BIOCHEMICAL AND MORPHOLOGICAL IMPACT OF INDUSTRIAL HERBICIDES OVER WILD WEED *CANNABIS SATIVA* AND INTERACTION WITH CYTOCHROME PROTEINS

Thesis Submitted for the Award of the Degree of

DOCTOR OF PHILOSOPHY

in

Botany

By

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2025

DECLARATION

I, hereby declare that the presented work in the thesis entitled "**Biochemical and Morphological impact of industrial herbicides over wild weed *Cannabis sativa* and interaction with Cytochrome proteins**" in fulfilment of degree of **Doctor of Philosophy (Ph.D.)** is outcome of research work carried out by me under the supervision of Dr. Anand Mohan, working as Professor, in the School of Bioengineering and Biosciences of Lovely Professional University, Punjab, India. In keeping with general practice of reporting scientific observations, due acknowledgements have been made whenever work described here has been based on findings of other investigator. This work has not been submitted in part or full to any other University or Institute for the award of any degree.

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CERTIFICATE

This is to certify that the work reported in the Ph.D. thesis entitled "**Biochemical and Morphological impact of industrial herbicides over wild weed *Cannabis sativa* and interaction with Cytochrome proteins**" submitted in fulfillment of the requirement for the award of degree of **Doctor of Philosophy (Ph.D.)** in the School of Bioengineering and Biosciences, is a research work carried out by Sabreen Bashir, 12014473, is bonafide record of her original work carried out under my supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.

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ABSTRACT

Cannabis sativa (Hemp crop) is renowned for its remarkable versatility and wide-ranging industrial applications. The plant serves numerous sectors including food production, paper manufacturing, pharmaceuticals, cosmetics, biofuel development, and textiles. Its adaptability makes it a valuable resource across these diverse industries. Cannabis is estimated to possess more than 550 molecules of bioactive compounds belonging to alkaloids, flavonoids, terpenoids, carotenoids, and cannabinoid classes. Currently, research on *Cannabis sativa* L. is growing in several disciplines like pharmaceutical research including cannabinoid therapeutics, agricultural science and cultivation, industrial and commercial applications, etc.

The textile industry is known to be one of the most environmentally burdensome sectors worldwide. Hemp fiber, originating from the Cannabis plant, has a long history of use in textiles and is a recyclable and renewable resource. Cannabis fiber shows great mechanical performance, is low cost, and environmentally sustainable, thus presenting as an appealing alternative to conventional fiber. *Cannabis sativa* L., plant's versatility in pharmaceutical and textile industry is highly vulnerable to various agricultural and environmental challenges, with herbicides posing a particular threat.

Cytochrome P450s are a critical enzyme family that plays a pivotal role in herbicide metabolism across various organisms. These enzymes are primary mechanisms for breaking down and providing resistance to herbicides in plants and other living systems. By catalysing hydroxylation reactions, cytochrome enables organisms to detoxify and metabolize herbicidal compounds, effectively reducing their harmful effects. This metabolic capability allows plants to modify and neutralize potentially damaging external chemicals, conferring a significant adaptive advantage in diverse environmental conditions. Beyond herbicide metabolism, cytochrome contributes to broader biological processes, including hormone synthesis, stress response, and production of defensive compounds that help organisms survive challenging environmental conditions.

In our current work, we studied the impact of two herbicides- glyphosate and metribuzin on the morpho-physiological and biochemical characteristics of cannabis plants. The secondary metabolite production analysis was carried out using Gas

Chromatography-Mass Spectrometry (GC-MS). Furthermore, *in silico* studies using molecular modelling and optimization via Density Functional Theory (DFT) of THCA synthase with glyphosate and metribuzin were performed, followed by molecular docking. Molecular docking was performed to validate the results obtained through GC-MS investigation for Δ9-Tetrahydrocannabinol (THC) for variation in its synthesis in different concentration groups. THC is the most powerful psychoactive cannabinoid, and thus, it becomes one of the primary focuses of our investigation.

Our study examines how herbicides affect both the yield and quality of fibers produced by *Cannabis sativa*. The content of cellulose and hemicellulose in fiber after treatment was calculated. The mechanical properties of the fiber from treated plants were analyzed using Universal Testing Machine (UTM), morphological features including the finer details of fiber were analyzed using Field Emission Scanning Electron Microscopy (FESEM), and cellulose crystallinity index was estimated using X-ray diffraction (XRD) techniques. Further, this study evaluates the impact of glyphosate and metribuzin on Cytochrome P450 enzyme content and its reduction activity occurring in a dose-dependent manner.

It was observed that both herbicides greatly impact overall plant productivity including primary and secondary metabolite production. Further, glyphosate treatment caused an increase in fatty acid synthesis while the contrary was observed in the case of metribuzin. Also, herbicide stress leads to the synthesis of cannabidivarol and cannabidiol, although they were absent in the untreated group. These findings provide crucial insights for optimizing agricultural practices in cannabis cultivation. In addition, molecular docking studies revealed that metribuzin binds to the same active channel as CBGA (THC precursor), while glyphosate binds at the entrance, thereby hindering THC production. These inputs have implications for manipulating cannabinoid profiles in pharmaceutical and other industrial applications.

Herbicide applications had notable effects on hemp fiber composition and characteristics. Low glyphosate concentrations reduced both cellulose and hemicellulose content, while low metribuzin levels slightly increased cellulose. High concentrations of either herbicide significantly decreased both cellulose and hemicellulose components. Mechanical testing and X-Ray Diffraction analysis showed that low glyphosate concentrations decreased fiber tensile strength, whereas low metribuzin concentrations improved it. However, high concentrations of both herbicides decreased tensile strength. Bast fiber content initially increased with low herbicide concentrations but declined at higher levels. Electron microscopy analysis demonstrated that higher herbicide concentrations progressively damaged fiber structure. Glyphosate primarily caused surface disruption, while metribuzin produced more extensive deterioration, including surface erosion and bubble formation at high doses. These results underscore the intricate impact of both herbicides on *Cannabis sativa* fiber characteristics, stressing the importance of judicious herbicide application when growing hemp for textile purposes.

The cytochrome results revealed a significant increase in Cytochrome P450 levels and reduction activity following herbicide exposure. Glyphosate treatment led to a maximum 2.05-fold increase in Cytochrome P450 content, while metribuzin induced a 1.35-fold rise compared to the control. Similarly, Cytochrome P450 reduction activity exhibited a progressive enhancement, with glyphosate and metribuzin showing 2.07-fold and 2.13-fold increases, respectively. These findings suggest that both herbicides trigger a metabolic response, likely involving oxidative stress mitigation and detoxification mechanisms. The greater induction by glyphosate highlights its pronounced effect on Cytochrome P450 activity, potentially influencing herbicide metabolism and plant stress tolerance.

This study, by meticulously examining the multifaceted impacts of glyphosate and metribuzin on *Cannabis sativa*, underscores the critical need for a nuanced understanding of herbicide interactions within this increasingly vital crop. The scope of this work extends beyond basic agricultural concerns, encompassing the manipulation of cannabinoid profiles for pharmaceutical applications, enhancement of cannabis fiber quality for textile industries, and the elucidation of plant detoxification mechanisms through Cytochrome P450 activity. This study provides insights into the biochemical adaptations of plants under herbicidal stress, emphasizing the role of Cytochrome P450 in detoxification pathways.

ACKNOWLEDGEMENT

First, all praises to Allah (SWT), the most merciful and the most beneficient, with whose sheer blessings I was able to complete my Ph. D. work.

I would like to thank all the people who contributed in one way or another to the work described in this thesis. First and foremost, I extend my sincere gratitude to my supervisor, Dr. Anand Mohan, Professor, Department of Botany, School of Bioengineering and Biosciences, Lovely Professional University, Punjab for accepting me into his group. I pay thanks to him for his enlightened guidance, valuable suggestions, and constant support throughout my journey. He has inspired me by being a living example of hard work and bringing out the best in me. I am heartily thankful to Dr. Anil Kumar, Staff Scientist, National Institute of Immunology, Delhi, for his support and for providing the resources during my research work.

Every result described in this thesis was accomplished with the help and support of fellow lab-mates and colleagues. I am highly grateful to my seniors Dr. Agrataben Vadhel, Dr. Akanksha Shukla, and Dr. Zeba Tabassum, whose keen scientific insight, knack for solving seemingly intractable practical difficulties and their ability to put complex ideas in simple terms were greatly beneficial to me. Besides, I would like to thank my juniors, Reenu Singh and Navneet kaur, for their help and support.

No journey of perseverance is ever walked alone, and I am deeply grateful to my friends Dr. Agrataben Vadhel, Dr. Natasha Kudesia, Saikrupa Adarthaiya, and Jeenu Cherian, who stood by me through every high and low of this endeavour. Their presence, whether through heartfelt conversations, shared laughter, or simple moments of reassurance, provided the strength I needed during the most challenging times. This accomplishment is as much theirs as it is mine.

A special note of gratitude to my roommate, Assima Firdoos, whose patience, kindness, and support have made this journey infinitely smoother. Sharing space with her has been more than just cohabitation—it has been a source of comfort, laughter, and much-needed moments of respite amidst the chaos of research. I want to extend my heartfelt gratitude to my wonderful friends Dr. Mankomal Arora, Dr. Qurat-ul-Ain, Dr. Shakira

Malik, Dr. Aaliya Mir whose companionship and unwavering care have been an integral part of this journey. Walking this path alongside individuals who understood the struggles, and celebrated even the smallest victories made all the difference. Their encouragement, insightful discussions, and ability to bring laughter into even the most stressful moments turned this academic pursuit into an experience I will always cherish.

I want to express my feelings of heartfelt gratitude towards my loving parents, Mr. Bashir Ahmad and Mrs Parveena Akhter and my sisters, Dr. Tabasum Mohi-ud-Din and Er. Seerat Bashir, who always stood there for me and supported and encouraged me in moments of gloom. It has been with their inestimable advices and prayers that I always got relit to work with renewed passion and energy.

Finally, I would like to thank everybody who was important to the successful realization of this work. I express my sincere thanks to department staff members who helped me during the degree period, but it is a lack of space not for regards which compels me to put the pen down.

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Abbreviation	Description
EPSPS	5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS)
ROS	Reactive oxygen species
Δ9-THC	delta-9-tetrahydrocannabinol
CBD	Cannabidiol
CBDV	Cannabidivarol
CBDA	Cannabidiolic acid
GST	c-glutamyl-cysteinylglycine
AMPA	aminomethylphosphonic acid
ALA	δ-aminolevulinic acid
GR	glutathione reductase
$NADPH_2$	nicotinamide adenine dinucleotide hydrogen phosphate
HBA	hydrogen bond acceptors (HBA)
HBD	hydrogen bond donors (HBD)
RB	rotatable bonds (RB)
MW	molecular weight (MW)

CHAPTER 1. INTRODUCTION

1.1. Introduction to Agriculture

In the ~200,000-year history of humans, one of the most important evolutionary and cultural transitions was the domestication of plants and animals (Larson et al., 2014). This change happened in at least six different parts of the world. It mainly occurred in warm areas having varied kinds of plants, animals, and cultures (Zizumbo-Villarreal et al., 2010). Agriculture encompasses a wide range of activities fundamental to providing food and other products through domesticated animals and crop plants to support the global human population. The term "agriculture" originates from two Latin words - "ager" meaning "field" and "colo" meaning "cultivate", together forming "agricultura" which signifies cultivation of land or soil tillage. However, agriculture includes livestock management like pastoralism, mixed crop-livestock farming, and activities like domestication, cultivation, vegeculture, horticulture, and arboriculture (Harris & Fuller, 2014).

With the cultivation of crops, however, came the challenge of managing weeds—unwanted plants that competed with crops for nutrients, sunlight, and water. Humans instantaneously recognized weeds as a problem as soon as they got acquainted with these unwanted plants around 10000 B.C. when they began the cultivation of crops (Vats, 2015). Early farmers relied on labour-intensive methods such as hand-weeding, tilling, and crop rotation to minimize weed interference. Over time, as agriculture expanded and food production became crucial for growing populations, traditional weed management methods proved insufficient, especially in large-scale farming. This led to the search for more effective and sustainable solutions (Leguizamón, 2024). The gradual advancement of agricultural practices and scientific understanding opened the way for discovering chemical weed control methods. The 19th and early 20th centuries saw significant progress in plant science, leading to the development of synthetic herbicides (Zimdahl, 2010). The introduction of compounds like 2,4-D in the 1940s revolutionized weed management by allowing selective control of unwanted plants without harming crops. This innovation significantly reduced labour, increased crop yields, and made modern large-scale farming possible. Thus, the discovery of herbicides was not an isolated event but a response to the long-standing challenge of weed control in agriculture. It represents the continuous evolution of farming practices, driven by the need for efficiency and productivity. The journey from manual weeding in ancient agriculture to the scientific development of herbicides highlights humanity's efforts to improve food production through innovation (Kraehmer et al., 2014). The evolution of weed control has been described in six stages by Baker Hay (Baker, 1974):

10000 B.C. – Manual weed removal by hand

6000 B.C. – Utilization of basic hand tools for tilling soil and weed destruction

1000 B.C. – Animal-drawn implements such as harrows

1920 A.D. – Machine-powered equipment including blades, cultivators, finger-weeders, harrows, rod-weeders, and rotary hoes

1930 A.D. – Biological weed control methods

1947 A.D. – Chemical herbicides including 2,4-D and MCPA

1.2. Discovery of Herbicides

The use of chemicals as herbicides is not new. To remove weeds from timber yards, railway tracks, and car roads, crude forms of chemicals like crushed arsenical ores, rock salts, sulfuric acid, oil wastes, and copper salts have been used for a very long time (Wehtje et al., 2015). The research that concluded that broadleaf weeds can be selectively controlled by using copper compounds and copper salt solutions in cereals was initiated back in 1900 by Schultz in Germany, Bolley in the United States, and Bonnett in France (Appleby, 2005; Bailey et al., 2010). Non-selective herbicides destroy all plants and leave the treated land toxic to plants for a long time. Early chemical herbicides couldn't be applied on cultivated fields because they harmed crop plants along with weeds. This limitation drove researchers to develop selective herbicides designed to target and eliminate only unwanted weeds while leaving the crops unharmed. Over time, the study of growth regulators as potential herbicides emerged as a key area of research. Figure 1.1 and Table 1.1 provide the classification of herbicides on a number of factors.

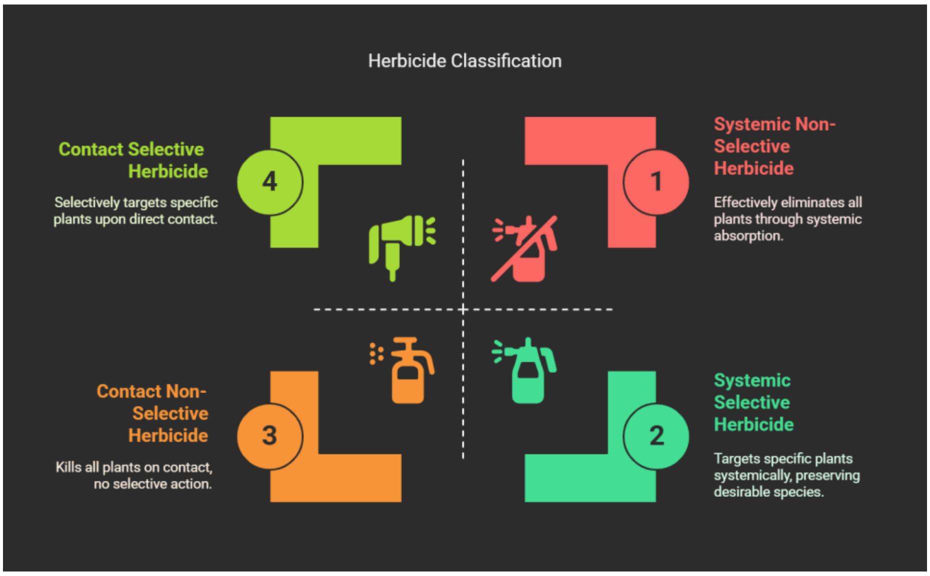


Figure 1.1: Representation of herbicide classification.

Table 1.1: Classification of Herbicides (Paul et al., 2024).

S.No.	Type	Properties
A. Based on Selectivity		
1.	Non-selective herbicides	These herbicides eliminate all or most of the plant species.
2.	Selective herbicides	Selective herbicides target and eliminate particular plant species while leaving desired vegetation undamaged.
B. Based on mode of action		
1.	Contact herbicide	Plant parts that are only chemically contacted are killed by these herbicides.
2.	Systemic/translocated herbicide	The roots or plant foliage absorb these herbicides, which are then transported throughout the plant.
C. Based on site of application		
1.	Soil-applied herbicides	These herbicides are directly sprayed to the soil.

