ANALYSIS OF THE DIVERSITY OF FOLIAR PATHOGENIC FUNGI IN *CANNABIS SATIVA* AND USE OF COPPER SULPHATE TO CONTROL PATHOGEN INFECTION

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ABSTRACT

Interaction between pathogens and cannabis plants may have the potential to cause significant yield losses in commercial cannabis growth facilities. Greenhouse studies indicated that fungal pathogens have the most severe impact on cannabis. Despite a considerable number of studies on fungal pathogens affecting cannabis, not many studies have focused on the role and efficacy of fungicides to control disease development. Copper sulphate has been suggested as an effective chemical against fungal pathogens.

In this study, we explored the diversity of fungal pathogens in the greenhouse settings and identified *Penicillium olsonii* to be the most abundant pathogen. We then studied the effect of copper sulphate as a fungicide to control the growth of *Penicillium olsonii* and found that copper sulphate could reduce the growth of *Penicillium olsonii in vitro* in a dose-dependent manner. This antifungal agent also effective in decreasing the amount of *Penicillium olsonii* on different cannabis cultivars *in vivo*.

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ANOVA: Analysis of variance
BP: base pair
CBD: cannabidiol
DNA: Deoxyribonucleic acid
EP: emerging pollutant
EPA: Environmental Protection Agency
ITS: Internal transcribed spacer
NCBI: National Center for Biotechnology Information
NTC: Non-template control
PCR: polymerase chain reaction
PDA: Potato dextrose agar
STB: Septoria tritici blotch
THC: Tetrahydrocannabinol

1. INTRODUCTION

1.1 *Cannabis sativa* plant

1.1.1 History of use

Cannabis has the longest recorded history of human use among plants. This plant provides food, seeds, oil, and fibre for ropes and nets. Our ancestors likely stumbled upon the euphoric properties of heated cannabis flowers and later on discovered its medicinal proerpties. People switched from harvesting cannabis to cultivating it and then started choosing strains for their fibre or delta-9-tetrahydrocannabinol (THC) content several thousand years ago [1].

In Xinjiang China, a body that was buried around 750 BC was found with a large cache of cannabis which shows the historical usage of cannabis in China [2]. Therefore, it is not surprising that the earliest registration of Cannabis in Chinese literature is dated to around 5000 years ago [3].

Dinesh (2021) demonstrated that in parts of South Asia, cannabis biotypes with high THC content were often used for medicinal and recreational purposes, creating a strong connection with social and religious rituals [4]. In temperate climates also, low-THC species were first grown for fiber and later also for edibles. It is estimated that hemp was already cultured in Central Asia around 11,700 years ago, near the Altai Mountains [5]. Southeast Asia has also been proposed as an alternative site for the primary domestication of hemp [6]. In contrast, it was not before the early 1600s that cultivation of cannabis has reached North America [7]

1.1.2 Cannabis and law

Cannabis gained widespread use for medicinal purposes until 1937 when the dangers of abuse led to a ban on marijuana in the United States of America [8]. In addition to that, cannabis was removed from the US Pharmacopoeia; with the introduction of the Controlled Substances Act in 1970; marijuana was classified as a Schedule I drug [9].

Canada was one of the first countries to criminalize cannabis and has included this plant in the list of prohibited "narcotics" in the register of opium and narcotics in 1923 [10, 11]. However, when cannabis criminalization was in its infancy, about 3.5% of the Canadian population reported having ever used cannabis, and 1% reported cannabis usage in the past 12 months [12]. Later studies showed that, for example, in 2004, 44.5% of Canadians over the age of 15 had tried cannabis in their lifetime, and 14.1% of them have used it in the past 12 months [13].

In October 2018, the recreational use of cannabis was legalized in Canada. The Cannabis Act states that the licensed production of cannabis is controlled by the federal government, while the distribution and sale of cannabis are regulated at the provincial level; that made Canada the only G20 nation to fully legalize cannabis [14].

1.1.4 Botanical aspects

Cannabis is a highly variable genus. It belongs to the *Cannabaceae* family, which also includes hop and Celtis berry [15]. The taxonomic classification of the cannabis plant has always been controversial. Schulte and Anderson described three hypothetical species, *Cannabis sativa* Linnaeus, *Cannabis indica* Lamarck, and *Cannabis ruderalis* Janisch [16, 17]. These three types differ fundamentally by their morphology and in the amount and content of the secondary metabolites [6]. Other research supports the classification in which the cannabis plant has two main subspecies, *Cannabis indica* and *Cannabis sativa*; they

can be also distinguished by their different morphological characteristics. Indica varieties represent short plants with broad, dark green leaves and contain a higher amount of CBD (cannabidiol) compared to *Cannabis sativa* plants, in which the THC content is higher, and they are mainly taller and have thin leaves with a light green colour [18, 19].

Cannabis is a wind-pollinated annual plant in which male and female plants grow as separate plants but in rare cases, the plant may grow as a hermaphrodite with male and female flowers on the same plant [20].

Morphologically, cannabis can reach a height of five meters or more in an outdoor growing season. It can be propagated from seed and cuttings in bright light and well-drained soil [19, 20]. The leaves are palm-shaped with 5-7 leaflets. The male flower is devoid of petals, usually with five yellowish leaves and five anthers sprouting pollen [15]. The female flower has single ovule and is filled with trichome structures representing the location of cannabinoids and terpenes production [15].

The female flowers produce most of the chemical constituents, including cannabinoids and terpenes. Hence, as soon as the flower is pollinated, the plant transfers its metabolic energy to seed production and not to the biosynthesis of cannabinoids and terpenes. To avoid this, in commercial cultivation, male plants are usually removed from indoor cultivation to prevent the plants from setting seeds [21].

Cannabis has been used from ancient times to the present day, and human selection of cannabis has resulted in changes in morphology, chemistry, and distribution of domesticated cannabis relative to their wild relatives [22].

Genetic-taxonomic analyses of the frequency of allozymes in various genes revealed that there was a prior geographic split between *Cannabis sativa* ("European"), *Cannabis indica* ("South Asian-African"), and *Cannabis ruderalis* ("Central Asian")[20, 23].

1.1.5 Cannabis secondary metabolites

Cannabis sativa is rich in trichomes, which are epidermal glandular projections that coat the leaves, bracts, and stems of the plant. These glandular trichomes contain the secondary metabolites [6, 24, 25].

The secondary metabolism of plants plays an important role in their survival in the environment. Secondary metabolites attract pollinators and defend plants against predators and disease [7].

1.1.5.1 Cannabinoids

The word cannabinoid refers to any chemical, regardless of structure or origin, that binds to cannabinoid receptors in the human body and brain. However, in the cannabis plant, cannabinoids are terpenophenolic compounds produced and accumulated mainly in the female glandular trichomes that are also known as phytocannabinoids. Phytocannabinoids are mainly found in the form of carboxylic acids, which decarboxylate over time or when heated by smoking or cooking [22].

Over 100 cannabinoids have been isolated from the cannabis [18]. The two main cannabinoids are cannabidiol (CBD) and delta-9-tetrahydrocannabinol (THC), which were discovered in 1963 and 1964, respectively [18, 26, 27].

The main difference between these two cannabinoids is that THC is believed to have a solid psychoactive effect, while CBD is claimed to even have an anti-psychoactive effect that controls or reduces the effects caused by THC. However, this claim is still controversial [28-30].

1.1.5.2 Terpenes

Cannabis contains a large number of monoterpene and sesquiterpene compounds, collectively called terpenes; these are natural chemical compounds found in plants and some animals [30]. Similarly to phytocannabinoids, they are mainly produced in trichomes of the cannabis plant [30].

