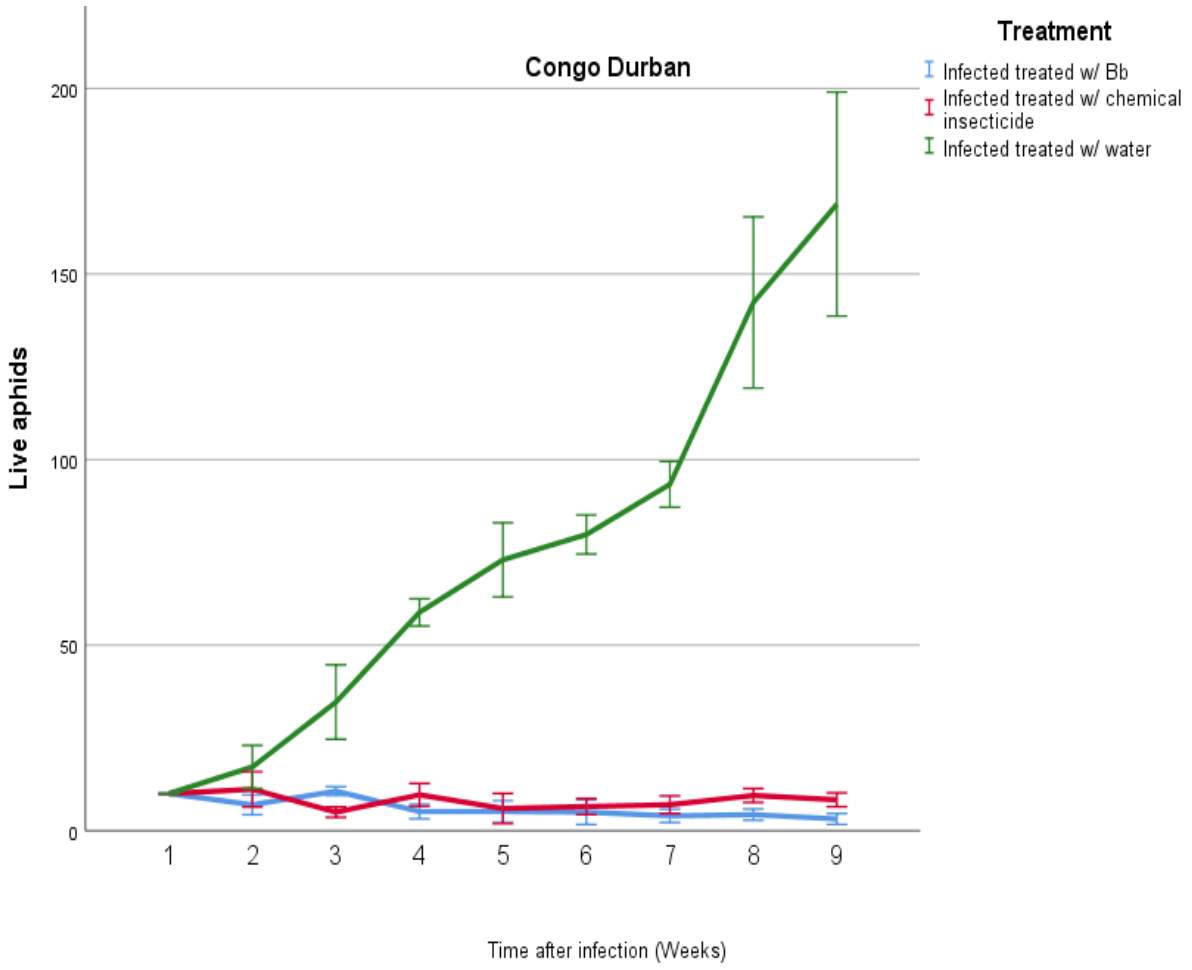


Figure 10. Effect of different treatments, on the aphid *P. cannabis* population in the cannabis variety Congo Durban under greenhouse conditions. Data represents mean aphid population counts per treatment (± SE) over the experimental period of 9 weeks after infection. Bb, *B. bassiana* 1 $x10^{7}$ conidia ml^{-1}.

Both chemical insecticide treatments and Bb, demonstrated effectiveness in limiting aphid growth across all three varieties. In GCC, chemical treatment kept aphid numbers relatively low, fluctuating between 2 and 21 aphids throughout the experiment. Bb treatment provided comparable control, with aphid counts staying between 1 and 15, showing minor variations but generally stable control. Perseid responded similarly, with aphid counts under chemical treatment ranging from 2 to 13, while the Bb treatment, maintained populations between 1 and 12 aphids. Congo Durban exhibited low aphid populations under both treatments as well, with counts between 2 and 20

aphids in the chemical treatment and between 1 and 12 aphids with Bb. The low aphid counts across all three varieties in the chemically and biologically treated groups suggest that both methods are effective for aphid control in cannabis, with Bb providing a viable, eco-friendly alternative to chemical insecticides.

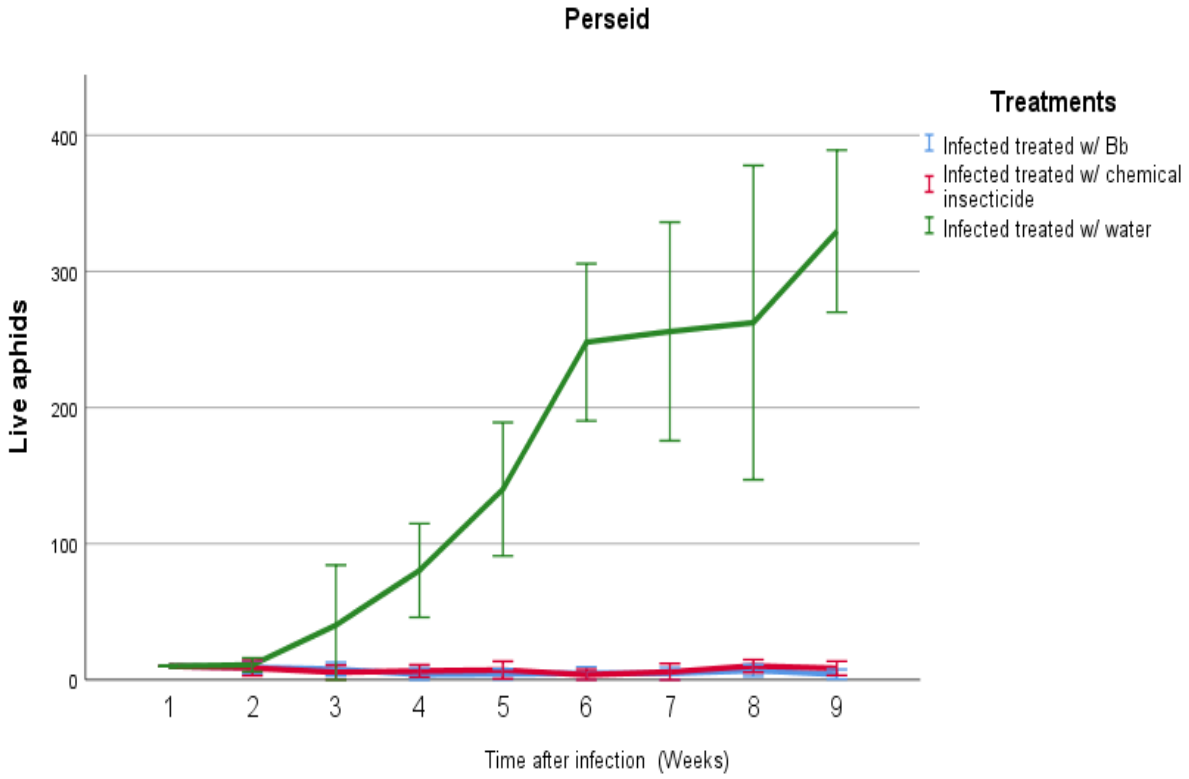


Figure 11. Effect of different treatments, on the aphid *P. cannabis* population in the cannabis variety Perseid under greenhouse conditions. Data represents mean aphid population counts per treatment (± SE) over the experimental period of 9 weeks after infection. Bb, *B. bassiana* 1 x10^{7} conidia ml^{-1}.

Overall, while untreated plants saw exponential aphid growth—most significantly in Perseid, followed by GCC and then Congo Durban—both chemical and Bb treatments were successful in maintaining low, stable aphid populations. Bb's comparable efficacy to chemical treatment across all three varieties highlights its potential as a sustainable control option in cannabis cultivation.

These findings demonstrate the importance of proactive aphid management, especially for more vulnerable varieties like Perseid, and underscore the effectiveness of both chemical and biological treatment options in controlling aphid populations under greenhouse conditions.