2.	Foliage-applied herbicides	These herbicides are applied to the plant parts.
D. Based on time of application		
1.	Pre-sowing or pre-planting herbicides	These herbicides have specific timing requirements - some must be applied to soil immediately before planting, while others need application several weeks prior to planting.
2.	Pre-emergence herbicides	These herbicides are applied in the window after seeds are sown but before crops, weeds, or both emerge from the soil.
3.	Post-emergence herbicides	Herbicides are administered following the sprouting of weeds, crops, or both plant types from the soil.
E. Based on ionic properties		
1.	Anionic herbicides	These herbicides are negatively charged and are proton acceptors.
2.	Cationic herbicides	These herbicides are positively charged and are proton donors.

1.2.1. Glyphosate: Glyphosate is a chemical compound with a complex history as an herbicide. Originally synthesized in 1950 as a metal chelator, it was discovered to have significant potential for killing unwanted plants by disrupting critical enzymatic processes in plant metabolism. Monsanto developed and marketed the compound under the trade name Roundup™, initially using it for weed control in various environments like agricultural fields, power lines, and train tracks. The herbicide's usage exploded with the introduction of Roundup Ready™ genetically engineered crops in the 1990s, which allowed for more flexible and extensive application (Gandhi et al., 2021). As its use became more widespread globally, scientific scrutiny increased, raising questions about its environmental and potential health impacts. Glyphosate's molecular mechanism of action makes it unique. It specifically inhibits the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS), which is critical in the shikimate pathway responsible for producing essential amino acids and compounds necessary for plant growth. This makes glyphosate a non-selective herbicide capable of affecting virtually all higher plants (Matozzo et al., 2020).

Initially considered relatively safe, glyphosate is a non-volatile chemical that doesn't easily evaporate or degrade. Its ability to quickly penetrate plant surfaces and translocate through plant tissues makes it exceptionally effective at controlling unwanted vegetation. However, its widespread use has prompted ongoing research into its broader ecological implications (Gandhi et al., 2021).

1.2.2. Metribuzin: Metribuzin is another selective triazinone herbicide widely used in agricultural crop production for controlling both grassy and broadleaf weeds. Chemically identified as 4-amino-6-tert-butyl-3-methylthio-1,2,4-triazin-5(4H)-one, this herbicide has gained significant attention for its ability to effectively manage dicotyledonous weeds (Hashem et al., 2011). The herbicide's primary mode of action involves a sophisticated mechanism of disrupting photosynthetic processes. By targeting Photosystem II (PSII), metribuzin competitively inhibits electron transport by binding to the QB site on the D1 protein, thereby interfering with electron transfer from QA to QB (Ma et al., 2020). This interference manifests through characteristic leaf yellowing and abscission and notably affects a wide range of plant species due to its non-specific nature (Kostopoulou et al., 2020a). Beyond its direct photosynthetic impacts, metribuzin demonstrates complex biochemical interactions. It significantly influences secondary metabolite biosynthesis by disrupting the mevalonate pathway and suppressing jasmonate production. These disruptions have profound implications for plant defence mechanisms, stress responses, and overall physiological resilience (Ganugi et al., 2021).

The herbicide's broad-spectrum effects underscore the critical need for careful and judicious application. Indiscriminate use could potentially compromise ecosystem balance by rendering non-target plant species more vulnerable to various biotic and abiotic stressors, thus highlighting the importance of strategic and thoughtful herbicide management.

1.3. Herbicide Contamination in Environment

Herbicide contamination has emerged as a critical concern in modern agriculture, impacting both target and non-target crops and ecosystems. Herbicides, while essential for managing weeds and enhancing crop yields, often leave residues in the environment, leading to a cascade of ecological and agricultural issues. These chemicals are not confined to their application sites; they migrate through runoff, leaching, drift, and volatilization, affecting non-target areas and organisms. This contamination presents challenges for sustainable agriculture, food safety, and biodiversity conservation (Fingler et al., 2017; Schreiber et al., 2018; Tsai, 2013).

In agricultural contexts, herbicide contamination can lead to toxicity in non-target crops, disrupting growth and productivity. Herbicide residues in soil may persist for varying periods depending on their chemical properties, environmental conditions, and microbial activity. For instance, herbicides like glyphosate and 2,4-D are known to remain active in the soil for weeks to months, potentially harming subsequent crop cycles or adjacent fields (Kanissery et al., 2020). The uptake of these residues by crops can lead to phytotoxicity, manifesting as stunted growth, chlorosis, or necrosis. This not only reduces crop yield but also compromises the quality of agricultural produce (Delcour et al., 2015; Pacanoski, 2015).

Non-target crops, particularly those in nearby fields, are highly vulnerable to herbicide drift and volatilization. Drift occurs when herbicides are carried by wind or air currents during application, while volatilization involves the evaporation of herbicides from treated surfaces into the atmosphere. Both processes result in the deposition of chemicals on unintended plants, causing damage to sensitive crops such as fruits, vegetables, and ornamentals. For example, dicamba, a commonly used herbicide, has been reported to cause widespread damage to non-target plants due to its high volatility and drift potential (Braschi et al., 2011; Gage et al., 2019). Such incidents highlight the risks of off-target movement, which can undermine the productivity of diverse cropping systems and lead to economic losses for farmers (Cioni & Maines, 2010).

The effects of herbicide contamination extend to soil health and its capacity to support crops. Persistent residues in soil disrupt the microbial communities responsible for nutrient cycling and organic matter decomposition. This affects soil fertility and the overall resilience of agricultural systems. Herbicides such as trifluralin and atrazine are known for their long-lasting residues, which not only impact soil quality but also pose risks to groundwater through leaching (Basu & Rao, 2020). Additionally, pre-emergence herbicides, designed to suppress weed germination, can unintentionally inhibit the germination of desirable plant species if they leach or drift into unintended areas (Pacanoski, 2015).

Herbicide contamination also interacts with the broader environment, indirectly affecting agriculture. For instance, contamination of water bodies through runoff introduces herbicides into aquatic ecosystems, where they can harm aquatic plants and reduce the availability of water for irrigation. This creates a feedback loop of environmental degradation and agricultural challenges. Furthermore, the persistent use of herbicides has led to the development of resistant weed species, necessitating higher doses or alternative chemicals. This intensifies the contamination of soils and water bodies, further affecting non-target crops and complicating weed management (Gressel, 2015; Peterson et al., 2018).

Thus, herbicide contamination in agriculture and its impact on non-target crops present multifaceted challenges. Addressing these issues requires adopting integrated weed management practices, improved application technologies, and stricter regulatory frameworks. By mitigating the risks of contamination and promoting sustainable practices, agriculture can balance the benefits of herbicide use with the imperative of environmental and crop protection (Damalas & Koutroubas, 2018; Meftaul et al., 2020; Parven et al., 2025).

1.4. *Cannabis sativa* as an Emerging Multipurpose Crop

Over the last decade, the prevailing agricultural practice of growing a limited number of crops in monoculture has faced substantial criticism due to its adverse impact on the environment. Both the recent European Union policy and the common worldwide trend emphasize the need for diversified cropping and the revival of numerous abandoned crops. Among such crops, *Cannabis sativa* L. has received heightened attention from farmers (Durak et al., 2021) which belongs to family Cannabaceae and is one of the earliest domesticated plants with a cultivation history spanning over 6000 years, substantiated by archaeological evidence. It is a multipurpose and most versatile crop, with nearly every part of the plant offering valuable applications across multiple industries (Abdollahi et al., 2020; Clarke & Merlin, 2016; de Andrade et al., 2023; Karche & Singh, 2019; Legare et al., 2022; Rehman et al., 2021; Réquilé et al., 2021; Viswanathan et al., 2020) and can thus be considered a fundamental cash crop. Its remarkable adaptability and rapid growth make it a cornerstone for advancing environmental, economic, and social sustainability (Kaur & Kander, 2023; Simiyu et al., 2022).

The current taxonomic classification of the *Cannabis sativa* L. is as follows (dos Santos & Romão, 2023).

Kingdom: Plantae (plants)

Subkingdom: Tracheobionta – Vascular plants

Superdivision: Spermatophyta – Seed plants

Division: Magnoliophyta – Flowering plants

Class: Magnoliopsida – Dicotyledons

Subclass: Hamamelididae

Order: Urticales

Family: Cannabaceae

Genus: *Cannabis*

Species: *sativa*

Cannabis seeds are a rich source of nutrients, including omega-3 fatty acids, proteins, and antioxidants. They are processed into oils, flours, and protein powders, widely used in culinary applications to enhance nutrition and promote heart health. These seeds are also incorporated into animal feed to boost the nutritional value of livestock products, such as eggs and milk, while their oil finds use in cosmetics for its hydrating and anti-inflammatory properties (Montero et al., 2023; Strzelczyk et al., 2023). Hemp seed oil additionally serves as a renewable base for paints, varnishes, and other industrial applications.

The stalk of the plant yields two primary materials: bast fiber and hurd (shives). Bast fiber, known for its durability, flexibility, and eco-friendliness, are used to produce textiles, ropes, high-quality fabrics, and paper. Compared to cotton, hemp fiber requires significantly less water and fewer pesticides, making it a sustainable alternative in the textile industry. The woody inner portion of the stalk- hurd, is used as animal bedding and as a raw material for construction products like hempcrete, an innovative and eco-friendly building material that offers excellent insulation and durability (Naeem et al., 2023).

Hemp flowers provide essential oils, particularly cannabidiol (CBD), which has gained prominence in the wellness and pharmaceutical sectors. CBD is used to manage conditions such as anxiety, inflammation, and neurodegenerative diseases, offering non-psychoactive therapeutic benefits (Farinon et al., 2020; Saifi et al., 2023). These oils are also integral to a variety of cosmetics and personal care products, including lotions, shampoos, and balms.

Hemp’s ecological benefits extend beyond its direct applications. Its dense canopy naturally suppresses weeds, reducing the need for herbicides, while its deep root system prevents soil erosion and aids in phytoremediation by removing heavy metals from contaminated soil (Adesina et al., 2020). Additionally, hemp is a highly effective carbon sink, capable of absorbing up to 22 tons of carbon dioxide per hectare annually, significantly contributing to climate change mitigation (Raihan & Tuspekova, 2022).

The industrial applications of hemp are vast (Figure 1.2), with over 25,000 products derived from this plant. It is used in bio-composites, construction insulation, carpets, home furnishings, biodegradable plastics, and renewable energy sources like biofuels. Cannabis-based materials are increasingly adopted in automotive components, paper production, and even animal feed, highlighting their broad industrial relevance (Kaur & Kander, 2023). In addition, Sustainable Development Goals (SDGs), including no poverty, zero hunger, and climate action, showcase their potential to address critical societal and environmental challenges (Raihan, 2023; Raihan & Bijoy, 2023).

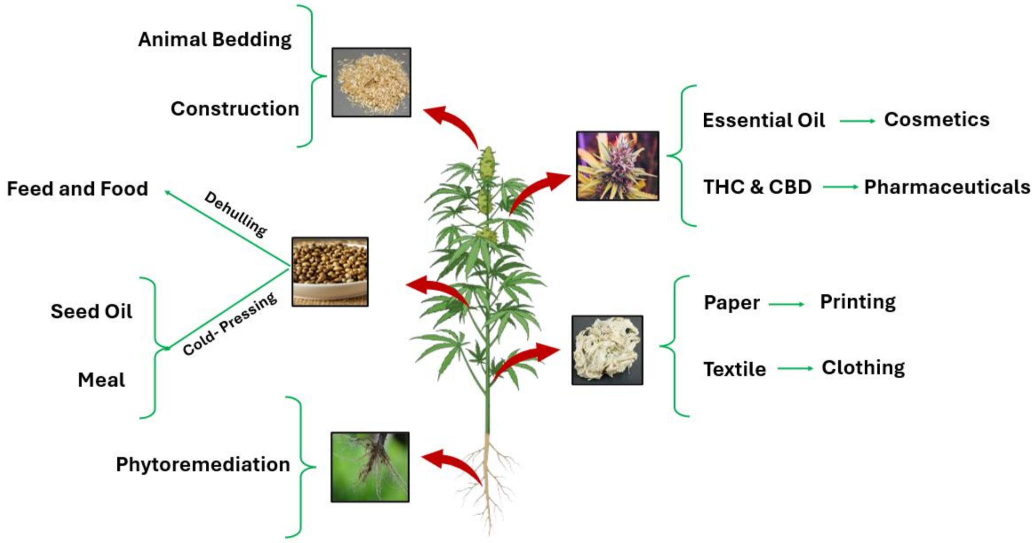


Figure 1.2: Various applications of the Cannabis plant.

1.5. Increasing Market Value of *Cannabis sativa*

The market value of cannabis has been experiencing exponential growth due to its diverse applications and increased global acceptance. In 2020, the global cannabis market was valued at $4.7 billion and is projected to reach $18.6 billion by 2027, with an average annual growth rate of 15.8% (Zimniewska, 2022). This expansion is fuelled by rising demand for cannabis-based products across multiple industries, including textiles, construction, food, and pharmaceuticals.

The Asia-Pacific region, in particular, has seen substantial growth in cannabis cultivation, driven by declining production costs and growing consumer demand for sustainable products. Similarly, the European Union has experienced a 60% increase in cannabis cultivation acreage between 2015 and 2022, with France producing 60% of the EU's cannabis output. This regional expansion has also encouraged significant investments in research and development to create innovative cannabis-based products, such as bioplastics and biofuels, further boosting market potential (Hemp - European Commission, 2024).

Moreover, the cannabis industry's increasing contribution to sustainability and economic growth aligns with global policy priorities. Legalization in numerous countries has opened up opportunities for farmers, entrepreneurs, and large-scale corporations to invest in this versatile crop. While legal cannabis markets are expected to grow significantly, the combined value of both legal and illicit global cannabis markets has been estimated between $214 billion and $344 billion (Wartenberg et al., 2021).

This rapid market growth not only highlights cannabis's economic potential but also underscores its role in advancing sustainability and innovation across various sectors. By leveraging its diverse applications and addressing consumer preferences for sustainable products, the cannabis industry is poised to play a critical role in the global bioeconomy (Raihan & Bijoy, 2023).

1.6. Development of Cannabis in India and the Rise of Cannabis-Based Companies

Cannabis sativa has a deep-rooted history in Indian culture, medicine, and spirituality. Ancient texts such as the *Atharva Veda* and *Sushruta Samhita* mention the use of cannabis for treating various ailments, including digestive disorders, anxiety, and pain. Traditionally consumed as *bhang*, cannabis was regarded as a sacred plant, used in religious rituals and Ayurvedic formulations (Balhara et al., 2022). However, during the British colonial era, the perception of cannabis began to shift. Increasing concerns about its intoxicating effects led to regulatory restrictions, which culminated in the implementation of the Narcotic Drugs and Psychotropic Substances (NDPS) Act in 1985. This law prohibited the use of cannabis resin and flowers, though the use of seeds and leaves (as in *bhang*) remained legal (Barman & Mishra, 2023).

In recent years, the global momentum toward cannabis legalization has influenced Indian policymakers and researchers to reassess its potential. The Indian government has allowed certain states—such as Uttarakhand, Uttar Pradesh, and Madhya Pradesh—to cultivate industrial hemp under controlled conditions. This hemp, a low-THC variety of cannabis, is being explored for its applications in textiles, nutrition, and bio-compostable materials (Malabadi et al., 2023). In parallel, institutions such as the CSIR-Indian Institute of Integrative Medicine (CSIR-IIIM) in Jammu have received licenses to conduct research on the therapeutic applications of cannabis. Notably, researchers there are investigating its use in treating conditions like cancer and epilepsy, as well as its antibiotic potential against drug-resistant pathogens.

These regulatory and research developments have catalyzed the emergence of several cannabis-based startups in India. A pioneering company in this space is the Bombay Hemp Company (BOHECO), founded in 2013. BOHECO has focused on promoting hemp as an industrial crop while also offering a wide range of products such as hemp protein powders, cold-pressed hemp seed oil, and topical wellness solutions. The company collaborates with farmers, scientists, and policymakers to build a sustainable cannabis ecosystem in India. Another major player, HempStreet, works at the intersection of Ayurveda and modern medicine (Nayak et al., 2023)

Startups like Cure By Design and Qurist are also contributing to the rapidly expanding cannabis wellness market. Cure By Design, based in Bengaluru, offers hemp-based nutrition products, pet wellness supplements, and a range of full-spectrum CBD oils. Qurist, on the other hand, focuses on clinical-grade cannabidiol (CBD) oils and partners with physicians to ensure safe and effective usage for conditions such as anxiety, inflammation, and neurological disorders. Another notable company is Delta Botanicals & Research, which aims to make India a hub for cannabis-based pharmaceuticals by focusing on clinical research and biopharma-grade cannabis products (Barman & Mishra, 2023).

Despite the positive momentum, challenges remain. The regulatory framework surrounding cannabis research and commerce in India is still ambiguous, which hinders large-scale investment and innovation. Social stigma also poses a barrier to public acceptance, despite growing evidence supporting cannabis's therapeutic benefits. Moreover, the lack of standardized guidelines for clinical studies complicates the work of investigators and ethics committees. Looking ahead, India stands at a critical juncture. With its favourable agro-climatic conditions, traditional knowledge systems like Ayurveda, and a rapidly evolving wellness industry, the country has the potential to become a global leader in medical cannabis and industrial hemp. Achieving this vision will require a coherent regulatory strategy, increased investment in scientific research, and public education to destigmatize the plant's medicinal uses.