Terpenes are the main components of essential oils and are responsible for the aromatic properties of cannabis [3]. They are responsible for cannabis colour, smell, and flavour.

Some of the most common terpenes found in cannabis flowers are α-pinene, b-pinene, linalool, limonene, myrcene, b-caryophyllene, and caryophyllene oxide [30, 31].

Many terpenes are considered bioactive molecules, which means that they can affect the body. This effect depends on the concentration of the terpene itself and how a person uses it. There is much research on the isolated terpenes themselves, and some can even be used for medicinal purposes. Many terpenes have shown beneficial effects on the body and could serve as medicines or alternative therapies. There is evidence that terpenes could increase the effect of cannabinoids [32]; the synergistic effect caused by the combination of terpenes and cannabinoids is called the "entourage effect" [14].

1.2 Plant pathogens

A plant pathogen is an organism that causes disease in a plant. All plants, from citrus fruits and cereals to ornamental shrubs and forest trees, are susceptible to plant diseases.

Pathogenic organisms in plants reduce farmers' ability to produce food, fiber, and biomass and, as a result, it harms the economy.

Plant pathogens include viruses, bacteria, fungi, and parasitic plants [33]. Plant diseases cause billions of dollars in direct and indirect losses every year and threaten world food

security. Therefore, responding to emerging plant pathogens requires scientific preparation and planned procedures to reduce the impact on the economy.

Successful interaction between the plant pathogens and a plant may result in an infection called a compatible interaction. On the other hand, a phytopathogen can attack the plants but fail to develop the proper infection - this is called an incompatible interaction [33]. As it happens, many genera of various plant pathogens invade the plant surface, but not all of these plant pathogens can cause plant diseases [33].

Most pathogens are highly specialized and only cause disease in a small number of hosts. Disease resistance in plants is formed in two ways. One is through preformed mechanisms – structures or chemicals. Another is through pathogen infection induced production of protective molecules, resulting in restriction of pathogen growth [34].

It must be mentioned that there is a difference between disease resistance and disease tolerance; disease resistance refers to the ability of host plants to control the severity of infection when environmental conditions are favourable for the pathogen. In contrast, disease tolerance is described as the ability to mitigate the negative effect of a pathogen or yield following infection by a plant pathogen, regardless of the level of pathogen presence [35].

1.2.1 Fungal pathogens in plants

Fungi are a highly diverse group of eukaryotic carbonaceous and heterotrophic organisms that have successfully occupied most natural habitats [36]. Fungi are much older than plants. The interaction between plant and fungus is thought to be as old as the evolutionary period of higher plants, predominantly terrestrial vascular plants [37-40]. Surprisingly,

most of the interactions between plants and fungi promote plant growth and development [41].

The fungus can act as a symbiotic partner that improves plant nutrition, acquiring soil resources (nutrients and water), and tolerance to stress. Plants, in turn, provide carbohydrates to the fungus [39, 42].

By contrast, pathogenic fungi are among the leading cause of plant diseases and can use different strategies to infect the plants; some fungi kill their hosts and feed on the dead matter (necrotrophs), while others colonize the living organisms (biotrophs) [43]. In the case of biotrophic pathogens, fungi can become active parasites, or they can associate with plants and become successful symbionts [39].

There has been a constant conflict between plants and fungal pathogens. Pathogens need to find innovative strategies to colonize their hosts successfully, and plants need to find new detection methods and more robust defence mechanisms to ward off pathogens.

The specialized morphological and biochemical strategies developed and used by fungi to interact with host plants have evolved convergently and divergently to contain complex components that utilize and control host pathways [39].

1.2.2 Mechanisms of fungal pathogenicity in plants

While the plant's defence system is generally very effective against pathogen infection, fungi can overcome these defences. Therefore, fungal pathogenicity genes are highly linked to the ability of the plant to recognize a foreign organism and develop defiance mechanisms. Many fungi go through a complex sequence of metabolic and developmental processes to infect a plant.

Some steps that may be critical for fungal pathogenicity are:

1) Adhesion to the plant surface in which the fungi contact and spread on a plant host through environmental mechanisms such as wind, water, insects, or through active growth such as fungi that infect roots [39, 44, 45].
2) Pre-penetration and at the same time germination on the plant surface and formation of infection structures. This includes spore germination, attachment of pathogens to host structures, and recognition events that are mediated by host and environmental signals [39, 44, 46].
3) Pathogen entry into the plant can happen through natural openings and wounds, or injuries that were caused by insects or through direct penetration that needs specialized penetrating structures such as appressoria [47, 48].
4) Infection and invasion, where the pathogens come into contact with host cells and can spread from cell to cell. In this step, the plant shows visible symptoms [39].
5) Reproduction, in which large numbers of fungal spores are produced from the host tissues [39].
6) Spore spread to other sensitive host surfaces or to new plants [39].
7) Dormancy, which is a step that allows the pathogen to survive under unfavorable conditions [49].

1.2.3 Plant antifungal agents

As mentioned earlier, plants produce a wide range of secondary metabolites, many of which exhibit antimicrobial activity. The chemical defence is the main strategy of plants against the fungi that enables plants to control invading microorganisms. Secondary metabolites that include preformed and stimulated antifungal proteins are believed to be important in plant protection as they are directly toxic to the fungus, and can also activate plant defences [50, 51].

One of the known groups of antimicrobial compounds is phytoanticipins that are released from their precursors after the microbial invasion [39]. Phytoalexins are another group of antimicrobial agents that are produced by plants not just in response to damage caused by microorganisms, but also in case of abiotic stresses such as temperature, UV radiation or heavy metals [52]. Phytoanticipins and phytoalexins have been recognized as phytoprotectants [50, 51].

Another plant protection molecule class is pathogenesis-related (PR) proteins. These plant proteins are usually present at low levels in different organs of plants; however, their production is increased after the pathogen attack occurs.

Saponins are another antimicrobial and antifungal agent produced in plants. Saponins are pre-formed glycosylated molecules. The presence or absence of saponins has been shown to correlate with disease development in various plant-fungal interactions [53].

1.3 Cannabis pathogens

Most of the cannabis that is used recreationally and medicinally is grown at high density in greenhouses or warehouse settings. The cultivation of cannabis in such a manner results in high incidences of infection with fungi, bacteria, viruses, and nematodes [54]. All production environments face challenges from plant pathogens. However, in some cases greenhouse systems share more diseases than outdoor in the cannabis industry [55]. Therefore, the ability to control this infection is very essential.

Plant pathogens that infect marijuana plants reduce crop growth by affecting the roots, crown, foliage, leaves, stems, and inflorescences. Fungus is the main group of cannabis pathogens that colonizes inflorescences during development or after harvest, in addition to internal tissues like endophytes [56].

As mentioned earlier, most cannabis pathogens are fungi and oomycetes, followed by viruses; bacterial pathogens are reported less frequently in cannabis [55].

Based on the recent studies, the most destructive root pathogens are *Fusarium* and *Pythium* species [57]. The crop losses caused by these two pathogens can be up to 30% of the overall losses [57]. On the other hand, pathogens that infect inflorescence cause huge damage to the crop because they directly infect and destroy buds, causing losses of up to 20%. *Penicillium* and *Golovinomyces* species colonize foliar and flower tissues and produce a large number of spores [57]. More importantly, the extensive development of fungi such as *Fusarium* and *Penicillium* in the inflorescences can lead to the accumulation of mycotoxins in the tissues; this causes additional health concerns for consumers [58-62] [56]

Furthermore, as revealed by Mckernan and colleagues (2015), many different fungal species were detected by molecular screening using internal transcribed spacer 2 (ITS2) in various cannabis samples [60]. Some of them were toxigenic *Penicillium* species: *P. paxilli, P. citrinum, P. common, P. chrysogenum, P. corylophilum.* Also, *Aspergillus* species such as *A. terreus, A niger, A. flavus, and A. versicolor. Eurotium repens* and *Cryptococcus liquefaciens* were also detected on cannabis [60]. Nevertheless, there is a lack of scientific research and knowledge on diseases of the cannabis plant, especially in areas where there are different climatic and ecological inconsistencies [63].

