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Medical cannabis - From cultivation and processing to market

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Despacho RT - 31 /2019 - Anexo 3

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Despacho RT - 31 /2019 - Anexo 4

Statement of integrity

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

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Cannabis medicinal - Do cultivo e transformação ao mercado

Resumo

Cannabis sativa é uma das espécies de plantas mais úteis e versáteis que tem sido explorada pelo homem desde há milénios. Atualmente, a cannabis medicinal está a ganhar força para uma variedade de fins, uma vez que as suas qualidades terapêuticas estão cada vez mais bem estabelecidas, tornando imperativa a produção de cannabis medicinal de alta qualidade. O cultivo da canábis passou do exterior para o interior, onde é possível controlar fatores como a luz e a temperatura, melhorando a produção e a qualidade do produto. Contudo, continuam a existir desafios na produção, como o aparecimento de estruturas masculinas em plantas feminizadas. O primeiro objetivo do trabalho foi compreender melhor o aparecimento destas estruturas e verificar se a temperatura é um fator associado. De acordo com os resultados existe uma relação direta entre o aumento da temperatura (entre 32 e 37 °C) e o aparecimento de estruturas masculinas nas plantas feminizadas. O segundo objetivo foi encontrar características observáveis indicadoras dos níveis de maturação e THC da planta ao longo do tempo de modo a evitar a quantificação por HPLC, reduzindo o uso de reagentes que afetam negativamente o ambiente. De facto, visualizações macro e microscópicas de características da flor e do tricoma, como cor e tamanho, nas primeiras semanas de trabalho, tornam possível ter ideia da evolução do teor em THC. O terceiro objetivo foi encontrar uma potencial utilização dos resíduos vegetais da empresa (folhas removidas), em linha com uma produção sustentável e responsável. Nesse sentido avaliou-se os efeitos fitotóxicos e antimicrobianos de um extrato metanólico desse material para uma eventual utilização como biopesticida no controlo de fitopatógenos causadores de doenças em culturas relevantes. Nos efeitos fitotóxicos realizados em mostarda verificou-se uma inibição do comprimento da raiz com 150, 300 e 450 µg/mL. Os ensaios antimicrobianos foram realizados com 2 fungos e 1 oomicete que afectam culturas de interesse em Portugal. Os resultados mostraram que o extrato inibiu ambos os fungos, *Diplodia cortícola,* maior eficiência, cerca de 40% comparativamente ao controlo, e *Colletotrichum acutatum*. Quanto à *Phytophtora cinanamomic* os resultados indicam que não há inibição. Da quantificação e identificação dos metabolitos secundários por HPLC e GC verificou-se que um grupo de terpenos poderá ser responsável pela atividade antimicrobiana do extrato, tornando promissora a abordagem ao aproveitamento de folhas de canábis descartadas.

Palavras-chave: Antimicrobiano, *Cannabis sativa* L., plantas feminizadas, extrato de folhas, sustentabilidade.

Medical cannabis - From cultivation and processing to market

Abstract

Cannabis sativa is one of the most useful and versatile plant species that has been exploited by man for millennia. Today, medicinal cannabis is gaining momentum for a variety of purposes, as its therapeutic qualities are increasingly well established, making the production of high-quality medicinal cannabis imperative. Production has moved from outdoors to indoors, where factors such as light and temperature can be controlled, improving production and product quality. Despite these advances, some production challenges remain, such as the appearance of male structures in feminized plants. The first objective of the work was to better understand the appearance of these structures and to see if temperature is an associated factor. According to the results, there is a direct relationship between an increase in temperature (between 32 and 37 °C) and the appearance of male structures in feminized plants. The second objective of the work was to find observable characteristics indicative of the plant's levels of maturation and THC over time to avoid quantification by HPLC, reducing the use of reagents that negatively affect the environment. In fact, macroscopic and microscopic visualizations of flower and trichome characteristics, such as color and size, in the first few weeks of work, make it possible to get an idea of the evolution of THC content. A third objective was to find a potential use for the company's plant waste (removed leaves), in line with sustainable and responsible production. To this end, the phytotoxic and antimicrobial effects of a methanolic extract of this material were evaluated for possible use as a biopesticide to control disease-causing phytopathogens in relevant crops. In the phytotoxic effects carried out on mustard, root length was inhibited at 150, 300 and 450 µg/mL. The antimicrobial tests were carried out on 2 fungi and 1 oomycete that affect crops of interest in Portugal. The results showed that the extract inhibited both fungi, *Diplodia corticola* and *Colletotrichum acutatum*, with greater efficiency against *Diplodia corticola*, around 40% compared to the control. As for *Phytophtora cinanamomic*, the results indicate no inhibition. The quantification and identification of the secondary metabolites by HPLC and GC showed that a group of terpenes may be responsible for the antimicrobial activity of the extract, making the approach to using discarded cannabis leaves promising.

Keywords: antimicrobial, *Cannabis sativa* L, feminized plants, leaves extract, sustainability

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1. Introduction

1.1. History of Cannabis

The species *Cannabis sativa,* also known as cannabis, is considered one of the oldest plants ever discovered and has been grown as a field crop for thousands of years (Pain, 2015).

The precise original geographic range of *Cannabis sativa* remains uncertain, although it is believed to have been located in central Asia (McPartland et al., 2019). The identification of ancient strains is complicated by the possibility of introgression from nearby domesticated plants (Clarke & Merlin, 2016). *Cannabis sativa* is one of the oldest cultivated crops, having been domesticated thousands of years ago. It was independently domesticated in Europe and northern Asia, primarily for fibre and occasionally for its edible seeds (Pain, 2015). While hybridization between European and Chinese strains has occurred, there are still landraces or cultivars that exhibit the genetic distinctiveness of the two types (Clarke & Merlin, 2016). Nevertheless, these northern strains, as well as related wild-growing plants, produce limited amounts of THC (tetrahydrocannabinol), the psychoactive compound that can cause intoxication used for recreational or therapeutic purposes (Clarke & Merlin, 2016).

Over the past millennium, cannabis consumption has become more prevalent in southern Asia than in any other region of the world. In Afghanistan and surrounding areas, a highly domesticated class of drug landraces was selected, which corresponds to what is now known as the "indica-type." In contrast, the "sativa-type" refers to a less domesticated but more potent drug plant that was grown in other parts of Asia (Clarke & Merlin, 2016). Over the centuries, the sativa-type strain of cannabis has been widely dispersed across various regions of the world (McPartland et al., 2019).

The domesticated indica-type strain has exhibited poor adaptability to natural environments, while the domesticated sativa-type strains have demonstrated greater resilience and similarity to their wild counterparts found in southern Asia (Clarke & Merlin, 2016). Consequently, the sativa-type strain requires significantly less maintenance in many outdoor settings. In addition to the differences in properties between the indica-type and sativa-type strains, the geographical distribution of the latter, which was considerably more extensive compared to the former, facilitated its widespread dissemination throughout history (Bonini et al., 2018).

Currently, the cannabis market, encompassing both legal and illegal sectors, is predominantly dominated by strains advertised as sativa-type. However, it is important to note that these strains often represent hybrids between the two classifications (Berardo et al., 2024). The identification of

materials, including both plants and drug preparations, as sativa-type is primarily based on their cannabinoid profile, which typically consists solely of THC or occasionally includes a limited amount of CBD (cannabidiol). On the other hand, materials identified as indica-type are characterized by the presence of appreciable CBD in addition to substantial THC (McPartland, 2017).

Despite years of use for several purposes, the horticultural classification of cannabis has still been extensively debated (Russo, 2007). There are more than 600 commercially available varieties of *Cannabis Sativa* (Raman, 1998) and no general agreement has been achieved on the taxonomic categorization of this plant. In 2021, the United States Department of Agriculture claims a taxonomic categorization of the *Cannabis sativa* L. species, as presented in Table 1 (USDA-NRCS, 2021).

Table 1. Taxonomic categorization of the *Cannabis sativa* L. species according to the United States Department of Agriculture.

Taxonomic Classification	
Kingdom	*Plantae* - Plants
Subkingdom	*Tracheobionta* – Vascular plants
Superdivision	*Spermatophyta* – Seed plants
Division	Magnoliophyta – Flowering plants
Class	*Magnoliopsida* – Dicotyledons
Subclass	*Hamamelididae*
Order	*Urticales*
Family	Cannabaceae - Hemp family
Genus	*Cannabis*
Species	*Sativa*

1.2. Evolution of the term cannabis

The term "Cannabis" is commonly used to refer to a genus of plants, with the accepted understanding that there is only one species within this genus, namely *Cannabis sativa* (Clarke & Merlin, 2016). Therefore, cannabis is often used as a shortened way of referring to *Cannabis sativa*. Historically, the term "cannabis" has been used in a broad sense, both as a noun and an adjective, encompassing various meanings. These include any preparations, whether drug-related or not, derived from cannabis plants, as well as considerations related to the chemical, medicinal, sociological, or commercial aspects of cannabis (Russo, 2007). However, in recent times, the term

"cannabis" has often been specifically used to refer to what was previously known as "marijuana." This refers to plants with high levels of THC, the psychoactive compound that can cause intoxication, and the associated drug preparations used for recreational or therapeutic purposes (Baker et al., 2003). It is worth noting that in certain jurisdictions, such as Canada, CBD is regulated or defined as falling under the term "cannabis." Additionally, synthetic cannabinoids may also be included within the scope of the term "cannabis" (Hartsel et al., 2016).

The term "Hemp" is a widely used term that refers to numerous plant species that are utilized as sources of fibre, with particular emphasis on forms of *C. sativa* that have limited THC content. Historically, "hemp" and "marijuana" have been used interchangeably (Datwyler & Weiblen, 2006). However, in recent times, hemp plants have gained significance as sources of oilseed and CBD. When hemp is cultivated for oilseed, it is classified as "oilseed hemp" or "hempseed." The term "industrial hemp" has been used to differentiate plants that are authorized for non-euphoric drug uses, including both fibre and oilseed (Small & Cronquist, 1976) . This phrase is commonly used in Canada and other jurisdictions to refer to plants that contain no more than 0.3% THC in the dried female reproductive parts. Such plants are treated less harshly than marijuana plants under the law. However, the term "cannabis" can overlap with both marijuana and hemp categories. For instance, in Canada, hemp plants that are used for CBD extraction are governed by "cannabis" legislation (Cherney & Small, 2016). Therefore, it is crucial to exercise caution when consulting literature, legislation, and regulations to avoid being misled by the usage of these terms.

1.3. Plant biology and special features

Cannabis sativa is a plant that typically completes its life cycle within a year, although certain female plants can be sustained for longer periods under controlled environmental conditions. The leaves of *Cannabis sativa* are easily recognizable due to their distinct arrangement of 5 to 9 leaflets (Figure 1.), making it one of the most widely recognized plants worldwide (Reed, 1914).

Figure 1. Cannabis leaves. Photos taken by the author at SMC+.

It is not possible to reliably differentiate between male and female plants based on their appearance until they reach a stage of maturity where they are capable of producing flowers (Figure 2.). In the flowering stage, male plants generally exhibit greater height (typically 10-15%) when compared to female plants (Clarke, 1999).

Figure 2. Cannabis female (A) and male (B) flowers on early stages of development. Photos taken by the author at SMC+.

In both male and female plants, most flowers are found in clusters. The flowers, characteristic of wind-pollinated plants, are small in size (measuring only a few millimetres in length) but abundant in number. They do not display showy petals or fragrance that would attract insect pollinators (Spitzer-Rimon et al., 2019). Male flower clusters possess a feathery appearance and acquire a yellowish hue due to the presence of pollen within the five stamens of the male flowers. Female flowers, on the other hand, consist of a minute ovary enclosed by a small bract (a rudimentary leaf) with protruding whitish stigmas, which are the receptive parts of the flowers for pollen. This gives the cluster of female flowers the appearance of being covered in whitish hairs (Figure 3) (Clarke, 1999).

Figure 3. Cannabis female flower. Photo provided by © SMC Therapeutic Health Center Production Lda.

Feminized cannabis plants are specifically bred to produce only female plants, as female plants typically have higher THC levels and are preferred for their cannabinoid-rich flowers (Owen et al., 2023). Feminized seeds are created through various breeding techniques to ensure that the resulting plants are predominantly female, being the formation of masculine structures generally considered an undesirable trait. However, it's important to note that feminized plants can

occasionally exhibit hermaphroditic characteristics, meaning they develop both male and female reproductive structures. This can happen due to genetic instability or stress factors such as light interruptions, nutrient deficiencies or environmental fluctuations. When this occurs, the plants may produce pollen sacs or stamens alongside their female flowers (Punja & Holmes, 2020).

Plants cultivated for fiber production, which are typically grown outdoors, exhibit a tall stature, often exceeding 2 meters in height and limited branching (Zheng, 2022). On the other hand, plants cultivated for oilseed production, which also grow outdoors, may resemble fibre plants, but recent breeding efforts have resulted in the development of shorter, less branched varieties, sometimes reaching just 1 meter high(Strzelczyk et al., 2022). In harsh outdoor conditions, wild plants, often classified as "weeds," may exhibit a stunted growth habit, with heights below 1 meter and minimal branching. However, under favourable conditions, some wild plants may develop into highly branched specimens, reaching heights exceeding 3 meters (R. C. Clarke, 1999). Wild plants are almost exclusively dioecious, with separate male and female individuals. Until the latter half of the 20th century, fibre and oilseed varieties also exhibited this trait, but recent breeding efforts have resulted in the development of many monoecious varieties, characterized by the presence of both male and female flowers on the same plant (Malabadi et al., 2023).

Most plant species have very small epidermal appendages on the aerial parts, termed “trichomes”, widely considered protective against pathogens and arthropod herbivores (Wagner, 1991). Trichomes are sometimes called “hairs,” since they are often hair-like. About 30% of flowering plants possess “glandular trichomes,” producing secondary chemicals, usually at the tip of the structure, often in distinctive head-like containers (Gülck & Møller, 2020). Although produced substances are often used by plants as protective agents, they are also used by humans as natural pesticides, food additives, fragrances and pharmaceuticals (Schuurink & Tissier, 2020).

Cannabis plants are covered by various types of non-glandular and glandular trichomes. There are two types of hair-like non-glandular trichomes in cannabis. Both types are unicellular and are located on both sides of leaves, but prominently on the abaxial side. Non-glandular trichomes on cannabis tend to point towards the tips of the stem or leaf, suggesting that their main function is to protect plants from the attack of pests, wind, and light (Dayanandan & Kaufman, 1976). Three types of glandular trichomes have been described in *C. sativa* based on the morphology of their surface, development, and physiology: bulbous, capitate-sessile, and capitate-stalked. Captite-stalked trichomes are the most studied and most important, since unlike the others, they increase

during flowering and are responsible for producing and accumulating phytocannabinoids and terpenes (Wang et al., 2021). This co-occurrence of cannabinoids and terpenes reflect common biosynthetic pathways for the two classes of chemicals (LaVigne et al., 2021). The biggest glandular heads burst on contact and can make the flower clusters quite sticky. The largest glandular trichomes and the greatest concentration of these occur in the buds (Figure 4).

Figure 4. Cannabis female buds (A) filled with trichomes (B and C). (B) Optical microscope image at 100x magnification provided by © SMC Therapeutic Health Center Production Lda. (C) Optical microscope image at 400x magnification. Photo taken by the author at © SMC Therapeutic Health Center Production Lda.

Cannabis strains have much larger clusters of female flowers than hemp or wild plants, reason why they produce greater amounts of cannabinoids (Mahlberg & Kim, 2004a). The buds are inconspicuous when young, but when mature, they constitute the basic product of commercial

environmentally controlled facilities (Livingston et al., 2020). They may contain 20% or more cannabinoids (on a dry weight basis). Small and much less frequent trichomes occur on the foliage, which may contain up to 5% cannabinoids (Jin et al., 2020). Very few trichomes are on the stems, twigs and roots, which are considered as waste because they contain few or no cannabinoids. Overall, for medical purposes cannabis, THC content can be 2.5% - 25% in the flowers, 0.2% - 6% in the leaves, 0.0% - 0.3% in the stems, 0.002% - 0.02% in the seeds and 0.0% - 0.003% in the roots (Jin et al., 2020).

1.4. Cannabis compounds: Cannabinoids, Terpenes and Flavonoids

The recreational and commercial value of drug forms of *Cannabis sativa* is determined by two classes of chemicals, namely phytocannabinoids and terpenes. These chemicals also play a role in determining the medicinal value of the plant for humans and other animals (Sandberg, 2012).

The term "cannabinoids" encompasses various classes of chemicals, including some that naturally occur in humans and have important physiological functions (Shahbazi et al., 2020). "Phytocannabinoids" refer to cannabinoids found in plants, particularly a class of chemicals with a C21 terpenophenolic structure that is primarily present in *Cannabis sativa* (Russo, 2011). Phytocannabinoids can be categorized into 11 types, which include: (-)-delta-9-trans-tetrahydrocannabinol (Δ9-THC), (-)-delta-8-trans-tetrahydrocannabinol (Δ8-THC), cannabigerol (CBG), cannabichromene (CBC), cannabidiol (CBD), cannabinodiol (CBND), cannabielsoin (CBE), cannabicyclol (CBL), cannabinol (CBN), cannabitriol (CBT) and miscellaneous cannabinoids (Gülck & Møller, 2020).

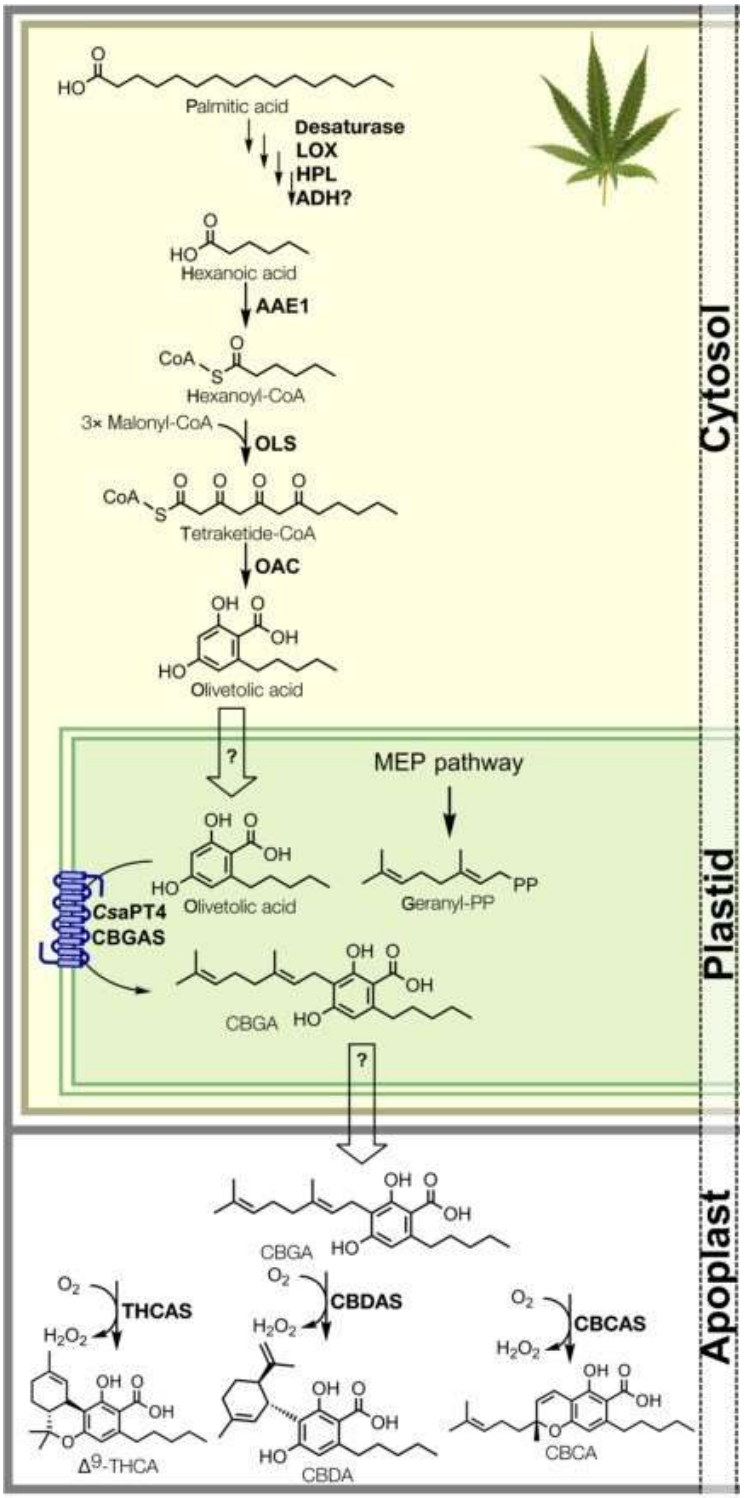


Figure 5. Phytocannabinoid biosynthesis in *Cannabis sativa*. Source: (Gülck & Møller, 2020).