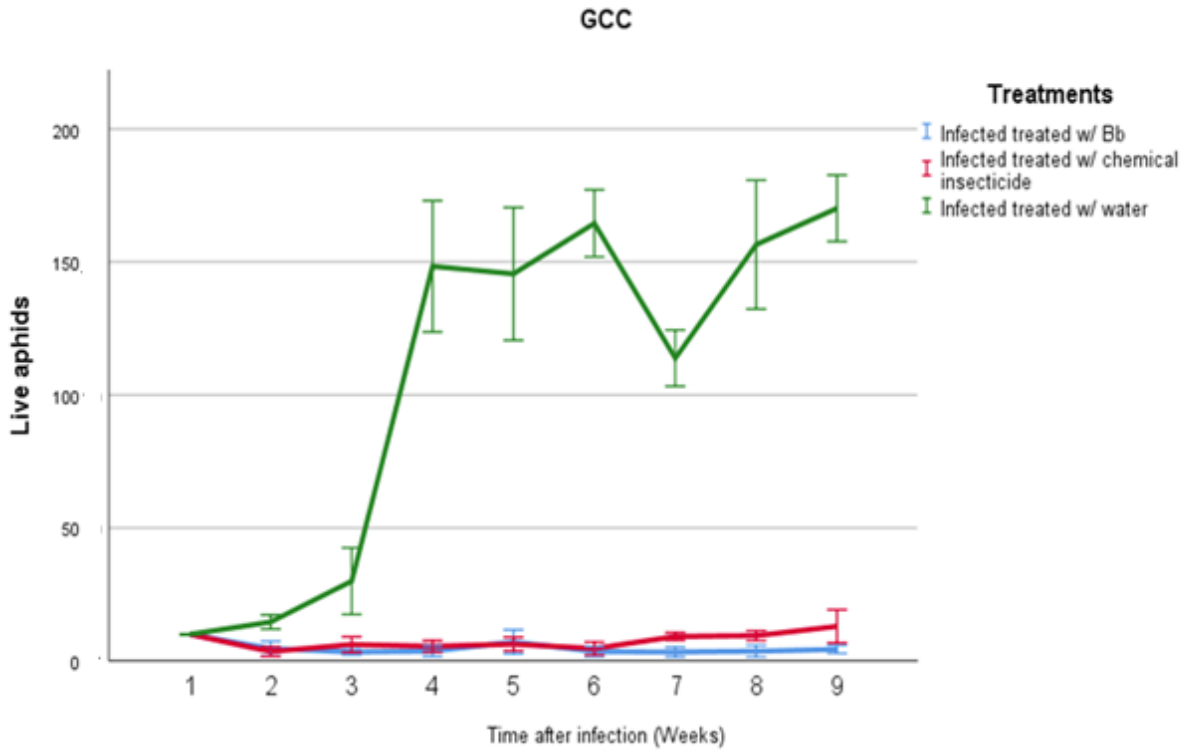


Figure 12. Effect of different treatments, on the aphid *P. cannabis* population in the cannabis variety GCC under greenhouse conditions. Data represents mean aphid population counts per treatment (± SE) over the experimental period of 9 weeks after infection. Bb, *B. bassiana* 1 x10^{7} conidia ml^{-1}.

5.4.3. Effects of aphid infection and treatment with *B. bassiana* on cannabinoid and terpene concentration.

The analysis of cannabinoid profiles in the THC-dominant cannabis variety Congo Durban, under various treatments, reveals intriguing trends in cannabinoid concentrations across infected

and non-infected plants treated with water, chemical insecticide, and *B. bassiana*. Data shows that THCa is the dominant cannabinoid across all treatments, with the highest concentration of THCa (19.68%) (Figure 13) in non-infected plants treated with Bb, while infected plants treated with chemical insecticide show the lowest concentration (16.52%). This suggests that Bb treatment might help maintain or even enhance THCa levels despite aphid infestation. Δ9-THC levels also vary, with non-infected Bb-treated plants having the highest concentration (7.07%) and infected, insecticide-treated plants the lowest (5.66%). This aligns with THCa patterns, implying that Bb treatment could positively influence Δ9-THC levels as well.

THCV a psychoactive cannabinoid concentration, is generally low, with the highest level (0.2%) (Figure 13) found in non-infected plants treated with water and undetectable levels in infected plants treated with Bb. This absence of THCV in infected Bb-treated plants may indicate that Bb treatment influences THCV production differently, though more data would be required to confirm this. CBGa, a precursor to THCa and CBDa is highest in non-infected Bb-treated plants (2.58%) and lowest in insecticide-treated infected plants (1.77%), mirroring the trends observed in THCa and Δ9-THC concentrations and further suggesting that Bb treatment might support higher cannabinoid levels compared to chemical insecticides.

CBG and CBC concentrations remain relatively stable across treatments, showing only minor variations. CBG is slightly higher in non-infected water-treated plants (0.23%) and lower in non-infected Bb-treated plants (0.1%), while CBC levels remain constant at 0.09% across all treatments, indicating that these cannabinoids may be less affected by infection or treatment type.

The total cannabinoid content shows the cumulative potency across all measured cannabinoids, with non-infected Bb-treated plants having the highest total concentration (29.52%), closely followed by infected Bb-treated plants (28.72%). Infected plants treated with chemical insecticide

have the lowest total cannabinoid concentration (24.39%), suggesting that chemical insecticide treatment may suppress overall cannabinoid production, while Bb treatment supports higher total cannabinoid levels, even with infection.

This cannabinoid profile analysis suggests that *Bb* treatment, especially in non-infected plants, is associated with higher levels of THCa, Δ9-THC, CBGa, and total cannabinoids compared to chemical insecticide treatment, which seems to reduce cannabinoid concentrations. This pattern highlights Bb as a potentially beneficial pest management alternative in cannabis cultivation, as it may help sustain or enhance cannabinoid production even in the presence of aphid infestation, unlike chemical insecticides, which could limit cannabinoid potency and thereby impact the therapeutic or psychoactive qualities of cannabis products.

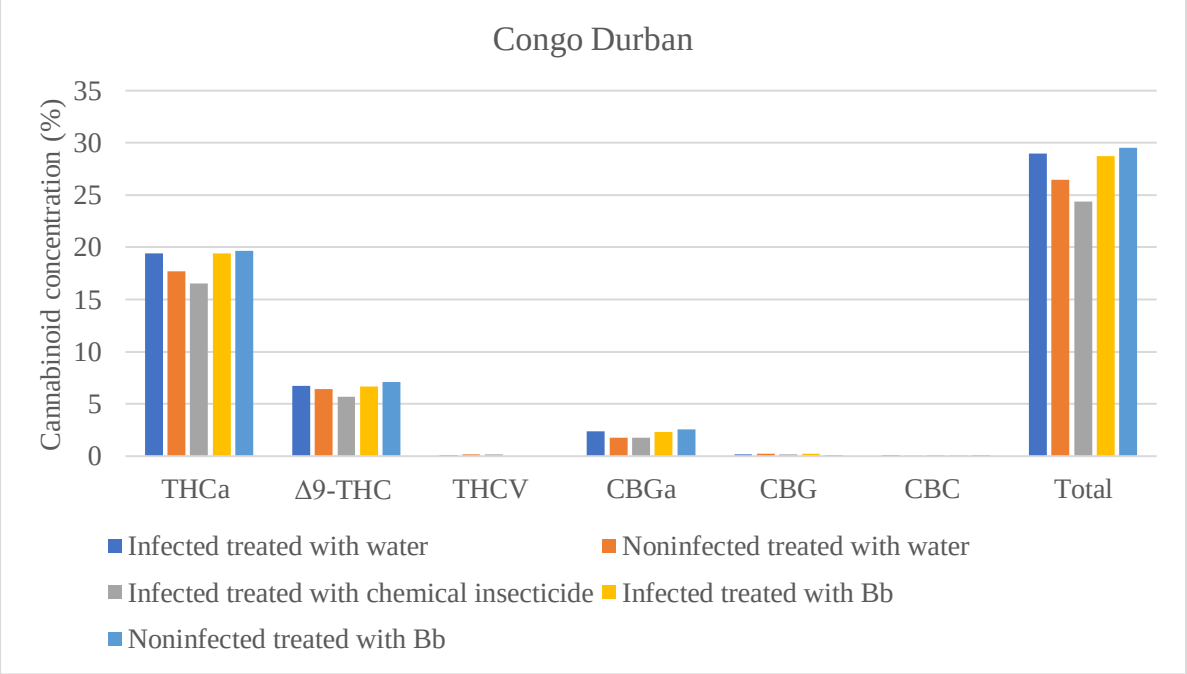


Figure 13. Cannabinoid profile of Congo Durban (THC dominant) cannabis plants: potency and concentration analysis (%) using USP <621> chromatography and HPLC-DAD quantification. Bb, *B. bassiana* 1 x10^{7} conidia ml^{-1}.

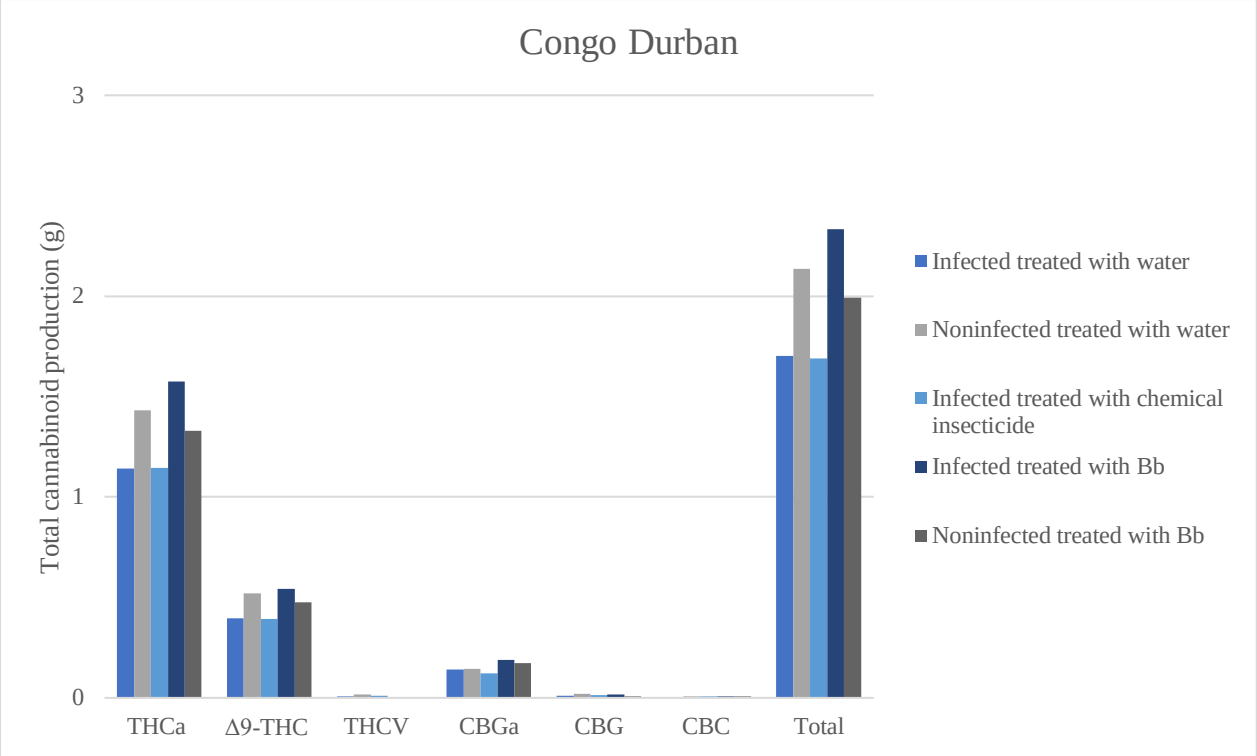


Figure 14. Cannabinoid profile of Congo Durban (THC dominant) cannabis plants: potency and total cannabinoid production (g) using USP <621> chromatography and HPLC-DAD quantification. Bb, *B. bassiana* 1 x10^{7} conidia ml^{-1}.