1.3.1 Penicillium species in cannabis and their effect on human health

One of the most common group of fungi found on cannabis are the *Penicillium* species [57]. Penicillium is a very diverse and cosmopolitan fungus; about 350 species are recognized within this genus and it was divided into four subgenera of *Aspergilloides*, *Penicillium*, *Furcatum,* and *Biverticillium* [61]. Recently, the first three subgenera have been considered in the genera *Penicillium* and the last one (*Biverticillium)* was redefined

to *Talaromyces* [61]. The most common species found in cannabis are *P. citrinum, P. olsonii, P. simplicissimum, and P. spathulatum* [64].

Penicillium contamination in the cannabis industry increases during post-harvest handling and pruning processes which causes physical damage to the plant material [56]. Data showed that inflorescences of some cannabis strains that were more susceptible to physical damage during propagation were generally more colonized by *Penicillium* strains [56].

Regarding human health, *Penicillium* species are rarely classified as pathogenic for human because they hardly grow at 37°C [61]. The most significant risk is from ingesting food contaminated with mycotoxins produced by various *Penicillium* species [65]. Several mycotoxins related to *Penicillium* species can be present in food; the most important are ochratoxin A and patulin, and to a lesser extent, cyclopiazonic acid [61]. While *Aspergillus* is known to be the main fungi that produces aflatoxins, several species of *Penicillium* were also found to produce aflatoxins [66]. Contamination of cannabis with *Penicillium* could cause infectious diseases such as penicilliosis that causes fever, dry cough, and skin lesions and many other symptoms [66]. Cannabis contamination with *Penicillium* as one of the most frequent fungi in the cannabis or with other microbial contaminants has limited the pharmacological use of phytocannabinoids found in the cannabis plant, thus there is great importance in controlling these contaminations [66].

1.4 Fungicide in plants

Since the dawn of civilization, various crop protection methods have been tried, but due to the co-development of plants and fungi, fungi overwhelmed the plants and caused a significant drop in yield [67]. Published literature revealed that nowadays, we lose around 10-23% of our crops before harvest, despite interventions, and another 10-20% after harvest

because of fungal disease [68, 69]. Even in case of the resistant varieties, fungicides still contribute greatly to pest control in conventional agriculture. Hence, for example, in 2020 the annual sales of pesticides in the European Union (EU) were almost 360,000 tones, and fungicides were the most sold group with 46% of all sold pesticides [70]. One of the examples that shows the high value of fungicides is the economic benefits that were achieved by controlling the *Septoria tritici* blotch (STB) as one of the most important foliar diseases in wheat. STB is caused by *Zymoseptoria tritici*, and data from the UK claimed that there would have been crop losses of 20% due to STB in the absence of fungicides, but these losses drop to 5-10% when control strategies were applied [71].

Fungicides target different processes in pathogens. Among the best-known modes of action are effects on plasma membrane integrity, microtubule cytoskeleton, and inhibition of mitochondrial respiration [69]. These processes are often targeted by fungicides that inhibit vital enzymes. The predominant group of these single-target fungicides are azoles, which inhibit ergosterol biosynthesis and thus affect plasma membranes and fungi organelles [72]. Other famous antifungal agents are strobilurins and succinate dehydrogenase inhibitors (SDHI) that interfere with the electron transfer chain in the mitochondrial respiration [72]. Along with azoles, these fungicides represent approximately 77% of the whole market [69, 72].

Although single target site fungicides are specific and effective in controlling fungal pathogens, there is a high risk of developing resistance. Indeed, point mutations in the active site of the target enzymes can generate strong resistance within a few years of field use [72]. This not only poses a challenge to crop protection strategies but also poses a threat to medicinal use of cannabis. An example of this is the emergence of drug-resistant strains of *Aspergillus fumigatus* that can cause infection in immunocompromised humans [73]. To

keep up with this fungal arms race, we must constantly develop new antifungal compounds. Ideally, these new fungicides should meet several criteria: (1) be effective against a wide range of pathogens that are frequently found on crops, (2) affect the essential processes of fungal pathogens in multiple ways, thereby reducing the risk of resistance development, (3) have low toxicity to non-target organisms, including humans, animals, and plants, (4) activate the plant's defense system and thus, prepare it for a possible attack by pathogens [69, 74].

In terms of fungicide pollution, one of the main problems in recent years has been the increasing presence of emerging pollutants (EPs) in the environment. EPs are naturally occurring or manufactured chemicals that have the potential to be released into the environment and have known or suspected adverse effects on human health and the environment [75]. One of the main risks of these compounds is their high leaching potential, especially for those with low hydrophobicity. This means that the pesticide moves through the soil rather than over the surface and can reach and contaminate the groundwater [75]. Metalaxyl-M (methyl *N*-(2,6-dimethylphenyl)-*N*-(methoxyacetyl)-d-alaninate) for example is one of the most popular and widely used fungicides in the world that is highly persistent, mobile and soil-leachable [76].

1.4.1 Pesticide contamination in cannabis

Cannabis plant and cannabis products can be contaminated with microbes, heavy metals, or pesticide residues. Microbiological contaminants are a potential threat to cannabis users. Bacteria and fungi while being dead can also trigger allergies and cause opportunistic infections in immunocompromised people [66]. While the use of pesticides is crucial for growing cannabis plants, human consumption of pesticides can have significant

consequences. Some of them are different malignancies, developmental problems, reproductive problems, neurological and endocrine disorders [77].

69% of pesticides used in cultivation have been shown to remain in cannabis when smoked and produce toxic pyrolytic by-products, suggesting that pesticide-contaminated cannabis may pose a significant toxicological threat to its users [78]. These include a wide variety of known substances and carcinogens, such as carbaryl, diuron, etoprophos, permethrin, propargite, and endocrine disruptors [79]. However, the prevalence and concentration of pesticides that are above the maximum residue limits in cannabis preparations remain uncovered and needs to be studied more [77].

Development of resistant microbes and the pollution that the current pesticides can cause to the environment show the importance of developing new effective agents that also have limited harmful effects on the environment. the fact that cannabis is a growing industry and fungi are one of the main threats to this plant, demonstrates the necessity of developing new pesticides and studying the probable effects that they can have on the environment and on human.

1.5 Copper sulphate

The use of copper in human civilization dates to the fifth and sixth millennia BC. Copper was one of the first used metals, probably because it was in a naturally occurring metallic form that did not require remelting [80]. The Smith Papyrus has mentioned the oldest documented medical use of copper [80, 81]. This Egyptian medical text was written between 2600 and 2200 BC and describes the use of copper to sterilize breast ulcers and drinking water. In the last century, copper has been recognized as an antimicrobial and now is added to fungicides for plants and animal diseases [82, 83].

$CuSO_4$ is a light blue salt that forms when the copper is treated in hot concentrated sulfuric acid (formation of sulphur dioxide). Copper sulphate is mainly used for agricultural purposes as a pesticide, germicide, food additive and soil additive. For example, in South Africa, copper sulphate-based fungicides used as a pre-treatment have delayed the rise of Anthracnose symptoms caused by fungi on mango and avocado trees [84].