The biosynthesis of cannabinoids, illustrated in Figure 5, involves the integration of key steps in polyketide and isoprenoid metabolism. The polyketide starter molecule, hexanoic acid, is likely generated from C18 fatty acids through sequential desaturation, peroxygenation, and cleavage into

a C6 and a C12 product by the action of desaturases, lipoxygenases, and hydroperoxide lyases Stout et al and supported by Livingston et al (Livingston et al., 2020; Stout et al., 2012). Hexanoic acid is converted into the activated thioester hexanoyl-CoA by acyl-activating enzyme 1 and is elongated with malonyl-CoA as a C2 donor in a reaction catalyzed by olivetol synthase and cyclized by olivetolic acid cyclase to produce olivetolic acid. The production of olivetolic acid from hexanoic acid takes place in the cytosol (Gülck & Møller, 2020). GPP (geranyl diphosphate) is synthesized by the MEP (plastidial non-mevalonate-dependent isoprenoid) pathway and is used by cannabigerolic acid synthase to prenylate olivetolic acid, forming the branch-point intermediate and the first cannabinoid compound CBGA (Cannabigerolic acid) (Fellermeier et al., 2001). CBGA is the direct precursor for the common cannabinoids which are decorated with an alkylic pentyl side chain. The transmembrane aromatic prenyltransferase CBGAS carries a plastid localization signal and remains to be determined into which plastid membrane it is integrated and whether its active site faces the inner or the outer side of that membrane. The flavoproteins Δ9-tetrahydrocannabinolic acid synthase and cannabidiolic acid synthase are secreted into the extracellular space and convert CBGA to Δ9-THCA and CBDA, respectively. These enzymes are responsible for catalyzing chemical reactions that modify the molecular structure of CBG to produce phytocannabinoids with different properties and effects (Gülck & Møller, 2020). The production of phytocannabinoids varies among different cannabis varieties, or strains, due to genetic variations in the enzymes involved in biosynthesis. This is what creates the great diversity of cannabinoid profiles found in cannabis plants (Gülck & Møller, 2020).

Currently, more than a dozen phytocannabinoids are undergoing intensive medical research (Maroon & Bost, 2018). However, the commercial cannabis industry is particularly interested in two natural phytocannabinoids: Δ9-THC and, to a lesser extent, the isomer Δ8-THC. These are the only phytocannabinoids that possess euphoric properties. CBD, on the other hand, significantly modifies or moderates the effects of THC, which is appreciated both recreationally and medicinally (Reekie et al., 2017). The cannabinoid profile of the most popular commercial cannabis strains is dominated by Δ9-THC, but many strains also contain CBD, and the percentages of these two cannabinoids are commonly advertised (Pennypacker et al., 2022). In living plants, cannabinoids exist primarily in the form of carboxylic acids, with a -COOH radical attached to the molecule. These acids decarboxylate into their neutral counterparts, losing the acidic -COOH radical and leaving an H atom, under the influence of light, time (such as prolonged storage), alkaline conditions, or when heated, as occurs when cannabis is smoked or cooked (e.g., in brownies) (Russo, 2011).

Terpenes are hydrocarbon compounds that are found in various plant species, including cannabis. Terpenes are volatile compounds that contribute to the distinct aromas and odors of different cannabis strains (Paduch et al., 2007). The variation in odor among cannabis strains is of significant commercial importance, as consumers have distinct preferences for specific odors. The terpene profile of a strain is therefore a crucial consideration in the cannabis industry (Sommano et al., 2020). Studies have also reported a role of terpenes in the medicinal properties of cannabis (Nuutinen, 2018). However, it is worth noting that further research is needed to fully understand the extent of their contribution. There are different types of terpenes in cannabis plants, including monoterpenes, sesquiterpenes, diterpenes and triterpenes. Some terpenes commonly found in cannabis strains include α-pinene, β-caryophyllene, β-myrcene, limonene, terpinolene, linalool, selina3,7(11)-diene, γ-selinene, 10-epi-γ-eudesmol, β-eudesmol, α-eudesmol, bulnesol and α-bisabolol (Radwan et al., 2021).

In addition to cannabinoids and terpenes, other compounds have been identified and ongoing studies have been done to explore their potential medicinal, therapeutic and industrial applications (Gonçalves et al., 2019). Flavonoids found in cannabis predominantly consist of flavones and flavonols (Panche et al., 2016). Their highest concentrations are observed in cannabis leaves, followed by inflorescence, while they are undetectable in roots, stems and seeds. The phenylpropanoid and flavonoid biosynthetic pathways are used in cannabis to synthesize its fundamental flavonoid structures (Bautista et al., 2021). Scientific investigations have revealed that canaflavins possess anti-inflammatory properties (Baron, 2018).

Each component of the cannabis plant may possess distinct medicinal and therapeutic values (Russo, 2011). The levels of cannabinoids, terpenes and flavonoids in cannabis are influenced by genetic factors and the cultivation environment. Consequently, the selection of genetics can significantly impact the production of these secondary metabolites through cultivation methods (Flores-Sanchez & Verpoorte, 2008).

1.5. Endocannabinoid system & Phytocannabinoids Therapeutic Effects

The endocannabinoid system (ECS), which plays a critical role in regulating human physiological processes by acting in central and peripheral nervous systemc, is composed of three main components: endocannabinoids, cannabinoid receptors and their synthesizing / degrading enzymes (Battista et al., 2012). Endocannabinoids are naturally compounds produced by the body and act as signalling molecules within the ECS. The two initial endocannabinoids identified were

anandamide (AEA) and 2-arachidonoylglycerol (2-AG), which are lipid-based molecules (Lu & Mackie, 2021). Two types of endocannabinoids receptors were also identified: cannabinoid receptor type 1 (CB1) and cannabinoid receptor type 2 (CB2). While CB1 receptors are primarily concentrated in the nervous system, particularly in the brain, CB2 receptors are predominantly located in peripheral organs and immune cells (Battista et al., 2012). The ECS-related enzymes are involved in the synthesis and degradation of endocannabinoids: amide hydrolase (FAAH) is involved in the degradation of AEA, whereas monoacylglycerol lipase (MAGL) is involved in the degradation of 2-AG (Lu & Mackie, 2021).

The discovery of cannabinoid receptors and the characterization of endocannabinoids, along with the identification of the ECS and its associated enzymes, sparked renewed scientific interest in cannabis (Legare et al., 2022). Over the last years, several studies have demonstrated a beneficial impact of phytocannabinoids on human health, namely in reducing the severity and incidence of convulsions and epileptic seizures, chronic pain, nausea and chemotherapy-induced vomiting (Burggren et al., 2019; Agarwal et al., 2019; Martínez et al., 2020; Poudel et al., 2021). The use of phytocannabinoids has also been associated with therapeutic capacity in neurodegenerative disorders as Multiple Sclerosis and Huntington's disease (Burggren et al., 2019, Agarwal et al., 2019; Martínez et al., 2020; Poudel et al., 2021). It is currently well known that upon cannabis consumption, cannabinoids interact with the ECS by binding to cannabinoid receptors (Crocq, 2022). Indeed, THC has a high affinity for CB1 receptors, especially those located in the brain, resulting in psychoactive effects. This interaction can impact various physiological processes, including memory, mood, pain perception, appetite, and sleep. It is also known that cannabis consumption can affect the production, release and degradation of endocannabinoids (Di Marzo & Piscitelli, 2015). In fact, THC can temporarily inhibit the enzymes responsible for the degradation of endocannabinoids, FAAH and MAGL, leading to increased levels of AEA and 2-AG in the body (Di Marzo & Piscitelli, 2015). On the other hand, CBD has a low affinity for both CB1 and CB2 receptors. However, higher doses of CBD can potentiate the lower doses of d-9-THC by enhancing the level of CB1R expression in the hippocampus and hypothalamus. The authors suggest that CBD potentiates the pharmacological effects of d-9-THC via a CB1R-dependent mechanism (Hutten et al., 2022). It's important to note that the effects of cannabis on the ECS and the overall physiological response depends on several factors, such as cannabis strain, method of cannabis consumption, cannabis dosage and individual differences (Kitdumrongthum & Trachootham, 2023). These scientific data have led several countries to revised their policies on Cannabis,

endorsing laws that allow its well-documented therapeutic use and decriminalizing or even legalizing it for recreational purposes (Pacula & Smart, 2017).

1.6. Cannabis in the modern world

In the 20th century, the issue of recreational cannabis use prompted the implementation of prohibition policies in most Western countries. Consequently, an extensive illegal drug trade emerged, resulting in a thriving black market that continues to dominate the cannabis consumption landscape (Pisanti & Bifulco, 2017). However, in the 1990s, certain nations began to recognize the medical benefits of cannabis, leading to the acceptance of its medical use. Subsequently, some countries also legalized its recreational use, albeit with the condition that cannabis plants be cultivated in highly secure indoor facilities rather than outdoors (Zuardi, 2006).

During the period of prohibition, amateur breeders selectively bred cannabis varieties that were highly productive and matured quickly, making them ideal for indoor cultivation in basements and garages. These varieties served as the foundation for the establishment of controlled environment cannabis facilities, which have now become multi-billion-dollar enterprises in several countries (Zheng, 2022).

However, while the origins of the cannabis industry can be traced back to the knowledge and strains developed clandestinely by amateurs, the scale and quality requirements of modern operations necessitate the involvement of professional scientific and technological expertise. In fact, recent years have witnessed significant advancements in this regard (Zheng, 2022).

1.7. Cannabis in Portugal

Portugal has made progressive strides towards the legalization and decriminalization of cannabis. In 2001, Portugal decriminalized the possession and use of cannabis, for personal use (SICAD, n.d.). By the law no. 30/2000 and decree-law no. 130-A/2001 instead of facing criminal charges, individuals found with small quantities of drugs are referred to a "dissuasion panel" comprised of legal, social and psychological professionals who evaluate the individual's situation and determine the most suitable course of action, which may involve educational programs or treatment. Concerning medical cannabis, Portugal implemented a medical cannabis program in 2018, Lei n.º 33/2018. The legislation permits patients with specific medical conditions to access medical cannabis products under specific circumstances. To qualify for medical cannabis treatment, patients must possess a medical prescription. Medical cannabis can only be prescribed for conditions where conventional treatment options have demonstrated ineffectiveness or significant

side effects. Some of the approved conditions for medical cannabis treatment in Portugal encompass chronic pain, spasticity associated with multiple sclerosis, chemotherapy-induced nausea and vomiting and Tourette syndrome (*DELIBERAÇÃO N.º 11/CD/2019*) The regulation of medical cannabis in Portugal is overseen by the Portuguese Health Ministry.

The cultivation, manufacture and distribution of medicinal cannabis in Portugal is regulated by INFARMED - Autoridade Nacional do Medicamento e Produtos de Saúde, I.P. (INFARMED, I.P.), the Portuguese National Authority of Medicines and Health Products (Decreto-Lei n.º 8/2019 de 15 de janeiro). To legally cultivate medicinal cannabis in the country, organizations must obtain a license from INFARMED, I.P, obtained after a rigorous assessment of the application by the Portuguese National Authority of Medicines and Health Products, which entails (*DELIBERAÇÃO N.º 11/CD/2019*):

- Appropriate facilities must be established that meet specific standards set out in legislation (Portaria n.º 83/2021 de 15 de abril, including requirements for security and hygiene.
- GACP and GMP guidelines: organizations should follow the Good Agricultural and Collection Practices (GACP), defined by the European Medicines Agency / Committee on Herbal Medicinal Products (European Medicines Agency, 2006), during the cultivation, harvesting, and drying of cannabis to ensure the quality and integrity of the plants. Good Manufacturing Practices (GMP), defined by European Commission / Health and Consumers Directorate-General (*EudraLex - Good Manufacturing Practice (GMP) Guidelines*, 2010), must be adhered to if involved in manufacturing of medicinal cannabis products, covering post-harvest processing operations, packaging, labelling, and quality control.
- Traceability is necessary to monitor the entire production process, and detailed documentation of all activities related to cultivation, manufacture and distribution is crucial for compliance with regulations.
- Quality testing of medicinal cannabis products is required to verify cannabinoid concentration, the absence of foreign material and contaminants like microorganisms, aflatoxins, pesticides and heavy metals.
- Personnel involved in the cultivation and manufacture of medicinal cannabis must be properly trained to follow established procedures.

- Waste Regulation and Safety: Specific regulations may apply to the treatment of cannabis waste. Additionally, safety measures, such as alarm systems and access control, are required.
- Compliance with Legislation: Complying with all national and local laws and regulations related to the cultivation and manufacture of medicinal cannabis is mandatory.

In relation to Good Agricultural and Collection Practices (GACP) and Good Manufacturing Practices (GMP), previously mentioned, these are European common quality guidelines for the production of pharmaceuticals and starting materials of herbal origin. While these two standards share some similarities, they differ in terms of their focus and application. The following are the key distinctions between GACP and GMP:

- Focus: GACP primarily pertains to the cultivation, harvesting and collection of medicinal plants or herbs. Its main objective is to ensure that the plant material is grown, harvested and collected under conditions that preserve its quality, safety, and effectiveness. GACP are particularly relevant in industries where the primary product is plant-based, such as the production of medicinal cannabis. On the other hand, GMP focuses on the manufacturing and processing of finished products, including medicinal products (GMP Part I) or active substances (GMP Part II) to be used for the production of medicinal products. GMP guidelines encompass the entire production process, from the utilization of raw materials leading to a final product. The aim is to ensure that each step is carried out under controlled and consistent conditions.
- Application: GACP is typically applied to the agricultural and horticultural aspects of production. It encompasses practices related to planting, growing, harvesting, drying, and storing plant materials, to be used as starting materials for pharmaceutical industry. The focus is on maintaining the quality and safety of the raw plant material. In contrast, GMP is applicable to medicinal products or active substances manufacturing facilities, encompassing processes such as post-harvest operations, packaging, labelling, and quality control procedures. It also addresses aspects such as process validation, equipment qualification and maintenance, personnel training, and documentation. The emphasis is on ensuring consistent and controlled manufacturing practices.
- Product Stage: GACP is relevant during the early stages of the product's life cycle, particularly when dealing with raw plant material. It is concerned with the cultivation and collection of plants or herbs. On the other hand, GMP becomes relevant during the later

stages of production, focusing on the processing and manufacturing of finished products. It is concerned with transforming raw materials into final products.

The distinction between companies that must comply with different GxP regulations is primarily determined by their production objectives, the type of product they manufacture, and the corresponding production methods. Consequently, the specific authorization or license issued by INFARMED, I.P. varies accordingly. For instance, even if three companies are involved in the sale of dried cannabis flower, it is important to note that these seemingly identical products are actually three distinct entities. Firstly, there is the dried flower that serves as raw material, which falls under the purview of Good Agricultural and Collection Practices (GACP). This type of production requires a cultivation authorization. Secondly, there is dried flower derived from GACP raw material, which can be further categorized into two types. The first type involves dried flower serving as an active pharmaceutical ingredient (API), which necessitates compliance with Good Manufacturing Practices (GMP) Part II. In this case, a manufacturing authorization for active substances is required. Alternatively, the second type encompasses dried flower that functions as a medicinal product itself. This particular category requires adherence to GMP Part I and mandates a manufacturing authorization for medicinal products. Therefore, while these three companies may appear to be engaged in the sale of dried cannabis flower, the variations in their production objectives, product types, and production methods necessitate different GxP regulations and corresponding authorizations/licenses issued by INFARMED, I.P.. This information is summed up in table 1 form annex 7 from Eudralex - Volume 4 - EU Guidelines to Good Manufacturing Practice Medicinal Products for Human and Veterinary Use (*EudraLex The Rules Governing Medicinal Products in the European Union*, 2008).

By following to GMP guidelines, cultivators of medicinal cannabis in Portugal can ensure that their products are manufactured under controlled conditions, meet quality standards, and are safe for patient use. These practices contribute to the overall integrity and credibility of the medicinal cannabis industry (Infarmed, 2023).

The GACP standards are lower than the GMP, which is why the objective of medical cannabis companies is to obtain their GMP certification, to the cost per gram of sale to the public is higher.

1.8. SMC- A Portuguese Medical Cannabis Company

SMC Therapeutic Health Center Production, Lda., hereinafter referred to as SMC+ started its activity in 2019, leveraging Swiss expertise and attracting investments from both Portuguese and

Swiss stakeholders. As a licensed entity, SMC+ specializes in the production of Medical Cannabis and boasts a team of highly qualified professionals from diverse backgrounds who are driven. The entire organizational structure adheres to the strictest standards of compliance mandated by the sector and relevant legislation. SMC+ distinguishes itself as one of the prominent companies committed to ensuring the provision of Premium Medicinal Cannabis to clients. In this sense, SMC+ seeks to establish successful strategic partnerships with leading research institutes and universities to promote cannabis-based pharmaceutical innovation. SMC+ also strives to be an ecologically responsible company, always trying to minimize the impact that the cannabis industry can have on the planet. SMC+ diligently follows the GACP standards and follows the GMP guidelines throughout all stages of production. To achieve the highest quality standards, SMC+ exclusively cultivates Medical Cannabis in indoor growing rooms (Figure 5).

Figure 6. SMC+ cannabis cultivation room. Photos provided by © SMC Therapeutic Health Center Production Lda.

It is important to note that indoor cannabis production offers distinct advantages that are not commonly found in other controlled environment crops (Zheng et al., 2021):

- Only female plants are cultivated, since male plants can pollinate the females leading to seed development and a reduced cannabis quality. The wind can carry cannabis pollen over long distances, making it easier to protect the female plants from male plants in indoor facilities.
- Most cannabis strains require uninterrupted long dark periods to induce flowering, like the autumn season outdoors. Indoor lighting can be artificially controlled to schedule flowering as desired, allowing for up to four annual crops compared to just one outdoor crop.
- The high level of security required by governments is more easily provided in indoor facilities than in outdoor fields.
- Certain cannabis "auto-flowering" varieties thrive under continuous light, which can be provided indoors, resulting in faster plant growth.
- Cannabis inflorescences and buds can be sticky and susceptible to contamination by insects and wind-blown soil outdoors. In contrast, controlled environments can produce extremely clean cannabis material (Y. Zheng, 2022).

In general, SMC+ works as follows (Figure 6):

1) Kown and understand the needs of the market concerning medical cannabis flowers, namely terpene and cannabinoid profile. The raw material (seeds or clones) is acquired according to the market assessment.
2) Cannabis Cultivation: Propagation (3-4 weeks) -> Vegetative Growth (2 weeks) -> Flowering (8-9 weeks) -> Harvest (1 day).
3) Cannabis Processing: Drying (1 week) -> Manual Trimming (4-5 weeks) -> Flowers Sampling for Analytical Purposes -> Primary and Secondary Packaging (1 day) -> Storage (while in stock) -> Expedition.

Over the Cultivation and Processing phases, In-Process Control (IPC) is made to monitor and if necessary to adjust the process and/or to ensure that cannabis product conforms to its specification.

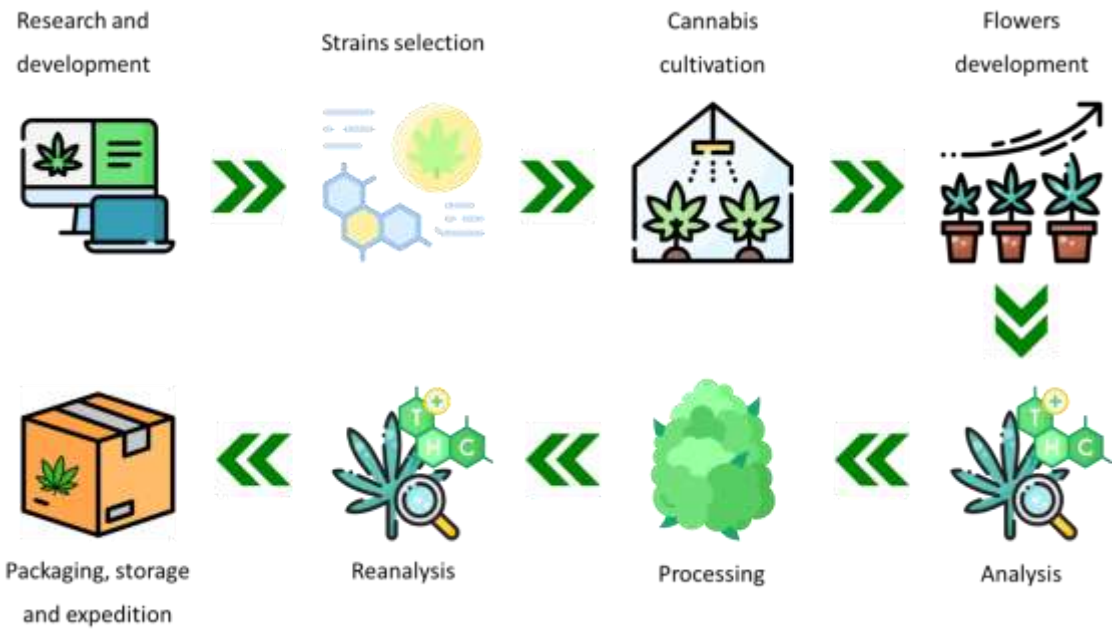


Figure 7. Process of Medical Cannabis production at SMC+.