1.7. Bioactive Compounds in *Cannabis sativa*

Cannabis sativa is a plant renowned for its chemical diversity, offering over 550 bioactive compounds that include cannabinoids, terpenes, flavonoids, fatty acids, and other phytochemicals. These compounds vary in composition depending on the plant part, environmental conditions, and genetic factors (Charitos et al., 2021; Yang et al., 2020).

The most significant bioactive compounds are cannabinoids, of which more than 100 have been identified. The primary ones are delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD) (ElSohly et al., 2017). THC is psychoactive and binds to CB1 and CB2 receptors in the endocannabinoid system, making it effective in pain relief, appetite stimulation, and managing nausea. It is widely used for treating chronic pain, muscle spasms, and certain neurological conditions (Shahbazi et al., 2020).

In contrast, CBD is non-psychoactive and has shown remarkable therapeutic potential due to its anti-inflammatory, neuroprotective, and anticonvulsant properties. Unlike THC, CBD acts as a CB2 receptor inverse agonist and a negative allosteric modulator of CB1 receptors, which contributes to its ability to alleviate inflammation, epilepsy, and anxiety (Fusar-Poli et al., 2009; Inglet et al., 2020).

Terpenes, another vital group of compounds, contribute to cannabis's distinct aroma and therapeutic properties. More than 100 terpenes have been identified in cannabis, with β-Caryophyllene, myrcene, and limonene being the most prominent (El Bakali et al., 2022). Β-Caryophyllene is a CB2 receptor agonist with strong anti-inflammatory and analgesic effects (Jha et al., 2021), while myrcene provides sedative and muscle-relaxant benefits (Surendran et al., 2021). These terpenes not only enhance cannabis's medicinal effects but also exhibit antimicrobial and antioxidant activities.

Flavonoids in cannabis, such as cannflavins A and B, add to its therapeutic potential (Izzo et al., 2020). These compounds demonstrate potent anti-inflammatory and antioxidant properties, helping to combat oxidative stress and chronic inflammation. Cannflavins are also linked to potential anticancer effects and may contribute to overall cellular health (Tomko et al., 2022). Additionally, cannabis contains essential fatty acids, particularly in its seeds, which are rich in linoleic acid and α-linolenic acid. These fatty acids are widely used in nutritional supplements, skincare products, and sustainable industries (Bouayoun et al., 2018).

Cannabis's bioactive compounds are extracted and formulated for pharmaceuticals, addressing conditions like epilepsy, arthritis, and neurodegenerative diseases (Adamczyk-Sowa et al., 2017; Bisogno et al., 2021).

Beyond medicine, cannabis finds applications in industrial and environmental fields. Cannabis varieties with low THC content are used to produce textiles, biodegradable plastics, and construction materials (Tang et al., 2016). Furthermore, cannabis's ability to absorb heavy metals and pollutants positions it as an effective tool for phytoremediation, aiding in soil detoxification and environmental restoration (Loffredo et al., 2021).

Thus, cannabis is a uniquely versatile plant with a remarkable range of bioactive compounds. These compounds- THC, CBD, terpenes, flavonoids, and others, make it valuable for healthcare, industrial innovation, and environmental sustainability. Its multifaceted applications continue to drive research and exploration in various fields.

1.8. Fiber from *Cannabis sativa*

The textile industry is known to be one of the most environmentally burdensome sectors worldwide (Franco, 2017). To address its impact, one of the proposed solutions is to emphasize the integration of sustainable materials in garment production (Resta et al., 2016). Cotton, which is the most used natural fiber, poses challenges due to its excessive water and agrochemical usage (Sandin & Peters, 2018). Therefore, there is a pressing need to identify and incorporate an economically viable alternative fiber to meet the growing demands of the textile industry while paving a way to protect the environment and manage our limited resources wisely (Kostic et al., 2008).

Hemp fiber, originating from the Cannabis plant has a long history of use in textiles. Hemp cell walls are made of cellulose, hemicellulose, pectin, and lignin, among other chemical constituents. These ingredients add to the fiber's overall characteristics. Hemp's essential component, cellulose nanofibers, act mechanically like other engineering materials, which makes them useful for a variety of applications like gel formation and plastic reinforcement (Schluttenhofer & Yuan, 2017). Hemp fiber is recyclable and renewable resource. It produces less residue and releases less carbon dioxide into the atmosphere when burned for disposal as compared to its absorbance while in growth period (Wang et al., 2007). In addition, hemp fiber can exhibit specific properties on par with or exceeding those of glass fiber in polymer composites (Sepe et al., 2018). Furthermore, hemp fiber is relatively inexpensive at \$0.5-\$1.5 per kg compared to E-glass (\$1.65-\$3.25 per kg) (Sahu & Gupta, 2020) and carbon fiber (\$8-\$11 per kg) (Thakur et al., 2014). Hemp fiber possesses high tensile strength and elastic modulus compared to other natural fibers like jute, sisal, cotton, banana, and bamboo (Figure 1.3) (Ali et al., 2018; Yan et al., 2014). Due to its superior tensile and elastic properties combined with cost competitiveness, hemp fiber shows tremendous potential to replace synthetic fibers as effective reinforcements in polymer composites across various applications. Therefore, the mechanical strength, affordability, and eco-friendly benefits of hemp fiber position it as a viable and sustainable substitute for traditional composite reinforcements (AL-Oqla et al., 2015).

Figure 1.3: Benefits of choosing cannabis fiber over other natural fibers.

1.9. Herbicide Stress on *Cannabis sativa*

The cultivation of *Cannabis sativa* presents unique challenges concerning herbicide contamination, particularly given the crop's significant ecological and economic value. While cannabis demonstrates natural competitiveness against weeds through rapid growth and high biomass production, certain cultivation scenarios, such as high early weed pressure or low-density planting, necessitate effective weed management strategies. However, the crop's sensitivity to herbicides, combined with limited research on herbicide selectivity and tolerance, poses significant challenges for cultivators.

Pre-emergence herbicides, including pendimethalin, s-metolachlor, and aclonifen, have shown promise in weed control but may induce temporary crop injuries under specific environmental and soil conditions (Jursík et al., 2020). These effects vary based on factors such as soil moisture, texture, and organic matter content. Despite initial setbacks such as reduced stand counts and transient leaf discoloration, cannabis plants often demonstrate recovery capabilities, ultimately achieving superior biomass and seed yields compared to untreated plots.

Research has indicated that herbicide exposure significantly impacts the secondary metabolite profiles of high-CBD cannabis varieties. According to a study, herbicide treatments resulted in more consistent and substantial reductions in total cannabinoid levels compared to other environmental stressors like flooding, wounding, and powdery mildew (Toth et al., 2021). Furthermore, research has shown that the application of paraquat at both lethal and sub-lethal rates generally led to reduced Δ9-THC concentrations, regardless of the plant's chronological or physiological age (Coffman & Gentner, 1980).

The impact of herbicides extends to the fiber parameters of *Cannabis sativa* as well. Recent studies indicate that weed-free conditions produce optimal results in terms of plant height, technical stem length, and fiber yield. While herbicides such as bentazone and bromoxynil demonstrate high phytotoxicity, resulting in decreased plant height, stem diameter, biomass, and fiber yields, others like pyroxasulfone and pendimethalin show minimal negative impacts on fiber quality (Kale et al., 2024).

Herbicides, therefore, while effective for weed control, pose significant ecological and crop-specific risks. Glyphosate residues have been reported in soils at concentrations up to 2 mg/kg and in surface waters exceeding 700 µg/L, raising concerns about persistence and non-target effects (Battaglin et al., 2014; Van Bruggen et al., 2018). Similarly, metribuzin exhibits leaching potential, with soil half-lives ranging from 30–60 days, depending on environmental conditions (USEPA, 2017). Such persistence can exacerbate phytotoxicity in sensitive species. Notably, Cannabis sativa is particularly vulnerable to certain herbicides. For example, Caplan et al. (2019) demonstrated that container-grown cannabis subjected to controlled drought and glyphosate stress exhibited altered physiology, with cannabinoid yields per unit area of THCA and CBDA reduced by up to 20–25% at sublethal herbicide doses. Likewise, Yep et al. (2020) reported that NaCl and glyphosate exposure during flowering caused a linear decline in THCA content of 0.026–0.037% per mM NaCl and significant reductions in plant growth parameters. In the case of metribuzin, even recommended field doses resulted in >70% biomass reduction in young hemp seedlings, confirming its strong phytotoxicity (Ortmeier-Clarke et al., 2022). These findings highlight that both glyphosate and metribuzin, while agronomically valuable, may compromise cannabis growth and secondary metabolism, underlining the need for focused physiological and biochemical assessments. These findings emphasize the importance of developing optimized herbicide regimes that consider specific soil and environmental conditions to support sustainable hemp cultivation practices.

1.10. Cytochrome P450 and Herbicide Metabolism

The metabolism of herbicides occurs in three distinct phases: Phase I (conversion), Phase II (conjugation), and Phase III (compartmentation). In Phase I, herbicides undergo nonsynthetic transformations such as oxidation and hydroxylation, which generally enhance polarity and modify the phytotoxicity of the original molecule. These Phase I reactions can also introduce functional sites for Phase II conjugation with glucose, amino acids, or c-glutamyl-cysteinylglycine (GST). During Phase III, secondary conjugation may take place, followed by the transport of xenobiotic conjugates to specific compartments, such as the vacuole or cell wall, where additional metabolism can proceed (Siminszky, 2006).

Cytochrome P450 monooxygenases and esterases are the primary enzymes involved in Phase I metabolism, catalysing oxidative and hydrolytic reactions of xenobiotics in plants. The initial evidence of plant P450 involvement in herbicide metabolism was documented by Frear in 1969, who investigated the metabolism of monuron in cotton seedling microsomal fractions. By the mid-1990s, a significant body of research had established that many herbicides undergo metabolism through P450 enzymatic activity in plants (Siminszky, 2006).

Plant P450 monooxygenases are intricate membrane-bound heme proteins composed of a protoporphyrin IX and an apoprotein that dictates substrate specificity. The heme group is anchored via a cysteine residue positioned approximately 15% from the carboxy-terminus. The core catalytic system comprises cytochrome P450 and a membrane-bound flavoprotein, NADPH-cytochrome P450-reductase, which facilitates the transfer of two reducing equivalents from NADPH to cytochrome P450. These enzymes were first identified in plants approximatly 15 years after their initial discovery in animal tissues. As monooxygenases, they catalyze the incorporation of one oxygen atom from molecular oxygen into the substrate, while the second oxygen atom is reduced to water. The typical reaction follows the scheme: $RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$. Plant P450s have been demonstrated to catalyse various reactions, including hydroxylations, epoxidations, heteroatom dealkylations, and oxygenations (Gorinova et al., 2005).

An extensive range of plant metabolism reactions are catalysed by the enzymes of cytochrome P450. There is an involvement of P450 in detoxification of herbicides through de-alkylation or hydroxylation, in addition to their role in physiological functions of the primary and secondary metabolites of plants (Khanom et al., 2019).

As cannabis cultivation expands globally for medicinal, recreational, and textile purposes, understanding the effects of commonly used agrochemicals on the plant's morphological, physiological, and biochemical characteristics becomes increasingly important. Among the herbicides used globally, glyphosate and metribuzin are widely applied (Kostopoulou et al., 2020a). While herbicide application is crucial in modern agriculture, it also presents potential risks to the plants.

Cannabis remains a relatively underdeveloped crop, and the limited understanding of cannabis-herbicide interaction impedes the development of optimized crop protection strategies. Despite its natural weed suppression abilities, researchers agree that weed control is essential for optimal fiber and grain yields, particularly at low plant densities (Amaducci et al., 2015; Carus & Sarmento, 2016; Petit et al., 2020). The increasing use of agrochemicals by producers aiming to maximize crop yields has largely been overlooked in cannabis research (Taylor & Birkett, 2020).

To address these research gaps, our study examines the impact of varying concentrations of glyphosate and metribuzin herbicide on cannabis plant's morphological and biochemical parameters, including effects on fiber quality from herbicide-stressed plants. We also analyse cytochrome P450's role by measuring changes in its content and reduction activity under different herbicide concentrations.

CHAPTER 2. REVIEW OF LITERATURE

Cannabis sativa L., belonging to the Cannabaceae family, is an annual dioecious flowering plant characterized by a strong taproot system (Andre et al., 2016). This versatile plant has been utilized by humans for over 5,000 years, making it one of the oldest known sources of food and fiber (Appendino et al., 2008). Native to western and central Asia, including areas like Russia, China, India, Pakistan, and Iran, *Cannabis sativa* flourishes in arid environments and along the natural margins of the Himalayas (Anwar et al., 2006; Babaei & Ajdanian, 2020). The plant's historical significance is underscored by its presence throughout human history, dating back to ancient times (Small, 2017).

While all *Cannabis sativa* plants share a common genetic heritage, they can vary significantly in their physical characteristics, chemical makeup, cultivation methods, and intended uses. Different terms and classifications often distinguish these distinct varieties (Visković et al., 2023). The term "marijuana" refers to cannabis cultivated for drug production, while "hemp" denotes cannabis grown for fiber production (Shams et al., 2020). The classification of cannabis types is primarily based on their THC (tetrahydrocannabinol) content and the THC/CBD (cannabidiol) ratio. Three distinct chemotypes have been identified: chemotype I (the drug type)- characterized by a high THC/CBD ratio (above 1), having psychoactive properties and is used in the production of drugs such as marijuana and hashish; chemotype II (the medium type)- exhibits a THC/CBD ratio close to 1 and possesses low or no psychoactive effects; chemotype III (hemp), containing less than 0.3% THC and has a low THC/CBD ratio (below 1), rendering it non-psychoactive and suitable for fiber and edible oil production. This diverse range of chemotypes underlies the plant's varied applications in both industrial and medicinal contexts (Kopustinskiene et al., 2022; Pellati et al., 2018).

2.1. Cannabis Market and Legalization

Significant shifts in legal and scientific landscapes have marked the history of cannabis research and regulation. William O'Shaughnessy's pioneering work initiated the scientific exploration of cannabis's chemical properties and medicinal potential. However, the early 20th century saw a trend towards criminalization, beginning with the

Pure Food and Drug Act of 1906 and the International Opium Convention of 1925, which imposed strict regulations on cannabis. Subsequent legislation, including the Uniform State Narcotic Drug Act (1925), Geneva Trafficking Conventions (1936), and the Marihuana Tax Act (1937), further restricted cannabis-related activities, although exemptions were made for medical and industrial use (Hussain et al., 2021). Following its obligations as a signatory to the 1961 UN Single Convention on Narcotic Drugs, India also enacted the Narcotic Drugs and Psychotropic Substances Act (NDPS) in 1985. This legislation prohibited the cultivation, production, and use of cannabis, alongside 70 other narcotic and psychotropic substances (Balhara & Mathur, 2014; Bhalla et al., 2015; Karki & Rangaswamy, 2023).