The effect of copper sulphate depends on the copper content. The mechanism by which preparations containing copper destroy fungi is not fully understood. Nevertheless, some reports revealed that the toxic effect of copper is attributed to its ability to denature cellular proteins and inactivate fungal enzymes [85]. To elaborate, as revealed by Borkow and Gabbay (2009), the antifungal effect of copper sulphate on conidia and linear growth can be attributed to copper ions, which can catalyze the production of hydroxyl radicals [86]. As a result, it leads to damage to lipids, proteins, DNA and fungus organelles [80, 87]. In other words, significant copper-induced disruption of membrane integrity results in loss of cell viability. Some additional reports inform that the toxicity of copper particles also depends on a combination of several factors, including concentration, exposure time, humidity, and temperature [88].

After antimicrobial efficiency tests, in February 2008, the United States Environmental Protection Agency (EPA) approved the registration of 275 antimicrobial copper alloys and by April 2011, that number had risen to 355 [82].

2. HYPOTHESIS

In this study, we hypothesized that copper (II) sulphate inhibits the growth of pathogenic fungi on cannabis plants without deleterious effects on plant growth and development.

The objectives of this work were:

1) analysis of the diversity of fungal pathogens detected on cannabis plants grown in the Hepler Hall greenhouse at the University of Lethbridge;
2) evaluation of the efficacy of copper (II) sulphate for controlling pathogenic fungi that affect cannabis plants in indoor growing.

3. MATERIAL AND METHODS

All experiments were carried out at the licensed facility (license number LIC-62AHHG0R77-2019) at Hepler Hall, at the University of Lethbridge, 4401 University Drive West, Lethbridge, AB T1K 3M4, Alberta, Canada.

3.1 Vegetative propagation

Healthy and disease-free stems were used to prepare stem cuttings; stems were selected and separated from the mother plants of the following varieties: Chemdawg RQOG, Aspice RBA, Kush Spice, GodBud, and Blue Rhino.10 to 15 cm long cuttings were made, and leaves from the center to the base of the stem were removed in order to concentrate the energy on rooting. A diagonal cut was made in the stem to maximize the surface area of the root zone. Subsequently, the cuttings were placed directly in clean water to prevent the formation of air bubbles in the stems.

To promote rooting, approximately 5 cm of the end of each stem was dipped in Wilson roots Liquid Stimulator which contained 0.4% Acid Indole-3-butynique and Etridiazole 0.01% rooting factors. Following this, cuttings were placed into starter blocks, which were prepared for propagation.

Rooting began in 2-3 weeks, and rooted plants were ready to transplant into larger, 20 x 20 cm pots. Plants were sprayed once with reverse osmosis water when they were placed in the chamber. The temperature was maintained at 25°C (day-night) throughout the growing period. To support vegetative growth, plants were exposed to long 24-hour photoperiods and were treated by Flora Series 3-part hydroponic-based nutrient system consisting of Flora Gro, Flora Micro and Flora Bloom.

3.2 Sampling and isolation of pathogens

Leaf samples of indoor cannabis plants (plants grown in greenhouses) were obtained from various strains of cannabis plants available in a licenced facility at the University of Lethbridge.

Samples showing suntan symptoms (discoloration) and asymptomatic samples were obtained at various times of plant growth. Plants were treated with appropriate nutrients and were provided with the same environmental and lighting conditions needed for commercial production.

Potato Dextrose Agar was made by adding 39 grams of dehydrated PDA powder (Cat# DF0013-17-6, ThermoFisher Scientific Company, Wilmington, DE) to 1000 ml of distilled water; dissolving by mixing and heating on a magnetic stirrer. It was then autoclaved for 20 minutes at 121°C. 25 samples from individual plants were put on Potato Dextrose Agar with 100 mg/L of streptomycin sulphate (Cat# 11860038, ThermoFisher Scientific Company, Wilmington, DE) which was prepared by dissolving 0.5 grams of streptomycin in 10 mL of sterile H_2O and filtering with a 0.22 µm syringe. All plates were stored at room temperature (23 ± 2°C) for 5 to 8 days. Emerging fungal mycelia were transferred to fresh PDA at least five times to have stable morphological colonies. Incubation was continued, until no changes in colour, shape and size of colonies were observed in the subsequent cultures.

3.3 Genomic identification of pathogens

One of the most used target sequences for fungal plant pathogens is the ITS regions of ribosomal DNA. ITS regions usually contain sufficient diversity in species levels. The ITS sequences of many fungi and oomycetes are available in databases, which makes them

easily accessible and provides useful reference material for the design of specific probes and primers.

Samples were sent to the University of Guelph Laboratory Services, Food and Agriculture Laboratory, Guelph, ON (www.guelphlabservices.com) for species identification by PCR (polymerase chain reaction) using ITS1 - ITS4 primers and the result sequences were compared with the corresponding ITS1-5.8SITS2 sequences from the National Center for Biotechnology Information (NCBI).

3.5 Evaluation of the effective concentration of copper (II) sulphate

Copper sulphate was prepared in eight different concentrations: 25 ppm, 50 ppm, 75 ppm, 100 ppm, 150 ppm, 200 ppm, 250 ppm and 300 ppm and added to PDA, which contained streptomycin sulphate to prevent any bacterial growth before transferring mycelium of penicillium to the Petri dishes. All plates were stored at room temperature (23 ± 2°C) for 12 days, and three independent repeats were performed.

3.6 Pathogenicity tests of *Penicillium olsonii*

A suspension of the spores was obtained by flooding a 2-week-old colony of *P. olsonii* on PDA with 15 ml distilled water, gently scraping the surface of the agar. It was then diluted to 250 ml with distilled water after adding 0.02% Tween-20. A spore suspension of approximately 10^9 spores per ml was prepared and sprayed on both groups of ten-week-old plants (control and experimental), and the plants were examined and observed every day for five days until they got ready for fungicide application (five days is approximately one growth cycle for the *Penicillium olsonii*). After this, one group of plants was treated with 100 ppm copper sulphate for 30 days, 2 times a week. Each individual plant was sampled and kept at -80°C for further studies.

3.7 Genomic DNA isolation from plant and fungi material

DNA extraction was performed for the quantitative polymerase chain reaction. DNeasy® Plant Mini Kit for purification of total cellular DNA from plant cells and tissues or fungi was used (Cat#69104). According to the manufacturer's instructions: 100 mg of the infected leaf tissue wet weight was fully ground by mortar and pestle in liquid nitrogen. Subsequently, 400 µl Buffer AP1 with 4 µl RNase A were added. After this, the mixture was incubated for 10 min at 65°C and mixed during incubation by inverting the tube every 2 min in order to lyse the cells.

130 µl Buffer P3 was added to the lysate, and the solution was mixed and incubated for 5 minutes on the ice. This step precipitates detergent, proteins, and polysaccharides. The lysate was centrifuged for 5 minutes at 20,000 x g. Then the supernatant was pipetted into the QIAshredder Mini spin column tube and centrifuged for 2 min at 20,000 x g to completely remove the most precipitates and cell debris. 1.5 volumes of Buffer AW1 were added to the cleared lysate and mixed by pipetting. 650 µl of the mixture was centrifuged for 1 min at 6000 x g, followed by adding 500 µl buffer AW2, again the same amount of buffer AW2 was added and centrifuged for 1 min at 20,000 x. 100 µl of Buffer AE was added directly onto the DNeasy membrane. Tubes were incubated for 5 min at room temperature, and then centrifuged for 1 min at 6000 x.