1.9. Prospecting antimicrobial proprieties of leaf extracts from plant waste envisaging of a circular economy and green agriculture: effect on phytopathogenic fungus and oomycete

Numerous pharmacological scenarios have been explored to investigate the potential therapeutic applications and bioactive mechanisms of crude cannabis extracts and purified compounds derived from C. sativa. These scenarios include the use of these extracts and compounds as anti-convulsive, analgesic, anti-anxiety and anti-emetic therapeutic drugs (Baker et al., 2003). While much research has focused on the psychoactive properties of cannabinoids (Andre et al., 2016), there is now a growing interest in the antimicrobial properties of compounds extracted from cannabis. This is due to the emergence of antimicrobial resistance as a significant threat to human health worldwide (Ferri et al., 2017), but also in the agrifood sector. To mitigate the human and economic impacts of this problem, as well as the emergence of new microbial pathogens, it is necessary to identify and elucidate new antimicrobial therapies (Vouga & Greub, 2016).

Given that agriculture is currently an area that is overexploited to meet humanity's basic living needs (Pimentel, 2012), there is a lot of research being carried out to make this area greener and less invasive for the planet (Wezel et al., 2014). One of these solutions involves using plant extracts instead of synthetic fungicides to combat phytopathogenic fungi (Gahukar, 2012). Synthetic

fungicides are very effective at fighting these pathogens, but at certain concentrations they can be phytotoxic and as being resistant to natural degradation, or even producing intermediates more toxic than the original compound, they contaminate and accumulate in soils, water tables thus jeopardizing human health. Furthermore, fungi can adapt to the synthetic fungicide, since it has a specific target, resulting in the emergence of resistant to the treatment (Damicone & Smith, 2009). Plant extracts, on the other hand, are characterized by having a vast array of secondary metabolites, many of which have antifungal properties (Gahukar, 2012). Additionally, the diversity of bioactive phytochemicals generally corresponds to different modes of actions and cellular targets, making the probability of raising resistance on the part of pathogens much lower when comparing with the synthetic fungicides.

Fungal plant pathogens have diverse lifestyles depending on their interaction strategies with the host plant. They can be classified into three main groups: necrotrophic, biotrophic, and hemibiotrophic. Necrotrophic fungi kill host plant cells and feed on the dead tissues. Biotrophic fungi colonize living plant tissues and use them as a source of nutrients. Hemibiotrophs initially establish a biotrophic relationship with the host plant but later kill the plant tissues and feed on them (Doehlemann et al., 2017). Within the biotrophic group, there are endophytes that establish biotrophic interactions without causing visible symptoms in plants. One example of this is mycorrhizas, which form mutualistic interactions with host plants and play a crucial role in terrestrial ecosystems (Lo Presti et al., 2015).

Phytopathogenic fungi can infect any part of the plant, with many developing in the aerial parts where symptoms are easily noticeable. However, there are also fungi that infect plants through the root system, making it challenging to detect initial symptoms (Coque et al., 2020). These phytopathogenic fungi have significant agronomic and economic impacts as they pose threats to agricultural production at all stages of growth, including post-harvest diseases. In this study, the focus is on phytopathogenic fungi and an oomycete, which is included for practicality and writing purposes (Lo Presti et al., 2015).

Limited research has been conducted on the antifungal properties of cannabis and its secondary metabolites. While the antibacterial activities of cannabis have been extensively studied, the antifungal effects have received less attention. However, some studies have reported that plant extracts derived from cannabis can effectively control pathogenic fungi (Berardo et al., 2024). For instance, Khan and Javaid (2020) demonstrated that different fractions prepared from *C. sativa*

leaf extract (from 1.562 to 200 mg mL^{-1}) were effective against *Aspergillus flavipes*, which are common human pathogenic fungi responsible for black mold in fruits and vegetables. Among them, *n*-butanol fractions showed the highest antifungal activity causing 68–82% growth inhibition in the pathogenic fungal biomass. Chloroform and *n*-hexane fractions also showed a remarkable reduction of 52–82% and 42–82% in *A. flavipes* growth over control, respectively (Khan & Javaid, 2020). In another study conducted by Tapwal (2013), the antifungal activity of aqueous extract of *Cannabis sativa*, *Parthenium hysterophorus*, *Urtica dioeca*, *Polystichum squarrosum* and *Adiantum venustum* was investigated against *Alternaria solani*, *Alternaria zinniae*, *Curvularia lunata*, *Rhizoctonia solani* and *Fusarium oxysporum* at different concentrations (5, 10, 15 and 20%). The leaf extract of cannabis was the most effective to restrict the growth of all the pathogens. At 20% concentration, 100% growth inhibition was recorded against *C. lunata,* followed by *A. zinniae* (59.68%), *A. solani* (53.53%), *F. oxysporum* (47.96%) and minimum 47.59% for *R. solani* (Tapwal et al., 2011).

The effect of cannabis leaf extract on fungi in general can vary depending on various factors such as the type of fungus, concentration of the extract and the specific compounds present in the extract. While there is some evidence to suggest that certain components of cannabis, such as cannabinoids and terpenes, may exhibit antifungal properties (Berardo et al., 2024; Khan & Javaid, 2020), it's important to note that the research in this area is still limited.

In this work we will focus on phytopathogenic microorganisms that affect important crops in Portugal but also of relevance worldwide, namely, *Diplodia corticola*, which severely affects *Quercus suber* (cork oak), *Colletotrichum acutatum*, a major cause of losses in *Fragaria* spp. (strawberries), and *Phytophthora cinnamomi*, a devastating oomycete with major impact in *Castanea sativa* (chestnut tree).

1.9.1. *Diplodia corticola*

Diplodia corticola is a type of ascomycete fungus that belongs to the Botryosphaeriaceae family (Muñoz-Adalia et al., 2022). It exhibits a hemibiotrophic lifestyle, transitioning from biotrophy to necrotrophy after causing stress in the host plant (Oliveira Fernandes, 2015). This fungus has the ability to produce secondary metabolites with phytotoxic, antibacterial and antifungal properties (Masi et al., 2016). While the exact mechanism of infection is not fully understood, studies suggest that phytotoxins, degradative and oxidative enzymes, and cytotoxic proteins play a role in the process (Félix et al., 2017).

Diplodia corticola affects various plant species, including grapevines, eucalypts and oak trees from the *Quercus* genus (Félix et al., 2017). We chose this species due to the economic value and ecological significance of cork oak forests in Portugal. The cork stopper sector alone represents a billion-euro turnover in Portugal. Cork oak forests are vital agro-forestry ecosystems rich in biodiversity and playing a crucial role in CO_2 sequestration, with a cork oak forest covering 30% of an area capable of fixing approximately 3.2 tons of CO_2 per hectare per year (APCOR, 2020). The decline of cork oak forests in the Iberian Peninsula poses significant ecological and economic challenges. *Diplodia corticola* is particularly associated with the decline of cork oak, causing canker disease. Symptoms of infection range from seedling mortality to necrosis, wilting, dieback of branches, and reduced phellem regeneration after cork extraction (Félix et al., 2017).

1.9.2. *Colletotrichum acutatum*

Colletotrichum acutatum is a fungus belonging to the *Phyllachoraceae* family (Peres et al., 2005). The fungus initially appears as white colonies that later become covered by pink to orange-coloured conidia. One of its most distinctive morphological characteristics is the acute ends of its conidia, although it can also have conidia with other shapes (Damm et al., 2012).

This ascomycete has a hemibiotrophic lifestyle, which means it depends on various factors such as the host, affected tissue and environment. For example, on apple trees, species belonging to the *C. acutatum* complex cause necrotrophic lesions on fruit but do not harm leaves. On orange trees, they exhibit a necrotrophic lifestyle on flowers and a biotrophic lifestyle on leaves (Baroncelli et al., 2017). Studies have shown that the fungus penetrates the cuticular layers of fruits, forming highly branched and differentiated hyphae. Once inside the cells, it colonizes and multiplies, producing conidia (Liao et al., 2012).

Colletotrichum acutatum is considered one of the top 10 most important fungal pathogens due to its economic impact. It affects a wide range of hosts, including strawberries, apples, peaches, grapes, almonds, citrus, lupin and olive trees (Tomas-Grau et al., 2019). Anthracnose, also known as bitter rot, is one of the main diseases caused by *C. acutatum* and is commonly associated with strawberries, apples, olive fruit, and chili peppers (Børve & Stensvand, 2006).

1.9.3. *Phytophtora cinnamomi*

Phytophthora cinnamomi is an oomycete that poses a significant threat to natural ecosystems and horticultural crops worldwide (Engelbrecht et al., 2021). Belonging to the kingdom Chromista, phylum Oomycota or pseudofungi, class Oomycetes, order Peronosporales, family

Peronosporaceae, this pathogen exhibits a hemibiotrophic lifestyle with both biotrophic and necrotic phases (Boughanmi et al., 2022). Initially identified on cinnamon trees in Sumatra in 1922, it is believed to have originated in Papua New Guinea but has now spread globally (Hardham, 2005). Although initially thought to be a fungus due to its growth patterns, it is also a filamentous microorganism, *Phytophthora cinnamomi* differs from true fungi in several ways. It spends most of its life cycle as a diploid, possesses a cell wall composed of cellulose and β-glucans instead of chitin, produces biflagellate zoospores and lacks the ability to synthesize sterols (Boughanmi et al., 2022). This oomycete is considered one of the top 10 plant pathogens among oomycetes, causing significant economic losses in over 76 countries (Chen et al., 2022).

Phytophthora cinnamomi infects a wide range of plant species (Andrade Lourenço et al., 2022). It primarily infects feeder roots, leading to root rot and impairing water uptake and transport to the shoot. This results in wilting and chlorosis of the leaves. The infection can either lead to rapid plant death or remain asymptomatic for extended periods (Hardham & Blackman, 2018). One particularly affected species is the chestnut tree, which occupies approximately 2 million hectares and plays a crucial role in European agriculture. Chestnut production has significantly declined since the 20th century, with losses amounting to around 300 million euros. The ink disease caused by *Phytophthora cinnamomi* is a major problem, particularly in Portugal (Boughanmi et al., 2022; de Andrade Lourenço et al., 2022).

1.10. Scientific problem

Cannabis sativa is one of the most useful and versatile of plant species that has been exploited by humans for millennia. The use of Cannabis plant for fuel, materials, textiles and medicinal purposes has been extensively reported over the years. Nowadays, Cannabis is gaining momentum for various medical purposes as their therapeutic qualities are becoming better established. In the last few years, the Cannabis global market has shown a rapid expansion, being estimated that the Cannabis market will be worth almost 60 billion euros in the next ten year in Europe alone.

Techniques for growing cannabis are increasingly advanced, however, throughout the process there are still some challenges that need to be overcome. One of these challenges is the emergence of male structures from feminized seeds. At SMC+, whose work is focused on developing flowers with high levels of THC, only female plants are valuable, as previously stated. Therefore, the use of feminized seeds (seeds that, after germinating, will produce purely female plants) is an added value for the company. However, during some cultivations, the emergence of male structures was observed in these plants. These male structures, if not controlled, can release pollen that can pollinate the female flowers, producing seeds, compromising the entire cultivation. Not only could THC levels drop, as the plant stops directing its energy towards the formation of secondary metabolites and starts channeling them into the formation of seeds, but it could also compromise the company's business, as it has a license to the sale of flowers and not seeds. After some research it was found that one of the causes for this event may be related to environmental factors. Therefore, the first objective of this work was to verify whether temperature, especially heat stress, can cause male structures to emerge in feminized plants.

Another of the cannabis industry's major concerns is its expenditure and excessive use of resources, so one of the main objectives is to make companies increasingly environmentally friendly. To this end, a study was also carried out to see if it would be possible to assess the maturation levels of female flowers without resorting to the use of HPLC, which involves the use of a lot of reagents and disposable material, essentially made of plastic, that cannot be used again. Therefore, it would be extremely enriching for the environment if this macro and microscopic visualization were sufficient to determine THC levels in plants throughout maturation.

Since sustainability is one of SMC's foundations, another aim of this research was also to assess whether methanolic extracts obtained from plant residues from cannabis plantations, in this case the leaves (Figure 8), have antifungal and anti-oomycetal activities, so that they are no longer just

a waste product, which used to be disposed of through expensive processes, but become a co-product with commercial value and a key product for a more sustainable and green agriculture. This is a major step forward for the company, as it is using a product that it "involuntarily" produces, thus obtaining more monetary value and, above all, becoming more environmentally friendly.

Figure 8. Residues (leaves) from cannabis plantations. Photos taken by the author at SMC+.

2. Materials and methods

2.1. Impact of temperature on the development of male structures in feminized plants of cannabis.

To understand the role of temperature in the development of male structures in plants from feminized seeds, flowers from 2 different batches of cannabis, Vanilla Frosting strain, were analysed. this variety is THC dominant (between 20% and 22%) and CBD < 1%. The supplier of this strain is Pharmaseeds. The first crop, with 2044 plants, was grown from September to November 2022 (flower induction at October 6th), while the second batch, with 1286 plants, was grown from March to May 2023 (flower induction at April 7th). Upon flower induction, all plants were monitored for 7 and 5 weeks, respectively. The development of male and female structures in each plant of both crops was assessed, where each plant was evaluated at least once a week, and represented on a grip map, in which each cell represents a plant. Plants with male structures were represented on the grip map by a red cell whereas the remaining cells illustrated plants with only female flowers. The temperature of the cultivation rooms was continuously registered by pre-installed temperature sensors (HIDROTEK KHT). The strength of association between high temperature and male structures development was calculated using the Spearman´s rank correlation method.

The impact of male structures development on the cannabinoid profile of cannabis flowers was also assessed. In this sense, on the last week of cultivation, a representative sampling of male and female flowers was performed and their levels of cannabinoids, namely Δ9-Tetrahydrocannabinol (Δ9-THC), Δ8-Tetrahydrocannabinol (Δ8-THC), Cannabidiol (CBD), Cannabinol (CBN), Cannabidiolic acid (CBDA), Δ9-Tetrahydrocannabinolic Acid (Δ9-THCA-A) and Cannabigerolic acid (CBGA), quantified by High Performance Liquid Chromatography (HPLC).

2.2. Assessment of flower development and maturation in cannabis by macro and micro visualization

Assessment of flower development and maturation in cannabis can be performed using macro and micro visualization techniques. These methods allow researchers and cultivators to examine the plant's growth stages, determine its readiness for harvest and analyse its reproductive structures. Macro visualization involves examining the plant, observing its growth patterns and tracking its developmental stages. This includes assessing the height, branching structure, leaf development and overall vigour of the plant. These macroscopical observations provide an initial assessment of

the plant's maturity and general health. Micro visualization involves the use of microscopes and binocular magnifiers to examine the finer details of the plant's reproductive structures and cellular development, specifically trichomes.

The cannabinoid profile, namely the THC pattern, of the flowers throughout their development and maturation was also characterized using HPLC.

2.3. Harvesting plant material and preparation of cannabis leaves extracts

Three prunings were carried out in a cannabis production room. The pruning took place on three different dates: March 3rd, March 16th, and March 25th. At the time of each pruning, leaf samples were taken. The collected leaves were washed with deionized water and then air-dried in the dark for 14 days. The leaves were crushed and ground to a fine powder using a blender. For the extraction of bioactive compounds, the powder was added to methanol (Fisher chemical, cod: u/4056/17 at 99.8% w/v) on a mass:volume proportions of 0.01 g/mL (m = 0.4 g; V v = 40 mL) and maintained under orbital agitation (Heidolph Multi Reax Vortex Mixer, 440 rpm), in the dark, for 72 hours. After that, 30 mL of the extract was centrifuge at 440 rpm, at room temperature, for 10 min, using a Domel Centrifuge Centric 350, and the supernatant was sterilized using a syringe (Agilent Technologies, Canada) and PFTE filter (0.20 μm; Agilent Technologies, Canada). The extract was stored in the cold at 4 °C until use.

As a standard procedure, since it is not permitted by law to transport any type of biological material with THC values higher than 0.2 % outside the production unit, the methanolic extracts were mixed, since the aim is to treat the waste, and then were evaluated to quantify the cannabinoids, specifically THC, by HPLC. As it was found that the THC levels were quite high, to continue the investigation, the extract were diluted (1:5 v/v), adding 5 mL of extract to 20 mL of MeOH, and further HPLC analysis was carried out to check that the new extract met the requirements. A schematic representation of the main steps of these procedures are depicted on Figure 9.

To obtain the dry methanolic extract, the solvent was evaporated at 35 °C, 60 rpm, under reduced pressure in a Buchi rotavapor R-200, with bath temperature at 36 °C, vacuum at 100 mbar and 160 rotations per minute. About 0.0301 g of dry extract was collected. Stock solutions were prepared with methanol, at a concentration of 6.02 mg/mL, for phytotoxic and antifungal assays.

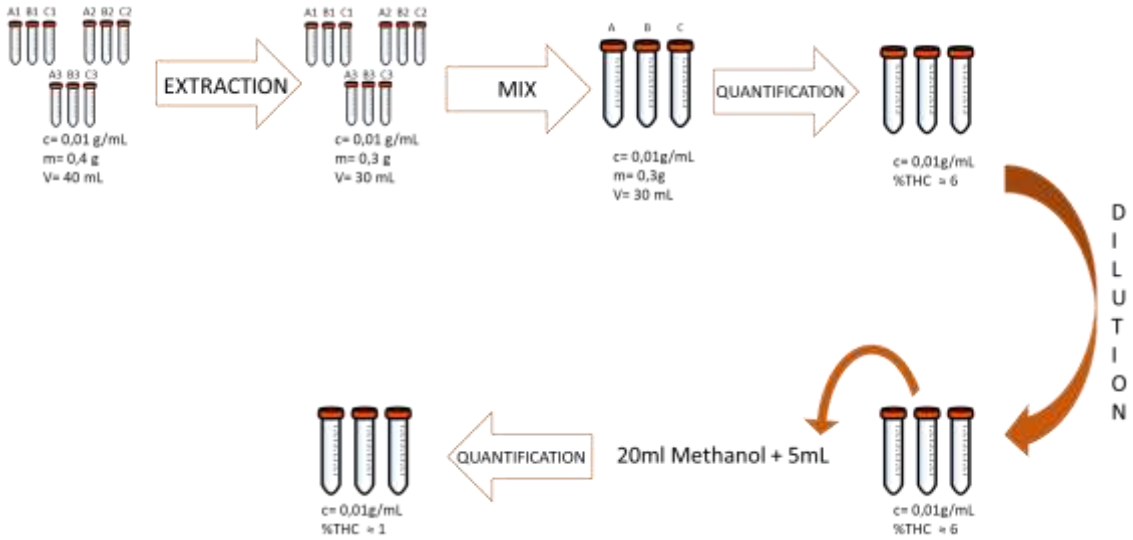


Figure 9. Schematic of the part of the preparation of the cannabis leaf extract carried out at SMC. Three replicates were carried out (A, B and C), at three different times when the leaves were collected (1, 2 and 3), which were ground into a fine powder. The compounds were then extracted with MeOH. The replicates were treated independently until the mixing phase, where the three moments were unified for the quantification of the cannabinoids. After quantification, a dilution was made to reduce the THC content.

2.4. Identification and quantification of classes of metabolites

2.4.1. Determination of cannabinoid by HPLC

Prior to analysis, 50 µL of extract was diluted with 950 µL MeOH(Fisher Chemical) and filtered using a syringe (Agilent Technologies, USA) and PFTE filter (0.20 µm; Agilent Technologies, USA) directly into 2 mL HPLC vials (Agilent Technologies, USA). Standard calibration solutions were prepared from individual commercially available certified reference standards(Δ9-THC, Δ8-THC, CBD and CBN were purchased at a concentration of 1000 µg/mL in MeOH and CBDA, Δ9-THCA-A and CBGA were obtained at a concentration of 1000 µg/mL in acetonitrile, obtained from LGC GmbH (Luckenwalde, Germany)), at 1 mg/mL each in organic solvent. Equal volumes of each reference standard were mixed and diluted with HPLC grade MeOH to a high concentration of 50 µg/mL. Serial dilutions were made to create a five-point calibration curve at concentrations of 50 µg/mL, 10 µg/mL, 5 µg/mL, 1 µg/mL and 0.5 µg/mL. Regenerated Cellulose (RC) filter membrane and a Hydrophobic Polytetrafluoroethylene (PTFE) filter membrane (both with 0.20 µm; Agilent Technologies, USA) were used to filter mobile phase A, 0.1% V/V formic acid (Fisher Chemical) in water (generated from an in-house ultrapure water

purification system from Biosan (Riga, Latvia)) and mobile phase B, 0.05% V/V formic acid in MeOH respectively.