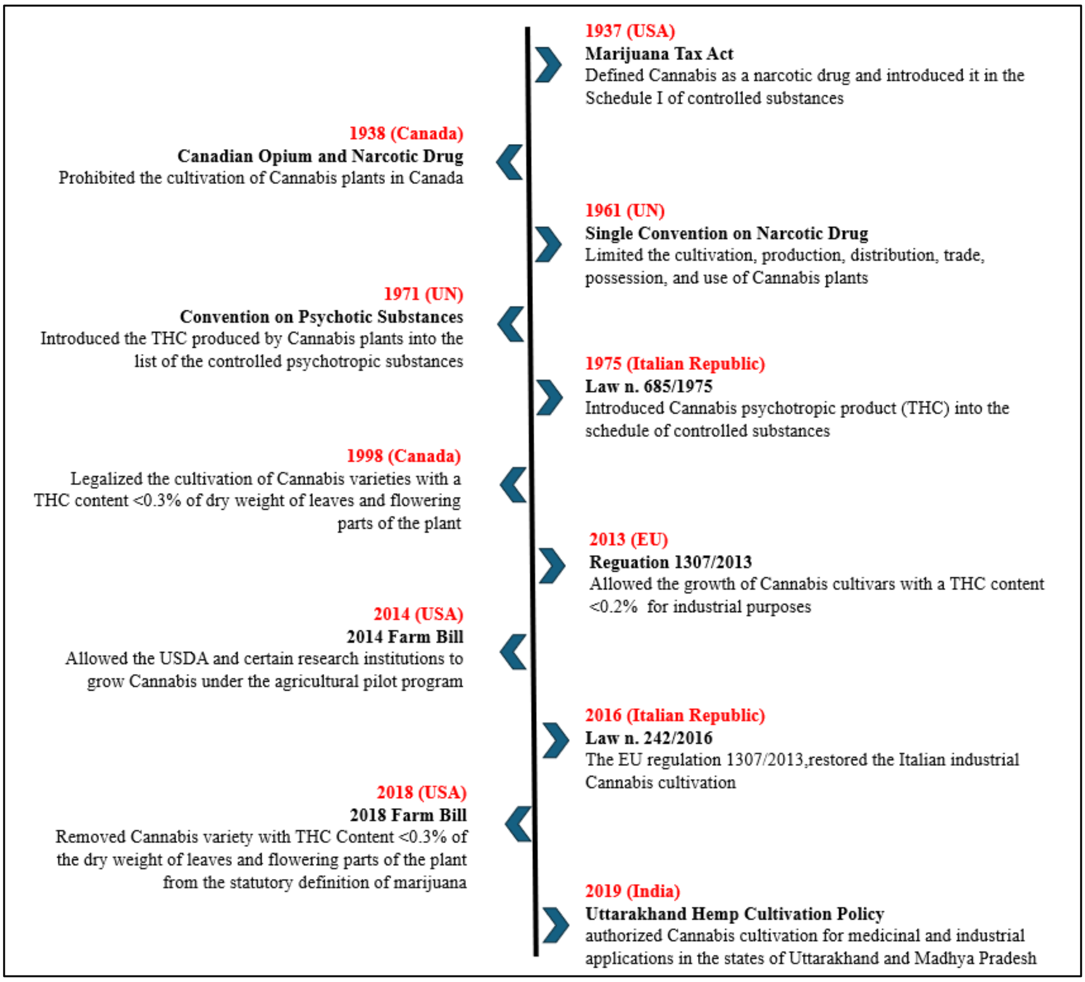
Despite legal constraints from 1937 to 1996, notable scientific advancements were made, including the identification of cannabidiolic acid in 1954 (Hanuš et al., 2016) and the isolation of THC in 1964 (Gaoni & Mechoulam, 1964). The discovery of cannabinoid receptors (CB1) (Devane et al., 1988) and CB2 (Munro et al., 1993), coupled with the Compassionate Investigational New Drug program (1978), initiated a shift towards its decriminalization. Post-1996 legalization efforts led to increased research, culminating in achievements such as the approval of first cannabis-based inhaler spray in 2005 (Pain, 2015; Perras C, 2005) and the draft cannabis genome in 2011 (van Bakel et al., 2011).

The Agricultural Act of 2014 marked a significant turning point by distinguishing hemp from marijuana and facilitating hemp cultivation and research. Currently, 33 US states and over 47 countries permit hemp cultivation for research and industrial purposes (Schluttenhofer & Yuan, 2017), while medicinal cannabis has been legalized in 16 countries (Ransing et al., 2021). The global trend toward decriminalizing cannabis use has influenced India for its legalization. In 2016, legislation supporting the medicinal and commercial use of cannabis was introduced in the Indian Parliament. Later, in 2019, the states of Uttarakhand and Madhya Pradesh approved cannabis cultivation for medicinal and industrial purposes (Figure 2.1) (*Bill for Legalised Supply of Opium, Marijuana Cleared for Parliament - Hindustan Times*, n.d.). This evolving legal landscape has contributed to the growth of both the cannabis drugs market and the fiber market.

The global cannabis drugs market, valued at USD 14.46 billion in 2023, is expected to grow to USD 51.02 billion by 2031, exhibiting a CAGR of 17.37% from 2024 to 2031. Simultaneously, the global cannabis fiber market, which stood at USD 7.55 billion in 2022, is projected to surge to USD 73.55 billion by 2030, with an impressive CAGR of 33.57%. This rapid expansion is driven by the increasing adoption of cannabis fiber in the clothing and paper industries, owing to its sustainability, high elasticity, and durability as a viable alternative to conventional materials (Omar, 2024). The cannabis market in India also shows promising financial potential. The market is expected to grow steadily at 1.84% per year, reaching US$765 million by 2029 (*Cannabis - India | Statista Market Forecast*, n.d.). These market trends reflect the growing acceptance and diverse applications of cannabis products in both medical and industrial sectors (Siddhi, 2023).

In India, Section 10 of the Indian Narcotic Drugs and Psychotropic Substances (NDPS) Act of 1985 empowers State Governments to license cannabis cultivation for medical and scientific purposes. While industrial hemp grows naturally throughout India, cultivation remains prohibited in many states. Nevertheless, India is emerging as a leader among developing nations working to revitalize this centuries-old industrial crop (Nath, 2022). Uttarakhand became India's first state to issue a hemp cultivation license to the Indian Industrial Hemp Association (IIHA) in 2018, allowing pilot-scale production. These permissions specifically authorize hemp cultivation for fiber to supply the textile industry. Uttar Pradesh has similarly legalized hemp cultivation, while Himachal Pradesh and Arunachal Pradesh are reportedly considering legalization options. Uttarakhand has established a policy under Section 14 of the NDPS Act, 1985, permitting industrial or horticultural cultivation of hemp plants. In 2016, it became the first Indian state to allow large-scale commercial hemp cultivation, with the Indian Industrial Hemp Association receiving a license to plant cannabis across 1,000 hectares. Notably, hemp offers significant agricultural advantages: it can be harvested within three months and requires minimal water. Consequently, in water-scarce regions like Uttarakhand, where conventional crop cultivation proves challenging, hemp presents a sustainable alternative (Karki & Rangaswamy, 2023).

Regulations governing Cannabidiol (CBD) in India operate under two frameworks. First, proprietary medicines manufactured under the State Ayush Regulation permit both Δ9-tetrahydrocannabinol (THC) and CBD compounds together, as Ayush laws only allow whole leaf plant extracts under the full spectrum approach. Other forms of isolation or broad-spectrum extracts complicate CBD's regulatory status, falling outside the Ayush regulatory jurisdiction (Nayak et al., 2023).



1937 (USA)
Marijuana Tax Act
Defined Cannabis as a narcotic drug and introduced it in the Schedule I of controlled substances

1938 (Canada)
Canadian Opium and Narcotic Drug
Prohibited the cultivation of Cannabis plants in Canada

1961 (UN)
Single Convention on Narcotic Drug
Limited the cultivation, production, distribution, trade, possession, and use of Cannabis plants

1971 (UN)
Convention on Psychotic Substances
Introduced the THC produced by Cannabis plants into the list of the controlled psychotropic substances

1975 (Italian Republic)
Law n. 685/1975
Introduced Cannabis psychotropic product (THC) into the schedule of controlled substances

1998 (Canada)
Legalized the cultivation of Cannabis varieties with a THC content <0.3% of dry weight of leaves and flowering parts of the plant

2013 (EU)
Reguation 1307/2013
Allowed the growth of Cannabis cultivars with a THC content <0.2% for industrial purposes

2014 (USA)
2014 Farm Bill
Allowed the USDA and certain research institutions to grow Cannabis under the agricultural pilot program

2016 (Italian Republic)
Law n. 242/2016
The EU regulation 1307/2013,restored the Italian industrial Cannabis cultivation

2018 (USA)
2018 Farm Bill
Removed Cannabis variety with THC Content <0.3% of the dry weight of leaves and flowering parts of the plant from the statutory definition of marijuana

2019 (India)
Uttarakhand Hemp Cultivation Policy
authorized Cannabis cultivation for medicinal and industrial applications in the states of Uttarakhand and Madhya Pradesh

Figure 2.1: Timeline of cannabis legalization.

2.2 Growing *Cannabis sativa* in India

Cannabis sativa has diverse applications across multiple industries, including textiles, medicine, cosmetics, rope manufacturing etc (Rupasinghe et al., 2020). The versatile fibers are even utilized in the production of artificial hair wigs (Andre et al., 2016b). The plant's seeds possess significant nutritional value and are processed into various food products. These seeds contain abundant protein, fiber, and essential fatty acids including omega-3 and omega-6 (Aggarwal, 2018). Research indicates they exhibit antioxidant properties and may alleviate symptoms of numerous ailments, potentially improving heart, skin, and joint health (Karki & Rangaswamy, 2023b).

Hemp seed has earned recognition as one of the most nutritionally complete food sources due to its exceptional nutritive profile (Shahzad, 2012). It can be consumed in multiple forms: whole, hulled (hempseed kernel), or as processed products including oil, flour, and protein powder. The seed contains 25-35% lipids with a uniquely balanced fatty acid composition, 20-25% highly digestible proteins rich in essential amino acids, and 20-30% carbohydrates, largely comprised of dietary fiber. Hemp seeds can be incorporated into numerous dishes, offering a natural and sustainable means to increase protein consumption. Like conventional food items, hemp is suitable for general consumption (Kuddus et al., 2013).

Hemp hearts- dehulled *Cannabis sativa* seeds effectively enhance protein intake. Hemp seed oil is extracted entirely (100%) from Cannabis seeds through cold-pressing methods. The seed is also valuable as a dietary supplement (14-24), providing carbohydrates (20-30%), dietary fiber (10-15%, approximately 20% being digestible), and minerals (4-6%) (Malabadi et al., 2023). Research suggests that other components, including trace amounts of terpenes and cannabinoids, may confer additional health benefits. Hemp powder represents an excellent plant-based protein solution, with a 30g serving delivering over 15g of protein (Nath, 2022).

The plant's stalks, seeds, and leaves are processed into diverse products spanning construction materials, textiles, paper, food, furniture, cosmetics, and healthcare items. Markets for nutraceuticals and health products are projected to expand considerably in coming years, driven by increasing health consciousness among consumers (Rupasinghe et al., 2020).

The state of Uttarakhand has developed a comprehensive licensing scheme allowing individuals and companies to obtain four types of permits: hemp farming, raw material storage, sales, and transport. Through public-private partnership models, the Uttarakhand government supports numerous Self-Help Groups (SHGs) and farmer collectives to develop the hemp industry. The state permits hemp cultivation for research and development purposes, with Section 10 rules being established by Uttarakhand, Uttar Pradesh, and Jammu & Kashmir. Several other states—including Goa, Karnataka, Orissa, and Jharkhand—are preparing similar frameworks (Joshi, 2019). Recent developments indicate significant progress toward the medicalization of cannabis in India. The Central Government has taken a notable step by commissioning a specialized council under the Ministry of Ayurveda, Yoga, Naturopathy, Unani, Siddha, and Homeopathy (AYUSH) to conduct comprehensive scientific research. This research initiative aims to systematically investigate and establish the therapeutic efficacy of cannabis for treating various medical conditions. This strategic move represents an important shift in policy approach, potentially paving the way for evidence-based integration of cannabis into India's traditional medical systems while maintaining regulatory oversight through established governmental structures (Karki & Rangaswamy, 2023).

Currently, brands and companies can legally import or export Cannabis plant-based medicines under Narcotic licensing and manufacture them under cover licenses. This represents the only legal pathway for incorporating Cannabidiol (CBD) into business operations. Any other methods of selling and distributing CBD tinctures or isolates would be deemed illegal. Legal distribution can only occur through approval from the Drugs Controller General of India (DCGI) under the Drugs & Cosmetics Act. Few companies operate in India's domestic hemp market, as the industry remains in the nascent stages constrained by national regulations. Notable startups include Bombay Hemp Company (BoHeCo), Health Horizons, Foxxy, Hempsters, Vedi, GreenJams, HempStreet, and NHempCo. Health Horizons aims to expand into textile manufacturing, recognizing hemp fiber's significant potential. These startups collaborate to develop domestic markets for innovative products including hempcrete, biofuels, and hemp paper (Malabadi et al., 2023).

Bangalore-based Namrata Hemp Company (NHempCo) promotes industrial hemp cultivation in southern Indian states. It was founded by the entrepreneurial couple Harshavardhan Reddy Sirupa and Namrata Reddy Sirupa, inspired by India's Make in India Campaign. The company has dedicated 15 acres in Alamuru village (Anantpur district, Andhra Pradesh) for organic industrial hemp cultivation in partnership with local farmer communities, with its headquarters in Bangalore and Karnataka. NHempCo has also established research and testing facilities to develop hemp-based products. NHempCo is part of India's growing hemp industry ecosystem, which includes companies like UKHI-Hemp Foundation, Cannabis Wellness, PIMCHA, Bombay Hemp Company (BOHECO), Global Hemp Solution India, Hemp Horizons, Happie Hemp, Satliva, Himalayan Hemp, and many others. These organisations collectively promote hemp cultivation, research, manufacturing, and advocacy throughout India and internationally (Malabadi et al., 2023).

The Indian Industrial Hemp Association (IIHA), alongside various organisations and companies, has worked to raise awareness about hemp's capabilities. Their educational efforts, highlighting the distinction between hemp and marijuana (specifically cultivated for psychoactive use), have contributed to improvements in the hemp market. With growing demand for hemp-based products and increasing health consciousness, industrial hemp presents opportunities for expanding both domestic and international markets. India possesses the potential to rapidly compete with countries like China in exporting industrial hemp products to North American and European markets, potentially providing alternative income streams for Indian farmers while supporting sustainable, eco-friendly product development (Tripathi & Kumar, 2022).

2.3. Drug Type Cannabis

The medicinal use of cannabis has a rich history dating back to ancient China. In 2737 BC, Chinese Emperor Shen Nung recorded the properties and medicinal applications of cannabis in his compendium of herbal remedies, marking one of the earliest documented uses of the plant in traditional medicine. From China, the knowledge and use of cannabis spread to India (Charitos et al., 2021; Crocq, 2020). The introduction of cannabis to Western medicine is often attributed to William O'Shaughnessy, who, while working in India in 1839, described the plant's pharmaceutical properties, thereby initiating its medical use in the West. By 1854, cannabis had gained recognition in the United States, as evidenced by its inclusion in the United States Dispensatory (Robson, 2001). It was freely available in Western pharmacies and remained a part of the British Pharmacopoeia for over a century, available in extract and tincture forms (Maccarrone et al., 2023).

Cannabis is a complex plant, containing more than 550 known chemical compounds. Among these, around 100 are identified as cannabinoids, a class of aryl-substituted meroterpenes (Bonini et al., 2018; Hanuš et al., 2016). The plant comprises eighteen chemical classes, including nitrogen compounds, amino acids, hydrocarbons, carbohydrates, terpenes, organic compounds, and fatty acids (Turner et al., 1980). Among these, the most important active compound in cannabis is the psychoactive cannabinoid delta-9-tetrahydrocannabinol (THC) (Atakan, 2012) and the non-psychoactive cannabidiol (CBD) (McCarberg & Barkin, 2007). THC's lipophilic structure allows it to cross the blood-brain barrier, contributing to its psychoactive effects (van den Elsen et al., 2014). The highest concentration of THC is found in the female inflorescences of the cannabis plant (Jin et al., 2020; Jin et al., 2021).

Various parts of the cannabis plant, including the flower, leaves, oil, and trichomes, have demonstrated a wide range of pharmacological properties. These effects include cytotoxic, antioxidant, antimicrobial, antihypertensive, antipyretic, and appetite-stimulating properties (Russo & Marcu, 2017). The antioxidant activity of flower extracts, particularly, has been associated with health-promoting and anti-aging properties. These extracts have been utilized in the treatment of numerous metabolic and chronic disorders, such as glaucoma, pain, cancer, cardiovascular diseases, liver disease, inflammation, depression, and metabolic syndrome (Nallathambi et al., 2017). This diverse array of potential therapeutic applications underscores the ongoing interest in cannabis as a source of novel treatments for various medical conditions.

2.4. Abiotic Stress on Cannabis

The effects of environmental stresses on *Cannabis sativa* L. have been extensively studied, with particular focus on drought, salinity, light, and temperature. These factors significantly influence the plant's growth, yield, and secondary metabolite production, including cannabinoids and terpenes.

2.4.1. Drought Stress

Drought stress has varying effects on cannabis, depending on its intensity and timing.

Morgan et al., (2024) observed that moderate drought (30–50% field capacity) had no significant impact on yield or cannabinoid content, whereas severe drought caused yield reductions of up to 25% and significantly lowered both THC and CBD levels. Cannabis under water deficit conditions in another study by Gill et al., (2022) showed decreased biomass production (by ~40% under severe deficit) and reduced total seed yield, though filled seeds were maintained, ensuring reproductive success. Water use efficiency increased by 20–25% due to reduced stomatal conductance and transpiration, and proline accumulation rose nearly 2-fold, suggesting osmotic adjustment under stress.

Caplan et al., (2019) demonstrated that container-grown cannabis subjected to controlled drought stress (midday water potential ≈ –1.5 MPa after 11 days without fertigation) exhibited increased concentrations of THCA and CBDA by 12% and 13%, respectively, compared to well-watered plants. Furthermore, yields per unit growing area of THCA, CBDA, THC, and CBD were 43%, 47%, 50%, and 67% higher, respectively, in drought-stressed plants.

Babaei & Ajdanian, (2020) in a study at the University of Tehran examined water deficit stress tolerance in 47 Iranian *Cannabis sativa* L. ecotypes, and reported that drought generally reduced dry matter production (up to 35% in sensitive ecotypes). However, drought tolerance indices such as geometric mean productivity (GMP), stress tolerance index (STI), and mean productivity (MP) effectively differentiated tolerant ecotypes like Darab and Isfahan, which maintained relatively higher biomass under stress.