Figure 14 shows the total cannabinoid production of THC-dominant Congo Durban cannabis plants, THCa, the acidic precursor to Δ9-THC, exhibited the highest concentration in infected plants treated with Bb (1.57 g), followed by noninfected plants treated with water (1.43 g). Infected plants treated with water or chemical insecticides showed reduced THCa levels (both 1.14 g), suggesting that Bb treatment may mitigate the negative impact of infection on THCa biosynthesis. Similarly, Δ9-THC levels were highest in infected plants treated with Bb (0.54 g), compared to noninfected water-treated plants (0.52 g) and lower concentrations in chemically treated or water-treated infected plants (both 0,39 g). These findings indicate that Bb treatment may enhance the conversion of THCa to Δ9-THC under infection-induced stress conditions.

Minor cannabinoids such as THCV, CBGa, CBG, and CBC also showed variations across treatments. THCV was highest in noninfected water-treated plants (0.01 g) and undetectable in infected plants treated with Bb. CBGa, a critical precursor in the cannabinoid biosynthetic pathway, was most abundant in infected plants treated with Bb (0.18 g), highlighting Bb's role in promoting precursor biosynthesis. Conversely, CBG levels peaked in noninfected water-treated plants (0.018 g), with slightly lower levels in infected plants treated with Bb (0.01 g). CBC concentrations were relatively stable across treatments but slightly elevated in Bb-treated infected plants (0.007 g).

Total cannabinoid production varied significantly across treatments. Infected plants treated with Bb exhibited the highest total production (2.33 g), followed by noninfected water-treated plants (2.13 g) and noninfected Bb-treated plants (1.99 g). Infected plants treated with chemical insecticides had the lowest total cannabinoid production (1.69 g), underscoring the potential negative impact of the chemical treatment on secondary metabolite synthesis. but also enhances overall cannabinoid production, particularly in stressed plants.

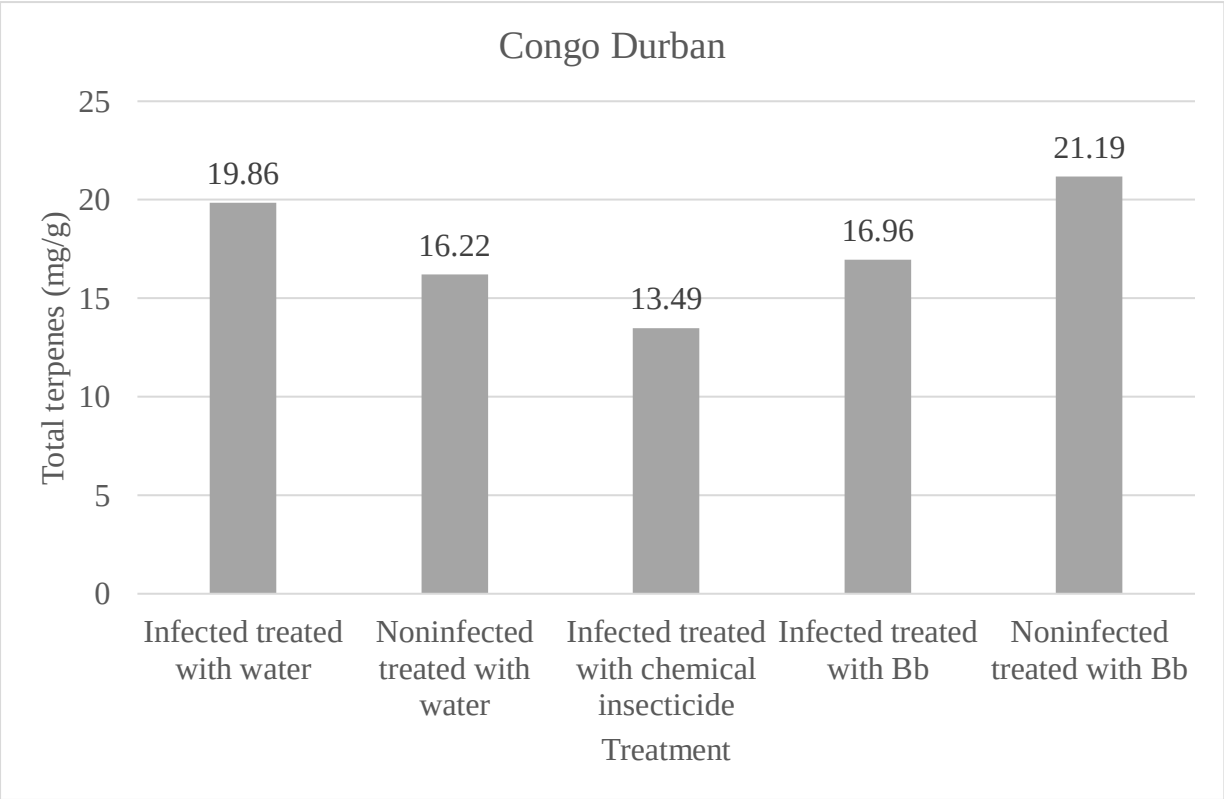


Figure 15. Terpenes profile of Congo Durban (THC dominant) cannabis plants: potency and concentration analysis (%) using USP <621> chromatography and HPLC-DAD quantification. Bb, *B. bassiana* 1 x10^{7} conidia ml^{-1}.

The terpene profile analysis Congo Durban cannabis plants (Figure 15), reveals notable differences in total terpene concentrations. These profiles, measured in mg/g, show that terpene levels vary according to treatment type and infection status. Non-infected plants treated with *Beauveria bassiana* exhibit the highest terpene concentration at 21.19 mg/g, suggesting that Bb treatment might positively impact terpene production. This finding aligns with the idea that Bb may not only help control pest populations but also support or enhance terpene synthesis.

Infected plants treated with water display a terpene concentration of 19.86 mg/g, which is also relatively high, indicating that while infection affects the plant, infection does not drastically reduce

terpene levels. This suggests that the plants retain significant terpene production capacity even when facing aphid infestation, provided no chemical insecticides are used.

Conversely, infected plants treated with chemical insecticide have the lowest terpene concentration, at 13.49 mg/g. This suggests that chemical treatments may inhibit terpene synthesis or negatively impact overall terpene profiles, potentially reducing the aromatic and therapeutic qualities of the cannabis. These results highlight a potential downside to using chemical insecticides on cannabis plants, as they might suppress the expression of key secondary metabolites like terpenes and cannabinoids like previously mentioned (Table 13).

Infected plants treated with Bb show a terpene concentration of 16.96 mg/g, which is higher than that of chemically treated plants but lower than that of non-infected Bb-treated plants. This indicates that while Bb treatment may support higher terpene levels even in infected plants, the infection itself still exerts some suppressive effect on terpene production. However, the fact that Bb-treated infected plants still have higher terpene levels than chemically treated ones underscores the potential advantage of Bb as a pest control method that is less detrimental to terpene synthesis. Non-infected plants treated with water have a terpene concentration of 16.22 mg/g, which is lower than that of infected water-treated plants but higher than infected, chemically treated ones.

Congo Durban's terpene profile analysis indicates that *B. bassiana* treatment, especially in non-infected plants, supports the highest terpene concentration among all treatments, making it a promising option for enhancing terpene potency in cannabis cultivation. Chemical insecticide treatment, on the other hand, once again is associated with the lowest metabolite levels, suggesting a potential negative impact on terpene synthesis. Thus, Bb appears to be a favorable pest management alternative that may enhance or sustain terpene production, even in the presence of

aphid infestation, compared to chemical insecticides, which could reduce the aromatic and therapeutic quality of the cannabis product.

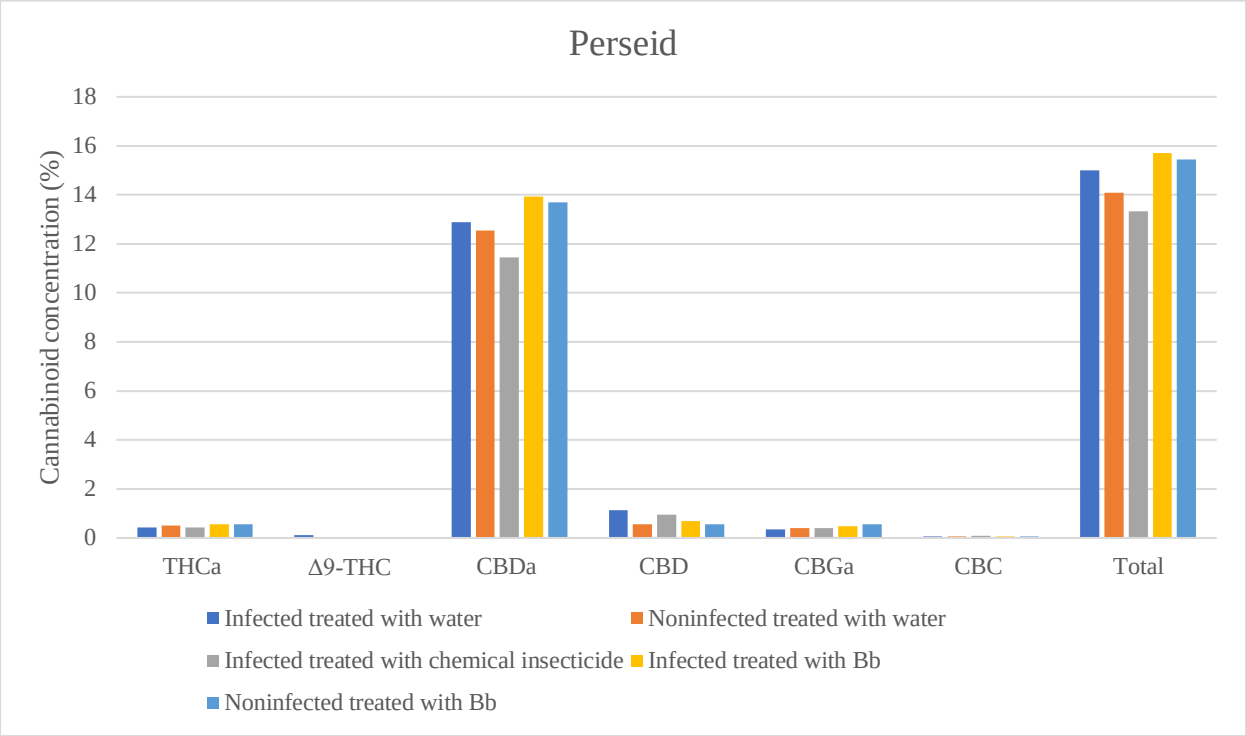


Figure 16. Cannabinoid profile of Perseid (CBD dominant) cannabis plants: potency and concentration analysis (%) using USP <621> chromatography and HPLC-DAD quantification. Bb, *B. bassiana* 1 x10^{7} conidia ml^{-1}.