An Agilent 1260 Infinity II LC system equipped with a quaternary pump, solvent degasser, column heater, and temperature controlled autosampler (Agilent Technologies, USA) was used for chromatographic separation. The column was an InfinityLab Poroshell 120 EC-C18, 4.6 x 50 mm, 2.7 μm with a guard column InfinityLab Poroshell 120 EC-C18, 4.6 x 5 mm, 2.7 μm (Agilent Technologies, USA) and was controlled at 50 °C. The mobile phases were programmed at a flow rate of 1 mL/min. The separation was achieved using a gradient as follows: 0 min, 60 % B; 1.0 min, 60 % B; 7.0 min, 77 % B; 8.2 min, 95% B, with a stop time of 9.5 min and a post run of 1.5 min. The autosampler was maintained at room temperature and the injection volume was set to 5 μL.

For the quantification of phytocannabinoids, the moisture content of samples was determined by loss on drying gravimetric method using the Mettler-Toledo HX240 Halogen Moisture Analyser. Samples were placed in weighing plates and were dried at a temperature of 105 °C until the shutdown criterion of 1 mg/90 s occurred. All samples (200 mg) were analysed in triplicate. The final amount of analyte [%] was calculated according to German Pharmacopoeia Cannabis Flos Monograph (DAB, 2018), as followed:

$$\frac{C_{r-a} * G_{r-a} * 100}{C_u * (100 - t)}$$

C_{r-a}= concentration in the test solution in milligrams per millilitre, calculated using function of the calibration solution I_a to I_f

G_{r-a}= concentration in RN in per cent

C_u= drug concentration in milligrams per millilitre

t= loss on drying of the drug per cent

2.4.2. Determination of terpene by GC

Initially, 0.2 g of crushed cannabis leaves were transferred to a GC vial. Standard calibration solutions were prepared from individual commercially available certified reference standards (Cannabis Terpenes Standard #1 (Catalog No. 34095) and Cannabis Terpenes Standard #2 (Catalog No. 34096) were purchased at a concentration of 2500 μg/mL in Isopropanol, obtained from Restek (Bellefonte, PA, USA)) at 2.5 mg/mL each in organic solvent. Equal volumes of each

reference standard were mixed to a high concentration of 1250 µg/mL. Serial dilutions were made to create a seven-point calibration curve at concentrations of 1250 µg/mL, 500 µg/mL, 200 µg/mL, 100 µg/mL, 50 µg/mL, 20 µg/mL and 10 µg/mL. 5 µL of each standard solution were then transferred, making a total of 10 µL, into the respective GC vials.

An Agilent 8860 GC system equipped with a FID detector (Agilent Technologies, USA) interfaced with a 7697A Headspace Sampler (Agilent Technologies, USA) was used for the analysis. Separation was accomplished on an Agilent VF-35ms column (30 m length, 0.25 mm i.d. and 0.25 µm film thickness). Injections were carried out in a split mode using a split/splitless liner (Agilent Technologies No. 5190-4047). The temperature programme starts at 60 °C, increases to 150 °C (at 45 °C/min) and holds for 0 min, then increases to 250 °C (35 °C/min) and holds for 0.5 min, for a total run of 10 min. 10 µl of each sample was injected with helium as a carrier gas (constant flow mode, 3 mL/min) using a split ratio of 100:1. Temperatures applied are 120 °C for injector, 140 °C for transfer line and 300 °C for FID detector.

To quantify the terpenes, areas of targets terpenes in the chromatograms were integrated for each identified compound to achieve the final amount of analyte [ppm].

2.5. Evaluation of bioactivities of cannabis leaves extract

2.5.1. Evaluations of the antimicrobial activity on phytopathogenic fungi and oomycete

Antimicrobial effects of the plant extracts were evaluated against two species of filamentous fungi: *Colletotrichum acutatum*, *Diplodia corticola* and one oomycete *Phytophthora cinnamomi*. (Table 2).

Table 2 Phytopathogenic fungi and oomycete used and their suppliers.

Species and strains	Suppliers
***Diplodia Corticola* CAA500**	Ana Cristina Esteves (Centre for Environmental and Marine Studies, University of Aveiro)
***Colletotrichum Acutatum* 15-015**	Pedro Talhinhas (Instituto Superior de Agronomia, School of Agriculture, University of Lisbon)
***Phytophthora Cinnamomi* PH107**	Helena Machado (National Institute of Agrarian and Veterinary Research)

In a flow chamber, 50 µL of extract was incorporated in 20 mL of melted Potato Dextrose Agar (PDA; Biolife) medium (final concentration of 15 µg/mL) at 50 °C, before plating. Two negative controls were also prepared, the volume of extract used (50 µL) was replaced by the solvent of the extract (solvent control), and for water (medium control). After solidification, a small portion (8-mm diameter) of fungal mycelium was obtained from the margins of a fully covered culture in a Petri dish and placed in the middle of the prepared Petri dishes, and incubated at 30 °C, in the dark. Four replicates of each condition were prepared. The growth of the filamentous microorganisms was evaluated daily by measuring two diameters of the mycelium colonies, in all plates, till the controls reach the margins of the Petri dishes. At this timepoint, the percentage of growth inhibition was calculated using the formula:

Inhibition (%) = $\frac{DC-DTi}{DC} * 100$, where DC is the mean mycelial diameter of the solvent control and DTi is the mycelial diameter of the replica i of a treatment. The mean % of inhibition of each treatment was estimated from the % of inhibition obtained for the respective replicates.

2.5.2. Evaluation of the phytotoxicity in vitro using mustard as a model

To test the phytotoxicity of cannabis leaf extract, 25 or 50 µL were incorporated into melted MS medium (2% w/v sucrose, 0.8% w/v agar; Murashige & Skoog, 1962) to prepare, respectively, 7,5 and 15 µg/mL final concentrations and poured into glass culture flasks. As methanol is inhibitory of seed germination (Mehrafarin et al., 2011) a control was prepared where the maximum volume of extract was replaced by using the same volume of solvent. Also, a negative control was prepared where the maximum volume of extract was replaced by sterile deionized water.

Seeds of mustard (*Sinapis alba* L.), variety white, were disinfected with 0,5 % (v/v) bleach (Moderna) for 20 min under agitation, and then washed thoroughly 3 times with sterile deionized water. In a flow chamber, the seeds were inoculated on the culture medium, 6 per flask, and 4 replicate flasks per condition. The flasks were maintained in a culture room under controlled conditions (temperature 23°C, photoperiod 16h and light intensity 6(36w)- 50 to 95 µmol/m2/s) and germination and early plantlet growth was monitored for 6 days. The number of germinated seeds were counted every day (seeds were considered germinated when the radicle had at least 1 mm length). The assay ended 6 days after inoculation and the germination percentage was calculated by the formula:

$$Germination\ percentage\ (\%) = \frac{Number\ of\ germinated\ seeds}{Total\ number\ of\ seeds} * 100$$

Also, plantlets biometric parameters were assessed, namely: hypocotyl length, root length, number of leaves and the longest leaf length.

2.6. Statistical analysis

A Spearmen correlation test was carried out to see if there was a relationship between the temperature peaks and the number of male structures per table using GraphPad Prism version 10.0.0 for Windows (GraphPad Software, Boston, Massachusetts USA, www.graphpad.com).

All the results are presented as the mean ± standard deviation (SD) of at least 3 independent replicates both in antimicrobial and phytotoxicity assays. Data was analysed by one-way and two-way ANOVA, after the homogeneity of variances between groups was verified with Bartlett's test, and followed by post-hoc tests for multiple comparisons using GraphPad Prism version 10.0.0 for Windows (GraphPad Software, Boston, Massachusetts USA, www.graphpad.com). The post-test Dunnett was used for comparisons between treatment groups and the control, and the post-test Tukey and Sidak were used for comparisons between all group means. When comparing with the control group the level of significance (p value) of each test is denoted in the figures using the asterisk notation [ns ($p > 0.05$) - non-significant, * ($0.01 < p \leq 0.05$) - significant, ** ($0.001 < p \leq 0.01$) - very significant, *** ($0.0001 < p \leq 0.001$) - highly significant, and **** ($p \leq 0.0001$) – very highly significant]. When comparing each mean with all the other means, the lettering notation was used, in which means with a common letter are not significantly different ($p > 0.05$).

3. Results and discussion

3.1. Impact of temperature on the development of male structures in feminized plants of cannabis.

In order to understand if abiotic factors such as temperature can or cannot influence the emergence of male structures/organs in plants originated from theoretically feminized seeds, two batches of cannabis, Vanilla Frosting variety, were monitored, both in the same growing room, and both also from seed. The first crop was grown from September to November, and flowering was induced on October 6th. The second crop was grown from March to May, and flowering was induced on April 7th.

For the first cannabis batch, Vanilla Frosting variety, Figure 10 A and B show, respectively, the number of plants with male structures per table in the growing room along time and the temperature variation throughout the flowering process. The mapping of the room was carried out to see if there was an agglomeration of plants with male structures in specific areas, due to the presence of microclimates (Figure 11).

The same analyses were carried out for the second batch where Figure 12 A and B show, respectively, the number of plants with male structures per table in the growing room along time and the temperature variation throughout the flowering process and Figure 13 is a representation map of the room.

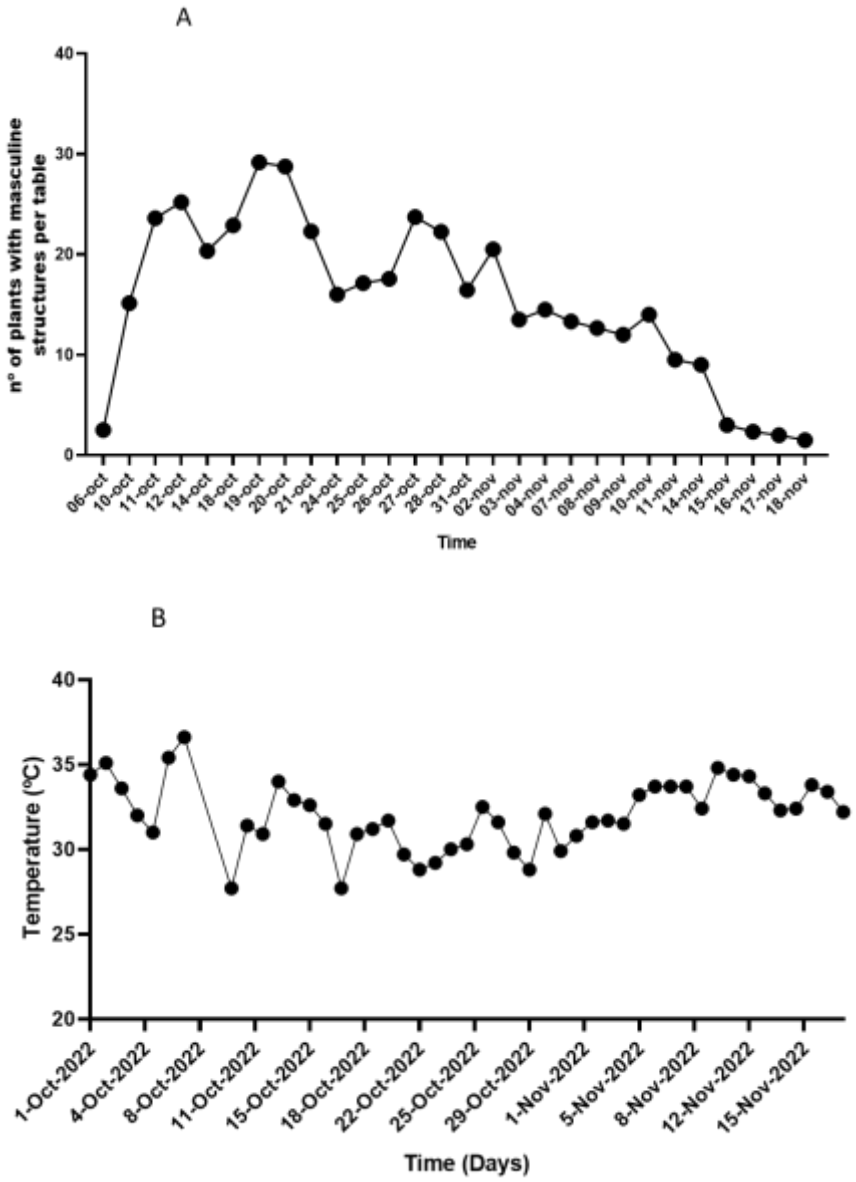


Figure 10. Average variation in the number of plants with male structures per table in the grow room over time in the first batch of cannabis, Vanilla Frosting variety. Variation in the maximum temperature reached in the grow room over the weeks of flowering, from 1st of October to 18th of November (B).

As it can be seen in Figure 10 A., in relation to the first batch of Vanilla Frosting, the presence of structures of any kind, either female or male, was not verified on the date of floral induction (October 6th), which is to be expected. Just four days after flower induction, an average of 15 plants per table already had male structures, alongside the female ones. This number increased to 25, then dropped briefly, followed by a further increase, where the highest number of plants with structures per table was observed, around 30 on average, on October 19th. Over the remaining

30 days, there was a downward trend in the number of plants with male structures, reaching values close to 0 on the final days of observation. As for the temperature, it initially shows large differences over the days, but towards the end of the growing season it tends to be more stable (Figure 10 B.)

When evaluating the 2 parameters, temperature and number of plants with male structures per table, an interesting feature was seen. On October 1st, the growing room reached a peak temperature of 35 °C, and about 11 days later, the first peak in the number of plants with male structures per table was reached. A new peak temperature was reached on October 7th, 37 °C, the highest during the whole process, and about 12 days later there was again a peak in the number of male structures per table in the room, around 30, the highest during the whole process. The temperature in the room, after decreasing, again reached a maximum temperature of around 34°C on October 13th, and around 13 days later, the number of plants with male structures per table also increased, peaking at around 24 plants with male structures per table.

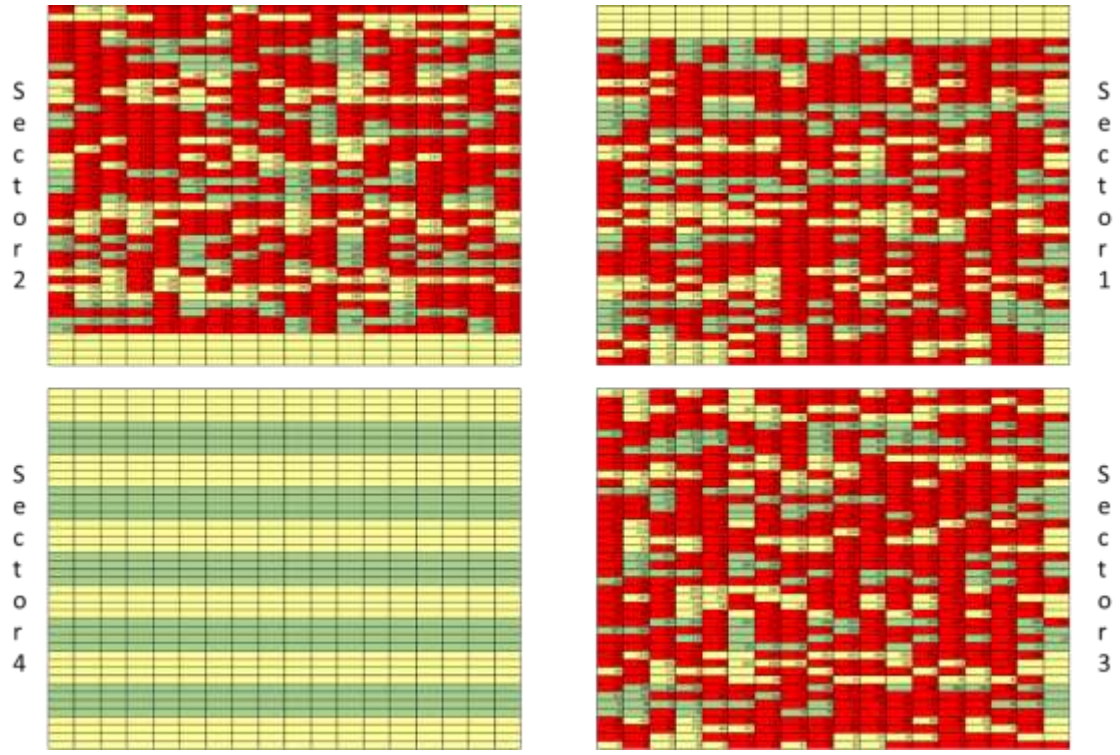


Figure 11. Representative diagram of the grow room. Of the four sectors available, only 3 were used, sectors 1, 2 and 3. Each sector is divided into 11 tables and each table holds an average of 67 plants. Each red cell represents plants that have developed male structures, the remaining cells represent plants that have only produced female flowers/structures.

In this first cultivation, out of 2044 plants, 1329 not only produced female flowers but also male structures. This corresponds to 65% of the entire crop. Initially it was thought that when mapping the plants that produced male organs, we would have zones with a greater agglomeration of plants with male structures than others, i.e., inside the same room there would be microclimates, specific zones where the temperature would be higher, which would enhance the emergence of these structures. However, after the mapping, it was verified the absence of positive or negatively affected zones and that the arising of these structures is random and, therefore, the tolerance of each plant to abiotic factors might be different (Figure 11).

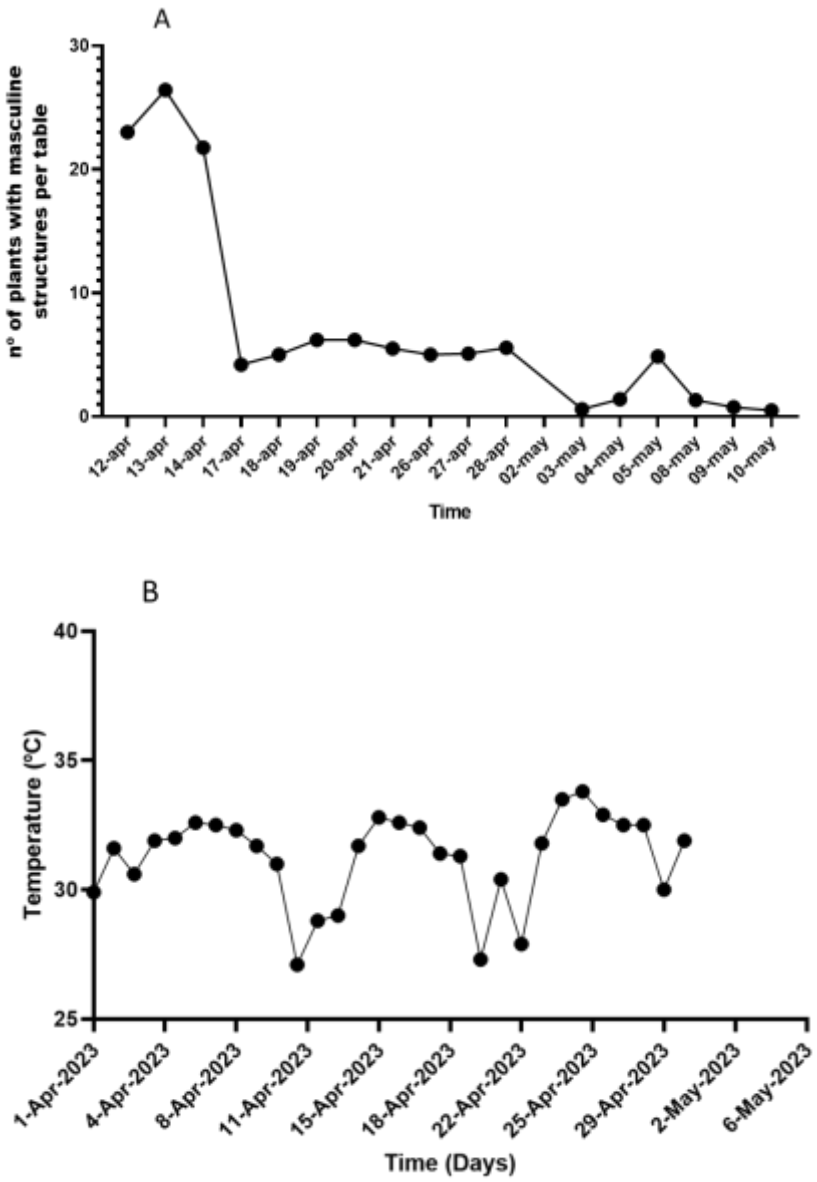


Figure 12. Average variation in the number of plants with male structures per table in the grow room over time in the second batch of cannabis, variety Vanilla frosting (A). Variation in the maximum temperature reached in the grow room over the weeks of flowering, from 1st of April to 1st of May (B).