3.8 qPCR-based quantification of *Penicillium olsonii*

Genomic DNA samples were subjected to a quantitative PCR, and the primer set (Table 1) was selected to detect and monitor the pathogen *Penicillium olsonii*. The qPCR reactions were performed using a qPCR detection System (Bio-Rad) in a final volume of 20 µL, including 10 µL SsoFast Evagreen supermix (Cat#172-5201, Bio-Rad Laboratories Inc., California, USA) and 1 µL of primer pairs (Table 1). The specificity of the primers

developed in this study was evaluated using BLAST, 1 µL of target DNA and 8 µL of RNase/DNase-free water.

Thermal cycling parameters consisted of an initial preheating step for 5 min at 95°C followed by 40 cycles at 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s for data collection and real-time analysis; then, the setpoint temperature was increased after cycle 2 from 72 to 95°C for 30 s in 0.5°C increments to construct a melting curve and perform data collection and analysis. Samples were run in triplicate. Each collection of samples was split across two 96-well plates, and each run included a non-template control (NTC) and positive extraction control. The ratio of *P. olsonii* to cannabis genomic DNA was calculated using the ΔΔCt method [89].

Table 1. Primers used to quantify *P. olsonii in* cannabis leaves.

Primers were commercially synthesized by Eurofins Genomics, Toronto, ON, CA.

Genes	***Seq Name***	***Seq 5' to 3'***	***Annealing °C***
EF-1α	Penicillium olsonii- For	GACACCTTGAACTCTGTCTGAA	60.8
	Penicillium olsonii-Rev	CATTTCGCTGCGTTCTTCATC	60.6
TUB	Cannabis-For	CTGAGGGCATGTCCTACATATC	62.7
	Cannabis-Rev	CCATGCAGTTTGGCTATGAAC	60.6

3.9 Analysis of spore numbers using a hemocytometer

The spore suspension was prepared by harvesting 100 mg of cannabis leaves per cultivar. Five ml H_2O was added, and spores were liberated by vortexing for 30 s at maximum speed. The spore solution was filtered to remove large debris, and spores were concentrated by centrifuging for 5 min 4000 x g. Hemocytometer (Cat# 0630010 Marienfeld, Lauda-Königshofen, Germany) and cover glass were cleaned with 70% ethanol. The suspension after adding 0.05 ml of Lactophenol blue (Cat# 113741, MilliporeSigma,ON, CA) was applied to the edge of the cover-slip and sucked into the void by capillary action, which completely filled the chamber with the sample. For each sample, spores were counted in eight of 1 mm^2 fields of the hemocytometer and placed under a microscope 10x objective to facilitate localization of the grid. After two minutes to settle down the spores in the chamber, counting was done by focusing on the large square consisting of 16 small squares at each corner.

The spores were counted within each small square and any positioned on the right hand or bottom boundary line while skipping the ones on the left border and upper boundary lines. This process was followed on each of the four large corner squares. Spores were counted using the equation: spores/ml = (n) x 10^4, where: n = the average of cell count on the four corner squares.

3.10 Statistical analysis

Data were analyzed using ImageJ and GraphPad Prism 8 software. One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test was done to analyze the ImageJ results of fungi covered area, and One-way analysis of variance (ANOVA)

followed by Tukey's multiple comparisons test was done to analyze qPCR results. Results were presented as the mean ± SD of data. Differences were considered statistically significant when $p < 0.05$.

4. RESULTS

4.1 *Penicillium olsonii* was the most frequent pathogenic fungi found in cannabis plant samples

Incubation of plant-derived fungal samples were continued until single clones with stable morphological characteristics were established. Eight different samples were then sent to the University of Guelph Laboratory service, Agriculture and Food Laboratory, Guelph, ON, for genomic identification using microbial ID by 16S/18S rRNA gene / ITS. Data were compared to corresponding ITS1-5.8SITS2 sequences from the National Center for Biotechnology Information (NCBI) GenBank database. Results revealed that *Penicillium olsonii* was the most common pathogen among the tested samples. Even though samples had different colony shapes and colours, five out of eight were identified as *Penicillium olsonii with at least >99.2% gene similarity* (Table 2). One of the samples was identified as 100% similar to the *Clonostachys rosea*, and two others were reported to be significantly similar to *Cladosporium* and *Rhizopus stolonifera* (Table 2).

Table 2. Microbial ID by 16S/18S rRNA gene / IT

SAMPLES	SPECIMEN TYPE SAMPLING	BEST SIMILAR SPECIES	SIMILARITY PERCENTAGE	SEQUENCE LENGTH (BP)
1	Media	*Penicillium olsonii*	>99.6%	>500
2	Media	*Penicillium olsonii*	>99.6%	>500
3	Media	*Penicillium olsonii*	>99.4%	>500
4	Media	*Clonostachys rosea*	100%	>500
5	Media	*Penicillium olsonii*	>99.2%	>500
6	Media	*Penicillium olsonii*	>99.2%	>500
7	Media	*Cladosporium*	>99.6%	>500
8	Media	*Rhizopus stolonifer*	99.8%	>500

4.2 Copper sulphate inhibits the growth of *Penicillium olsonii in vitro*

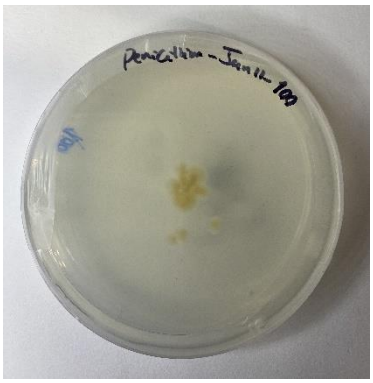
Single colonies of *Penicillium olsonii* were transferred to fresh PDA media containing different concentrations of $CuSO_4$ (25-300 ppm) and incubated for 12 days at ambient room temperature (23-25°C). After the incubation period, the growth of fungi was observed, and photos were taken (Figure 1). Photos were analyzed, and covered areas were calculated by ImageJ. Results showed that copper sulphate can reduce the growth of *P. olsonii* in a dose-dependent manner (Figure 1). While copper sulphate did not have any inhibitory effect on the tested fungi at the lower tested concentrations (25 and 50 ppm), it was significantly cytotoxic for the fungal pathogen from the concentration of 75 ppm (Figure 2A). The inhibition ratio was also calculated, and it was revealed that at 250 ppm the copper sulphate ceases *P. olsonii* growth by more than 99%.