Figure 16 shows that THCa concentrations were relatively consistent across all treatments for the Perseid variety, with slight variations; the highest levels were found in noninfected plants treated with water (0.5%) and in the infected plants treated with Bb (0.55%) and noninfected plants treated with Bb (0.56%), suggesting that *B. bassiana* may have a slight boosting effect on THCa production, particularly in the noninfected plants. Δ9-THC was present only in trace amounts (0.12%) in the infected plants treated with water, with all other treatments showing no detectable levels, reinforcing the predominance of CBD-related compounds in this strain and indicating that infection and treatments do not significantly affect the synthesis of Δ9-THC under these conditions.

CBDa was the most abundant cannabinoid in the profile, with the highest concentrations observed in the infected plants treated with Bb (13.92%) and noninfected plants treated with Bb (13.7%). This suggests that *B. bassiana* treatment may enhance the synthesis of CBDa, possibly as part of a plant defense response or metabolic adaptation. Chemical insecticide treatment showed slightly reduced CBDa levels (11.45%), indicating that insecticides might inhibit CBDa production compared to Bb treatments. CBD concentrations were relatively low across all treatments, with the highest concentration found in the infected plants treated with water (1.14%), followed by infected plants treated with chemical insecticide (0.95%), but generally, the Bb treatments showed lower CBD concentrations, particularly in noninfected *Bb*-treated plants (0.56%) (Figure 16).

CBGa levels were also low, with the highest concentration in noninfected plants treated with Bb (0.57%). CBC concentrations were similarly low, with minimal variation across treatments, indicating that CBC is not strongly influenced by the treatments applied.

The total cannabinoid concentration was highest in the infected plants treated with *Bb* (15.71%) and noninfected plants treated with Bb (15.45%), suggesting that *B. bassiana* significantly enhances the total cannabinoid content, particularly in infected plants, compared to the other treatments, which showed lower total cannabinoid concentrations, with chemical insecticide treatment resulting in the lowest total (13.32%). This suggests that chemical insecticides could suppress cannabinoid biosynthesis.

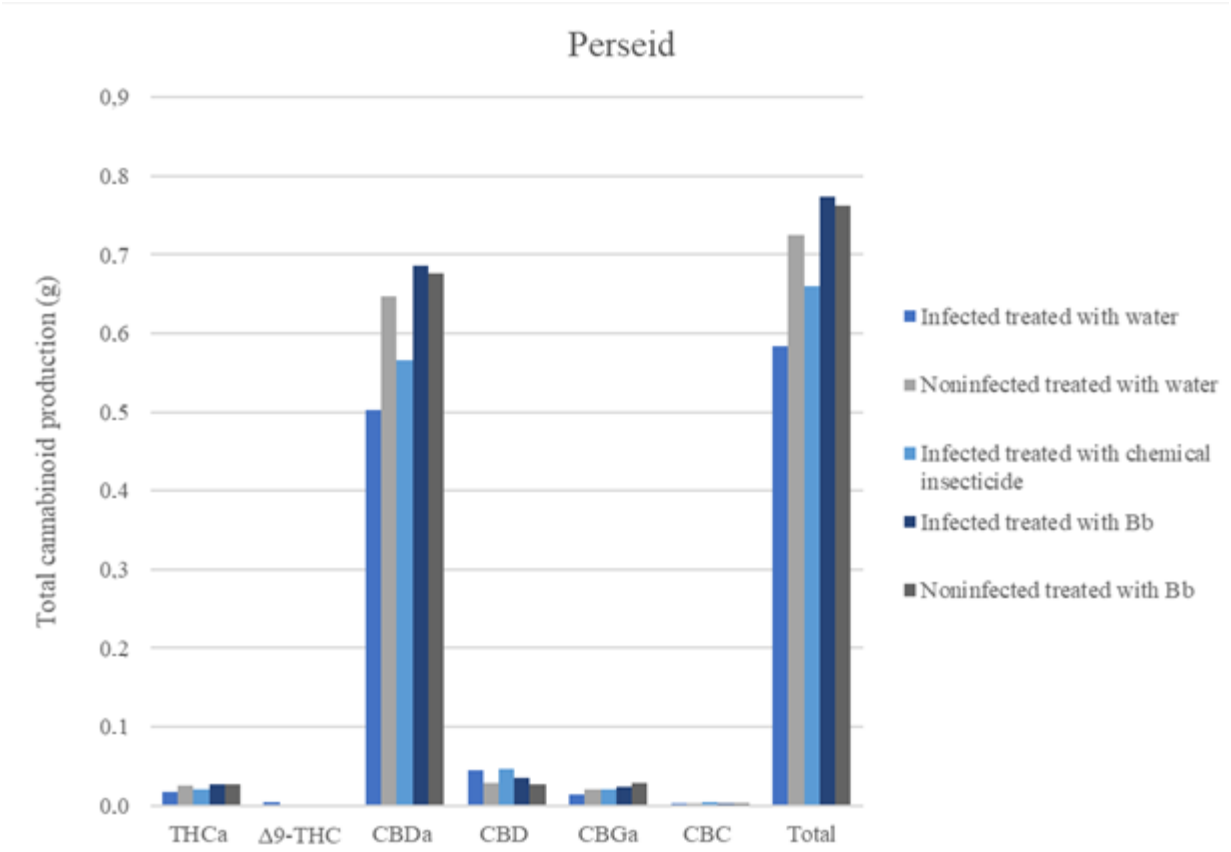


Figure 17. Cannabinoid profile of Perseid (CBD dominant) cannabis plants: potency and total cannabinoid production (g) using USP <621> chromatography and HPLC-DAD quantification. Bb, *B. bassiana* 1 x10^{7} conidia ml^{-1}.

The analysis of total cannabinoids of Perseid shown is figure 17. THCa, the acidic precursor to THC, was most abundant in infected plants treated with Bb (1.57 g). Noninfected water-treated plants exhibited the next highest concentration (1.43 g), while noninfected Bb-treated plants had slightly reduced levels (1.32 g). Infected plants treated with water or chemical insecticide showed comparatively lower THCa concentrations (around 1,14 for both), indicating that Bb treatment effectively enhances THCa accumulation, particularly under stress conditions.

Δ9-THC, the psychoactive decarboxylation product of THCa, was highest in infected plants treated with Bb (0.54404 g) and noninfected water-treated plants (0.52 g). Lower levels were observed in

noninfected Bb-treated plants (0.47 g), while the least Δ9-THC production occurred in infected water-treated (0.39 g) and insecticide-treated (0.39 g) plants. The results suggest that Bb positively influences the conversion of THCa to Δ9-THC, enhancing overall potency.

Minor cannabinoids such as THCV, CBGa, CBG, and CBC displayed distinct patterns across treatments. THCV was detectable in all treatments except Bb-treated plants, with the highest levels in noninfected water-treated plants (0.016 g). Bb treatment completely suppressed THCV production, indicating a potential alteration in minor cannabinoid biosynthesis pathways. CBGa, a crucial precursor in the cannabinoid biosynthetic pathway, showed the highest concentration in infected Bb-treated plants (0.18 g), followed by noninfected Bb-treated plants (0.17 g). Infected plants treated with water or chemical insecticides produced lower amounts of CBGa (0.14 g and 0.12 g, respectively). CBG concentrations peaked in noninfected water-treated plants (0.018 g), with slightly lower levels in infected Bb-treated plants (0.017 g). CBC concentrations were relatively stable across treatments, with the highest levels observed in infected Bb-treated plants (0.007 g) and the lowest in noninfected Bb-treated plants (0.006 g).

The total cannabinoid production was highest in infected Bb-treated plants (2.33 g), followed by noninfected water-treated plants (2.13 g) and noninfected Bb-treated plants (1.99 g). Infected plants treated with water or chemical insecticides exhibited the lowest total cannabinoid yields (1.70 g and 1.69 g, respectively). These results indicate that Bb treatment significantly enhances total cannabinoid production, particularly in infected plants, likely through its role as a biocontrol agent and its interaction with plant stress pathways.