As it can be seen in Figure 12 A., in the second batch of cannabis, variety Vanilla frosting, flowering was induced on April 7th 2023, but the first assessment of male structures was only carried out on April 12th, because, as seen earlier, it was around 5 days after flowering was induced that was reached a more significant amount of these structures. After reaching a maximum of around 25 plants with male structures per table on April 13th, this number drops radically to around 5 plants after just 3 days, which remains until the end of the crop, reaching 0. The maximum temperatures reached in the room during this cultivation batch were lower than the first, which at times exceeded

35 °C and in this second batch never reached it. As represented in figure 12 B, after a few days of stable temperatures, there was an accentuated decline on April 10th, followed by a further rise in temperature until April 14th, when the peak of maximum temperature was reached. As was the case with the first batch, after about 12 days the number of male structures per table increased. On May 3rd, no male structures were detected on the plants, but 12 days after that temperature peak, an average of 5 plants with male structures per table were detected.

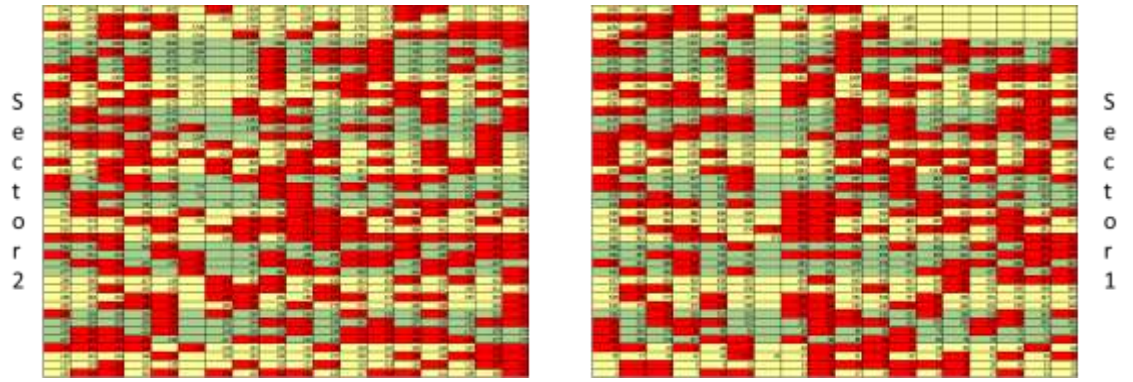


Figure 13. Representative diagram of the grow room. Of the four sectors available, only 2 were used, sectors 1 and 2. Each sector is divided into 11 tables and each table holds an average of 67 plants. Each red cell represents plants that have developed male structures, the remaining cells represent plants that have only produced female flowers/structures.

In this second batch, out of 1286 plants, 534 not only produced female flowers but also male structures. This corresponds to 41,5% of the entire crop. Just as was seen in the first cultivation batch, there are no specific areas of agglomeration of plants with male structures in this one, refuting the idea that there are microclimates in the grow room (Figure 13). However, in this second batch, the percentage of plants with male structures is considerably lower. This may be directly related to the temperature because, as can be seen in Figure 10 B and Figure 12 B, the maximum temperatures reached in the first cultivation batch were higher than those reached in the second batch.

Taking into account the results obtained in the 2 independent batches, it appears to be a direct relationship between high temperatures and the arising of male structures in feminized plants since after around 12 days of reaching a very high temperature peak, there is also a peak in the number of male structures in the plants per table. A Spearmen correlation test was carried out to see if there was a relationship between the temperature peaks and the number of male structures per

table. Taking into account the high temperature peaks mentioned and the number of plants with male structures verified around 10-13 days later, the r value in Spearmen's test was 1, showing that there is a relationship between these 2 factors.

In terms of the differences in THC levels between plants with male structures and plants without male structures, no significant differences were found. This is because the male structures are removed before they are able to pollinate the female flowers, preventing the formation of seeds.

The occurrence of hermaphroditic inflorescences, characterized by the presence of masculine structures in female plants during the cultivation of cannabis for medical purposes poses a challenge for growers. This is because the formation of seeds as a result of this spontaneous development diminishes the quality of the harvested flower (Punja & Holmes, 2020). The allocation of resources by the female plant towards pollen production, followed by seed production, can lead to a significant decrease (up to 56%) in the levels of secondary metabolites in the pollinated flowers compared to unfertilized female flowers (Meier & Mediavilla, 1998). Consequently, inflorescences that contain seeds are of inferior quality and are often unsuitable for sale.

The factors which trigger this change in phenotype have not been extensively researched. This is due, in part, to the restrictions placed by government or regulatory agencies on conducting research experiments on flowering cannabis plants, which reduces the opportunity to conduct the types of controlled experiments that are needed to elucidate the basis for hermaphroditism.

In a recent study, applications of silver thiosulfate induced male flower formation on genetically female hemp plants (Lubell & Brand, 2018). These findings demonstrate that changes in growth regulator levels in treated plants can impact hermaphroditic flower formation.

Numerous studies have indicated that epigenetic mechanisms may play a role in the regulation of sex determination in hemp, in addition to genetic factors (TRUŢĂ et al., n.d.). The phenotypic manifestation of sex expression in hemp can be influenced at the transcriptional or post-transcriptional level, without any changes in the DNA or alterations in chromatin structures (Soldatova & Khryanin, 2010). These studies have also provided evidence for the involvement of endogenous phytohormones in the regulation of genetically determined sex types, as well as the potential for modification by exogenous factors such as ions, phytohormonal treatments, and environmental conditions like photoperiod (TRUŢĂ et al., n.d.). External factors have a significant impact on the modulation of sex expression in monoecious plants, where both staminate and

pistillate flowers are induced in varying proportions. Schaffner (1921) reported that female hemp plants grown under low light winter conditions produced male flowers, whereas those grown under normal spring conditions did not exhibit male flower development (Schaffner, 1921).

The phytohormones gibberellins, auxins, ethylene and cytokinins play a role on the expression of sex in many monecious and diecious systems (TRUŢĂ et al., n.d.). The influence of temperature on cannabis male flower production in feminized plants can be attributed to hormonal regulation. This stress can potentially lead to the expression of genes responsible for the formation of male reproductive organs. While feminized plants are bred to be predominantly female, they can still possess genetic traits that make them more susceptible to stress-induced hermaphroditism.

It's important to maintain a stable and optimal temperature range for cannabis cultivation to minimize stress and the risk of hermaphroditism. Ideal temperatures for cannabis cultivation typically range between 20 °C to 30 °C during the day and slightly cooler at night (Zheng, 2022). By providing a controlled and consistent temperature environment, growers can reduce stress on feminized plants and minimize the chances of masculinization.

In summary, while temperature alone may not directly induce the formation of masculine structures in feminized cannabis plants, high temperatures can contribute to overall stress levels, which can increase the likelihood of stress-induced hermaphroditism. Creating a stable and optimal temperature environment is crucial for maintaining the desired female phenotype and preventing the development of male or hermaphroditic traits.

To maintain high THC levels in feminized plants, it is crucial to monitor them closely and identify any signs of hermaphroditism or the formation of masculine structures. Prompt removal of any male reproductive parts can help prevent pollination and ensure that the female plants continue to produce high-quality flowers with desirable THC levels.

Various techniques can be used to detect and remove male structures, including visual inspections, magnification tools and employing trained personnel to identify and remove any potential pollen sacs or stamens. By ensuring that feminized plants remain free of male structures, growers can optimize THC production and maintain the desired cannabinoid levels in their crops.

3.2. Assessment of flower development and maturation of cannabis plants. by macro and micro visualization

Assessing maturation and flower development in cannabis can be done through macro and micro visualization techniques. These methods allow researchers and cultivators to examine the plant's growth stages, determine its readiness for harvest, and analyse its reproductive structures. Table 3 shows flower, trichomes and THC levels of cannabis throughout 7 weeks of flowering.

When plants are induced to flower, their THC levels are practically zero since, as already mentioned, most of the trichomes that produce cannabinoids are found in the flowers. Only 3 weeks after flowering, can be observed some flowers, small ones, with very few trichomes and therefore with THC levels still low, around 2.04%.

Four weeks after flower induction, there has been a significant increase in the size of the flowers and the number of trichomes, but these are not yet mature, which is reflected in the slight increase in THC levels, which are now at 4.05%.

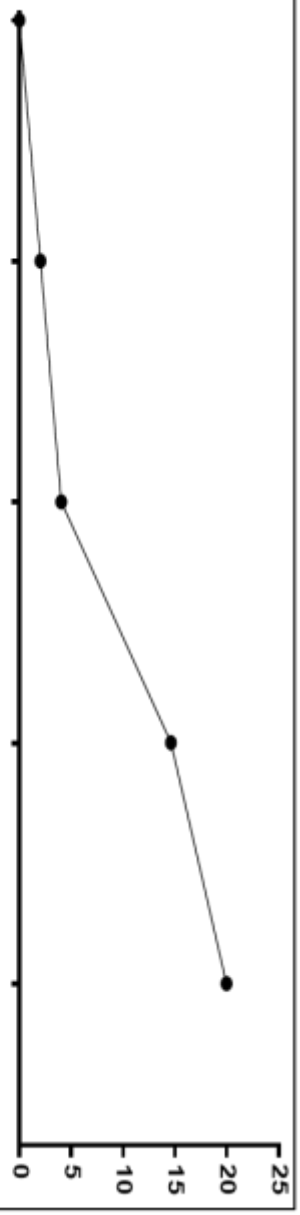
In the 6th week, there was again an increase in flower size and in the size and number of trichomes. On the other hand, the trichomes now have a cloudier color, because the concentration of cannabinoids, especially THC, is significantly higher, now reaching around 14.65%. Macro visualization techniques allow the examination of trichomes to determine their color, density, and maturity. Generally, there is a transition of the trichomes from clear to cloudy and finally to amber as the plant matures. The appearance of cloudy trichomes is often an indicator of optimal cannabinoid production and a suitable time for harvest (Mahlberg & Kim, 2004).

At the end of the 7th weeks, when the flowers were harvested, they appeared to have shrunk a little in size, due to the normal compaction process. As for the quantity and physiology of the trichomes, there are no significant differences in either parameter, but at the level of quantification by HPLC, the values are considerably higher, around 20.00%.

Over the first 6 weeks, it is possible to assess the levels of THC through macro and microscopic visualization because the differences not only in the flowers but also in the trichomes are easily detectable. Not only by the increase in the size of the flowers and the increase in the number and size of the trichomes, but also by the difference in the shade of the flowers, which over the weeks take on a more purple hue and the trichomes change from a more transparent shade to a milkier

white hue. However, the differences between the 6th and 7th weeks are not easily detectable and can only be gauged using HPLC.

Table 3 Development and maturation of the inflorescence. The first column refers to the week of the inflorescence, the second is a photo of the state of the inflorescence macroscopically, the third column refers to the microscopic state, especially of the trichomes (optical microscopy images at 40x magnification, on the 3rd and 7th weeks, and 100x magnification, on the 4th and 6th weeks) and the last column refers to the exact value of THC levels.

	Flowers	Trichomes	%THC
Flower induction			
3rd week			
4th week			
6th week			
7th week			

3.3. Evaluation of bioactivities of cannabis leaf extract

3.3.1. Phytotoxicity of cannabis leaf extract – preliminary assay

Before considering formulations of plant extracts with antifungal capacity to be used for green agriculture, we must first assess the phytotoxic potential of extracts at concentrations that show antifungal activity. For this, we tested the phytotoxicity of the MeOH extract of *Cannabis sativa* leaves on seeds of white mustard (*Sinapis alba* L.), using 2 concentrations 7,5 and 15 µg/mL, also preparing the respective controls, where a volume of 50 µL of MeOH 99% (solvent control), and the negative control where 50 µL of water was added. The assay lasted for 6 days and was composed of 2 parts: i) the observational non-destructive daily analysis of the evolution of germination (percentage of germinated seeds over the 6 days) (Figure 14); and ii) a destructive sampling on the last day of the assay where root length, number of leaves, and hypocotyl length were measured (Figure 15).

To study the phytotoxic effect of the extract in germination of plants, mustard (*Sinapis alba* L.) was used as a model species of dicotyledons plants. These plants are ideal for studying toxicity because they are sensitive to broad range of chemicals (Palm et al., 2022), specially to bioactive ingredients of herbicides (referencia) which globally make them good models for phytotoxicity tests.

Also, mustard is a plant species exhibiting high sensitivity to bioactive ingredients of herbicides (Sekutowski, 2011), so it was thought to be a good indicator in phytotoxic bioassays.

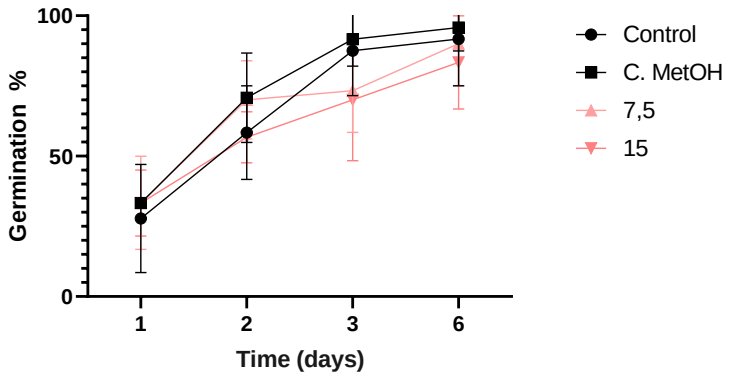


Figure 14. Germination efficiency (%) of mustard seeds. The influence of the extracts at two different concentrations, 7,5 µg/mL and 15 µg/mL on seed development is evaluated and compared with the respective controls. Data presented as the mean of four independent replicates ± SD. The statistical analysis was performed using one-way ANOVA and Tukey's test for multiple comparisons. The absence of statistical notation means that no significant differences were registered for any comparison.

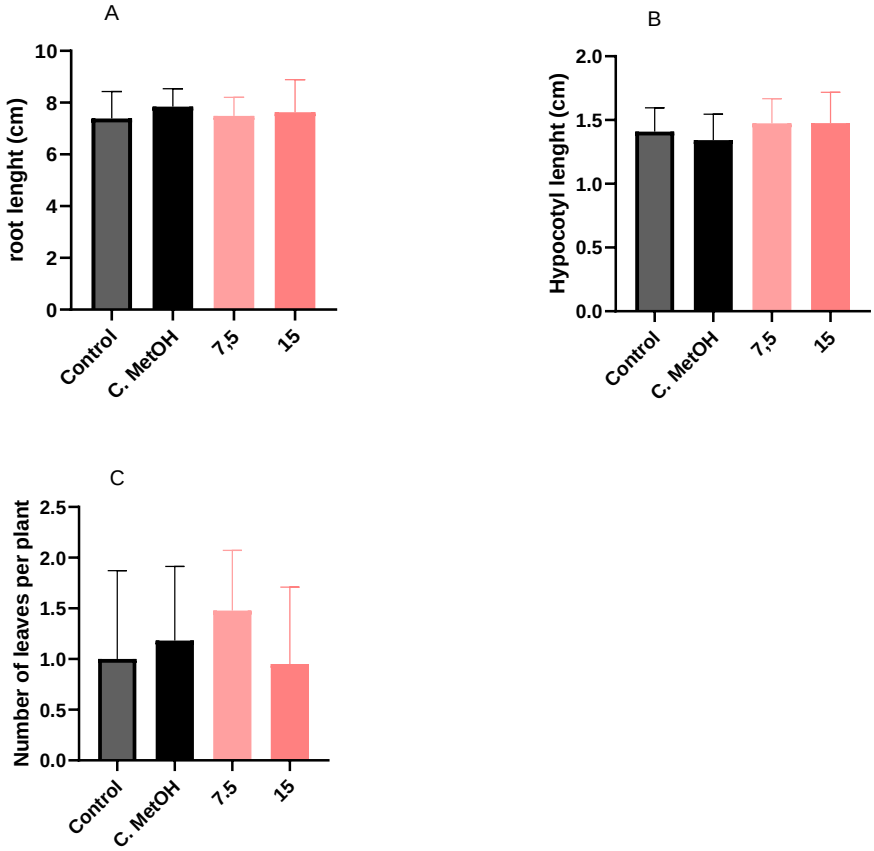


Figure 15. Influence of the extracts at different concentrations of 7,5 and 15 µg/mL compared to the respective controls in biometric parameters of 6-day mustard plantlets root length (A), hypocotyl length (B), number of leaves (C). Data is presented as the mean of four independent replicates ± SD. The statistical analysis was performed using one-way ANOVA and Tukey's test for multiple comparisons. The absence of statistical notation means that no significant differences were registered for any comparison.

Analysing figures 14 and 15, it is possible to verify that both the % germination and all the biometric variables showed no significant differences when comparing the four conditions. These results showed that the volume of MeOH used in the assay was adequate, not impacting significantly on seed germination and early plant growth when comparing with the negative control, and that the extract at the concentrations tested did not show phytotoxic activity.

3.3.2. Antimicrobial activity of cannabis leaf extract against phytopathogenic fungi and oomycete- Preliminary essay

3.3.2.1. Effect of cannabis leaf extract on *Diploidia corticola* mycelial growth

The antifungal properties of the cannabis leaf extract was assessed against *Diplodia Corticola*. The extract was incorporated into the PDA medium at a final concentration of 15 µg/mL and then a mycelium disk was placed in the middle of the Petri dishes as described in M&M (2.5.1). The antifungal activity was evaluated through measurements of the diameter of the mycelium colony (Figure 16),and calculating the percentage of inhibition of fungal growth (Figure 17). Figure 18 depicts representative plates of each condition taken on days 1, 3, 5 and 7.

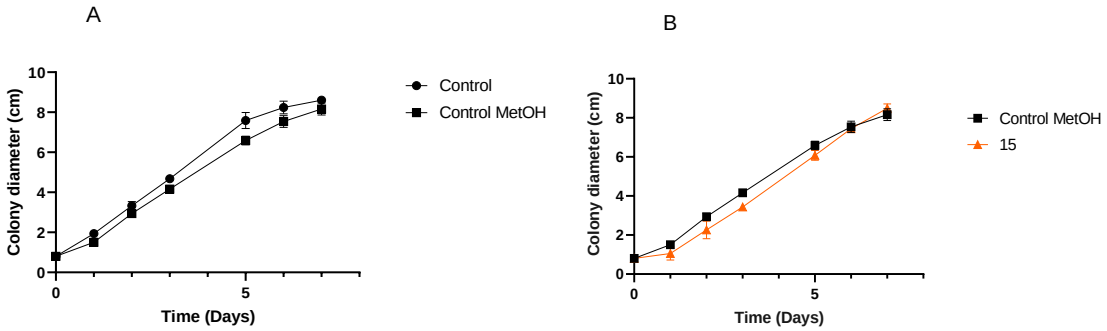


Figure 16. Antifungal effect of cannabis leaf extract on *Diplodia corticola*. Comparison between water control and the MeOH control to see if MetOH has an inhibitory effect on growth (A). Comparison between the MeOH control and the extract at a concentration of 15 µg/mL to check whether the extract has an inhibitory effect (B). Data is presented as the mean of four replicates ± SD. The statistical analysis was performed using two-way ANOVA (time and treatments as factors) and Sidak's test for multiple comparisons (see figure 18 for detailed results).

Since the extract is diluted in MeOH, which inhibits fungal growth, it was necessary to make a comparison between the water control group and the MeOH control group. And as we can see and according to the statistical analysis there are significant differences, from day 1 to 7, the growth speed of the fungus in the water control group is higher than the MeOH control. The figure 16 shows that the extract grows at a slower rate than the MeOH control group, since day 1 to day 5 where there were significant differences.

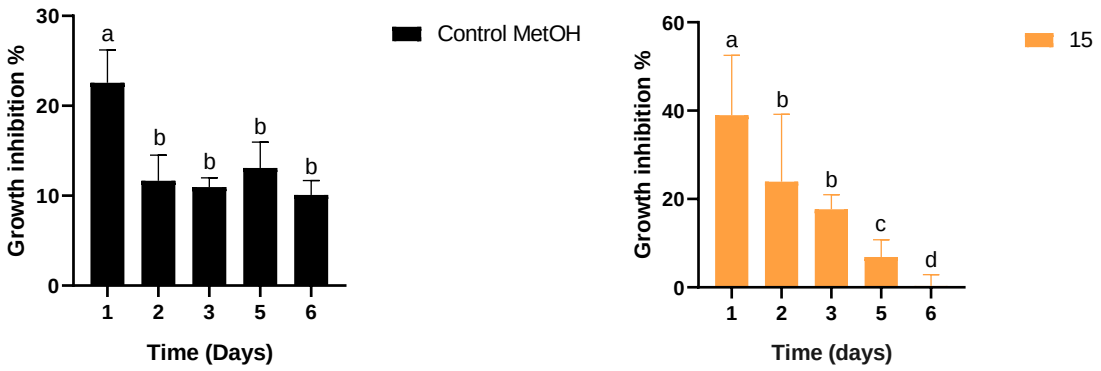


Figure 17. Inhibitory effect of cannabis leaf extract on mycelial growth of *Diplodia corticola* on solid PDA medium. Percentage of growth inhibition was determined over 7 days of incubation by the action of MeOH and by action of the extract on a concentration of 15 µg/mL. Data is presented as the mean of four replicates ± SD. The statistical analysis was performed using one-way ANOVA and Tukey test for multiple comparisons.