Sheldon et al., (2021) focused on identifying water-saving traits among hemp cultivars and their influence on cannabidiol (CBD) potency. They demonstrated that water scarcity dramatically affects cannabis plants' transpiration rates (by 30–40%), crucial for growth and overall health. As soil moisture decreases, many cannabis cultivars struggle to maintain optimal water usage, potentially leading to reduced growth and yield. High vapor pressure deficit conditions, often associated with drought, can cause excessive water loss in some cannabis varieties, further stressing the plants. These water-related stresses can impact the plant's ability to produce valuable compounds like CBD by up to 15% in sensitive cultivars, potentially reducing the crop's economic value.

In contrast, a study by Asadi et al., (2023) aimed to identify drought-tolerant ecotypes along with medicinal and industrial populations. Findings revealed a genotype-dependent response to drought: THC levels increased under 50% soil moisture, with the Qom ecotype recording the highest THC concentration (1.63%), whereas CBD levels were maximized under well-watered conditions, with the Rasht ecotype showing the highest CBD concentration (2.15%).

2.4.2. Salinity Stress

Salinity stress has been shown to have significant impacts on cannabis growth and cannabinoid production. Yep et al., (2020) reported that increasing NaCl concentrations (1–40 mM) during the flowering stage in hydroponic and aquaponic systems caused a linear decline in cannabinoid content, with THCA decreasing by 0.026% per mM NaCl in aquaponics and 0.037% per mM NaCl in hydroponics. Growth and physiological traits were significantly impaired at 40 mM NaCl in hydroponics, whereas aquaponics conferred partial tolerance.

Another study by Beheshti et al., (2023) that female cannabis plants tolerated salinity up to 75 mM NaCl, where biomass increased by nearly 10% compared to control, but were more sensitive than male plants at higher salinity (≥100 mM). Salinity stress enhanced the accumulation of phenols (by ~30%), flavonoids (by ~25%), proline, and soluble carbohydrates in both sexes, though the increase was more pronounced in females.

A recent study Rabbani et al., (2023) found that high salinity levels (8 dS $m^{-1} \approx 80$ mM NaCl) significantly reduced plant height (–35%), shoot weight (–42%), root weight (–38%), total chlorophyll (–29%), and relative water content (–20%) in hemp. Ion imbalance was evident, with decreased K^+, Mg^{2+}, and Ca^{2+} and increased Na^+ and Cl^- levels in leaves.

Formisano et al., (2024) investigated the effects of saline irrigation on cannabis, particularly at higher salinity levels (4.0 and 6.0 dS m^{-1}). These treatments significantly reduced biomass by 15–22% due to nutrient imbalances, but surprisingly increased CBD content by ~18% at 6.0 dS m^{-1}. Application of a plant-based biostimulant improved nitrogen uptake and partially mitigated salt-induced growth reduction. Yuan et al., (2024) investigated the impact of high salt stress (200 mM NaCl) on cannabis inoculated with arbuscular mycorrhizal fungi (AMF). The study showed that high salt stress had a negative impact on hemp, even when inoculated with AMF. Under these conditions, plant physiology and biochemistry were negatively affected, and the synthesis of secondary metabolites was reduced by 25-30%, despite some alleviation of growth inhibition. This high salt concentration caused ion toxicity and oxidative damage, which AMF inoculation could not fully counteract. While AMF generally helps plants cope with stress, at extreme salt levels, its positive effects were limited, and overall plant health declined. This highlights that high salt stress can overwhelm the beneficial effects of AMF in hemp.

2.4.3. Light and Temperature Stress

The response of *Cannabis sativa* to light intensity, temperature, and CO_2 concentrations has been studied in detail. Chandra et al., (2008) found that light intensities exceeding 1500 μmol $m^{-2}s^{-1}$ and temperatures above 30°C negatively impacted photosynthesis and water use efficiency. Low CO_2 levels (250 μmol mol^{-1}) led to a significant decline in photosynthesis and water use efficiency, while causing an increase in transpiration and stomatal conductance. A study by Chandra et al., (2011) found that different cannabis varieties showed varying responses to temperature, with the drug-type variety HPM from Mexico exhibiting the highest rate of photosynthesis between 30°C and 35°C. Heat stress at 40°C caused significant reductions in photosynthesis across all varieties. Lydon et al., (1987) studied UV-B radiation effects in cannabis and revealed no significant physiological or morphological differences among UV-B treatments. However, in drug-type plants, the concentration of Δ9-THC increased as the UV-B dose intensified.

Jenkins, (2021) evaluated the impact of narrow bandwidth UV-A light and a combination of blue and red light on cannabinoid and terpene production during the final two weeks of flowering. Two of the three cannabis cultivars tested showed increased THC production, particularly under UV-A light, though the effects on terpene profiles and yield varied.

2.4.4. Combined Stresses and Other Factors

Park et al., (2022) examined the impact of short-term environmental stresses on cannabinoid production in young, immature flowers. Herbivory, excess heat, and drought negatively impacted cannabinoid production, with drought being the most significant constraint (70-80% reduction in CBD and THC. Darigh et al., (2022) explored the effects of simulated microgravity on *Cannabis indica* callus cells. The treatment increased callus biomass, induced gene expression related to cannabinoid synthesis and enhanced the production of cannabinoids such as CBG and CBD, while reducing THC concentration.

These studies demonstrate the complex interactions between environmental stresses and cannabis growth, yield, and secondary metabolite production. Understanding these relationships is crucial for optimizing cultivation practices and maximizing desired cannabinoid profiles in both medical and industrial applications of *Cannabis sativa* L.

2.5. Fiber Type Cannabis

The textile industry, valued at approximately 1 trillion USD, is one of the largest global industrial sectors (Harsanto et al., 2023). However, it is also among the most polluting, contributing significantly to water pollution, greenhouse gas emissions, and land use (Leal Filho et al., 2022). The rise of fast fashion in the late 20th century, driven by low-cost clothing production, has led to a sharp increase in garment manufacturing and, consequently, a surge in textile waste from both producers and consumers (Bick et al., 2018).

The accumulation of textile waste presents a major environmental challenge, with around 87% of textile products ending up in landfills or being incinerated as of 2022. In the European Union (EU), this equates to an annual waste generation of 11 kg per person (Arafat & Uddin, 2022). In response to the urgent need for reducing carbon emissions and promoting sustainability, efforts have intensified to develop durable, reusable, and recyclable textiles (Kamaruddin et al., 2021).

Among the promising alternatives, industrial cannabis (hemp) stands out as a key contributor to sustainable textile production (Fordjour et al., 2023). Currently, hemp holds a 0.3% market share in textile fibers (Mariz et al., 2024). As a cost-effective natural fiber, hemp is priced at \$0.5–\$1.5 per kg, making it a more affordable alternative to E-glass (\$1.65–\$3.25 per kg) and carbon fiber (\$8–\$11 per kg) (Thakur et al., 2014; Yan et al., 2014).

Cannabis fiber production has been conducted for centuries, with end uses ranging from textiles and papers to ropes and sails. Notably, cannabis paper was used in the first copies of the Bible (Ahmed et al., 2022). Its fiber is recognized as one of the strongest and stiffest available natural fibers, possessing high tensile strength and elastic modulus compared to other natural fibers such as jute, sisal, cotton, banana, and bamboo (Abdalla et al., 2023). These superior mechanical properties, combined with its cost-effectiveness, make hemp fiber a promising sustainable alternative to conventional composite reinforcements (Behera et al., 2021).

The combination of hemp fiber's mechanical performance, low cost, and sustainability advantages positions it as a potential replacement for synthetic fibers in polymer composites across various applications. As the textile industry seeks to address environmental concerns and move towards more sustainable practices, industrial hemp stands out as a viable and promising alternative fiber source.

2.6. Abiotic Stress on Natural Fibers

Natural fibers, derived from plant sources such as cotton, flax, hemp, jute, and coir, are highly susceptible to abiotic stress factors. These environmental stresses, including drought, salinity, extreme temperatures, UV radiation, and pollution, significantly influence fiber yield, quality, and structural composition. Various studies have documented that salt stress can adversely impact fiber characteristics, with reductions observed in fiber strength, length, and maturity, though an increase in fiber fineness has also been noted under these conditions. Longenecker, (1974) found that as sodium ion concentration increased, micronaire values exhibited a declining trend, indicating a reduction in fiber maturity. Environmental stressors such as increased electrical conductivity (EC) have been shown to influence key physiological processes, including cellulose deposition, photosynthesis, and sugar transport, all of which are integral to fiber development. Yfoulis & Fasoulas, (1973) reported that elevated EC levels hinder cellulose deposition, leading to a reduced cross-sectional area and subsequently lower production of mature fibers. Interestingly, Abdelraheem et al., (2019) observed that under a moderate salt level (0.42%), there was an increase in fiber length accompanied by a decrease in fiber fineness and elongation, suggesting that salt stress may have complex effects depending on its severity and specific conditions.

The timing of stress exposure is also a critical factor influencing fiber quality. For instance, Marani & Amirav, (1971) conducted experiments with two American cotton cultivars, Acala 4–42 and Deltapine Smooth Leaf, which were subjected to early stress during the flowering season and again shortly after flowering. Their results indicated no significant effect on fiber quality due to early stress exposure; however, stress imposed after the flowering stage led to diminished fiber quality.

The study by Gören & Tan, (2024) assessed ten homozygous cotton lines under two irrigation conditions: well-watered (100% field capacity) and water-deficit (50% field capacity). Key traits, such as boll weight, fiber length, and strength, were measured using HVI instruments, to evaluate genotype performance and the impact of water stress on fiber quality traits. The results evaluated that water-deficit conditions alter lint percentage, boll weight, and fiber strength in cotton, with fiber length and uniformity often declining under drought stress. While some genotypes, like LTDS-116 and LTDS-128, show resilience under stress by maintaining fiber strength, others experience substantial quality degradation.

Johnson et al., (2002) investigated the impact of soil spatial variability on cotton "LA 887" variability, fiber output, and quality in South Carolina. The study emphasized that soil pH and nutrient levels- particularly phosphorus, potassium, and magnesium affect fiber yield and maturity, with lower soil pH and nutrient imbalances often leading to reduced fiber quality. Their study also suggests that areas with higher moisture and organic matter may yield shorter, less mature fibers. Spatial analysis reveals that soil and fiber properties exhibit distinct patterns, where high variability within a field may necessitate site-specific management to optimize fiber quality across different environmental conditions.

In a study by (Kwiatkowska et al., 2023) flax was grown under controlled conditions and settings with various irrigation levels- such as 25%, 35%, and 45% field soil moisture, in Poland. The study concludes that drought stress negatively affects flax fiber quality by reducing the linear density, length, and tenacity of fiber. Flax plants subjected to lower soil moisture (25% and 35% field capacity) yielded fiber with reduced thickness and lower overall maturity compared to those grown under optimal conditions (45% field capacity). Fiber density and length were both significantly diminished under drought, indicating that adequate water is essential for developing high-quality flax fibers. Moreover, SEM imaging showed that fiber under drought stress had a reduced pectin coating, which resulted in thinner fiber.

Khalequzzaman et al., (2024) in their study found that reduced soil moisture, particularly at 50% of field capacity, has been shown to decrease critical fiber quality parameters of cotton, impacting both the physical and structural attributes of the fiber. Fiber length, an important determinant of textile strength and smoothness, was declined by approximately 6–7% in drought-stressed plants compared to those with optimal water availability. Similarly, fiber strength was reduced by 6–12%, diminishing the durability and tensile qualities of the fiber, which are essential for producing resilient textile products. Drought stress also affected fiber uniformity, with a decrease in the uniformity index from 83.1% to 81.4%, indicating increased variability in fiber thickness. Additionally, micronaire values, which indicate fiber fineness and maturity, increased from 4.7 to 5.0 mg/inch under water-limited conditions. This rise in micronaire reflects a coarser fiber texture, which can impact the fabric’s overall quality and processing efficiency.

Sahu et al., (2019) found that saline conditions during boll development negatively affect fiber characteristics, with substantial reductions in fiber length, strength, and fineness. These adverse effects are attributed to the accumulation of ions, specifically sodium (Na^+) and chloride (Cl^-), which disrupt nutrient uptake and cellulose deposition, a critical factor in fiber development. Under salt stress, salt-sensitive cultivars exhibit higher chloride accumulation in leaves, which is associated with reduced fiber maturity and a decrease in staple length and fiber strength. Meanwhile, an increase in micronaire values- a measure of fiber fineness and maturity, indicates a trend toward coarser fiber quality under saline conditions.

A study by Gao et al., (2021) explored the effects of abiotic stress, specifically drought and elevated temperature, on cotton fiber quality, with a focus on two cultivars: the heat-sensitive Sumian 15 and the heat-tolerant PHY370WR. The findings indicate the detrimental impact of combined stress on fiber quality, with varying responses observed between the heat-sensitive and heat-tolerant cultivars. Both individual and combined abiotic stresses significantly reduced fiber quality attributes, such as length, strength, and micronaire values, although elevated temperatures occasionally mitigated the adverse effects of drought on fiber strength. The findings suggest that fiber quality, especially fiber length, is highly sensitive to the intensity of drought stress, with elevated temperatures exacerbating these losses.

Another study by Kwiatkowska et al., (2024) examined the effects of drought stress on C*annabis sativa* L. fiber quality through a three-year controlled experiment. By manipulating soil moisture levels, researchers found that drought negatively impacts fiber characteristics. Specifically, drought reduced fiber linear density but did not significantly change fiber strength. Chemical analysis revealed increased wax and fat content in fiber, potentially as a water conservation mechanism. Thermal and structural analysis showed lowered decomposition temperature and reduced fiber crystallinity under drought conditions. The research highlights the potential challenges drought poses to cannabis fiber production for textile and industrial applications, demonstrating how environmental stress can compromise crop quality.

2.7. Impact of Glyphosate Stress on Biochemical Parameters

2.7.1. Photosynthetic Pigments

Glyphosate, a widely used herbicide, has been shown to have significant effects on photosynthesis and related processes in plants. While glyphosate does not directly inhibit photosynthesis, it indirectly affects this crucial process through various mechanisms.

Glyphosate primarily acts by inhibiting the shikimate pathway, which is essential for the biosynthesis of aromatic amino acids, carotenoids, chlorophylls, and fatty acids (Duke & Dayan, 2018). This inhibition can lead to a cascade of effects on photosynthetic processes. The first stage of photosynthesis takes place in the thylakoid membranes of chloroplasts, where light excites photosynthetic pigments, primarily chlorophylls, triggering electron flow (Rohacek et al., 2008). Several studies have reported that glyphosate application leads to decreased chlorophyll content in plants, likely due to either chlorophyll degradation or inhibited biosynthesis (Mateos-Naranjo et al., 2009; Zobiole et al., 2011). Glyphosate may indirectly suppress chlorophyll synthesis by reducing Mg levels in leaves (Cakmak et al., 2009), which affects the role of Mg chelatase in incorporating Mg into the porphyrin structure, a critical step in chlorophyll production (Tanaka & Tanaka, 2007).

Due to its strong cation-chelating properties, attributed to its carboxyl and phosphonate groups, glyphosate can form complexes with essential nutrients in plant tissues, making them biologically unavailable for processes like photosynthesis (Cakmak et al., 2009). Interestingly, Reddy et al., (2004) suggested that aminomethylphosphonic acid (AMPA), a degradation product of glyphosate, may primarily contribute to the inhibition of chlorophyll biosynthesis, though the exact mechanism remains unclear.

Research by Serra et al., (2013) found that AMPA exposure reduced glycine, serine, and glutamate levels in plants, which could, in turn, limit δ-aminolevulinic acid (ALA) and chlorophyll production, as these amino acids are essential for ALA biosynthesis. In plants lacking glyphosate oxidoreductase (GOX) and not exposed to AMPA, glyphosate-induced reductions in chlorophyll content may result from impaired nitrogen assimilation, leading to lower glutamate levels and disrupted ALA and chlorophyll synthesis (Zobiole et al., 2011).

Further studies, such as that of Maldani et al., (2021) on Vicia faba, Phaseolus vulgaris, and Sorghum bicolor, demonstrated that glyphosate application negatively impacted seed germination, seedling efficiency, photosynthetic pigments, and amino acid content. Similarly, Percival, (2017) found that glyphosate tolerance in three Acer species, measured by reductions in leaf chlorophyll content, was linked to the concentrations of carotenoids and xanthophyll-cycle pigments in leaf tissues.

Investigations by Gomes et al., (2016) into the differential effects of glyphosate and AMPA on *Salix miyabeana* showed that while both compounds led to decrease in chlorophyll content and photosynthetic rates, they did so through different mechanisms. AMPA primarily disturbed chlorophyll biosynthesis, whereas glyphosate induced chlorophyll degradation through increased reactive oxygen species (ROS) production.