A. Control

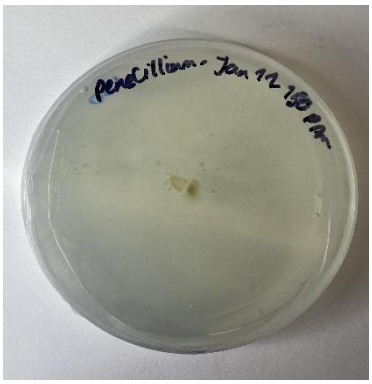
B. 25 ppm $CuSO_4$

C. 50 ppm $CuSO_4$

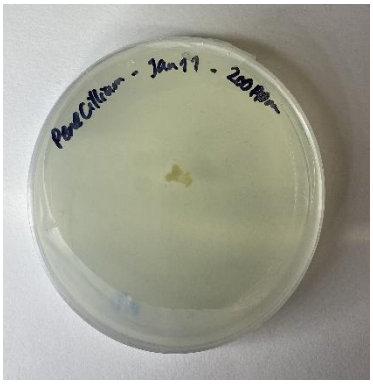
D. 75 ppm $CuSO_4$



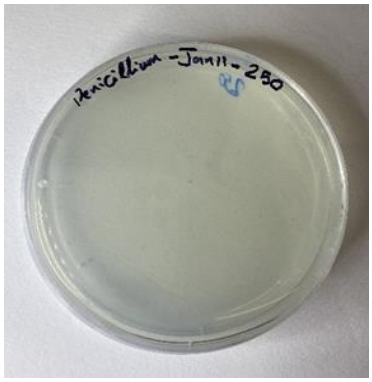
E. 100 ppm $CuSO_4$



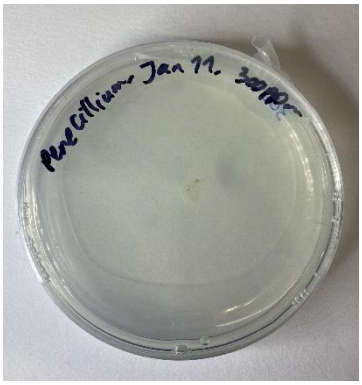
F. 150 ppm $CuSO_4$



G. 200 ppm $CuSO_4$



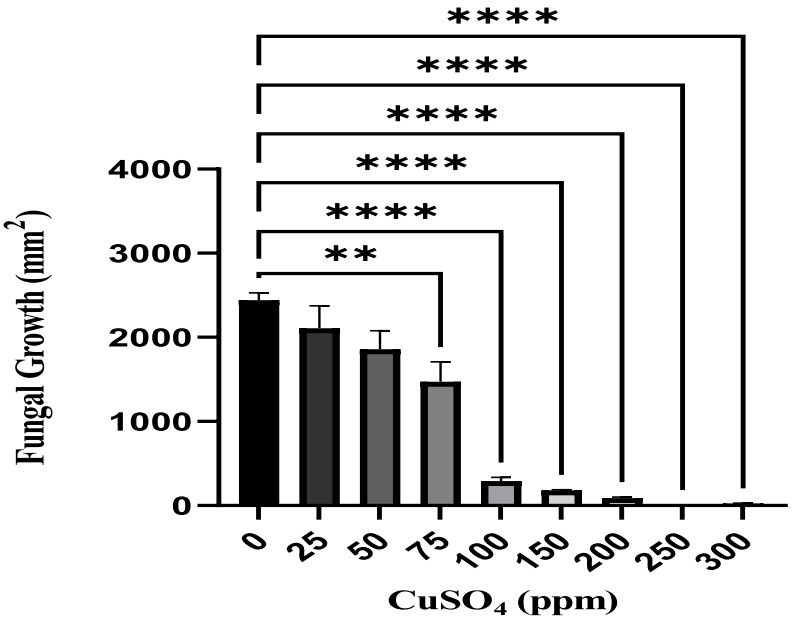
H. 250 ppm $CuSO_4$



I. 300 ppm $CuSO_4$

Figure 1. The growth of *Penicillium olsonii* isolated from cannabis leaves in the PDA medium containing $CuSO_4$. (A- I) The growth of single colonies of *Penicillium olsonii* after 12 days of incubation on the PDA medium containing a range of $CuSO_4$ concentrations (0- 300 ppm).

A.



B.

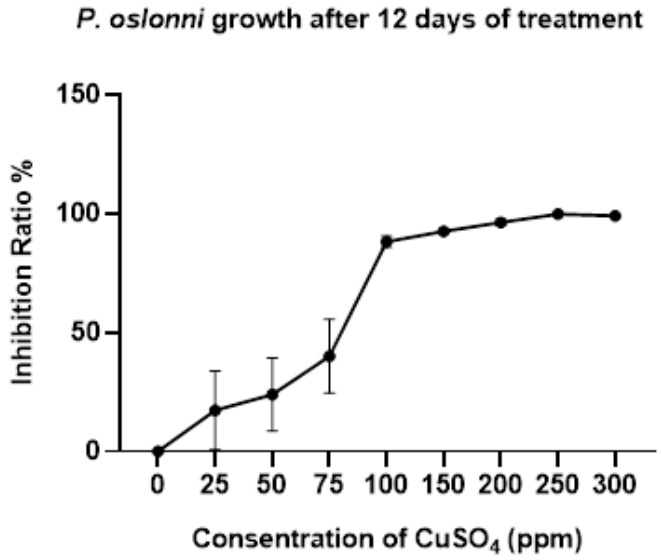


Figure 2. The effect of different concentrations of $CuSO_4$ on the growth of isolated *Penicillium olsonii* from cannabis leaves. (A) The effect of nine different concentrations of copper sulphate (0-300 ppm) on the growth of the isolated fungi after 12 days of incubation. (B) The percentage of inhibition of fungi growth in response to nine different concentrations of copper sulphate (0-300 ppm) after 12 days of incubation. Significant differences between groups are marked with: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

4.3 qPCR analysis of the effect of copper sulphate treatment in vivo

In this study, a qPCR assay was developed to detect and quantify a load of plant pathogen (*P. olsonii*) in four different cannabis cultivars. The target sequences for both the gene of interest, the host genes and primers in this research are summarized in Table 1.

Different c_t values of *P. olsonii* in different samples determined that copper sulphate was effective against the foliar fungal pathogen. The c_t values showed a difference in the

treatment and control groups of all four tested cultivars with all the controls having lower c_t values than the treatment groups (Figure 3). Kush Spice, GodBud and Blue Rhino showed the best response to the treatment as they had the highest difference between the control and treatment groups. Copper sulphate had less effect on Chemdawg RQOG and Aspice RBA did not show any significant response to the treatment (Figure 3).

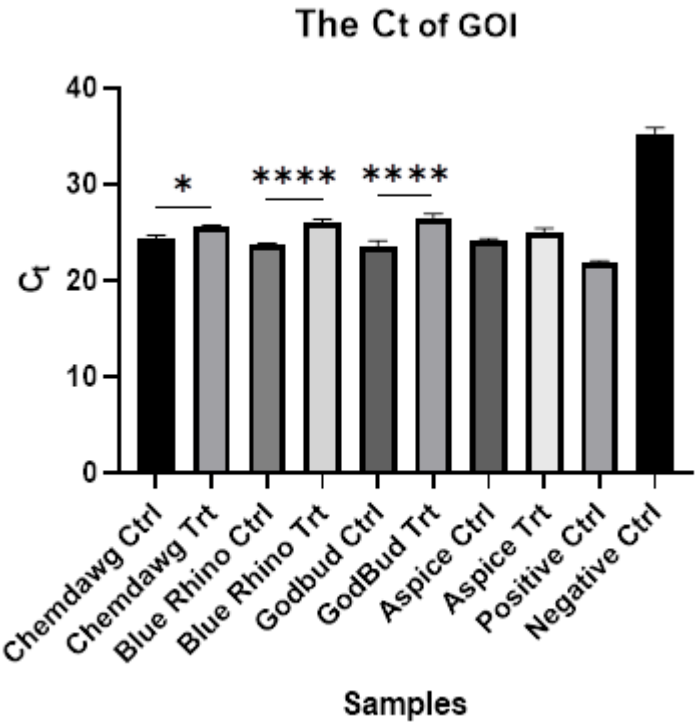
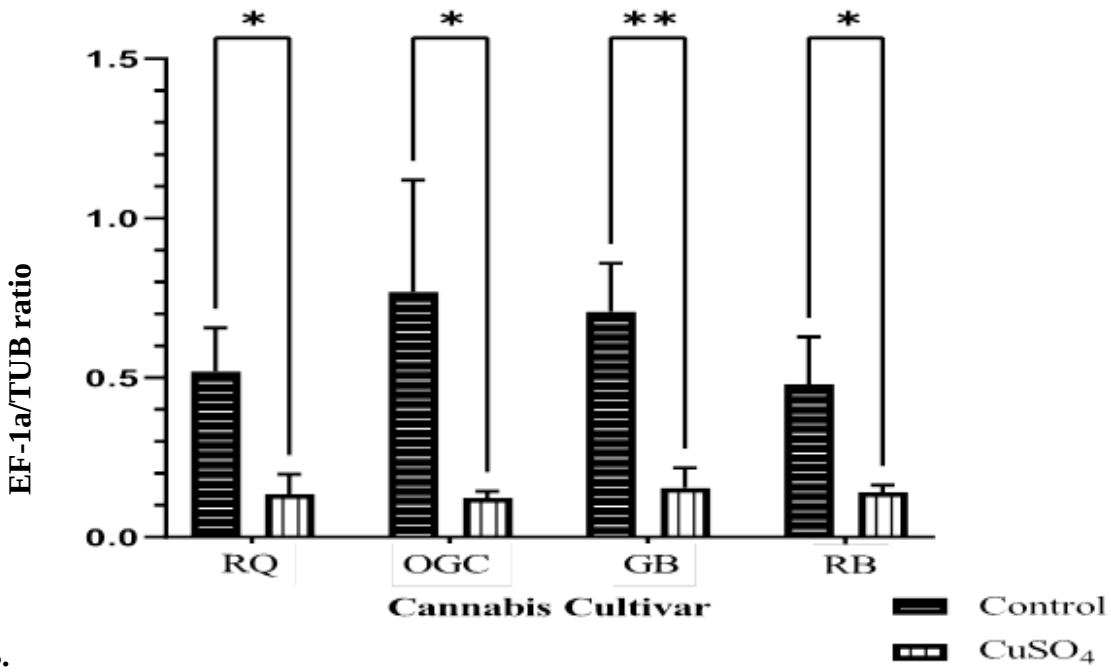