In the figure 17 and according to the statistical analysis, it can be seen that the percentage of inhibition of the MeOH control group compared to the water control group reached its maximum after the first day of incubation, around 22%. After this day, inhibition dropped to 10%, around half, where it remained throughout the test, with no significant differences. In the figure, it can be seen that the percentage of inhibition of the extract treatment compared to the MeOH control group also reached its maximum after one day of incubation, around 40%. After that day, the percentage of growth inhibition decreases over time, becoming almost zero on the 6th day of the test.

The results suggest that the extract was able to act against the fungus but the decrease in inhibition over time suggests that somehow, possibly, the fungus adapted to the presence of the extract. Another explanation could be that the extract can easily degrade, however we think that is a more remote one since it's a relatively short assay.

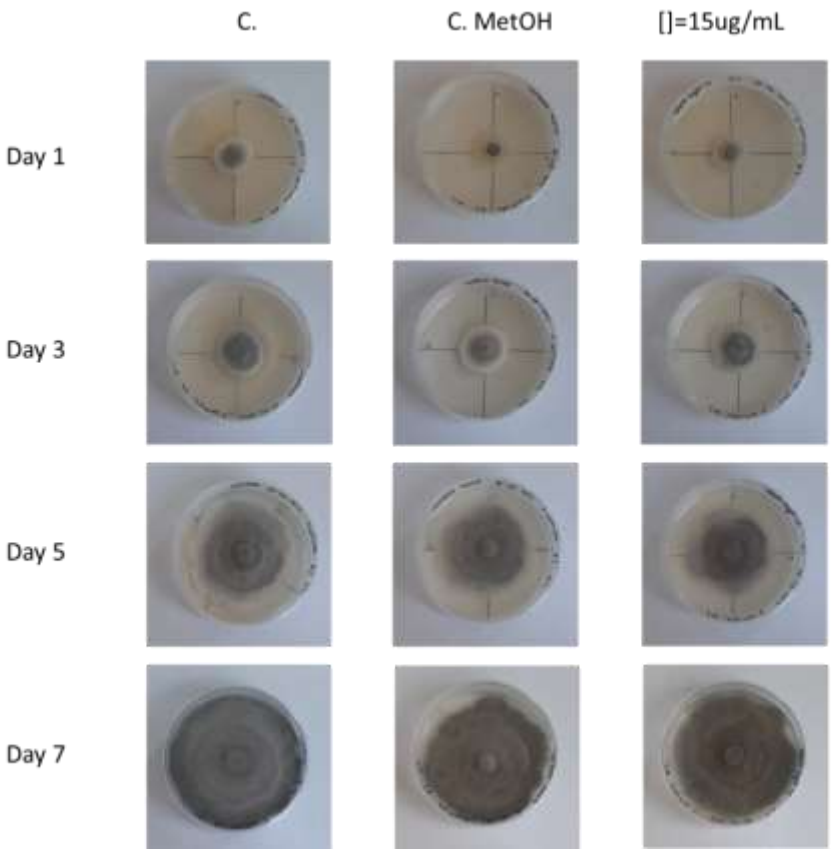


Figure 18. Photographs of *Diplodia corticola* plates throughout the antifungal assay of cannabis leaf extract. In the first column are images of the control with water after 1,3, 5 and 7 days, respectively, in the second column are images of control with MeOH of plates after the same time points, and in the last column are images of the treatment of the treatment in plates after the same time points.

In the figure 18, the images are in accordance with the statistical data. It is possible to see that the diameter of the MeOH control group is smaller than the water control group and in turn the extract treatment is smaller than the MeOH control group. This can be seen on days 1, 3 and 5. On day 7, the plates from the 3 groups are already more equivalent.

3.3.2.2. Effect of cannabis leaf extract on *Colletotrichum acutatum* mycelial growth

The antifungal properties of the cannabis leaf extract was assessed against *Colletotrichum acutatum*. The extract was incorporated into the PDA medium at a final concentration of 15 µg/mL and then a mycelium disk was placed in the middle of the Petri dishes as described in M&M (2.5.1). The antifungal activity was evaluated through measurements of the diameter of the mycelium colony (Figure 19). Figure 20 depicts representative plates of each condition taken on days 1, 3, 5 and 7.

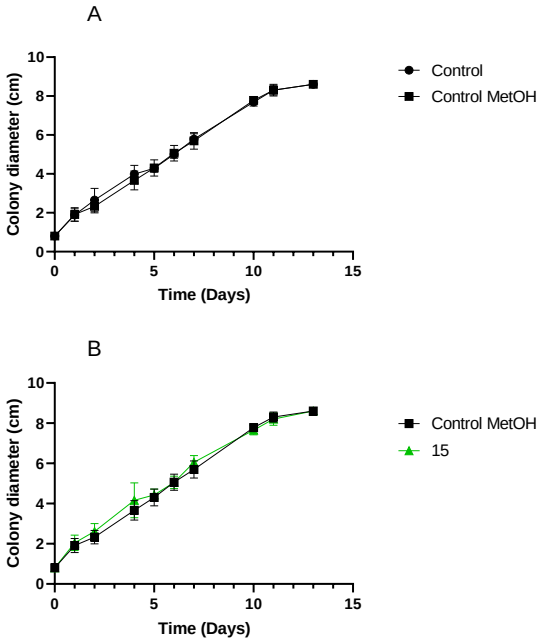


Figure 19. Antifungal effect of cannabis leaf extract on *Colletotrichum acutatum*. Comparison between water control and the MeOH control to see if MeOH has an inhibitory effect on growth (A). Comparison between the MeOH control and the extract at a concentration of 15 µg/mL to check whether the extract has an inhibitory effect (B). Data is presented as the mean of four replicates ± SD. The statistical analysis was performed using two-way ANOVA and Sidak's test for multiple comparisons. The absence of statistical notation means that no significant differences were registered for any comparison.

During the assay, and according to the statistical analysis, there were no significant differences in the growth of fungi in contact with the extract or the MeOH control when compared to the control plate for the tested concentration (Figure 19). To our knowledge, there are no reports of tests with this extract against *Colletotrichum acutatum*.

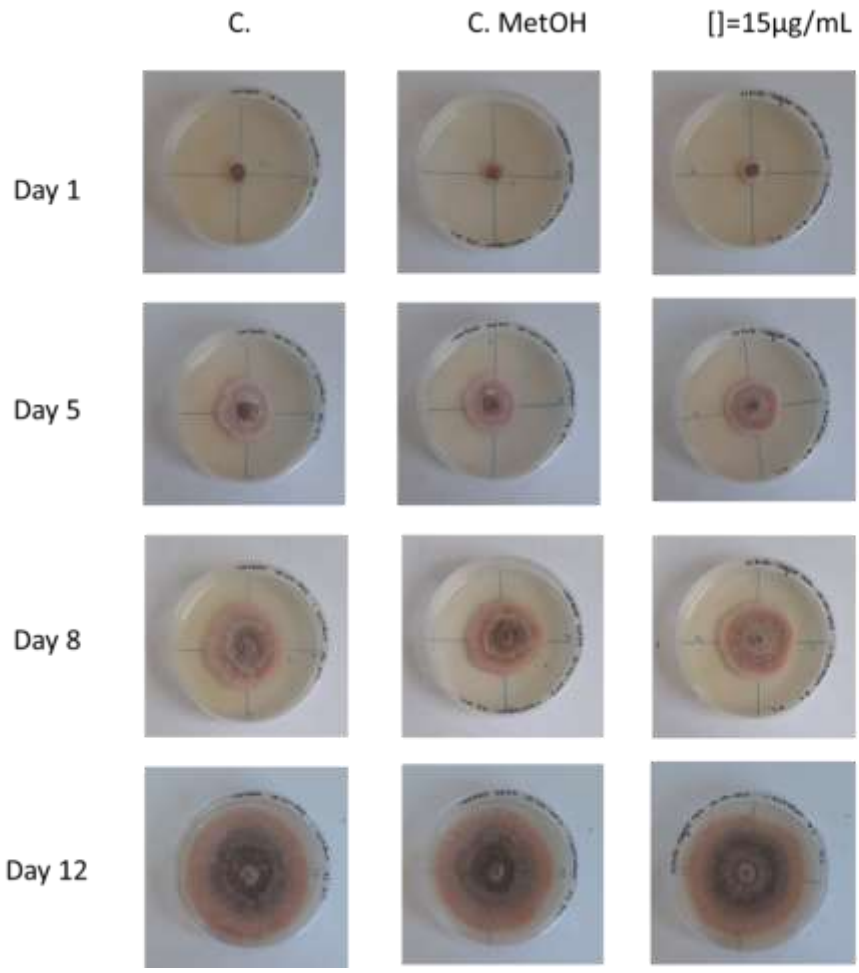


Figure 20. Photographs of *Colletotrichum acutatum* plates throughout the antifungal assay of cannabis leaf extract. In the first column are images of the control with water after 1,5, 8 and 12 days, respectively, in the second column are images of control with MeOH of plates after the same time points, and in the last column are images of the treatment of the treatment in plates after the same time points.

On Figure 20, and concordant with the quantitative analysis, it is possible to see that no noticeable differences were found in mycelial colony growth and neither on type, shape, and colour of the colonies among treatments and treatments and the control.

3.3.2.3. Effect of cannabis leaf extract on *Phytophthora cinnamomic* mycelial growth

The antifungal properties of the cannabis leaf extract was assessed against *Phytophthora cinnamomic*. The extract was incorporated into the PDA medium at a final concentration of 15 µg/mL and then a mycelium disk was placed in the middle of the Petri dishes as described in M&M (2.5.1). The antifungal activity was evaluated through measurements of the diameter of the mycelium colony (Figure 21). Figure 22 depicts representative plates of each condition taken on days 3, 4 and 7.

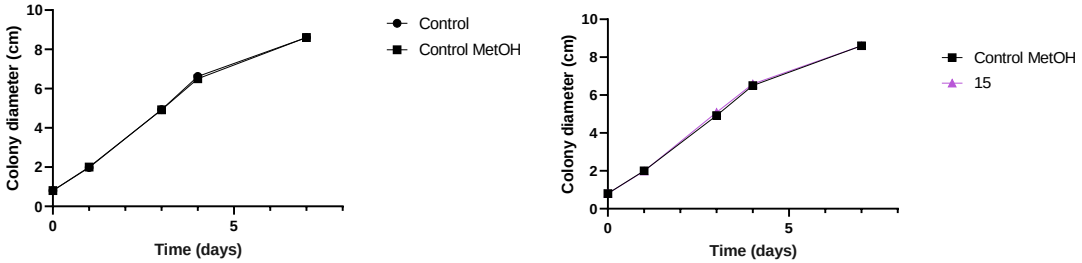


Figure 21. Effect of cannabis leaf extract on against *Phytophthora cinnamomic* growth. Comparison between water control and the MeOH control to see if MeOH has an inhibitory effect on growth (A). Comparison between the MeOH control and the extract at a concentration of 15 µg/mL to check whether the extract has an inhibitory effect (B). Data is presented as the mean of four replicates ± SD. The statistical analysis was performed using two-way ANOVA and Sidak's test for multiple comparisons. The absence of statistical notation means that no significant differences were registered for any comparison.

During the assay, and according to the statistical analysis, there were no significant differences in the growth of fungi in contact with the extract or the MeOH control when compared to the water control plate for the tested concentration (Figure 21). To our knowledge, there are no reports of tests with this extract against *Phytophthora cinnamomic*.

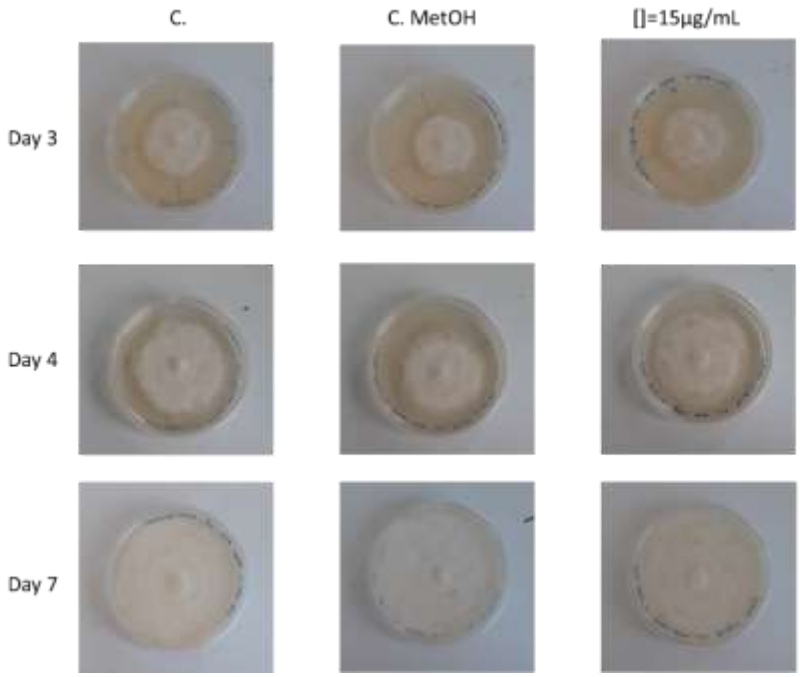


Figure 22. Photographs of *Phytophthora cinnamomic* plates throughout the antifungal assay of cannabis leaf extract. In the first column are images of the control with water after 1,5, 5, 8 and 12 days, respectively, in the second column are images of control with MeOH of plates after the same time points, and in the last column are images of the treatment in plates after the same time points.

On Figure 22, and concordant with the quantitative analysis, it is possible to see that no noticeable differences were found in mycelial colony growth and neither on type, shape, and colour of the colonies among treatments and treatments and the control.

In general, the extract used for these tests, although there is some initial inhibition of the fungus *Diplodia Corticola*, we can see that its effect on the other fungi is not significant and can even be considered null. This is probably because the concentration used was very low (15 µg/mL).

To carry out a new assay testing higher concentrations, SMC provided higher volumes of diluted standard extract so that it would be possible obtain higher extract dry mass solvent extraction. This time it was possible to obtain 1.7241 g of dry extract, which was diluted in 9.5 mL of MeOH, to prepare a stock solution of 180 mg/mL.

The 2 types of assays, phytotoxic and antimicrobial, were carried out again, now testing new final concentrations of 150, 300 and 450 µg/mL. For the negative control, a volume of 50 µL of MeOH was used. Only the MeOH control was used since the previous tests showed that there were no significant differences between the treatment and the 2 types of controls, water and MeOH, in two cases, but not in all the phytopatogens tested.

3.3.3. Phytotoxicity of cannabis leaf extract – testing higher concentrations

This new test was carried out exactly as described in the preliminary test, with both the procedures and the response variables analysed (3.3.1). The only differences were that, in this assay, only the MeOH control was used and the new extract concentrations to be tested are 150, 300 and 450 µg/mL.

The percentage of germinated seeds over the 6 days are depicted in Figure 23, and the biometric variables root length, hypocotyl length, number of leaves per plant and larger leaf length are represented on Figure 24.

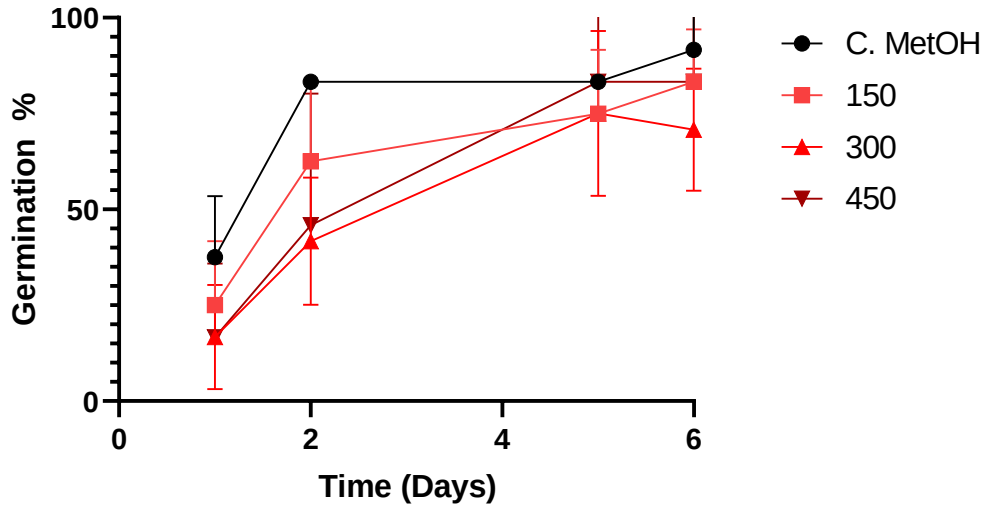


Figure 23. Germination efficiency (%) of mustard seeds. The influence of the extracts at two different concentrations, 150, 300 and 450 µg/mL on seed development is evaluated and compared with the respective controls. Data presented as the mean of four independent replicates ± SD. The statistical analysis was performed using one-way ANOVA and Tukey's test for multiple comparisons. The absence of statistical notation means that no significant differences were registered for any comparison

Regarding the germination process (Figure 23), only on the second day there were significant differences between the control group and the two highest concentrations, 300 µg/mL and 450 µg/mL. After that, there were no further differences revealing that the extract at the higher concentrations tested delayed the onset of germination, but it didn't reduce seed viability allowing a similar germination (%) at the end of the experiment.

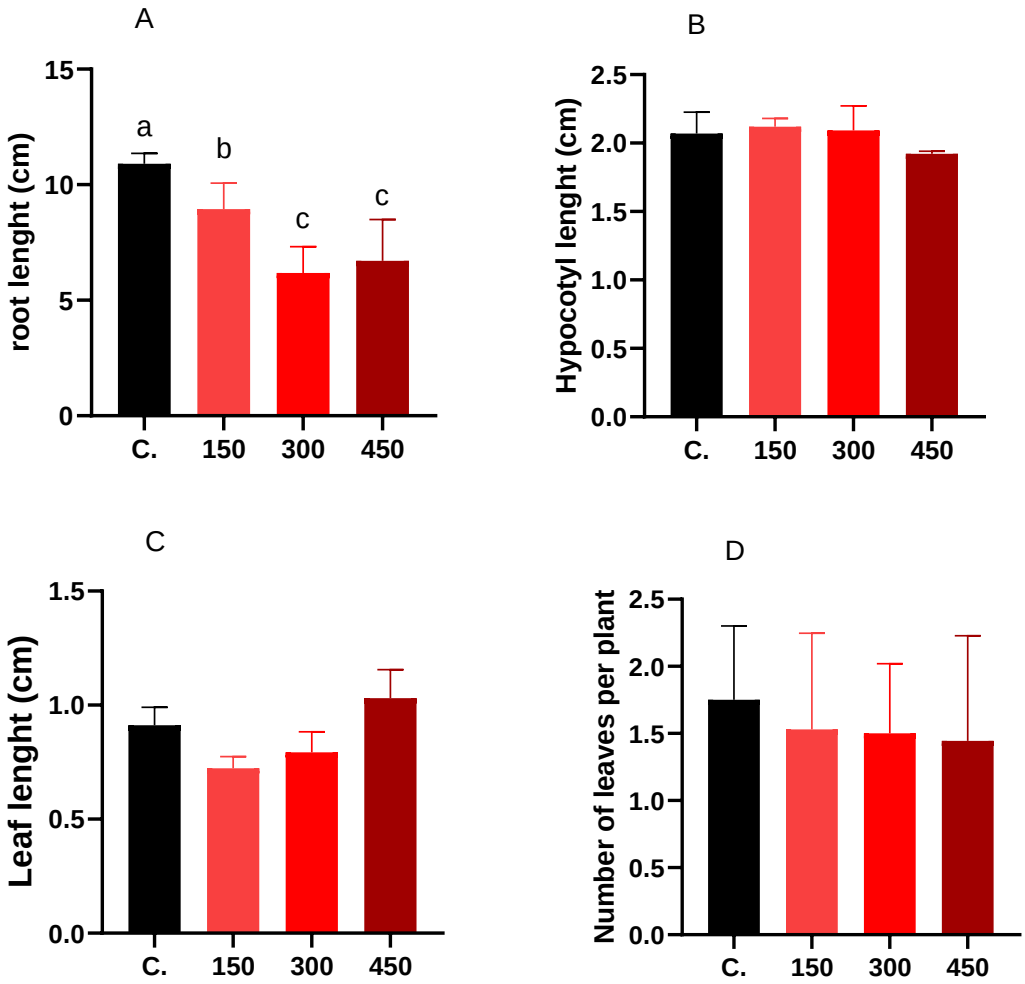


Figure 24. Influence of the extracts at different concentrations of 150, 300 and 450 µg/mL compared to the respective controls in biometric parameters of 6-day mustard plantlets root length (A), hypocotyl length (B), leaf length (C) and number of leaves (D). Data is presented as the mean of four independent replicates ± SD. The statistical analysis was performed using one-way ANOVA and Tukey's test for multiple comparisons. The absence of statistical notation in Figure 25 B, C and D means that no significant differences were registered for any comparison.