Sandmann et al., (2006) concluded that glyphosate's effects on photosynthesis go beyond chlorophyll reduction. It can also directly lower carotenoid content by inhibiting plastoquinone biosynthesis, a crucial co-factor for key enzymes involved in carotenoid production. Silva., (2014) demonstrated that Raphanus sativus plants treated with glyphosate exhibited reduced photosynthetic efficiency and increased cellular membrane damage, resulting in a decline in chloroplast pigment content. Another study by Yanev & Kalinova, (2020) on tobacco plants showed that glyphosate application led to a slight reduction (6% to 18%) in the rate of photosynthesis compared to untreated plants, likely due to decreased content of photosynthetic pigments.

Kaeoboon et al., (2021) researching on microalgae, specifically Chlorella species, revealed that increasing glyphosate concentrations had a negative linear effect on chlorophyll and carotenoid levels. Glyphosate may compromise thylakoid membrane integrity and indirectly inhibit chlorophyll synthesis by reducing Mg availability. Furthermore, a study by Hernando et al., (1989) demonstrated that glyphosate functions as a photosynthetic electron transport inhibitor, specifically suppressing the activity of Photosystem II (PSII) in *Chlorella pyrenoidosa*. Research by Krenchinski et al., (2017) on glyphosate-resistant (RR) soybean cultivars demonstrated a significant linear decline in chlorophyll index with increasing glyphosate dose. This effect is attributed to glyphosate's ability to chelate metal cations like Fe, Mn, and Mg, which are crucial for chlorophyll biosynthesis (Coutinho & Mazo, 2005; Lazar, 2003).

Ahsan et al., (2008) in their proteomic studies on rice leaves exposed to glyphosate revealed significant alterations in protein expression profiles. Notably, glyphosate treatment led to a significant reduction in the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), a crucial enzyme in photosynthesis.

Thus, while glyphosate does not directly inhibit photosynthesis, its effects on various biochemical pathways and cellular processes indirectly lead to significant impacts on photosynthetic efficiency, chlorophyll content, and overall plant health. These effects are observed across a wide range of plant species and can vary depending on the plant's resistance to glyphosate and its ability to metabolize the herbicide.

2.7.2. Antioxidant Enzymes

The impact of glyphosate on plant antioxidant systems and oxidative stress has been extensively studied across various plant species. Superoxide dismutase (SOD) and catalase (CAT) are primary antioxidant enzymes that play crucial roles in protecting cells against the harmful effects of free radicals. SOD catalyses the conversion of superoxide radicals to hydrogen peroxide (H_2O_2) and oxygen, while CAT further converts H_2O_2 into water and oxygen, completing the detoxification process initiated by SOD (Yalçin & Çavuşoğlu, 2022).

Research has shown that glyphosate application can lead to increased oxidative stress in plants, triggering various physiological responses. de Freitas-Silva et al., (2017) reported that glyphosate administration in *Arabidopsis thaliana* resulted in increased oxidative stress, oxidized proteins, and induced antioxidant enzymes, including elevated CAT activity. This suggests that plants may increase SOD and CAT enzyme levels as a defence mechanism against excessive free radical production caused by glyphosate exposure.

A study by Kültiğin, (2011) on *Allium cepa* L. revealed that glyphosate exposure at concentrations of 100, 250, and 500 mg L^{-1} produced toxic effects. The study found significant changes in several parameters, including root length, germination percentage, seedling weight, and malondialdehyde (MDA) levels. Notably, all treatment doses significantly enhanced lipid peroxidation, leading to increased MDA levels ($P<0.05$). Research by Beker et al., (2018) on *Zea mays* L. investigated glyphosate's impact on antioxidant enzyme activity and lipid peroxidation over a 10-day period. The research documented increased activity of several antioxidant enzymes—peroxidase (POD), ascorbate peroxidase (APX), superoxide dismutase (SOD), catalase (CAT), and glutathione-S-transferase (GST)—at both 5 and 10 days after treatment. In contrast, glutathione (GSH) and glutathione reductase (GR) activities showed a decrease by day 10. The study also found that malondialdehyde (MDA) content, a marker of lipid peroxidation, progressively increased throughout the experimental period. These alterations in antioxidant enzyme activities and elevated MDA levels were considered indicative of glyphosate-induced cytotoxicity in *Zea mays* L.

Shopova et al., (2021) investigated how brassinosteroids might influence winter wheat's

(*Triticum aestivum* L.) physiological responses to glyphosate stress. The research found that applying glyphosate significantly elevated levels of stress markers including proline and malondialdehyde (MDA), indicating oxidative damage in the plants. While glyphosate treatment caused only minor increases in superoxide dismutase (SOD) and catalase (CAT) activity, it triggered substantial increases in glutathione reductase (GR) and guaiacol peroxidase (POX) activities. The researchers suggested that the enhanced activities of POX and glutathione S-transferase (GST) likely play roles in the plant's mechanisms for detoxifying the herbicide.

These studies collectively demonstrate that glyphosate exposure in plants triggers complex responses in antioxidant enzyme systems. The general trend shows increased activities of enzymes like SOD, CAT, POD, and APX, likely as a protective mechanism against oxidative stress. However, the specific responses can vary depending on the plant species, glyphosate concentration, and duration of exposure. The consistent increase in MDA levels across studies indicates that despite the upregulation of antioxidant enzymes, glyphosate still induces significant oxidative damage in plant tissues. These findings underscore the importance of understanding the intricate balance between oxidative stress and antioxidant defence mechanisms in plants exposed to glyphosate.

2.7.3. Protein Synthesis

Glyphosate, a widely used herbicide, exerts its primary mode of action through the inhibition of the enzyme 5-enolpyruvylshikimic acid-3-phosphate synthase (EPSPS). This enzyme plays a crucial role in the shikimate pathway, which is essential for the production of aromatic amino acids- tyrosine, phenylalanine, and tryptophan (Mondal et al., 2017). These amino acids are fundamental for protein synthesis and various primary and secondary metabolic processes in plants. The inhibition of EPSPS by glyphosate leads to a cascade of physiological effects in plants. One of the primary consequences is the depletion of aromatic amino acids, which in turn impairs the synthesis of functional proteins (Burke & Bell, 2014). This effect is particularly pronounced in meristematic tissues, where the inhibition of branched-chain amino acid synthesis causes significant injury (Mondal et al., 2017).

Studies on various plant species have provided insights into the specific effects of glyphosate exposure on the metabolism of amino acids. Wang, (2001) demonstrated in purple nutsedge (*Cyperus rotundus*) that glyphosate treatment led to rapid accumulation of shikimic acid and a significant decrease in tryptophan levels in sprouted tubers. A noteworthy observation in the study was that purple nutsedge shoots exhibited shikimic acid accumulation without corresponding reductions in the three aromatic amino acids. This finding indicates that the toxic effects of glyphosate in purple nutsedge shoots might be primarily linked to the quick buildup of both glyphosate and shikimic acid, rather than to a deficiency in aromatic amino acids as commonly assumed.

Research by Dor et al., (2017) on the parasitic plant *Phelipanche aegyptiaca* demonstrated that glyphosate treatment resulted in dose-dependent shikimic acid accumulation and an increase in total free amino acid content. This increase in free amino acids suggests an inability to synthesize complete proteins or an indirect effect leading to protein degradation.

Gomes et al., (2017) showed in willow (*Salix miyabeana*) plants, glyphosate exposure led to H_2O_2 accumulation beyond the plant's tolerance limit, potentially causing protein carbonylation - an irreversible oxidative process affecting lysine, arginine, proline, and threonine side chains.

A comparative study by Orcaray et al., (2010) of glyphosate and ALS inhibitors on pea plants revealed that glyphosate treatment caused an early accumulation of total free amino acids, similar to ALS inhibitors. This increase was attributed to enhanced protein turnover, where protein degradation outpaced synthesis. Notably, glyphosate initially decreased the proportion of aromatic amino acids within 24 hours of treatment, followed by a subsequent increase, suggesting a compensatory mechanism possibly related to proteolysis. The study also observed an increase in the proportion of branched-chain amino acids (valine, leucine, isoleucine) following glyphosate treatment.

Shilo et al., (2016) in their research on *Phelipanche aegyptiaca*, using a glyphosate-resistant tomato as a host plant, provided detailed insights into the temporal effects of glyphosate on amino acid metabolism. The study observed a decline in parasite tissue viability within 40 hours after treatment, accompanied by shikimate accumulation starting at 24 hours post-treatment. Free levels of phenylalanine and tyrosine decreased by 48 hours after treatment, indicating a deficiency in these essential compounds. Interestingly, tryptophan levels remained similar to the untreated control, suggesting differential effects of glyphosate on the pathways leading to various aromatic amino acids.

The inhibition of EPSPS by glyphosate leads to complex alterations in plant amino acid metabolism. These effects include the accumulation of shikimic acid, changes in free amino acid pools, and disruption of protein synthesis. The specific impacts can vary depending on the plant species, tissue type, and duration of exposure. While the primary effect is on aromatic amino acid synthesis, secondary effects on protein turnover and oxidative stress also play significant roles in glyphosate's overall impact on plant physiology. These findings underscore the complex nature of glyphosate's mode of action and its far-reaching effects on plant metabolism.

2.7.4. Carbohydrates

Studies on the effects of glyphosate on plant carbohydrate metabolism reveal significant disruptions, primarily due to glyphosate's impact on photosynthetic processes. Vital et al., (2017) in their study in sunflower plants, showed glyphosate exposure resulted in considerable reductions in carbohydrate levels, affecting total soluble sugars, reducing sugars, non-reducing sugars, and starch content. Upon exposure to higher glyphosate doses, carbohydrate reserves in the plants showed dramatic reductions. Specifically, compared to untreated control plants, soluble sugars decreased by up to 72%, reducing sugars by 71%, and non-reducing sugars by 73%. The most severe impact was observed in starch content, which plummeted by up to 92%. This depletion of carbohydrates correlates with glyphosate-induced impairments in photosynthetic efficiency, which limits the production of these essential compounds. The decrease in carbohydrates under glyphosate stress affects not only the energy supply and growth of plants but also disrupts essential metabolic pathways, as these carbohydrates provide critical carbon sources for various physiological and structural functions. Consequently, glyphosate-induced carbohydrate reduction illustrates the herbicide’s extensive impact on plant energy balance and overall metabolism.

Salman et al., (2016) examines the impact of glyphosate, a common herbicide, on the biochemical attributes of the cyanobacterium *Oscillatoria limnetica*. The study found that glyphosate exposure led to a significant reduction in carbohydrate content in *Oscillatoria limnetica*. The inhibition was concentration-dependent, with the greatest decrease observed at the highest glyphosate concentration (20 mg/L), where carbohydrate content dropped by 12.223%. This reduction is likely due to the disruption of photosynthetic processes, particularly in Photosystem II, where glyphosate exposure impairs chlorophyll and accessory pigments essential for energy transfer. As a result, photosynthetic efficiency declines, reducing carbohydrate synthesis within the cells.

Beker et al., (2018) examines the impact of glyphosate on carbohydrate content in *Zea mays*. Over the course of the study, it was observed that carbohydrate levels in *Zea mays* decreased progressively, with the most significant reductions occurring at higher glyphosate concentrations and later observation times (days 5 and 10). This reduction can compromise the plant's ability to perform essential physiological activities, ultimately stunting growth and potentially affecting crop yields.

2.8. Impact of Metribuzin Stress on Biochemical Parameters

2.8.1. Photosynthetic Pigments

Metribuzin, a commonly used herbicide, has been thoroughly researched for its impacts on photosynthesis and plant development in different species. This herbicide primarily works by inhibiting Photosystem II (PSII) in the photosynthetic process. Specifically, metribuzin attaches to the QB-binding site on the D1 protein complex within the chloroplast thylakoid membrane, which interrupts the electron transport system (ETS) between QA and QB (Sherwani et all., 2015). This interruption triggers a series of effects, including the blockage of carbon dioxide fixation, ATP generation, and nicotinamide adenine dinucleotide hydrogen phosphate ($NADPH_2$) production—all essential components for various biochemical processes involved in plant growth and development (Lambreva et al., 2014).

When metribuzin inhibits the electron transport chain (ETC), it prevents QA reoxidation, which leads to the formation of triplet-chlorophyll and subsequently singlet-oxygen. These reactive molecules initiate lipid peroxidation, causing oxidation of lipids in the bilayer and other proteins, ultimately generating reactive oxygen species (ROS) (Roach & Krieger-Liszkay, 2014). The resulting oxidative stress causes the loss of chlorophyll and other pigments like carotenoids from cell membranes, which exposes cells and organelles to harsh conditions and eventually leads to their collapse and disintegration (Santabarbara, 2006).

Studies across various plant species have consistently demonstrated the negative impact of metribuzin on photosynthetic processes. Research by Kumar et al., (2022a) on *Vigna radiata* leaves showed a significant decline in photosynthetic pigments at metribuzin concentrations of 200-1000 mg L^{-1}, with chlorophyll and carotenoids reduced by up to 82.4% and 73.1%, respectively, at the highest concentration.

Agostinetto et al., (2016) studied metribuzin application in wheat crops, which led to significant reductions in photosynthetic rate, stomatal conductance, and transpiration at 24 and 120 hours after spraying compared to control plants. Silva et al., (1991) observed in soybean plants, that metribuzin negatively impacted net photosynthetic rates, particularly at doses of 0.8 kg a.i./ha^{-1}. The herbicide also decreased dry weight of plant parts and leaf area, with a more pronounced effect on root dry weight compared to shoot biomass.

Comparative studies of metribuzin with other herbicides have provided insights into its relative phytotoxicity. In a study by Zaidi et al., (2005) on greengram (*Vigna radiata*) inoculated with *Bradyrhizobium* sp., metribuzin showed a higher degree of phytotoxicity compared to glyphosate, fluchloralin, and 2,4-dichlorophenoxy-acetic acid (2,4-D). A reduction of 33.3% in seed yield at a metribuzin concentration of 0.5 1 µg a.i. g^{-1} and 55.5% at 1 µg a.i. g^{-1} was observed, and it also resulted in significant reduction nodulation and grain protein content.

The effects of metribuzin extend beyond crop plants to aquatic ecosystems. Wilkinson et al., (2015) in their study on *Halophila ovalis* demonstrated that metribuzin, along with other PSII herbicides, inhibited photosynthetic activity ($\Delta F/Fm'$). Notably, metribuzin required 48 hours to reach maximum inhibition, resulting in significantly lower IC50 values and indicating increased potency with longer exposure times.

Research on natural periphyton communities by Gustavson et al., (2003) revealed that metribuzin significantly impact photosynthetic activity at lower concentrations compared to standard phytoplankton tests. The effect concentration (EC50) of metribuzin decreased with longer exposure times, highlighting higher sensitivity with prolonged exposure. While photosynthetic activity showed near-full recovery after 48 hours in herbicide-free water, different periphyton groups exhibited varying responses, with chlorophytes being severely affected and showing poor recovery compared to diatoms and cyanobacteria.

Balakumar & Mahesh, (2023) in cyanobacteria, specifically *Phormidium* and *Chroococcus* species showed that metribuzin significantly inhibits chlorophyll *a* content and reduced carbohydrate, protein, amino acid, and lipid levels in a dose- and time-dependent manner. *Phormidium* showed greater sensitivity to metribuzin compared to *Chroococcus*, with noticeable reductions observed at concentrations as low as 2µg.

The impact of metribuzin on photosynthesis and plant growth can be mitigated to some extent by various factors. Asadzadeh et al., (2024) showed the use of plant growth-promoting Pseudomonads isolates and filter cake was found to effectively decrease the extent of damage caused by metribuzin in spinach plants. These treatments enhanced photosynthetic pigments and growth components, reducing the negative effects of metribuzin application.

The metribuzin exerts its herbicidal effects primarily through the inhibition of Photosystem II, leading to a cascade of physiological changes that ultimately result in reduced photosynthetic efficiency and plant growth. Its impacts are observed across a wide range of plant species and can extend to aquatic ecosystems. The severity of these effects depends on factors such as concentration, exposure time, and the specific physiology of the target organisms. While metribuzin remains an effective tool for weed control, its use must be carefully managed to minimize unintended consequences on non-target species and ecosystems.

2.8.2. Antioxidant Enzymes

Research on the effects of metribuzin on plant physiology has demonstrated significant oxidative stress, particularly at higher herbicide doses. A study by Karimmojeni et al., (2022b) examining the impact of various dosages of metribuzin (0, 250, 500, 750, 1000, and 1250 g ha^{-1}) on morphological and physiological characteristics of *Echinacea sp.* from different regions in Iran found that metribuzin exposure increased oxidative stress markers, particularly malondialdehyde (MDA), a key indicator of lipid peroxidation. At the highest metribuzin dose (1250 g ha^{-1}), *E. purpurea* from the Shiraz population exhibited elevated MDA levels (up to 9.14), suggesting increased lipid peroxidation compared to other species and populations, which showed lower MDA levels under the same conditions. These findings indicate that metribuzin treatment is associated with heightened oxidative stress in *E. purpurea*, as evidenced by increased MDA content in leaves, pointing to potential damage in cellular membranes due to lipid peroxidation.