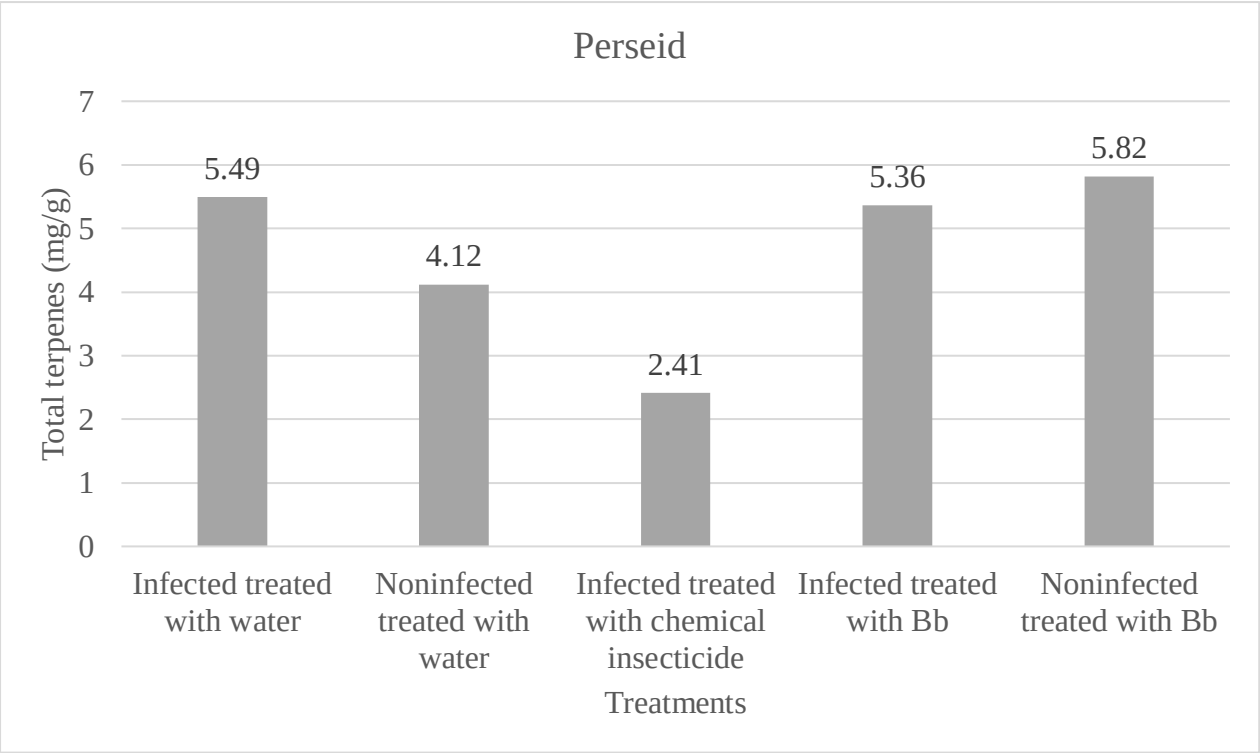


Figure 18. Terpenes profile of Perseid (CBD dominant) cannabis plants: potency and concentration analysis (%) using USP <621> chromatography and HPLC-DAD quantification. Bb, *B. bassiana* 1 x10^{7} conidia ml^{-1}.

The terpene profile analysis of Perseid cannabis plants shows distinct variations across the different treatment groups. The highest concentration of total terpenes was observed in the noninfected plants treated with *B. bassiana* (5.82 mg/g), followed closely by the infected plants treated with distilled water (5.49 mg/g) (Figure 18). This suggests that Bb treatment, particularly in noninfected plants, may enhance terpene production, likely as part of the plant's response to the biocontrol agent or the metabolite production of the microorganism. Infected plants treated with Bb also showed a relatively high concentration of total terpenes (5.36 mg/g), which further supports this notion.

In contrast, infected plants treated with chemical insecticide had the lowest total terpene concentration (2.41 mg/g) (Figure 18), indicating again that chemical insecticides might suppress the production of terpenes, potentially due to their negative effects on plant metabolism or stress response pathways as mention before for Congo Durban variety (Figure 15). Noninfected plants treated with water had a total terpene concentration of 4.12 mg/g, which is lower than that of the infected plants treated with distilled water, suggesting that infection might induce a slight increase in terpene production for this variety.

Overall, the analysis highlights the impact of different treatments on terpene synthesis, with *B. bassiana* emerging as a treatment that enhances the production of terpenes, while chemical insecticide treatment appears to have a suppressive effect on terpene levels. These findings suggest that biocontrol agents like Bb could not only aid in pest management but also enhance the aromatic and potentially therapeutic properties of cannabis strains such as Perseid.

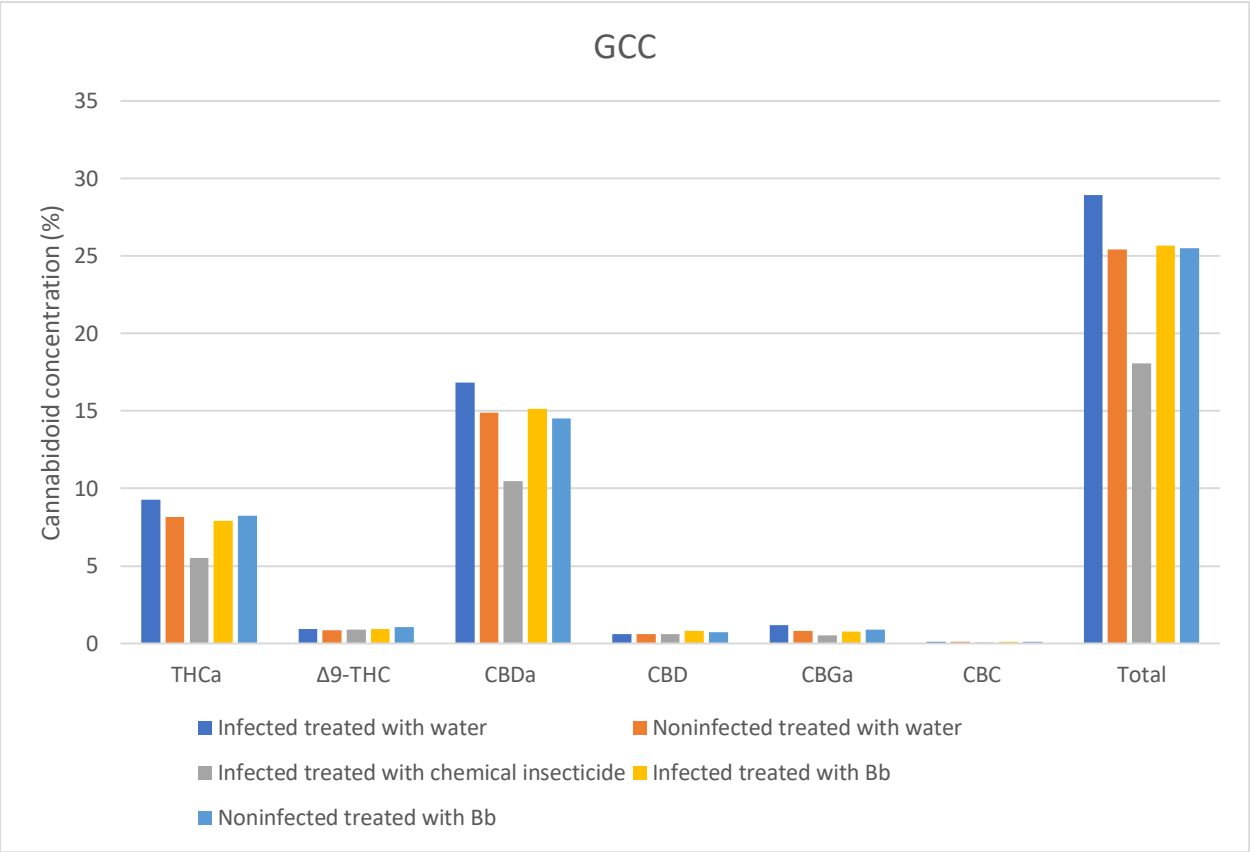


Figure 19. Cannabinoid profile of GCC (CBD ≈ THC) cannabis plants: potency and concentration analysis (%) using USP <621> chromatography and HPLC-DAD quantification. Bb, *B. bassiana* 1 x10^{7} conidia ml^{-1}.

The concentration of THCa showed some variation across treatments. The highest THCa concentration was observed in the infected plants treated with water (9.27%) (Figure 19), while the infected plants treated with chemical insecticide exhibited the lowest THCa content (5.52%). These results suggest that insecticide treatment may reduce THCa production, while water treatment could have a more neutral or slightly beneficial effect. The *B. bassiana* treatments resulted in THCa concentrations of 7.93% (infected) and 8.24% (noninfected), indicating that Bb treatment generally supports THCa levels but does not drastically alter its concentration compared to water treatment.

For Δ9-THC levels were generally low but consistent across the different treatments, with noninfected plants treated with *Bb* showing the highest Δ9-THC concentration (1.05%) (Table 19). The increase in Δ9-THC in the noninfected Bb group suggests that *B. bassiana* may slightly enhance the conversion of THCa to Δ9-THC, potentially due to a stress-related metabolic response. Chemical insecticide treatment showed Δ9-THC concentrations similar to those in water-treated plants, reinforcing the idea that insecticides have minimal impact on THC levels in this particular strain.

CBDa was the dominant cannabinoid in all treatments, with the highest concentration observed in the infected plants treated with water (16.85%) (Figure 19). The reduction in CBDa concentration in the infected plants treated with chemical insecticide (10.46%) suggests that insecticides may inhibit the production of this cannabinoid for this cannabis strain. The other treatments, including those treated with Bb, showed more moderate reductions, with the noninfected plants treated with distilled water and the infected plants treated with *Bb* showing CBDa levels around 14.9% and 15.14%, respectively.

CBD data showed relatively low and consistent concentrations across all treatments, with a slight increase in CBD levels in the infected plants treated with Bb (0.79%) (Figure 19). This indicates that Bb treatment may promote a minor increase in CBD production, although the overall CBD content remains low compared to CBDa. The consistent presence of CBD across all treatments further suggests that GCC plants have a balanced production of both CBD and THC, though CBD is less prominent than its acidic precursor.

The highest concentration of CBGa was observed in the infected plants treated with distilled water (1.17%) (Figure 19), and the lowest was in the infected plants treated with chemical insecticide

(0.51%). Similar to other cannabinoids, Bb treatment led to a moderate increase in CBGa (0.77% for infected and 0.89% for noninfected plants), suggesting that Bb might slightly enhance the production of CBGa, a precursor to cannabinoids like CBD and THC, which could have implications for overall cannabinoid production. Furthermore, the highest levels of CBC observed in the infected plants treated with Bb (0.1%) and the noninfected plants treated with Bb (0.1%). This slight increase in CBC levels under Bb treatment indicates a potential modulation of this cannabinoid in response to the biocontrol agent, but the overall concentration remains low across all groups.