Regarding the biometric variables (Figure 24) obtained in the destructive final sampling, there were only significant differences in root length. Significant differences were found between the control and all the extract treatments, and differences between the treatment with the lowest concentration (150 µg/mL) and the two treatments with the highest concentrations 300 and 450 µg/mL. The large standard deviation (SD) estimated for treatment 450 µg/mL took us to a closer look to the respective replicates.

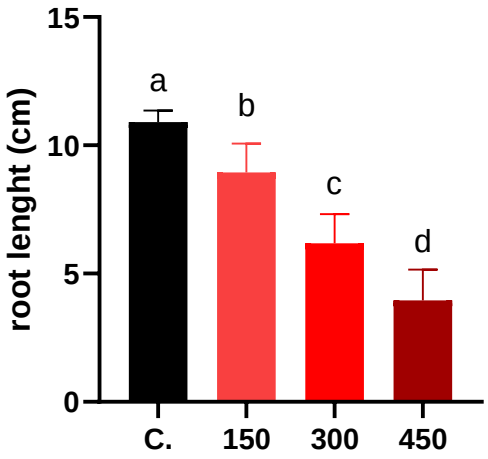


Figure 25. Influence of the extracts at different concentrations, 150 µg/mL, 300 µg/mL and 450 µg/mL, compared to the respective control, on root length removing two flasks of the 450 µg/mL treatment.

Of the 4 replicates made, plantlets from 2 showed roots considerably smaller than all the other treatments and from the other two behaved more like the control group, which may be due to pipetting errors (obstruction of the tip with extract residues). When those two replicates that behaved more like the control group were eliminate, a dose-dependent result was obtained (Figure 25). These results need further confirmation, but if proven, would suggest that cannabis leaf extract may have bioactive/allelopathic compounds exerting an inhibitory action on root growth and development. The allelopathic compounds may also serve as natural herbicides for weed control in a sustainable manner (Chung et al., 2000).

3.3.4. Antimicrobial activity of cannabis leaf extract against phytopathogenic fungi and oomycete - testing higher concentrations

3.3.4.1. Effect of cannabis leaf extract on *Diploidia corticola* mycelial growth

The antimicrobial properties of cannabis leaf extract against phytopathogens were again assessed in *Diplodia Corticola*. This new test was carried out exactly as described in the preliminary test, with both the procedures and the response variables analyzed (3.3.2). The only differences were that, in this assay, only the MeOH control was used and the new extract concentrations to be tested are 150, 300 and 450 µg/mL.

The antifungal activity was evaluated through measurements of the diameter of the mycelium colony (Figure 26) and calculating the percentage of inhibition of fungal growth (Figure 27). Figure 28 depicts representative plates of each condition taken on days 1, 3, 5 and 7.

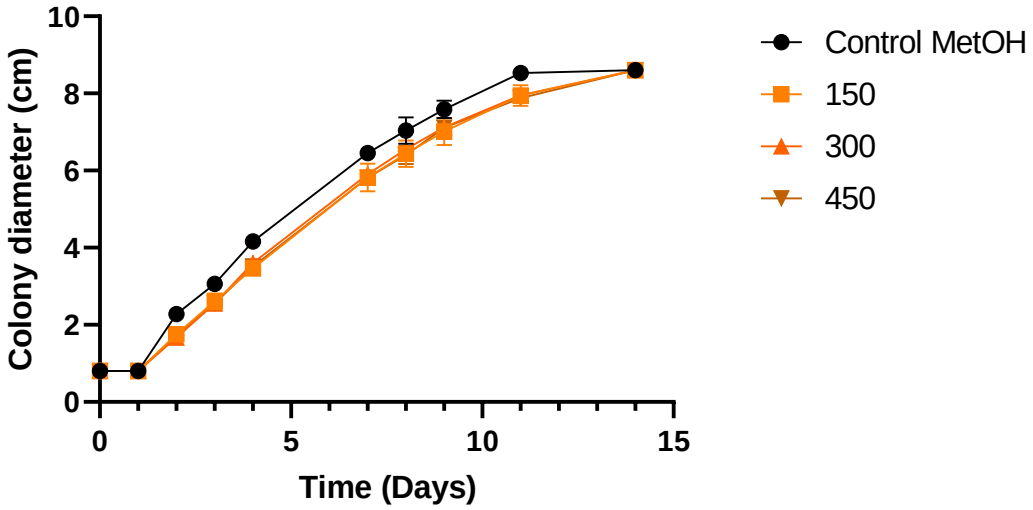


Figure 26. Antifungal effect of cannabis leaf extract on *Diplodia corticola*. Comparison between the MeOH control and the extract at a concentration of 150, 300 and 450 µg/mL to check whether the extract has an inhibitory effect. Data is presented as the mean of four replicates ± SD. The statistical analysis was performed using two-way ANOVA (time and treatments as factors) and Sidak's test for multiple comparisons (see figure 28 for detailed results).

As can be seen in Figure 26, the control group has a higher growth rate than the treatments, reaching the maximum diameter, 8.6 cm, 4 days earlier than the treatments. There are significant differences between the treatments and the control group, but there are no differences between the treatments. These significant differences are seen from the second day to the eleventh day.

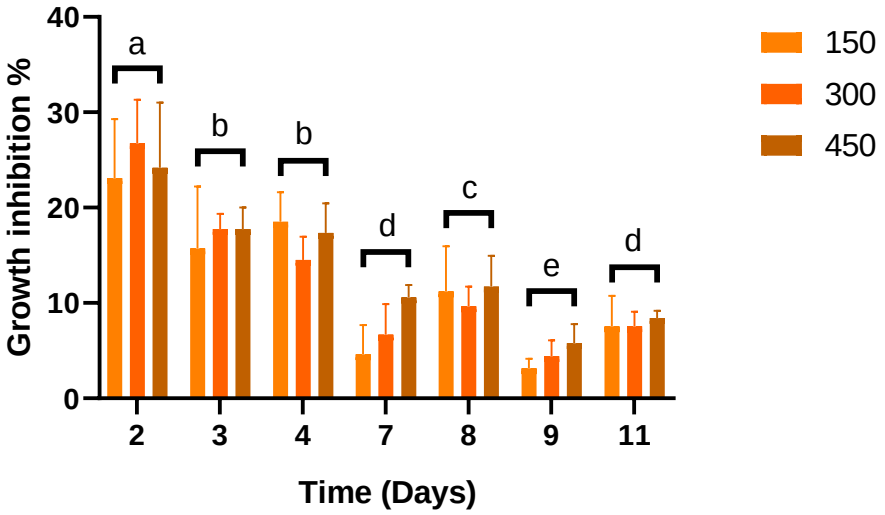


Figure 27. Inhibitory effect of cannabis leaf extract on mycelial growth of *Diplodia corticola* on solid PDA medium. Percentage of growth inhibition was determined over 7 days of incubation by the action of the extract on a concentration of 150, 300 and 450µg/mL. Data is presented as the mean of four replicates ± SD. The statistical analysis was performed using two-way ANOVA and Sidak's for multiple comparisons. As the factor "concentration" was non-significant, the statistical results are shown only for factor "time" using the lettering notation.

The figure 27 shows the inhibitions of the treatments compared to the control group. In the two-way ANOVA carried out, it was verified once again that there were no significant differences between the treatments, with only significant differences as the days went by. It was on the second day that the greatest inhibition was seen throughout the process, around 30 % compared to the control group. Over the days, this inhibition decreases reaching 10%.

Once again, the results suggest that the extract was able to act against the fungus but the decrease in inhibition over time suggests that somehow, possibly, the fungus adapted to the presence of the extract. Another explanation could be that, or the extract can easily degrade, however we think that is a more remote one since it's a relatively short assay.

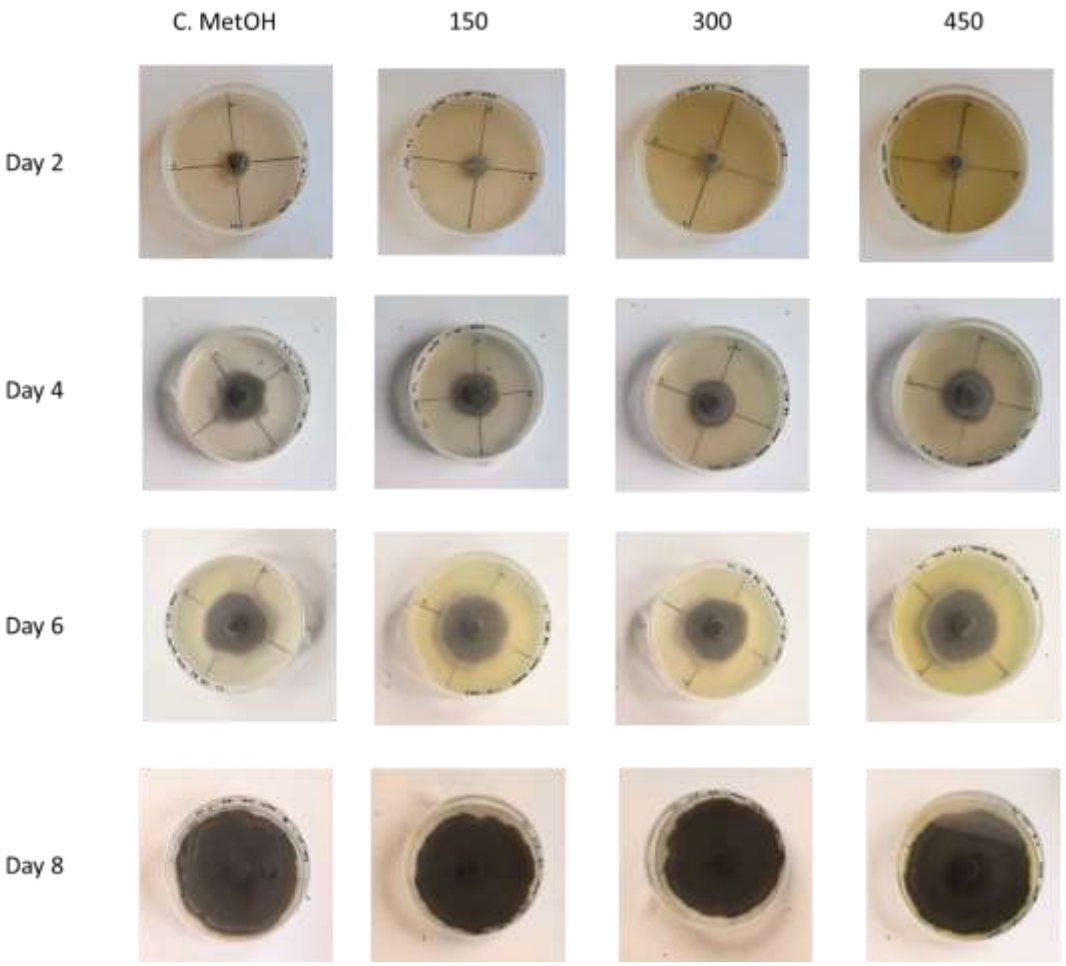


Figure 28. Photographs of *Diplodia corticola* plates throughout the antifungal assay of cannabis leaf extract. In the first column are images of MeOH control with water after 2, 4, 6 and 8 days. In the second, third and fourth column are images of the plates with 150, 300 and 450 µg/mL, respectively, taken at the same time points.

Regarding the photographs presented in the figure 28, although the statistical data show us that there is inhibition from the treatments compared to the control group this inhibition is not easy to see it to the naked eye. Both color and shape are quite identical. On day 2, when the percentage of inhibition is greater, it is not so easily detected by naked eye. however, on day 4, it is possible to observe that the treatments are slightly smaller than the control group. On the remaining days, the differences are no longer easily detected by naked eye.

3.3.4.2. Effect of cannabis leaf extract on *Colletotrichum acutatum* mycelial growth

The antimicrobial properties of cannabis leaf extract against phytopathogens were again assessed in *Colletotrichum acutatum*. This new test was carried out exactly as described in the preliminary test, with both the procedures and the response variables analyzed (). The only differences were that, in this assay, only the MeOH control was used and the new extract concentrations to be tested are 150, 300 and 450 µg/mL.

The antifungal activity was evaluated through measurements of the diameter of the mycelium colony (Figure 29) and calculating the percentage of inhibition of fungal growth (Figure 30). Figure 31 depicts representative plates of each condition taken on days 3, 9 and 15.

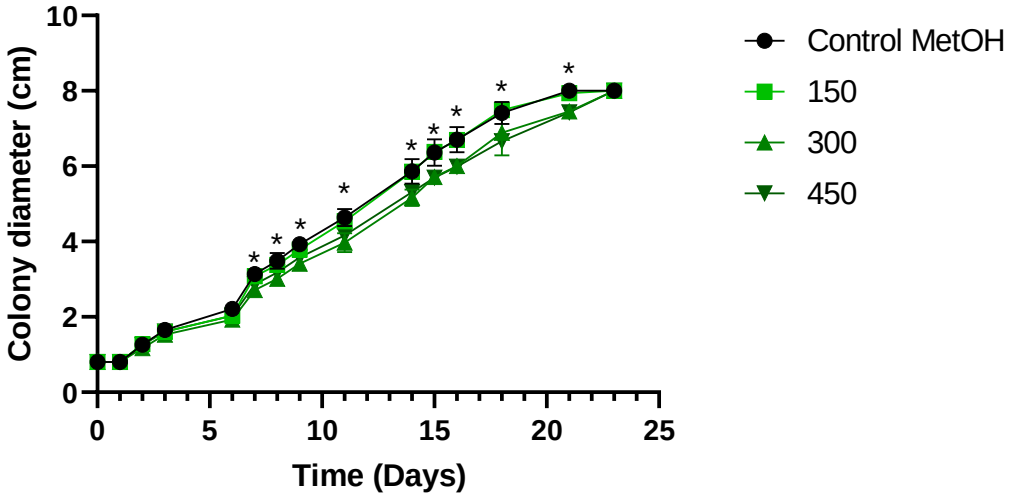


Figure 29. Antifungal effect of cannabis leaf extract on *Colletotrichum acutatum*. Comparison between the MeOH control and the extract at a concentration of 150, 300 and 450 µg/mL to check whether the extract has an inhibitory effect. Data is presented as the mean of four replicates ± SD. The statistical analysis was performed using two-way ANOVA (time and treatments as factors) and Sidak's test for multiple comparisons (see figure 31 for detailed results). The atherists indicate moments in the trial where the control group and the 150 g/mL treatment had equal growth trends, different from the 300 and 450 g/mL treatments which also had equal growth trends.

As can we see in figure 29, Initially, during the first 5 days, both the control and treatment groups grew at similar rates, with no significant differences between them. After this period, the treatment with a concentration of 150 µg/mL had the only moment on day 6 when growth showed significant differences with the control. After day 6 and until the end of the essay, it behaved in exactly the

same way as the control group. On the other hand, the treatments with the highest concentrations, 300 and 450 µg/mL, always grew at the same rate as each other (with no significant differences), but at a slower rate compared to the control group and the 150 µg/mL treatment, until the 21st day of the trial.

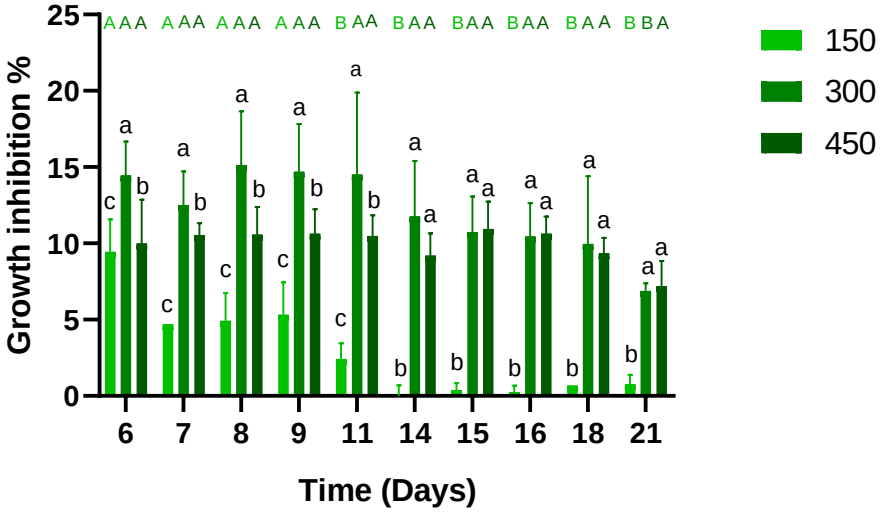


Figure 30. Fungal growth inhibitory effect of cannabis leaf extract on mycelial growth of *Colletotrichum acutatum* on solid PDA medium. Percentage of growth inhibition determined along 21 days of incubation by the action of the extract on a concentration of 150, 300 and 450 µg/mL, compared to the control. Data is presented as the mean of four replicates ± SD. The statistical analysis was performed using two-way ANOVA and Sidak's test for multiple comparisons. As both factors "time" and "concentration" were significant (p< 0,0001), lowercase letters represent significant differences between treatments for each day and capitals letters with color represent significant differences for each treatment over time.

Regarding the percentage inhibition of the treatments in relation to the MeOH control group, the two-way ANOVA carried out showed that there were significant differences between concentrations on each day and at each concentration throughout the trial. It can be seen that the 150 µg/mL treatment has an inhibition of around 10% on the 6th day of the test, but this quickly drops to 5% on the 7th day, which is no longer significant, and continues to decrease throughout the test, even reaching 0. As for the 350 and 400 µg/mL treatments, we see that they have slightly different inhibitions from the 6th to the 11th day, but from the 14th to the 21st day there are no significant differences. As for their variations in inhibition percentage over time, we see that the 300 µg/mL treatment is practically constant throughout the test, with around 15% inhibition, with only a slight

decrease on day 21. The 400 µg/mL treatment shows a constant percentage of inhibition, around 15%, as it does not show any significant differences (Figure 30).

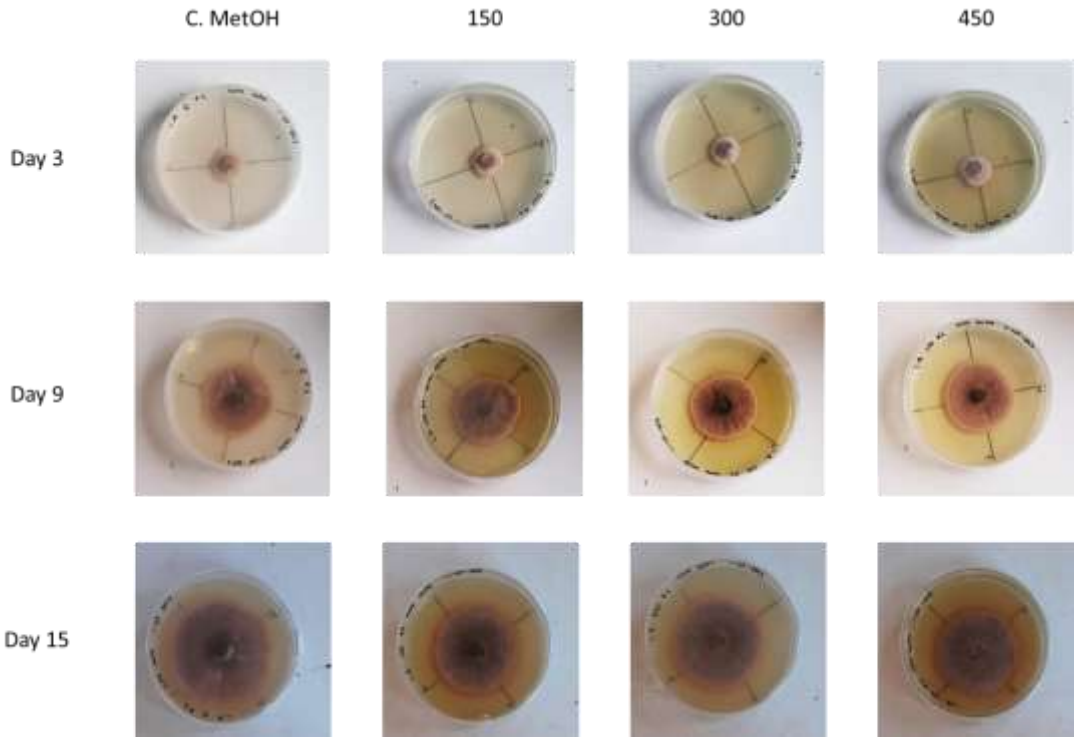


Figure 31. Photographs of *Colletotrichum acutatum* plates throughout the antifungal assay of cannabis leaf extract. In the first column are images of MeOH control with water after 3, 9 and 15 days. In the second, third and fourth column are images of the plates with 150, 300 and 450 µg/mL, respectively, taken at the same time points.