Figure 3. Analysis of the gene of interest (EF-1α) using qPCR in the control and $CuSO_4$ treated samples. Ct values of four different tested cultivars after 30 days of treatment with 100 ppm of $CuSO_4$ two times a week. The DNA was extracted from each individual plant and the primers were designed for both cannabis and *P. olsonii.* Significant differences between groups are marked with: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

A.


*
*
**
*
1.5
1.0
0.5
0.0
EF-1a/TUB ratio
RQ
OGC
GB
RB
Cannabis Cultivar
Control
CuSO4

B.

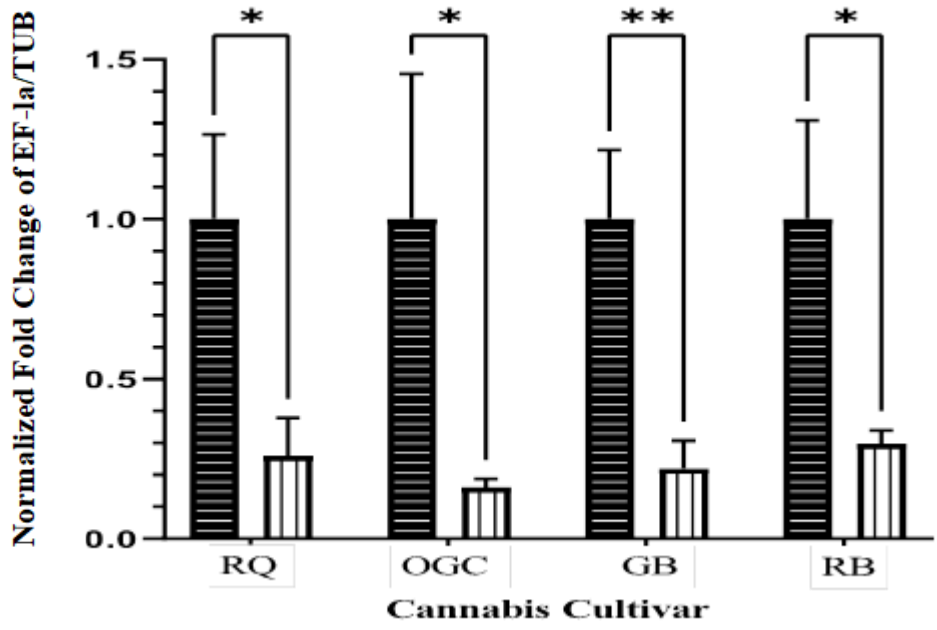

*
*
**
*
1.5
1.0
0.5
0.0
Normalized Fold Change of EF-1a/TUB
RQ
OGC
GB
RB
Cannabis Cultivar

Figure 4. qPCR analysis of the level of *P. olsonii* relative to cannabis genomic DNA. (A) The gene expression level and (B) the fold change in the amount of *Penicillium olsonii* present in indoor cannabis cultivars were determined by qPCR. The quantified amount of *P. olsonii* relative to cannabis genomic DNA correlated well with the amount of fungal biomass in treatment and control groups.

The level of *P. olsonii* shows that control samples have more fungal biomass compared to treatment groups. qPCR confirms the efficacy of $CuSO_4$ against *P. olsonii*. Significant differences between groups are marked with: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 (Figure 4).

4.4 Spore counting data confirm the results from the qPCR analysis.

Data revealed that there are differences in cannabis cultivars' responses to the copper sulphate treatment. We confirmed the results of qPCR by microscopic method and found that they agree with qPCR - CuSO4 is effective in reducing the growth of *Penicillium olsonii* on the cannabis grown in the greenhouse (Figure 5). We have tested 100 ppm of $CuSO_4$ on 4 different cultivars over 30 days applying it two times a week. Results indicate that GodBud and Blue Rhino showed the best response to the treatment, while treatment of Chemdawg and Aspice RBA showed a less significant reduction of fungal growth.

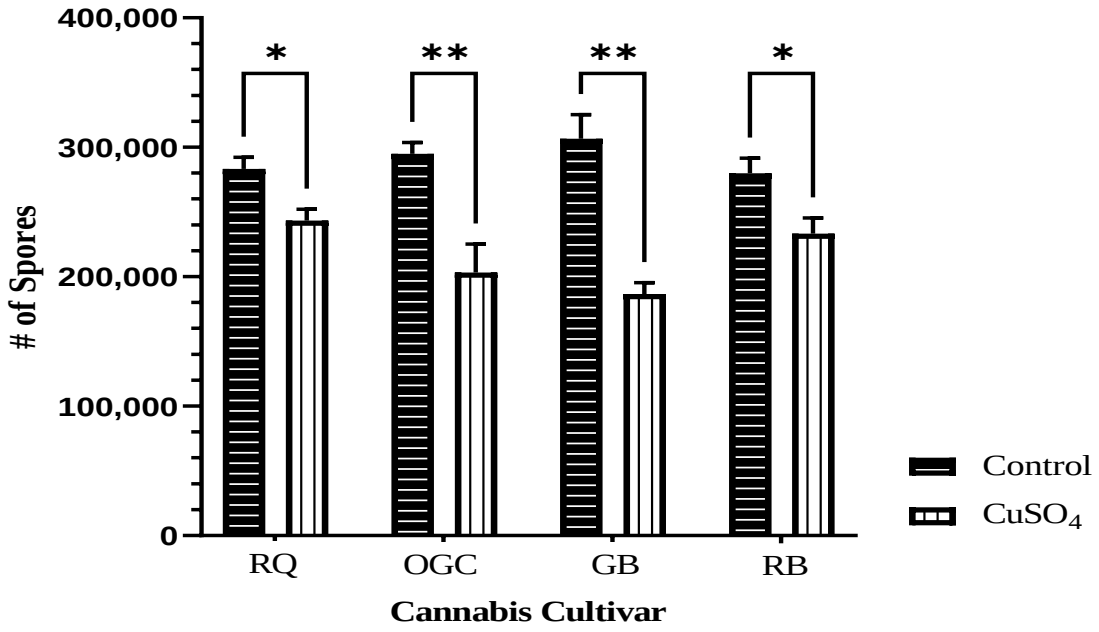


Figure 5. Analysis of *Penicillium olsonii* infection by spore count.