The total cannabinoid concentration was highest in the infected plants treated with water (28.93%) (Figure 19), suggesting that water treatment, while allowing the infection to persist, may support the highest overall cannabinoid production for this cannabis variety. The chemical insecticide treatment resulted in the lowest total cannabinoid concentration (18.07%), indicating a suppressive effect on cannabinoid biosynthesis. The total cannabinoid content in the Bb-treated plants was slightly reduced compared to the distilled water-treated infected plants but remained higher than in the chemical insecticide-treated group, with concentrations ranging from 25.52% to 25.68%.

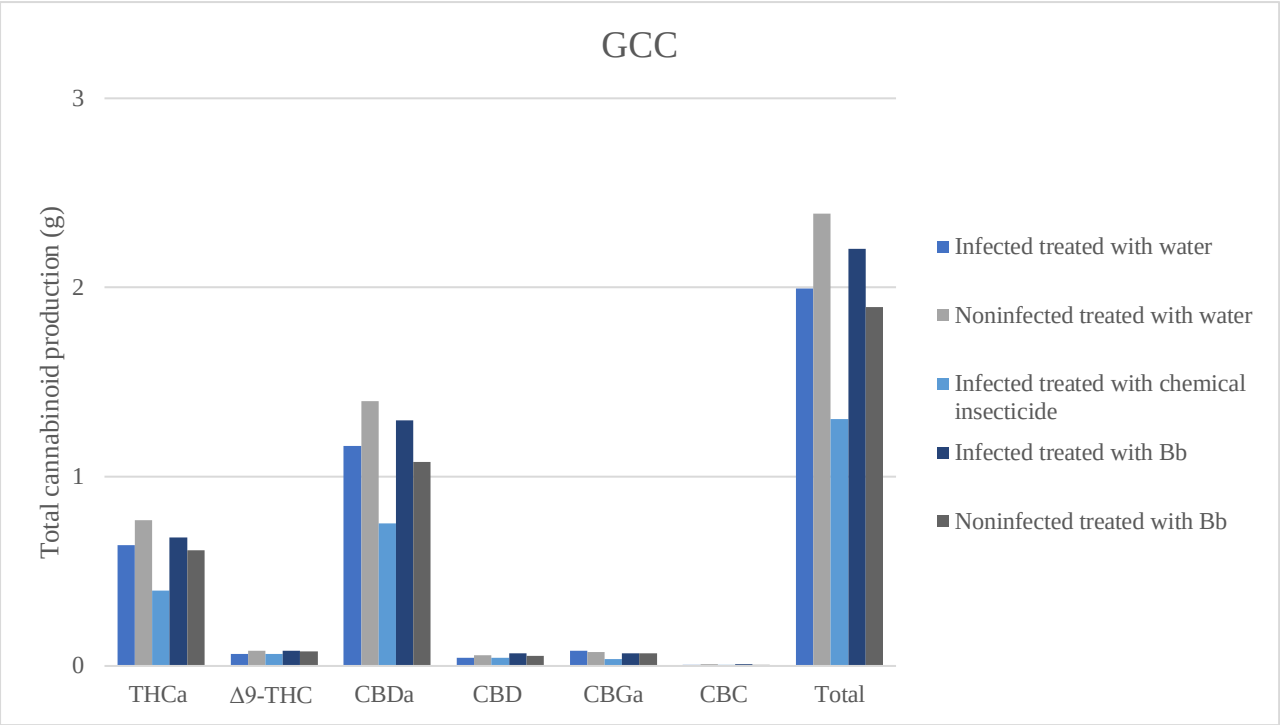


Figure 20. Cannabinoid profile of GCC (CBD ≈ THC) cannabis plants: potency and total cannabinoid production (g) using USP <621> chromatography and HPLC-DAD quantification. Bb, *B. bassiana* 1 x10^{7} conidia ml^{-1}.

The cannabinoid profile of GCC (CBD ≈ THC) cannabis plants was evaluated to and is shown in figure 20. THCa levels were highest in noninfected water-treated plants (0.76 g), indicating optimal conditions for this precursor's biosynthesis in the absence of stressors. Among infected plants, Bb-treated samples exhibited the highest THCa concentration (0.68 g), outperforming those treated with water (0.63 g) or chemical insecticides (0.39 g). Noninfected plants treated with Bb showed moderate THCa levels (0.61 g), suggesting that Bb mitigates the reduction in THCa associated with stress.

Δ9-THC, derived from THCa, followed a similar pattern, with the highest levels detected in Bb-treated infected plants (0.08151 g) and noninfected water-treated plants (0.080 g). Noninfected Bb-treated plants also demonstrated relatively high Δ9-THC production (0.078 g). Infected plants

treated with chemical insecticides or water exhibited the lowest Δ9-THC levels (0.06 g and 0.064 g, respectively). These findings highlight Bb's ability to enhance Δ9-THC synthesis under stress conditions.

CBDa, the acidic precursor to CBD, was the dominant cannabinoid across all treatments, with the highest concentration observed in noninfected water-treated plants (1.40 g). Infected Bb-treated plants showed the second-highest CBDa levels (1.29 g), followed by infected water-treated plants (1.162 g). Plants treated with chemical insecticides demonstrated the lowest CBDa concentration (0.75 g), indicating a negative impact of chemical treatments on CBDa biosynthesis. Noninfected Bb-treated plants also showed reduced CBDa levels (1.078 g) compared to their water-treated counterparts.

CBD levels were highest in infected Bb-treated plants (0.067 g), suggesting that Bb not only enhances CBDa production but also facilitates its conversion to CBD. Noninfected water-treated plants also displayed elevated CBD levels (0.057 g), while the lowest CBD concentrations were observed in infected plants treated with water (0.042 g) or chemical insecticides (0.044 g).

CBGa, a precursor in the cannabinoid biosynthetic pathway, showed its highest levels in infected water-treated plants (0.080 g). Infected Bb-treated and noninfected Bb-treated plants demonstrated slightly lower CBGa levels (0.066 g and 0.066 g, respectively), while plants treated with chemical insecticides had the lowest concentration (0.036 g). CBC concentrations were consistent across treatments, with the highest levels in infected Bb-treated plants (0.008 g) and the lowest in insecticide-treated plants (0.005 g).

The total cannabinoid production was highest in noninfected water-treated plants (2.39042 g), followed by infected Bb-treated plants (2.20 g). Noninfected Bb-treated plants produced slightly

lower totals (1.89 g). Plants treated with chemical insecticides exhibited the lowest total cannabinoid production (1.30 g), further emphasizing the detrimental effects of chemical treatments on cannabinoid biosynthesis.

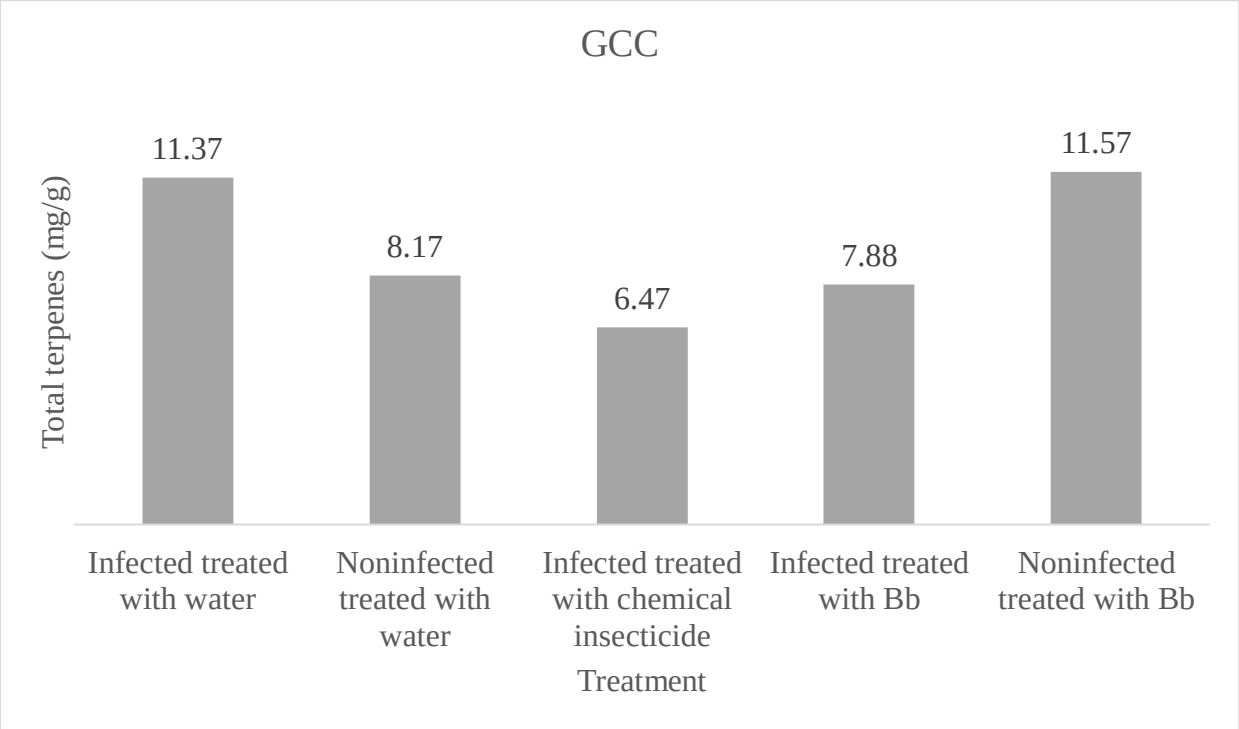


Figure 21. Terpene profile of GCC (CBD ≈ THC) cannabis plants: potency and concentration analysis (%) using USP <621> chromatography and HPLC-DAD quantification. Bb, *B. bassiana* 1 x10^7 conidia ml^{-1}.