Regarding the photographs presented in the figure 31, although the statistical data show us that there is inhibition in the treatments with greater concertation compared to the control group and the treatment of 150 µg/mL, this inhibition is not so noticeable to the naked eye. Both the size, color and shape are quite identical.

3.3.4.3. Effect of cannabis leaf extract on *Phytophthora cinnamomic* mycelial growth

The anti-oomycete potential of cannabis leaf extract was tested against *Phytophtora cinnamomic* and the assay was as above described for the antifungal evaluation. Over 8 days, the diameters of the mycelial colonies grown in MeOH control and 150, 300 and 450 µg/mL plates were measured (Figure 32). Figure 33 depicts representative plates of each condition taken on days 3, 9 and 15.

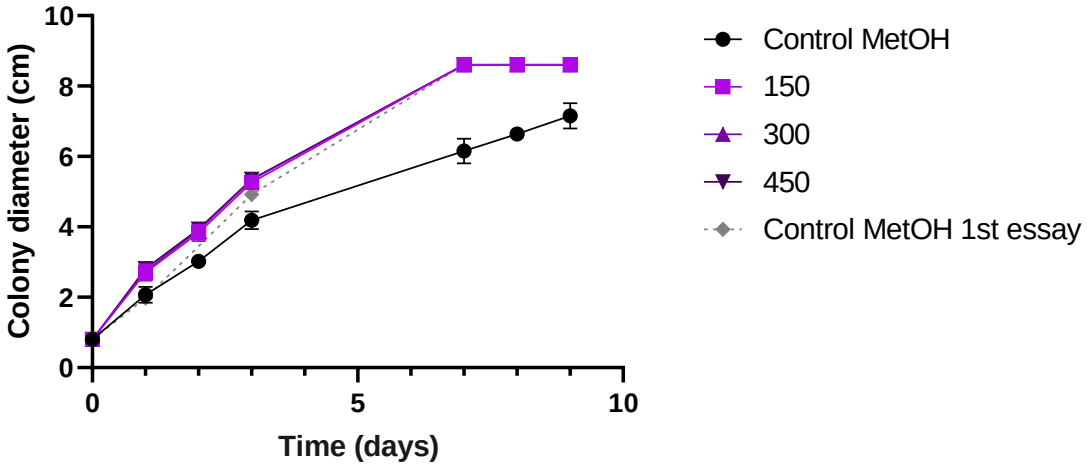


Figure 32. Anti-oomycete effect of cannabis leaf extract on *Phytophthora cinnamomic*. A mycelium disk of the fungus was placed in the middle of Petri dishes with PDA . Percentage of growth inhibition determined along 8 days of incubation by the action of the extract on a concentration of 150, 300 and 450 µg/mL, compared to the control. Data is presented as the mean of four experiments ± SD. No statistical tests were carried out as the control group did not have normal growth patterns as in the previous essay (3.3.2.3).

The figure 32 shows that the treatments, with no significant differences between them, and control groups grew at different rates. After 2 days of incubation, the mycelial colonies were already larger in all three treatments than the control, and after 8 days, the colonies had reached full grown, 8.6 cm, while the controls were clearly smaller, around 6.5 cm. In a first impression this led us to think that the cannabis leaf extract was enhancing the growth of the oomycete, however, the growth dynamics of the control did not seem regular. When plotting the control curve obtained in the preliminary assay, it was possible to verify that all the replicates had reached their maximum size after 8 days (Figure 20, dashed line). Therefore, the extract did not enhance the growth of the oomycete, it was the control group that did not develop normally for some reason that we cannot easily identify.

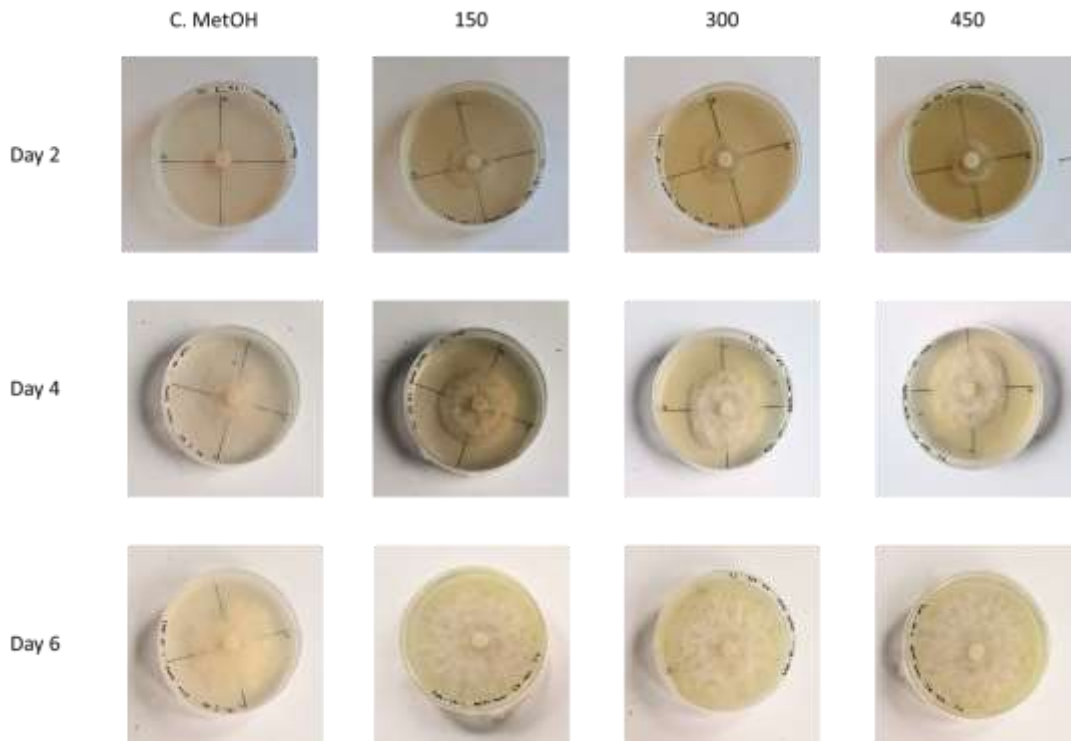


Figure 33. Photographs of *Phytophthora cinnamomic* plates throughout the anti-oomycete assay of cannabis leaf extract. In the first column are images of MeOH control with water after 2, 4 and 6 days. In the second, third and fourth columns are images of the plates with 150, 300 and 450 µg/ml, respectively, taken at the same time points.

In figure 33 it is already possible to verify that the control group is smaller than the treatments on the 2nd day of the trial. This is even more visible on the 4th day of the trial, where it is not only possible to see that the control group is smaller than the treatments, but it is also possible to see that the density of the treatments is higher.

3.4. Identification and quantification of classes of metabolites

3.4.1. Phytocannabinoid profile

In this work, in order to quantify and identify the phytocannabinoid profile present in the leaf extract developed, an HPLC assay was carried out. Only the phytocannabinoid THC-A was identified and at a percentage of 7.63%, in the leaf extract made for the phytotoxicity and antimicrobial tests. This concentration is relative to biomass of vegetative material to make the extract, and it means that

in 1 g of biomass of vegetative material to make the extract here are 0.0763 g of THC-A. This amount is considerable high since the usually amount is around 0.2% and 6% (Jin et al., 2020)

3.4.2. Terpene profile

Part of the leaves that were crushed to prepare the extracts were set aside to be used for the identification and quantification of terpenes using gas chromatography. The results are shown in figure 34.

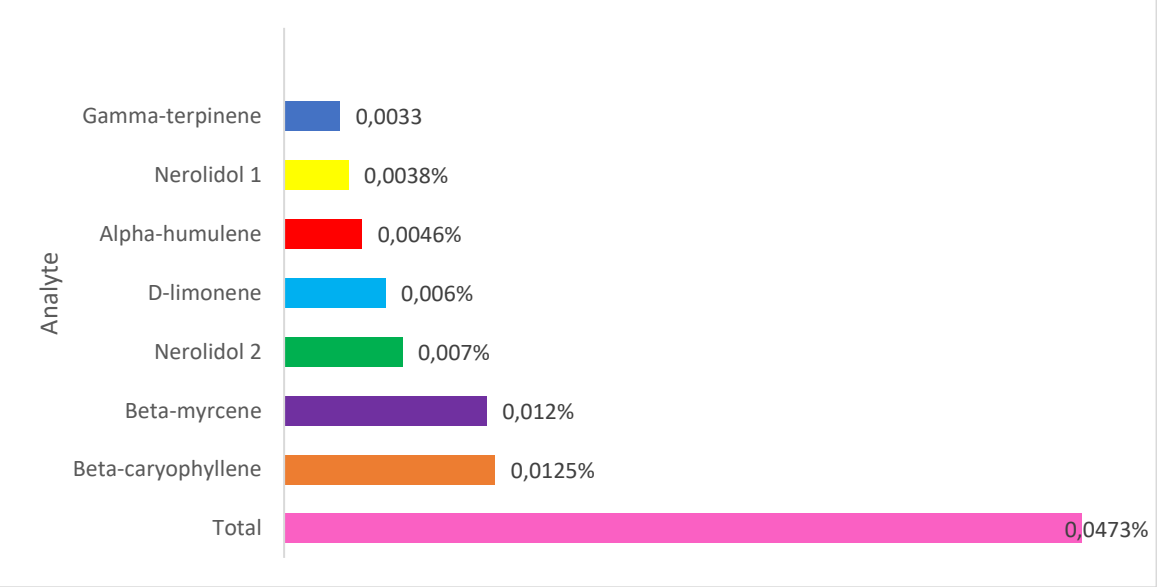


Figure 34. Terpene summary profile of cannabis leaf extract on wt% (total weight percentage). Data is presented as the mean of six independent measurement.

As presented in the figure 34, a total of 0.0473% of terpenes was quantified and it was possible to identify the presence of 7 different terpenes in different percentages. The terpenes β-caryophyllene and β-myrcene display the highest percentages, 0.0125% and 0.0120% respectively. Since the ratio between β-caryophyllene and β-myrcene is lower than 2, in this case 1.04, it means there is a co-dominance (Sarma et al., 2020). β-myrcene and β-caryophyllene are among the most commonly abundant terpenes found in a large set of samples (Sarma et al., 2020). This was followed by Nerolidol 2 and d-limonene with a percentage of 0.0070% and 0.0060%. Finally, alpha humulene, Nerolidol 1 and Gamma-terpinene were also identified in percentages of 0.0046, 0.0036, 0.0033, respectively.

As evidenced, regarding the phytotoxicity of the extract, it didn't interfere with germination or with various biometric parameters of the plants. There were only significant differences for root length in the second essay, where was observed a decrease in root length as the concentration increased. As for the phytotoxic activity of extracts from medical cannabis plantations (high THC levels), further research is needed as no reports on the subject were found. However, several researches has already been carried out to evaluate the phytotoxic activity of cannabis leaf extracts from industrial hemp plantations (THC levels > 0.3%), which showed that these extracts affected the germination of various seeds of different species and also affected the length of roots and leaves (Mahmoodzadeh et al., 2015; Pudełko et al., 2014).

In a study led by Pudełko, in which toxicity of hemp flowers extract in germination of several plants was evaluated. In the test, the concentrations used in these investigations were much higher compared to our test, with the highest concentration tested being 100 mg/mL and the lowest 1 mg/mL. The results showed that the germination of oilseed rape was concentration dependent. Low concentrations stimulated the germination of rape, while high significantly reduced. Higher concentrations of hemp extract resulted in serious disorders in lupine seedlings growth, roots elongation and gravitropism. Rape produced significantly shorter roots already at 2.5% concentration of hemp extract and all other test species at 10% concentration (100 mg/mL) (Pudełko et al., 2014).

In the study led by Mahmoodzadeh, the extracts made of shoot parts of cannabis showed inhibitory effects to lettuce seed germination indices. Extract made of the shoot parts, in concentration of 75 and 100% (100 mg/mL) significantly affected mentioned factors, while the lower two concentrations did not show a significant effect (Mahmoodzadeh et al., 2015).

Given that our tests are in line with those carried out, this could mean that the phytotoxic effect of the extract may not be related to phytocannnabidnoids but to another class of bioactive compounds. This is because our strain of cannabis used to make the extracts is of the medicinal type, i.e. with THC <0.3% and a low percentage of CBD, whereas the strain of cannabis used in the other investigations is of the industrial hemp type, i.e. with THC levels >0.3% and higher CBD values. Another type of bioactive compounds probably has this phytotoxic capacity, such as flavonoids (Samanta et al., 2011).

In the same study led by Mahmoodzadeh, it was analysed the yellow lupine seeds response on hemp extract on a molecular level, being analysed the expression of genes of the isoflavonoids

synthesis pathway: phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS) and isoflavone synthase (IFS). It was concluded that as the concentration of the extract increased, there was an increase in the transcription of genes related to isoflavone synthase pathway (Mahmoodzadeh et al., 2015). Based on this, it can be deducted that the component that may be affecting the normal growth of the roots is probably flavonoids.

As far as the antimicrobial essays, the results show that cannabis leaf extract does have antimicrobial activity, in this case antifungal activity. This activity was only visible in *Diplodia corticola* in the two tests carried out, i.e. at all concentrations, and in *Collecetrituim acutatum* there was only growth inhibition in the second test, at the two highest concentrations, 300 and 450 µg/mL. In the test with *Diplodia corticola*, it can be seen that for both situations, the extract loses its effect over time as the % inhibition decreases with time suggesting that somehow, possibly, the fungus adapted to the presence of the extract. Another explanation could be that, or the extract can easily degrade, however we think that is a more remote one since it's a relatively short assay. On the other hand, in *Collecetrituim accutatum* here is a slight decrease in the activity of the extract, but nothing very significant since the % inhibition remains practically consistent throughout time. As for the oomycete *Phytophthora cinnamomic*, the cannabis extract did not affect its growth, at any of these concentrations.

Initially, it was thought that the antimicrobial activities of the extract might be related to THC levels, however a study carried out by Berardo refuted our thoughts. In his study, 5 varieties of cannabis, C1 to C4 with high levels of THC compared to CBD, while variety C5 has equal levels of THC and CBD, were used to check their antimicrobial activity. As can be seen from the results shown all cannabis varieties were effective to different degree against Gram (+) and Gram (–) bacteria as well as on spore germination and vegetative development of phytopathogenic fungi. These effects were not correlated to the content of major cannabinoids such as CBD or THC, but with the presence of a complex terpenes profile (Berardo et al., 2024).

Terpenes are known to contribute to the fragrance and flavour of cannabis flowers. The particular mixture of these compounds will determine the viscosity of cannabis resin which represents an advantage as the notable stickiness of cannabis exudations traps insects (McPartland et al., 2000). Some of them exert antimicrobial effects (Guimarães et al., 2019). A 'phytochemical polymorphism' seems operative in the plant (Baser & Buchbauer, 2009), as production favours

agents such as limonene and pinene in flowers that are repellent to insects (Nerio et al., 2010), while lower fan leaves express higher concentrations of bitter sesquiterpenoids that act as anti-feedants for grazing animals .

Monoterpenes usually predominate (limonene, myrcene, pinene), but these headspace volatiles (Hood et al., 1973), while only lost at a rate of about 5% before processing, do suffer diminished yields with drying and storage (Turner et al., 1980) resulting in a higher relative proportion of sesquiterpenoids (especially caryophyllene), as also often occurs in extracts, like in this case.

The monoterpene β-myrcene is one of the major constituents in EO (essential oils) and the most potent aromatic flavour component of EOs of many plant species like verbena (*Verbena officinalis*) and mango (*Mangifera indica*) (Chalchat & Garry, 1996; Malundo et al., 1997). It has been shown that EOs containing myrcene can have antifungal properties against plant pathogenic fungi in previous studies. In a study carried out by Albayrak, the compound β-myceren was isolated to evaluate its antifungal activity against phytopathogenic fungi. It was found that in order to inhibit the growth of the fungus by around 50%, the minimum concentration required would be around 12.5 µg/mL (Albayrak et al., 2023).

As far as β-caryophyllene is concerned, the isolated compound has not yet been tested on phytopathogenic fungi, but in previous investigation it has been found to inhibit fungal growth in combination with other terpenes(Jassal et al., 2021).

4. Final considerations

The use of Cannabis plant for fuel, materials, textiles and medicinal purposes has been extensively reported over the years. In fact, there are evidence of Cannabis plant healing properties dating as early as 400 A.D. Nowadays, Medical Cannabis are gaining momentum for various medical purposes, as their therapeutic qualities are becoming better established. Growing cannabis in closed facilities has allowed for a better control of variables such as temperature, humidity and light. This makes cultivation a faster, more productive process with fewer problems. SMC is now facing two challenges, one relating to the presence of male structures in feminized plants that reduces the total content in THC, and the other related to the destination of their plant residues (discarded leaves).

From the systematic monitorization performed, one important conclusion was that temperature has a significant impact on cannabis production male flowers through its influence on sex determination. Higher temperatures tend to promote the development of male flowers on feminized plants. Understanding how temperature determines this alteration in the differentiation of male flowers would help cultivators manipulate environmental conditions to optimize their desired yield. If removed before pollination these structures don't affect THC levels.

Regarding finding observable plant characteristics indicators of plants flower's maturation and THC level over time, in order to avoid extraction and quantification procedures, hence, to reduce the use of materials and reagents that negatively affect the environment, it was concluded that in the first few weeks of work it was possible to do this through macroscopic and microscopic visualizations of flower and trichome characteristics, such as color and size. However, in the last few weeks, these characteristics are already similar enough to be perceptible to the naked eye, making it necessary to resort to HPLC. Although there were no significant differences to the naked eye, HPLC showed that THC levels rose significantly from week 6 (14,65%) to week 7 (20%).

As for the plant waste, the idea was to try to give them commercial value, instead of paying for their destruction. The first approach was to study their antimicrobial properties against phytopathogenic fungi, so that cannabis leaves could be used as a source of biofungides for agriculture. Although synthetic fungicides play an important role in agriculture by preventing the spread of disease and consequent loss of many important crops, they carry many associated risks that urgently need to be reduced. For this reason, the need arises to look for safer, more effective and less hazardous alternatives for the environment and human health. Plants, due to the

secondary metabolites present in their constitution, appear as an alternative to this type of products. In the first assay, at a concentration of 15 µg/mL, the extract was only able to inhibit the growth of *Diplodia corticola*. However, in the second assay, at higher concentrations (150, 300 and 450 µg/mL) the extract was able to inhibit the growth of *Diplodia corticola* and *Colletotrichum acutatum*. In the case of *Diplodia corticola*, all the concentrations had practically the same percentage of inhibition, and as in the first test, this inhibition decreased over time, suggesting that the extract was no longer having an effect, either because it had degraded or because the fungus had adapted in some way. As for *Colletotrichum acutatum*, the 150 µg/mL treatment behaved in a similar way to the control group, with no significant inhibition, while the 300 µg/mL treatment behaved in a similar way to the 450 µg/mL, with inhibition of around the same level throughout most of the test.

In order to understand if the extract would have toxic effects on plants, we tested its phytotoxicity on mustard seeds (*Sinapis alba* L.) and it was possible to show that in the in the first assay, 7.5 and 15 µg/mL, there were no significant differences in any of the metrics evaluated. However, in the second assay, it was found that as the concentration increased (150, 300 and 450 µg/mL), there was a decrease in root length.

Through the results obtained in the identification and quantification of phytocannabinoids and terpenes, and considering previous research, we were able to conclude that the antimicrobial activity of the extract is more related to the terpene profile than to THC levels. As for phytotoxicity, it doesn't seem to be related to phytocannabinoids either, but to another class of metabolites, flavonoids.

5. Future perspectives

With regard to the appearance of male structures in feminized plants, it would be interesting in to see if the appearance of these structures recurs in all plants with temperature high peaks, or if there are plants that don't differentiate them again after the first temperature rise. It would also be interesting to understand the molecular basis of these responses and whether there are other factors that, besides temperature, are influencing this plant response. Resorting to clonal propagation instead of using seeds, would ensure greater homogeneity in the room, making it easier and more accurate to monitor.

It would be of interest to explore various extraction methods, solvents, and harvesting periods to prepare leaf extract, as these factors can influence the composition of the extracts and hence its bioactivities. The potential cannabis leaf extract to be incorporated into natural fungicide formulations has been identified, and knowing its compounds present in the extract, would be relevant to know how they affect the fungus. Once the composition is known, it would important to identify the specific compounds responsible for the antifungal activity by testing pure compounds. Investigating the induction of resistance in fungi by the extract would highlight the advantages of natural products over synthetic fungicides. This can be achieved by studying the doses required for resistance emergence after application of synthetic fungicides and the extract. Phytotoxicity tests on adult plants would provide insight into the potential effects of field application on crops. *Ex situ* assays on various plant species, such as strawberry leaves or apples, would be crucial in determining the extract's applicability in the field. Expanding the action zone of the extract by testing it on more phytopathogenic fungi but also on other target organisms would also enhance the product. If commercialization of cannabis leaf extract is considered, toxicity to non-target organisms, including humans, would need to be tested using animal models.

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