A study by Almeida et al., (2019) showed that in *Chlamydomonas reinhardtii*, metribuzin exposure causes a concentration-dependent increase in reactive oxygen species (ROS), which leads to oxidative damage, particularly within chloroplasts. Although metribuzin does not appear to significantly impact glutathione (GSH) levels, it activates other antioxidant mechanisms, suggesting the involvement of alternative defence pathways to counteract ROS. Furthermore, increased lipid peroxidation (LPO) indicates damage to cell membranes, signalling oxidative stress and destabilization of cellular structures, though LPO levels tend to decrease at higher metribuzin concentrations, possibly due to adaptive cellular responses.

Study by Kumar et al., (2022a) evaluating metribuzin-induced stress responses in *Vigna radiata* has shown that exposure to this herbicide significantly impacts biochemical parameters, particularly oxidative stress markers. In their experiment examining the effects of metribuzin after 7 days of treatment, *V. radiata* leaf tissues exhibited an increase in lipid peroxidation, as well as elevated activity of antioxidant enzymes. These findings suggest that metribuzin induces oxidative stress in *V. radiata*, triggering lipid damage and activating antioxidant defence mechanisms to counterbalance the increased reactive oxygen species.

A study Agostinetto et al., (2016) examining the oxidative stress caused by metribuzin in wheat plants, report several biochemical alterations indicative of cellular stress. Metribuzin exposure has been associated with an increase in MDA levels, a marker for lipid peroxidation, which indicates significant oxidative damage to cell membranes.

Additionally, metribuzin-treated plants show variable hydrogen peroxide (H_2O_2) accumulation, an oxidative stress marker that suggests increased production of reactive oxygen species (ROS). In response to this oxidative challenge, key antioxidant enzymes, including SOD and CAT, are activated to mitigate ROS effects. SOD activity initially rises to detoxify superoxide radicals by converting them to H_2O_2, which CAT subsequently breaks down. However, over time, metribuzin exposure has been shown to reduce CAT activity, potentially leading to an imbalance in ROS regulation and further oxidative stress. These findings highlight the role of metribuzin in inducing oxidative damage and the complex antioxidant responses it triggers in wheat plants.

2.8.3. Protein Synthesis

A study by Nemat et al., (2008a) has demonstrated the differential effects of metribuzin on nitrogen metabolism enzymes in young cereal seedlings. Research has shown that when the recommended field dose of metribuzin was applied to wheat and maize seedlings at 10 days of age, it exhibited varying degrees of impact on key nitrogen assimilation enzymes. While the activities of nitrate reductase and nitrite reductase were only marginally affected, the herbicide substantially inhibited the activities of glutamine synthetase (GS) and glutamate synthase (GOGAT). The differential inhibition of these enzymes has significant implications for nitrogen metabolism. The strong inhibition of GS and GOGAT suggests that metribuzin treatment leads to impaired ammonia assimilation, which consequently results in decreased protein synthesis. This observation is further supported by the elevated levels of soluble nitrogen and amino acids found in metribuzin-treated seedlings, which appear to originate from the degradation of existing proteins.

2.8.4. Carbohydrates

Razieh, (2012b) has shown metribuzin exposure to significantly impact carbohydrate metabolism in wheat (*Triticum aestivum*). In this study, the effects of metribuzin on the activities of certain enzymes along with its impact on carbohydrate content of *Triticum aestivum* L. cv. Zarrin were determined. Thirty days old plants were treated with 20ml metribuzin at recommended field dose (1.01 kg ha^{-1}) through foliar application, and the plants were harvested after 7 days. Results demonstrated that metribuzin-treated wheat plants exhibit a significant reduction in the levels of total water-soluble carbohydrates, including glucose, mannose, and xylose, in the shoots. This reduction is directly linked to the herbicide’s interference with photosynthetic activity, leading to limited carbohydrate production and storage. Carbohydrates are crucial for various plant physiological processes, including energy provision, structural integrity, and as precursors in metabolic pathways. When carbohydrate levels drop, plants experience an energy deficit, which affects their ability to maintain growth, repair, and cellular metabolism.

Mona & Chung, (2013) explains that carbohydrate content is one of the most affected parameters in response to herbicide application. Research on the pre-emergence effects of metribuzin indicates that this herbicide can significantly alter carbohydrate metabolism and nitrogen accumulation in plants. Metribuzin application has been found to suppress carbohydrate levels, impacting both soluble sugars and polysaccharides. This inhibitory effect on carbohydrates coincides with a reduction in photosynthetic efficiency, pigment biosynthesis (chlorophyll), and nucleic acid content.

A study by Razieh, (2012)investigated the effects of metribuzin herbicide on wheat (*Triticum aestivum* L. cv. Zarrin) with a focus on various physiological parameters including carbohydrate content. The research found that metribuzin application significantly reduced the content of water-soluble carbohydrates in wheat plants. This decrease in carbohydrate content was linked to metribuzin's mode of action, which disrupts photosynthesis by inhibiting electron transport between the primary and secondary acceptors of Photosystem II (PSII).

2.9. Role and Mechanism of Cytochrome P450 in Herbicide Metabolism

Cytochrome P450 monooxygenases (P450s) are heme-thiolate proteins that play a crucial role in various monooxygenation and hydroxylation reactions. These enzymes are essential in herbicide metabolism, contributing to herbicide selectivity in crops and resistance mechanisms in weeds. With approximately 30 P450s identified to have herbicide-metabolizing functions across diverse plant species, their role in agricultural systems is pivotal yet complex due to variations in their function, expression, and regulation (Dimaano & Iwakami, 2021; Yu & Powles, 2014).

2.9.1. Mechanism of Cytochrome P450-Mediated Herbicide Metabolism

Cytochrome P450 enzymes typically introduce an oxygen atom into hydrophobic herbicide molecules, making them more reactive and hydrosoluble. This metabolism primarily involves reactions such as alkyl-hydroxylation, N-demethylation, O-demethylation, and aryl-hydroxylation (Schuler & Werck-Reichhart, 2003). Some reactions, such as NIH-shift (a rearrangement of hydrogen atoms on aromatic rings), are unique to plant P450s (Dimaano & Iwakami, 2021).

2.9.1.1. Role in Herbicide Selectivity

Cytochrome P450s contribute to herbicide selectivity by metabolizing specific herbicides more rapidly in tolerant crops than in susceptible weeds. For example:

(a) CYP81A6 in Rice: This enzyme metabolizes bentazon, a photosystem II (PSII) inhibitor, rendering it inactive. It also confers tolerance to sulfonylurea herbicides like bensulfuron-methyl and metsulfuron-methyl. Downregulation of CYP81A6 results in increased sensitivity to these herbicides, underscoring its critical role (Pan et al., 2006).

(b) CYP72A31 in Rice: Found primarily in *indica* varieties, this P450 confers tolerance to acetolactate synthase (ALS)-inhibiting herbicides such as bispyribac-sodium. The absence or mutation of CYP72A31 in japonica rice results in sensitivity to these herbicides (Saika et al., 2014)

(c) CYP81A9 in Corn: This enzyme metabolizes the sulfonylurea herbicide nicosulfuron, contributing to herbicide tolerance (Choe & Williams, 2020).

2.9.2. Role in Herbicide Resistance

P450 enzymes are also implicated in herbicide resistance in weeds, often leading to cross-resistance against multiple herbicides. Examples include:

(a) CYP81A12 and CYP81A21 in *Echinochloa phyllopogon*: Overexpression of these genes confers resistance to a broad range of herbicides, including ALS inhibitors, acetyl-CoA carboxylase (ACCase) inhibitors, and 1-deoxy-D-xylulose 5-phosphate synthase (DXPS) inhibitors. These enzymes metabolize a diverse array of herbicides, posing a significant challenge to weed management (Dimaano et al., 2020; Guo et al., 2019).

(b) CYP71C6 in Wheat: This enzyme metabolizes sulfonylurea herbicides and is involved in the biosynthesis of allelochemicals, showcasing a dual role in herbicide metabolism and plant defences (Gardin et al., 2015).

Cytochrome P450 enzymes play a dual role in agriculture promoting crop selectivity and herbicide tolerance while complicating weed resistance management. Further research into their catalytic mechanisms, structural properties, and regulatory networks will unlock their full potential in advancing sustainable agricultural practices and ensuring global food security (Dimaano and Iwakami, 2020; Yu and Powles, 2014).

Table 2.1: Role of different Cytochrome enzymes in herbicide metabolism

Enzyme	**Plant Species**	**Herbicides Metabolized**	**Reaction/Role**	**References**
CYP81A6	Rice	Bentazon, sulfonylurea herbicides (e.g., metsulfuron-methyl)	Metabolizes herbicides into inactive forms, conferring tolerance.	(Lu et al., 2015; G. Pan et al., 2006)
CYP72A31	Rice (*Indica*)	ALS inhibitors (e.g., bispyribac-sodium)	Confers tolerance; mutation leads to herbicide sensitivity.	(Saika et al., 2014)
CYP81A9	Corn	Nicosulfuron	Increases herbicide tolerance in corn.	(Choe & Williams, 2020)
CYP81A12	*Echinochloa phyllopogon*	ALS inhibitors, ACCase inhibitors, DXPS inhibitors	Contributes to cross-resistance by metabolizing multiple herbicides.	(Iwakami et al., 2019)

CYP81A21	*Echinochloa phyllopogon*	Same as CYP81A12	Similar role to CYP81A12 in weed resistance.	(Guo et al., 2019; Iwakami et al., 2019)
CYP71C6	Wheat	Sulfonylurea herbicides	Contributes to herbicide detoxification and resistance.	(Xiang et al., 2006)
CYP76B1	*Helianthus tuberosus*	Phenylurea herbicides	Di-N-demethylation of phenylureas, leading to detoxification.	(Robineau et al., 1998)
CYP81E22	Soybean	Bentazon	Identified as a causative gene for herbicide sensitivity in soybean.	(Kato et al., 2020)
CYP71AH11	Tobacco	Chlorotoluron	Participates in alkyl-hydroxylation and N-demethylation.	(Dimaano & Iwakami, 2021)
CYP76C2	Rice	Pelargonic acid	Catalyzes omega-hydroxylation of herbicides, promoting detoxification.	(Imaishi & Matumoto, 2007)
CYP1A1, CYP2B6, CYP2C19	Transgenic rice	Atrazine, metolachlor	Enhance herbicide tolerance in transgenic rice plants.	(Kawahigashi et al., 2006)
CYP81B2	Tobacco	Chlorotoluron	Performs alkyl-hydroxylation and N-demethylation.	(Dimaano & Iwakami, 2021)

Cytochrome P450s in *Cannabis sativa* and Their Roles Under Stress

Cytochrome P450 monooxygenases (CYPs) constitute one of the largest and most functionally diverse protein families in plants, catalyzing essential reactions in hormone signalling, detoxification, and secondary metabolism. While their significance has been extensively documented in model crops such as Arabidopsis, rice, and wheat, emerging genomic and transcriptomic evidence now demonstrates that *Cannabis sativa* harbours a similarly diverse and stress-responsive CYP repertoire.

A recent genome-wide analysis identified 221 CYP genes encoding 225 proteins in Cannabis, grouped into nine clans and 47 families, distributed across all chromosomes. This represents one of the largest CYP complements described in any non-model crop. Promoter analyses revealed that a majority of these *Cannabis sativa* CYPs contain cis-elements responsive to drought, salinity, light, hormones, and wound stress, strongly suggesting that they are inducible under abiotic challenges. In silico docking further revealed that several *Cannabis sativa* CYPs (e.g., CYP215, CYP213, CYP217, CYP14) exhibit strong binding affinities with ALS-inhibiting herbicides, highlighting their potential contribution to xenobiotic detoxification and herbicide tolerance (Kaur et al., 2024).

Transcriptomic studies reinforce the view that Cannabis P450s are stress-responsive. Under drought, genome-wide RNA-Seq analysis identified 1,292 differentially expressed genes (DEGs) in hemp, with enrichment of oxidation–reduction processes and the ABA signaling pathway, where multiple CYPs are typically involved (Gao et al., 2018). Likewise, drought combined with uniconazole treatment revealed transcriptional modulation of CYP genes linked to gibberellin and ABA metabolism, underscoring the central role of P450s in hormone-mediated stress adaptation (Jiang et al., 2021). In salinity stress, comparative transcriptomics identified over 10,000 DEGs, with strong representation of phenylpropanoid biosynthesis and hormone signaling pathways, both P450-rich networks (Zhang et al., 2021). These data clearly indicate that abiotic stresses induce reprogramming of Cannabis CYP expression, likely affecting both detoxification capacity and specialized metabolism.

At the baseline level, a comprehensive transcriptome atlas of Cannabis tissues revealed that many CYPs are highly expressed in glandular trichomes and floral tissues, the primary sites of cannabinoid and terpenoid biosynthesis (Braich et al., 2019). This provides a mechanistic link between P450 activity and secondary metabolite regulation in Cannabis. Given that cannabinoids and flavonoids are themselves modulated by abiotic stress (e.g., drought-induced shifts in THC/CBD ratios), it is plausible that CYPs mediate these responses by altering metabolic fluxes within stress-responsive pathways.

Taken together, these findings demonstrate that *Cannabis sativa* possesses a large and stress-responsive cytochrome P450 superfamily with clear roles in hormone metabolism, secondary metabolite biosynthesis, and herbicide detoxification. Although functional validation of individual *Cannabis sativa* CYPs under stress remains limited, current genomic and transcriptomic resources provide a strong foundation. A forward-looking priority will be the integration of RNA-Seq, qPCR, and metabolomic approaches to identify stress-inducible CYP isoforms that directly regulate cannabinoid biosynthesis or herbicide tolerance. Such studies will not only advance fundamental understanding of Cannabis stress biology but also enable breeding or metabolic engineering strategies aimed at enhancing resilience and maintaining phytochemical quality under challenging environmental conditions.

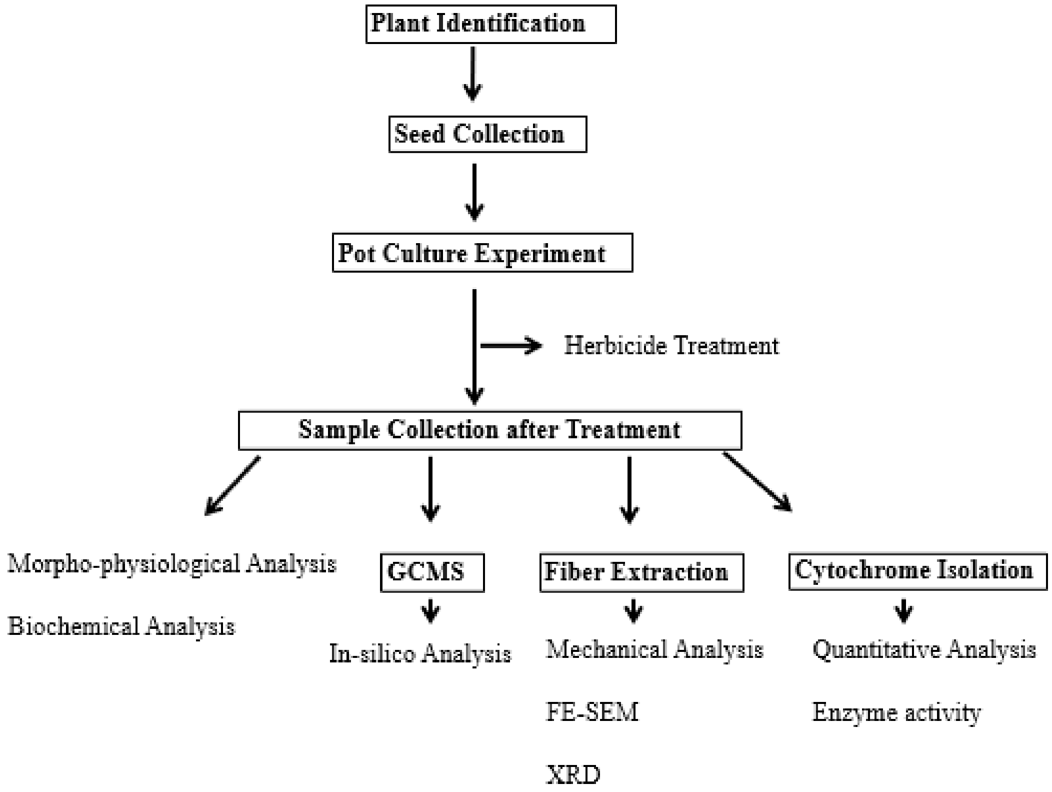
2.10. Hypothesis

This study aims to investigate the impact of glyphosate and metribuzin on *Cannabis sativa* to understand their effects on plant productivity, secondary metabolite synthesis, and fiber quality. We hypothesize that these herbicides alter cannabinoid profiles by interfering with THC biosynthesis, potentially offering insights into controlled cannabinoid manipulation for pharmaceutical applications. Additionally, we propose that herbicide exposure affects fiber composition and mechanical properties, influencing its suitability for textile production. By examining Cytochrome P450 activity, we aim to understand the plant's detoxification response and stress adaptation mechanisms. This research will provide crucial insights into optimizing *Cannabis sativa* cultivation under herbicidal stress for both industrial and pharmaceutical applications.