The terpene profile of *GCC* revealed that the highest total terpene concentration was observed in the noninfected plants treated with *B. bassiana* (11.57 mg/g) (Figure 21), closely followed by the infected plants treated with water (11.37 mg/g). The infected plants treated with distilled water also showed a relatively high level of total terpenes, which could be a result of the plant's natural response to infection or a metabolic shift induced by the stress of the infection. On the other hand, infected plants treated with the chemical insecticide had the lowest terpene concentration (6.47

mg/g), indicating that chemical insecticide treatment may suppress terpene production, potentially due to its impact on plant metabolism and stress response pathways on this cannabis variety.

Noninfected plants treated with distilled water exhibited a total terpene concentration of 8.17 mg/g, which is lower than the infected plants treated with water but still significantly higher than the levels observed in the insecticide-treated plants. Infected plants treated with Bb also showed a moderate terpene concentration (7.88 mg/g) (Figure 21), which is higher than the chemical insecticide-treated plants but lower than that of the noninfected plants treated with Bb.

CHAPTER 6: DISCUSSION

6.1. Isolation and identification of entomopathogenic fungi

Among the isolates, genera morphologically consistent with *Trichoderma*, *Penicillium*, *Aspergillus*, *Beauveria*, and *Metarhizium* were identified. Notably, isolate F2, consistent with *Beauveria* spp., exhibited a white to pale cream colony color and a cottony texture, with conidia observed as small, oval to globose structures in dense clusters. Meanwhile, isolate F12, linked to *Metarhizium* spp., showed green to olive colonies with a granular texture. Microscopically, F12 isolate presented cylindrical conidia in brush-like arrangements, a typical feature of the genus (Afifah et al., 2022)

The isolation methodology aligns with established protocols, such as using sterile conditions, PDA media, and incubating serial dilutions to promote fungal growth while suppressing bacterial contamination. Similar studies have successfully employed these techniques for isolating entomopathogenic fungi from infected insects, demonstrating their utility in biological pest control. For instance, (Ullah et al., 2022) has shown that EPF can be isolated from dead or infected insects collected in two distinct environments: a greenhouse and agricultural fields. Ullah et al. (2022) also used morpho-taxonomic identification and revealed that two isolates from the agricultural samples corresponded to EPF species: *Beauveria bassiana* and *Metarhizium anisopliae*. These fungi were identified based on their cultural characteristics, growth patterns, and microscopic features. Both species, are known for their potential virulence against insect pests such as *Myzus persicae* and *Spodoptera frugiperda*.

The molecular analysis results provide robust confirmation of the morphological identification of two entomopathogenic fungi. This identification was achieved through sequencing the internal transcribed spacer (ITS). The phylogenetic analysis, showed that the sequences of these isolates had 99% homology with previously characterized *B. bassiana* and *M. anisopliae* isolates in the GenBank database.

The results align with similar findings reported in other studies (Akrich et al., 2023), where *B. bassiana* was isolated and identified as an EPF associated with cowpea aphid *Aphis craccivora*. The study similarly used ITS sequencing and phylogenetic analysis to confirm the fungal identity, with the sequences showing 99% homology to GenBank records. In that case, the *B. bassiana* isolate was included in a phylogenetic tree for comparative analysis. These findings highlight the reliability of ITS sequencing combined with phylogenetic methods for EPF identification and classification.

6.2. Entomopathogenicity bioassay

The pathogenicity results of *B. bassiana* and *M. anisopliae* against *P. cannabis* are consistent with studies of EPF as biological control agents for aphids (Vu et al., 2007). At a concentration of 1×10^7, both isolates demonstrated high effectiveness, with 100% mortality by the 10th day after treatment (DAT). This aligns with prior research showing that high conidial concentrations typically yield higher aphid mortality rates. For instance, studies on *Aphis gossypii* (Bayındır Erol et al., 2020) and *Aphis craccivora* (Saranya et al., 2010) treated with EPF, including *B. bassiana* and *M. anisopliae*, reported mortality rates of 80-100% at comparable concentrations and timelines, emphasizing dose- and time-dependent effects on pest mortality.

The faster infection progression and higher mortality rate by *B. bassiana* at 7 DAT in our study are supported by prior evidence suggesting that *B. bassiana* often has a slightly faster infection cycle compared to *M. anisopliae*. For instance, research on diamondback moth larvae and aphid species observed quicker onset of mortality with *B. bassiana*, although *M. anisopliae* displayed comparable effectiveness over longer durations (Shehzad et al., 2021). Another study (Omar et al., 2021) showed that while *M. anisopliae* performs comparably to *B. bassiana* over time, its efficacy may depend more on specific environmental conditions, such as humidity and temperature. In field evaluations, both fungi successfully reduced pest populations, but *B. bassiana* often achieved faster initial mortality and higher spore viability on the insect host. These findings underscore *B. bassiana's* potential as a faster-acting biocontrol agent under controlled conditions.

Microscopic observations of fungal development corroborate the mode of action of these fungi. Initial spore adhesion and germination on the aphid cuticle by the third DAT, followed by extensive tissue colonization and hyphal outgrowth (Figure 6), are hallmark features of EPF pathogenicity. These processes mirror findings in other reports, where fungal growth from insect cadavers produces new infectious conidia, underscoring the fungi's potential for population-level suppression of aphids (Ma et al., 2023).

Our results reinforce the utility of EPF as sustainable alternatives to chemical pesticides, especially given their specificity and reduced environmental impact. Studies have shown that *B. bassiana* and *M. anisopliae* are effective against diverse aphid species and other pests, achieving mortality rates similar to or better than some chemical treatments under comparable conditions. These findings suggest that deploying *B. bassiana* at 1×10^7 conidia/mL in greenhouse or field conditions could effectively control *Phorodon cannabis* populations while promoting alternative pest management strategies.

6.3. Bioassay

6.3.1. Effects of aphid infection on cannabis plants growth parameters

The results of the present study show the impact of *P. cannabis* infection and subsequent treatments with *B bassiana*, a chemical insecticide and negative control with distilled water on cannabis plant growth align with existing research demonstrating how aphid infestations and biological controls influence plant development. Aphid infection, with *P. cannabis*, has been shown in this study to significantly reduce plant height and biomass, as observed in infected plants treated with distilled water. This underscores the detrimental effects of unmanaged aphid infestations, including nutrient depletion and the introduction of plant stress, which can severely impair photosynthesis and overall growth.

The comparable effectiveness of *B. bassiana* and the chemical insecticide we used in mitigating biomass loss highlights the potential of EPF as a sustainable alternative in integrated pest management (IPM). Similar studies on tomatoes and other crops have reported that *B. bassiana* not only controls pest populations but can also enhance plant resilience and growth, sometimes even stimulating beneficial traits such as root and shoot development under certain conditions (Britt & Kuhar, 2021; Sui et al., 2023). Root length was significantly greater at 3, 4, and 5 days post-sowing ($p < 0.05$), and plant height was significantly higher at 7, 14, and 21 days post-emergence. Field experiments demonstrated improvements in tomato yield. Fruit quantity under the Bb treatment increased by 22.9–28.0% compared to controls and *Bacillus cereus* (Bc). The findings suggest that *B. bassiana* enhances tomato growth and yield characteristics (Sui et al., 2023). The fungal colonization of plant tissues, a phenomenon referred to as endophytism, may contribute to reduced pest-induced stress and improve nutrient uptake, as seen in these studies with other host plants .

The modest reductions in plant height and biomass in infected cannabis plants treated with *B. bassiana* and chemical insecticides suggest that while these treatments manage aphid populations effectively, they may not fully reverse the stress induced by initial infestations. This pattern mirrors findings in tomato and maize crops (Sui et al., 2023), where biocontrol agents reduced pest damage but plant growth metrics were slightly lower compared to uninfected controls. These results collectively emphasize the dual role of EPF, like *B. bassiana* in pest suppression and potential plant growth support. The comparable performance to chemical insecticides also highlights its suitability for cannabis cultivation, where residue concerns and regulatory constraints are significant.

6.3.2 Effects of *B. bassiana* on aphid population

The results of this study align with existing research on the efficacy of *B. bassiana* as a biological control agent and its potential as an alternative to chemical insecticides in managing aphid populations. Bb is a widely studied entomopathogenic fungus that infects and kills a range of insect pests, including aphids, through spore adhesion and subsequent colonization of the insect cuticle (Ortiz-Urquiza & Keyhani, 2013). Studies have shown that Bb formulations can effectively reduce pest populations in various crops (Homayoonzadeh et al., 2022), supporting its performance in controlling *P. cannabis* observed in the current study.

The low and stable aphid populations observed in Bb-treated plants of all three cannabis varieties are consistent with findings in other studies, which reported that Bb achieves effective and sustained control of aphid populations under greenhouse conditions (Nielsen & Hajek, 2005; Rasool et al., 2021). Specifically, aphid counts remained below 15 aphids per plant over the 9-week period, a level comparable to that achieved with chemical insecticides, which maintained populations under 21 aphids. This demonstrates that Bb provides a comparable level of pest

suppression, an important factor in considering it as an alternative to synthetic insecticides in cannabis production.

The study also highlighted varietal differences in *P. cannabis* susceptibility. Untreated plants showed significant population increases, with Perseid experiencing the most rapid growth (exceeding 350 aphids by week 9). This aligns with prior research suggesting that host plant characteristics, such as nutrient composition and secondary metabolite profiles, can influence aphid reproduction and survival (Mcpartland & Sheikh, 2018); this report showed that plants with higher amounts of essential oil extract production, which are rich in terpenoids, were more effective against infection by small arthropods like mosquitoes, aphids, and spider mites. The relatively moderate growth in GCC and slower growth in Congo Durban may be attributed to varietal differences in resistance mechanisms, such as metabolite defenses, which have been documented in related crops (Mcpartland & Sheikh, 2018).