Number of spores extracted from *Cannabis sativa* after copper sulphate (100 ppm) or control-treated foliar applications biweekly for four weeks ($n = 3$). Multiple unpaired *t*-tests were performed with a False Discovery Rate correction (Q = 5%). Significance is indicated with: * $p < 0.05$, ** $p < 0.01$.

5. DISCUSSION

Various plant pathogens such as bacteria, viruses, and fungi can infect cannabis plants and reduce crop yield by affecting roots, crowns, and foliage. Fungi, however, are the main group of cannabis pathogens affecting plant development and reducing the quality of the produce [56].

Interactions between fungi pathogens and cannabis have been reported repeatedly in several studies [57, 63, 90]. Despite a considerable number of studies on several fungal pathogens in cannabis, not many studies have focused on the role and efficiency of the fungicides [57, 91]. However, fungicide research is important to be done to decrease the yield losses, improve the quality of the plant and as a result, improve the plant product.

In this research, we have analyzed the diversity of fungal pathogens in cannabis in an indoor facility at the University of Lethbridge and studied the effect of copper sulphate as a fungicide to control one of the most common fungi pathogenic fungi.

To achieve this goal, it was first necessary to identify the common agents of the disease in cannabis plants. Single colonies were isolated from the infected cannabis leaves. Using ITS regions demonstrated that five out of eight of the tested samples identified as *Penicillium olsonii* (Table 2). Other isolated fungi were *Clonostachys rosea*, *Rhizopus stolonifera and Cladosporium* (Table 2). The most frequently isolated fungus from diseased cannabis in this study was identified as *P. olsonii*. These results correlate with cannabis disease research by Punja (2021), who also reported the *Penicillium olsonii* as one of the main foliage pathogens in cannabis in indoor environments [57]. However, in another research *Alternaria alternata* and *Fusarium oxysporum* were identified as the main cannabis pathogens [63]. This difference could be explained in part by the variation in environmental

factors such as location and climate, highlighting the importance of local research and data collection.

We evaluated the antifungal potential of several concentrations of $CuSO_4$ dissolved in the PDA medium (in vitro) to model the agricultural delivery (in vivo). Our study showed that $CuSO_4$ significantly inhibited the growth of *Penicillium olsonii*. Not all the tested concentrations (25-300 ppm) showed such an effect; however, the antifungal effect of $CuSO_4$ increased as the concentration increased. 100 ppm was the most cost-effective concentration with a high rate of fungal growth inhibition. At 100 ppm, $CuSO_4$ inhibited growth by 88% (Figure 2). To our knowledge, this was the first time that $CuSO_4$ was tested specifically on *Penicillium* in cannabis.

Another study has tested $CuSO_4$ on *Botrytis cinerea* and *Sclerotinia sclerotiorum* as pathogens in Cucumber while $CuSO_4$ inhibited their growth by 58.82 and 77.44%, respectively, at 4000 ppm *in vitro* [92]. Moreover, the same study was done on *Colletotrichum gleosporioides* in mango; the results showed 66.3% reduction in conidia germination at 0.8 ppm [84]. These wide ranges of $CuSO_4$ concentrations were also tested in another study showing that different concentrations of copper-based fungicides are needed to have at least 50% growth inhibition [93]. In comparison with our results, the mentioned studies have also demonstrated the efficacy of $CuSO_4$ on the tested fungi pathogens in different plants.

To be able to develop knowledge on the efficacy of $CuSO_4$ against *Penicillium olsonii* as a pathogen in cannabis, qPCR was developed for quantification of *P. olsonii* target genes in treated and untreated groups of plants. qPCR results (c_t values) were obtained from each different plant sample, and the fold change was calculated using $\Delta\Delta C_t$ method.

From the studies, it is evident that 100 ppm $CuSO_4$ is effective in controlling the foliar fungi pathogen in cannabis in the greenhouse when it was applied two times a week.

Different cannabis cultivars might have different susceptibility to the same fungus pathogen; this may cause them to have various responses to the fungicides as well [91]. Similar results were also observed in watermelon in which the same fungicide didn't have the same effect on different tested varieties [94]. Our results showed that different cultivars respond to the treatment differently; in this case, Kush Spice and Blue Rhino showed the best result.

6. CONCLUSION AND FUTURE STUDIES

The cannabis boom in Canada is reaching full swing. At the same time, the research on agricultural and medicinal cannabis is still lagging. Cannabis is typically grown at a high density in a contained indoor environment. Such conditions lead to high pathogen pressure. Particularly pathogenic fungi are the main pathogen in the cannabis industry resulting in a significant decrease in yield and sometimes even a loss of the entire crop or recall of various products.

This study has focused on the identification of foliar fungal pathogen in cannabis in an indoor environment and the antifungal effect of copper sulphate against *Penicillium olsonii*. The first limitation of our study was that besides *Penicillium olsonii* other pathogens were also present in different parts of the plant such as the root, and internal tissue which makes an impact on the plant and the efficacy of the fungicide in this research. Furthermore, in this study, we used samples for fungal isolation and identification only from one cannabis facility. However, it is important to examine the diversity of fungi pathogens in different areas and facilities in order to obtain more comprehensive results.

The following objectives were addressed in this master thesis:

Isolating and identifying fungal pathogens from the commercial operation greenhouse. *Penicillium olsonii* was the main pathogen.

$CuSO_4$ can reduce the overall *P. olsonii* as a pathogen in indoor cannabis cultivation.

A range of concentrations of $CuSO_4$ from 25 to 300 ppm was used to test its effectiveness against *P. olsonii*. The results demonstrated that 100 ppm did not cause any physiological changes during the vegetative cycle; however, it significantly inhibited the growth of *P. olsonii* in cannabis. Furthermore, plants could tolerate it well when applied two times a week. $CuSO_4$ was found to be more effective for a specific cultivar.

This work allowed us to identify the cultivars that have the most and the least responsive to $CuSO_4$. The degree of sensitivity to the fungicide was monitored using qPCR. Blue Rhino and Kush Spice had the highest responses to the treatment whereas Aspice did not show a significant difference after treatment with $CuSO_4$.

Future research, possibilities, and goals may include identifying the concentrations of $CuSO_4$ to be used at the flowering stage as well as controlling infection in clones propagated by cutting. In this experiment, the maximum tolerable concentration of $CuSO_4$ in the vegetative cycle of the plants was tested. It might be beneficial to test whether the pathogen infection can be controlled at different stages of cannabis growth.

Another important study that can be done is to isolate different fungal pathogens in cannabis and study the effect of copper sulphate on them, alone and in combination.

Moreover, identifying whether the flower yield and the secondary metabolites profile are affected by exposure to $CuSO_4$ would be highly beneficial. Although we do not predict any changes in the cannabinoids and terpenoids profile in response to the treatment with $CuSO_4$, it is still worthwhile to test it.

Furthermore, it would be good to identify the extent to which copper ions accumulate in flowers because they are sold to the public; the ICP-MS analysis can address this goal.

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