However, the slightly higher standard deviation of the mean aphid populations under Bb treatments compared to chemical insecticides in some varieties, such as GCC, suggests that environmental factors (e.g., humidity and temperature) may influence Bb efficacy and conidial germination (Vu et al., 2007). Key areas for improvement include refining application methods and ensuring the successful establishment of *B. bassiana* in the plants. Strategies like optimized humidity and temperature, and provisioning sugar-based food sources (e.g., honeydew),could enhance fungi establishment and infection capacity. Technological advancements, such as LED lighting, CO_2 supplementation, and climate-control strategies, also introduce uncertainties regarding their impact on biocontrol efficacy (Vu et al., 2007). Emerging systems like vertical and indoor farming further highlight the need to assess how these innovations influence EPF performance (Harmon, 2009).

This underscores the need for careful application timing and environmental monitoring when using Bb in commercial settings.

Our results are supported by a robust body of literature on the effectiveness of Bb and in controlling aphid populations (Javed et al., 2019; Juliya, 2020; Mweke et al., 2018). Bb offers a sustainable and eco-friendly alternative with comparable efficacy to chemical treatments. The rapid proliferation of aphids in untreated plants, especially in Perseid plants, highlights the necessity of effective pest management strategies tailored to varietal susceptibility. Future studies should explore combining Bb with other IPM approaches, such as predatory insects or resistant cultivars, to enhance pest control outcomes in cannabis production and test different application techniques under different temperature and humidity conditions.

6.3.3. Effects of aphid infection and treatment with *B. bassiana* on cannabinoid and terpene concentration

The interaction between pest infestation, pest management strategies, and secondary metabolite production in cannabis represents a complex interplay of stress responses and biosynthetic regulation. This study evaluated the effects of a *B. bassiana* isolate, on the cannabinoid and terpene profiles of three cannabis varieties Congo Durban (THC-dominant), Perseid (CBD-dominant), and GCC (balanced CBD and THC) under aphid infestation. The results demonstrate that Bb offers significant advantages in sustaining or enhancing secondary metabolite production, particularly in comparison to the chemical insecticide used, which appeared to suppress or negatively affect these pathways.

For the THC-dominant Congo Durban variety, Bb treatment significantly enhanced THCa and Δ9-THC levels, particularly in non-infected plants, where the highest concentrations of THCa (19.68%) and Δ9-THC (7.07%) were observed. Infected plants treated with the chemical insecticide showed the lowest concentrations of these cannabinoids, with THCa at 16.52% and Δ9-THC at 5.66%. These findings align with previous studies that suggest biocontrol agents can elicit induced systemic resistance in plants, activating metabolic pathways associated with secondary metabolite production (MacWilliams et al., 2023). This research underscores the potential of *C. sativa* chemical and physical defenses in sustainable pest management strategies for hemp cultivation, while also highlighting the need for further exploration of these intricate mechanisms.

Interestingly, Bb-treated infected plants maintained relatively high cannabinoid levels compared to chemically treated ones, highlighting Bb's ability to mitigate the stress impacts of aphid infection on biosynthesis of cannabinoids. However, minor cannabinoids like THCV showed inconsistent patterns, with undetectable levels in some Bb-treated infected plants, suggesting that Bb might modulate minor pathways differently, a phenomenon also noted in other plant-fungi interactions (Rodriguez et al., 2009). Although there is not much research on the interactions of EPF with cannabis plants to facilitate understanding of these interactions, there are reports on other microorganisms and their direct interactions with host metabolism. Endophytic fungal isolates have been shown to confer disease resistance against virulent pathogens, nematodes, and insects, and this resistance has been correlated with increased concentrations of phenolic metabolites (Rodriguez et al., 2009).

In the CBD-dominant Perseid variety, Bb treatment similarly supported higher CBDa levels, with infected and non-infected plants treated with Bb showing concentrations of 13.92% and 13.7%, respectively. In contrast, chemical insecticide control reduced CBDa levels to 11.45%. This

enhancement under Bb treatment aligns with research suggesting that beneficial microorganisms can enhance plant metabolic activity, possibly by modulating hormonal signaling and defense pathways (Pieterse et al., 2012). Total cannabinoid content in Bb-treated plants was the highest among all treatments, particularly in infected plants, which reached 15.71%. These results in CBD and THC-dominant cannabis varieties probe the effectiveness of *B. bassiana* as a good option to be used in conjunction with insecticidal soaps and horticultural oils, the main two options for Canadian growers to control cannabis pests (Lemay et al., 2022).

In the balanced GCC variety, the highest total cannabinoid content (28.93%) was found in water-treated infected plants, suggesting that natural stress responses might enhance secondary metabolite synthesis, as has been documented in studies on stress-induced biosynthesis of cannabinoids and terpenes (Kostanda & Khatib, 2022). However, Bb treatments maintained robust cannabinoid profiles, with total concentrations around 25.52–25.68%, despite aphid infection. The chemical control, however, resulted in the lowest cannabinoid content (18.07%) again as in the other two varieties, mirroring findings in other crop systems where synthetic insecticides negatively impacted secondary metabolites due to their interference with key enzymatic pathways. Moreover, EPF offer a promising alternative to traditional chemical pesticides in cannabis cultivation, particularly given the potential impact of conventional pesticides on cannabinoid and metabolite production. The use of chemical pesticides or plant growth regulators during cultivation or storage raises significant concerns regarding product safety. These concerns are amplified for medical cannabis consumers, who may be more vulnerable to harmful pesticide residues or their byproducts (Atapattu & Johnson, 2020).

Additionally, the manufacturing process for cannabis products, such as oils and concentrates, can intensify these risks. Processing plant material often concentrates pesticide residues in the final

extracts. In addition, when cannabis is consumed via smoking or vaping, pesticide residues or their pyrolysis byproducts may interact with the pyrolysis products of the plant itself, potentially forming more toxic compounds. In some cases, highly toxic pyrolysis products may form exclusively from pesticide residues during the smoking process (Atapattu & Johnson, 2020).

Such risks highlight the importance of minimizing pesticide use in cannabis cultivation. By contrast, EPF provides a safer, eco-friendly solution that aligns with the need to maintain cannabinoid and metabolite integrity while protecting consumer health. The adoption of EPF can also reduce the need for ultra-trace pesticide residue analysis, which is currently a focus in ensuring the safety of cannabis products.

The observed increase in terpene concentrations in Bb-treated plants, even under aphid infection, highlights even more the potential of biological control agents to enhance plant secondary metabolite production. These results align with findings from other researchers (Bezerra et al., 2021) who proved that insect feeding damage induced the cotton plants to synthesize some of the volatile compounds they released. However, the source and nature of the factor(s) responsible for both increased production and release of these volatile compounds, as well as their biosynthesis of the induced compounds, was not revealed (Paré & Tumlinson, 1997). They also mentioned that biocontrol agents and stress-related interactions could trigger volatile organic compound synthesis through the activation of defense defense pathways. The consistent enhancement of terpene profiles across all tested varieties—such as the Congo Durban, Perseid, and GCC—underscores Bb's role in bolstering plants' biochemical defenses. This contrasts with the suppressive effects of chemical insecticides on terpene production, as seen in the lower concentrations in chemically treated plants. Such findings emphasize the ecological and agricultural benefits of biocontrol strategies in managing pest stress while maintaining or enhancing the quality of plant secondary metabolites.

Further studies could expand on the specific molecular mechanisms through which Bb mediates these effects.

6.4. Conclusion

Overall, Bb demonstrated a consistent ability to sustain or enhance secondary metabolite production in cannabis under both pest-free and aphid-infested conditions. The results suggest that Bb lead to increased synthesis of cannabinoids and terpenes, thereby improving the plant's aromatic and therapeutic properties. Conversely, the chemical control used in this research suppressed secondary metabolite production across all varieties, potentially through their negative impacts on plant metabolism and stress-response pathways, as has been widely reported in agricultural studies (Aktar et al., 2009; Moulins et al., 2018). These findings highlight the potential of Bb as a sustainable pest management strategy in cannabis cultivation, offering dual benefits of pest control and quality enhancement. Future research should focus on elucidating the molecular mechanisms underlying these effects and optimizing Bb formulations and application protocols for large-scale cannabis production.

6.5. Future directions

Future research on the use of EPF as biological control agents in cannabis cultivation should focus on optimizing their application protocols and scaling their use for commercial production. Refining environmental parameters such as temperature, humidity, and light conditions will be essential to maximize fungal spore germination and infection efficacy. Developing advanced formulations, such as oil-based or encapsulated spores, could improve the life and application effectiveness of EPF. Further investigations should also explore their integration with other IPM

strategies, including the use of predatory insects, insecticidal soaps, and resistant cannabis cultivars, to create synergistic and holistic approaches to pest control.

Another key direction is understanding the interaction between EPF and cannabis plants, including their potential for endophytic colonization and how this influences pest resistance and abiotic stress tolerance. Molecular and metabolomic studies are needed to elucidate the pathways through which Bb enhances secondary metabolite production, such as cannabinoids and terpenes. Additionally, varietal responses to EPF treatment should be assessed to identify specific traits in cannabis cultivars that influence the fungi's efficacy and the quality of the plant's secondary metabolites.

Advanced bioassays under controlled and field conditions should evaluate the long-term sustainability of pest suppression by EPF across multiple growth cycles, including their efficacy against other pests like spider mites and whiteflies. Dose-response studies will be important to determine optimal concentrations for pest control without adverse effects on plant health. Scaling up this research to include field trials is crucial for validating laboratory findings and understanding the practical performance of EPF in commercial cannabis production. These trials should also assess the economic viability of EPF use compared to chemical pesticides, considering factors like pest suppression efficacy, yield improvement, and regulatory compliance.

From a molecular perspective, genomic and transcriptomic analyses could reveal the genetic mechanisms underlying the pathogenicity of fungi such as *B. bassiana* and *M. anisopliae*. This would provide insights into how these fungi influence plant metabolic pathways, particularly those related to cannabinoid and terpene biosynthesis. On the regulatory front, research should address safety concerns, including residue analysis and compliance with standards for medical and

recreational cannabis, while also exploring consumer perceptions of cannabis products grown with biological pest management. Overall, the present research directions aim to establish entomopathogenic fungi such as *B. bassiana* and *M. anisopliae* as sustainable, effective, and scalable solutions for cannabis pest management, offering dual benefits of pest suppression and quality enhancement for the growing cannabis industry.

CHAPTER 7: REFERENCES

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