2.11. Objectives

1. Pot culture experiment performance on *Cannabis sativa* with differential exposure of herbicides.
2. Analysis of morphological and biochemical parameters after exposure of herbicides.
3. Extraction and analysis of plant fiber after treatment.
4. Analysis of herbicide interaction with cytochrome P450 in treated plants.

2.12. Schematic Representation of Present Study



CHAPTER 3. MATERIALS AND METHODS

3.1. Chemicals and Glassware

All glassware, including beakers, flasks, Petri dishes, pipettes, and test tubes utilized in the current investigation, were manufactured by Borosil. The chemicals and solvents used were of analytical grade, obtained from HiMedia Laboratories Private Limited, Sigma Aldrich Specialties Private Limited and Loba Chemie India, Merck, and CDH Fine Chemicals. All aqueous solutions were produced in double-distilled water (DDW) to ensure consistency and reproducibility of results.

3.2. Instruments Used

The instruments used during the study along with their models have been mentioned in Table 3.1.

Table 3.1: Detail of instruments used during the study.

Instrument	Model
Cooling Centrifuge	Rota 4V/Fm
UV-Vis Spectrophotometer	LI-2800 Ex
Gas Chromatograph	Shimadzu, GCMS-TQ8040 NX
BOD Incubator cum Shaker	Labfit
XRD	Bruker, D8 Advance
Zwick Roell Universal Testing Machine	Zwick Roell, Germany Static UTM Z010
FESEM	JEOL, JSM-7610F
Ultracentrifuge	Beckman Coulter, Optima TM L-100K Ultracentrifuge
Hot air Oven	NSW- 143

3.3. Plant Material and Growth Conditions

Cannabis sativa L. seeds were collected from a roadside plant. The herbarium was prepared and sent to a national referral facility: Janaki Ammal Herbarium CSIR-IIIM. Taxonomist Dr. Sumeet Gairola performed the plant identification at the Council of

Scientific and Industrial Research- Indian Institute of Integrative Medicine (CSIR-IIIM) Jammu, and it was given accession number 26832 (Figure 3.1).

Janaki Ammal Herbarium (RRLH)
CSIR-Indian Institute of Integrative Medicine
Jammu and Kashmir

Flora of Phagwara, Punjab
Coll No. 53083
Name Cannabis sativa L.
Family Cannabaceae
Place of Collection Phagwara
Altitude 234m
Habitat Roadside
Remarks 31·2560°N 75·7051°E
Date 22/6/2022
Coll Agustaben Vadhel and Shaheen Bashir

Figure 3.1: Herbarium of *Cannabis sativa* L. (Accession number – 26832)

The healthy and uniform seeds were surface sterilized in NaClO (0.1%) for 15 minutes, then rinsed and soaked with distilled water for an additional 20 minutes. The seeds were germinated in plastic pots containing soil that was not previously treated with any type of chemical. The pots were kept in a greenhouse under controlled conditions at a mean temperature of 24-28°C in an agricultural field at Lovely Professional University, Phagwara, Punjab (234 m.a.s.l, 31.2560° N, 75.7051° E). Different concentrations of glyphosate and metribuzin were used to treat the plants. Both glyphosate (a.i. 40.6% w/w, Agri Venture) and metribuzin (a.i. 88% w/w, TATA) herbicides used in the current experiment were of industrial grade. To prevent cross-contamination between treatments during herbicide application, each pot was individually removed from the experimental area and treated separately with the designated herbicide concentration before being returned to its original position. For 2nd objective, the experiment comprised the following treatments: T0 (control: 0 mL/L for glyphosate; 0 g/L for metribuzin), T1 (X/8: 0.625 mL/L glyphosate; 0.25 g/L metribuzin), T2 (X/4: 1.25 mL/L glyphosate; 0.5 g/L metribuzin), T3 (X/2: 2.5 mL/L glyphosate; 1 g/L metribuzin), and T4(X: 5 mL/L glyphosate; 2 g/L metribuzin), where X (1L/acre for glyphosate and 300g/acre for metribuzin) is the recommended herbicide dose. Glyphosate samples were collected after 3 days (de Freitas-Silva et al., 2020) of treatment and metribuzin samples after 7 days (Kumar et al., 2022b) of treatment for morphological analysis and were stored at -20°C for biochemical analysis. For 3rd objective, the experiment comprised of the following treatments: T0 (control: 0 mL/L for glyphosate; 0 g/L for metribuzin), G1 (X/8: 0.625 mL/L glyphosate), G2 (X: 5 mL/L glyphosate), M1(X/8: 0.25 g/L metribuzin), and M2 (X: 2 g/L metribuzin) for glyphosate and metribuzin respectively. Each treatment was replicated three times. After maturation (approximately 10 weeks), plant stems were cut just above the soil and brought to the laboratory for further analysis. For 4th objective, the experiment comprised of following treatments: T0 (control: 0 mL/L for glyphosate; 0 g/L for metribuzin), T1 (X/8: 0.625 mL/L glyphosate; 0.25 g/L metribuzin), T2 (X/4: 1.25 mL/L glyphosate; 0.5 g/L metribuzin), T3 (X/2: 2.5 mL/L glyphosate; 1 g/L metribuzin), and T4(X: 5 mL/L glyphosate; 2 g/L metribuzin). Samples were collected after 3 days and stored at -80°C for further analysis.

3.4. Morphological Data

The observation of various morphological parameters in the form of visible plant injury, relative water content, and above-ground fresh and dry biomass was taken.

3.4.1. Visible Plant Injury

The phytotoxicity was evaluated visually.

3.4.2. Fresh and Dry Weight

The fresh weight (FW) of plants was recorded immediately after harvesting, whereas dry biomass was determined by placing the plants in an oven at 80 ± 1.5°C and weighing them after 5 days (Elangovan et al., 2023).

3.4.3. Measurement of Leaf Water Relations

Leaf relative water content (RWC) was measured following the standard method proposed by Barrs and Weatherly (Barrs & Weatherley, 1962) using the formula:

$\text{RWC}\% = (\text{FW}-\text{DW})/(\text{TW}-\text{DW}) \times 100$

Where FW represents fresh leaf weight, DW is dry weight, and TW is the turgid weight recorded after 24 hours of floating in distilled water at 4°C in darkness.

3.5. Biochemical Analysis

3.5.1. Chlorophyll Estimation

Chlorophyll content was estimated by the method given by Arnon, (1949).

Reagent:

- Acetone (80%)

Procedure: A 0.1 g sample of fresh plant tissue was homogenized in a chilled pestle and mortar with 4 mL of 80% acetone. The homogenized material was then centrifuged at 13,000 rpm for 20 minutes at 4℃. The resulting supernatant was collected for chlorophyll content analysis. The absorbance of the supernatant was measured at 645 nm and 663 nm using a UV-Vis spectrophotometer.

Calculations:

$$\text{Chl a} = 12.7\ (\text{A663}) - 2.69\ (\text{A645})\ \frac{V}{1000 \times W}$$

$$\text{Chl b} = 22.9\ (\text{A645}) - 4.68\ (\text{A663})\ \frac{V}{1000 \times W}$$

$$\text{Total Chl} = 20.2\ (\text{A645}) + 8.02\ (\text{A663})\ \frac{V}{1000 \times W}$$

Where V = Volume of plant extract, W = Fresh weight

3.5.2. Total Carotenoid Estimation

Total carotenoid content was estimated by the method given by Maclachlan & Zalik, (1963).

Reagent:

- Acetone (80%)

Procedure: A 0.1 g sample of fresh plant tissue was homogenized in a chilled pestle and mortar with 4 mL of 80% acetone. The homogenized material was then centrifuged at 13,000 rpm for 20 minutes at 4°C. The resulting supernatant was collected for carotenoid content analysis, with absorbance measured at 470 nm using a UV-Vis spectrophotometer.

Calculation:

$$\text{Carotenoid} = \frac{1000\ (A470) - 1.8\text{Chl}\ a - 85.02\text{Chl}\ b}{198}$$

3.5.3. Anthocyanin Content Estimation

Total anthocyanin content was determined by the method given by Mancinelli, (1984).

Reagents:

- Absolute methanol
- Distilled water
- HCl

Procedure: A 0.1 g sample of fresh plant tissue was crushed in a chilled pestle and mortar with 3 mL of an acidified methanol extraction mixture (methanol: water: HCl, 79:20:1). The homogenized material was then centrifuged at 13,000 rpm for 20 minutes at 4°C. The resulting supernatant was collected for anthocyanin content analysis, with absorbance measured at 530 nm and 657 nm using a UV-Vis spectrophotometer.

Calculation:

$$\text{Anthocyanin} = \text{A530} - 0.25(\text{A657})$$

3.5.4. Protein Content Estimation

Protein estimation was performed using the method given by Lowry et al., (1951).

Reagents:

- Reagent A – 2% sodium carbonate in 0.1 N sodium hydroxide.

- Reagent B – 0.5% copper sulphate in 1% potassium sodium tartarate.
- Reagent C – 50 mL of reagent A and 1 mL of reagent B (prepared before use)
- Reagent D – FC reagent.
- Protein solution (stock standard) – 50 mg of BSA dissolved in distilled water to make a volume of 50 mL. Protein working standard solutions were prepared by diluting the stock standard.

Procedure: A 0.1 mL aliquot of the sample and standard was pipetted into a series of test tubes. The volume in each tube was adjusted to 1 mL using distilled water, with a separate tube containing 1 mL of distilled water serving as the blank. 5 mL of reagent C was then added to each tube, mixed thoroughly, and allowed to stand for 10 minutes. Subsequently, 0.5 mL of reagent D was added, shaken well, and incubated in the dark at room temperature for 30 minutes. The development of a blue color was observed, and absorbance readings were recorded at 660 nm using a UV-Vis spectrophotometer.

Calculations: A graph of absorbance vs. concentration was plotted using the standard protein solutions. The protein content in the sample was determined by extrapolating its absorbance value from the standard curve. The final protein concentration was expressed as mg/g tissue.

3.5.5. Total Soluble Carbohydrate Estimation (TSC)

Total soluble carbohydrate was estimated by a method given by Islam et al., (2021).

Reagents:

- Ethanol
- Anthrone reagent
- Glucose

Procedure: A 25 mg sample of freeze-dried leaves was homogenized in 5 mL of 95% ethanol and subsequently centrifuged at 3500 rpm for 10 minutes. After collecting the supernatant, the remaining pellets were re-suspended in 70% ethanol and subjected to the same centrifugation process. The combined supernatants were stored at 4°C for the determination of total soluble carbohydrate (TSC) content. Then, 0.1 mL of the extract was mixed with 1 mL of anthrone reagent (prepared by dissolving 200 mg of anthrone in 100 mL of 72% sulfuric acid). The mixture was heated at 100°C for 10 minutes, cooled, and the TSC was estimated by measuring the absorbance at 625 nm using a glucose standard curve.

3.5.6. Determination of Sucrose

Sucrose content was estimated by the method given by van Handel, (1968).

Reagents:

- KOH
- Anthrone reagent
- Sucrose

Procedure: A 25 mg sample of freeze-dried leaves was homogenized in 5 mL of 95% ethanol and centrifuged at 3500 rpm for 10 minutes. The supernatant was collected, while the pellets were re-suspended in 70% ethanol, and the process was repeated. The combined supernatants were used for sucrose determination. 0.2 mL of the supernatant was mixed with 0.1 mL of 30% KOH and heated at 100°C for 10 minutes. After cooling to room temperature, 3 mL of anthrone reagent (prepared by dissolving 150 mg of anthrone in 100 mL of 70% sulfuric acid) was added. The samples were cooled for 10 minutes, and absorbance was measured at 620 nm. The sucrose concentration was then determined using a standard curve.

3.5.7. Antioxidant Enzyme Activity

A 0.1 g sample of fresh plant material was crushed in a pre-chilled pestle and mortar using 3 mL of 100 mM potassium phosphate buffer (PPB, pH 7.0). The homogenized material was then centrifuged at 13,000 rpm for 20 minutes at 4°C. The resulting supernatant was collected for the analysis of various antioxidant activities.

3.5.7.1. Peroxidase (POD): Guaiacol peroxidase was estimated according to the method given by Pütter, (1974).

Reagents:

- Phosphate buffer (0.1M, pH 7.0)
- Guaiacol solution – 20mM

- H_2O_2 solution – 12.3mM

Procedure: A 50 µL aliquot of enzyme extract was added to a reaction mixture containing 1 mL of 50 mM sodium phosphate buffer (pH 5.5), 1 mL of 0.3% H_2O_2, and 0.95 mL of 0.2% guaiacol. The change in absorbance at 470 nm was recorded to determine peroxidase (POD) enzyme activity. The enzyme activity was calculated using an extinction coefficient of 25 $mM^{-1}cm^{-1}$.

Calculation:

$$\text{Specific Activity (mol U mg}^{-1}\text{ protein)} = \frac{\text{Unit Activity (U min}^{-1}\text{ g}^{-1}\text{ FW)}}{\text{Protein Content (mg g}^{-1}\text{ FW)}}$$

3.5.7.2. Catalase (CAT) Activity: The activity of catalase was determined according to the method of Aebi et al., (1983).

Reagents:

- Phosphate buffer (100 mM, pH 7.0)
- Hydrogen peroxide (150 mM)

Procedure: For catalase (CAT) activity, 200 µL of enzyme extract was added to a reaction mixture containing 1.5 mL of 50 mM sodium phosphate buffer (pH 7.8), 300 µL of 0.1 M H_2O_2, and 1 mL of distilled water. The change in absorbance at 240 nm per minute was recorded to determine CAT activity. The enzyme activity was calculated using an extinction coefficient of 6.93×10^{-3} $mM^{-1}cm^{-1}$.

Calculation:

$$\text{Specific Activity (mol U mg}^{-1}\text{ protein)} = \frac{\text{Unit Activity (U min}^{-1}\text{ g}^{-1}\text{ FW)}}{\text{Protein Content (mg g}^{-1}\text{ FW)}}$$

3.5.7.3. Malondialdehyde (MDA): The level of MDA was determined using the method given by Heath & Packer, (1968).

Reagents:

- Trichloroacetic acid (TCA) – 0.1%
- Thiobarbituric acid (TBA) – 0.5%

Procedure: A 0.1 g fresh leaf sample was homogenized with 5 mL of 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged at 5,000 rpm. 1 mL of the supernatant was mixed with 6 mL of 20% (w/v) TCA containing 0.5% (w/v) thiobarbituric acid (TBA). The mixture was then heated at 95°C for 30 minutes and immediately cooled on ice. The optical density of the supernatant was measured at 532 nm, with a correction for non-specific absorbance by subtracting the absorbance at 600 nm. The malondialdehyde (MDA) content was determined using an extinction coefficient of 155 $mM^{-1}cm^{-1}$.

Calculations:

$$\text{MDA} = \frac{\text{Absorbance} \times \text{Total volume} \times 1000}{\text{Ext coeff} \times \text{Sample volume} \times \text{weight of plant tissue}}$$

3.6. Secondary Metabolite Estimation: The secondary metabolites were estimated using GCMS studies via method described by Vadhel et al., (2023).

Reagents:

- Absolute Methanol

Procedure: 1g of dried leaf powder was extracted at room temperature for 24 hours in 100 mL of methanol by placing it in shaking incubator. The mixture was then paper-filtered and kept at room temperature until the excess solvent evaporated. After getting the extract, it was kept at 4°C in an airtight container for secondary metabolite estimation. The extract was then reconstituted in methanol and filtered through 0.22 micron nylon syringe filter (Moxcare labware) before injecting into the GC. The analysis was conducted at Lovely Professional University's Central Instrumentation Facility using a Shimadzu gas chromatograph instrument model, GCMS-TQ8040 NX, equipped with a mass detector. 1µL of sample was injected into GC via autosampler. The injector was used in splitless mode. Additionally, the injector was set at a temperature of 250°C. The compounds were separated using an SH-RXi-5Sil MS capillary column and crosslinked to a 30 m × 0.25 mmID, 0.25 µm df (similar to 5% diphenyl/95%dimethyl polysiloxane). The column temperature was initially set at 40°C and then it was raised to 300°C at a rate of 7°C per minute and maintained for three minutes. The carrier gas utilized was helium (flow rate= 1 mL min^{-1}). The National

Institute of Standards and Technology's spectrum database was used to find and identify the components in the extract. Within the m/z range of 40 to 1000, detection was carried out using the Q3 scan acquisition mode. The proportion of an individual component is expressed as the relative peak area percentage of the total peak area percentage.

3.7. Molecular Docking Studies

3.7.1. Protein 3D Structure Preparation: The protein 3D structure of the *Cannabis sativa* L. tetrahydrocannabinolic acid synthase (THCA synthase) was acquired from the Protein Data Bank (PDB ID 3VTE), with a resolution of 2.75 Å, having no mutation (Shoyama et al., 2012). The flavine adenine dinucleotide (FAD) ligand was kept as it is responsible for the enzyme's catalytic activity, and the protein was cleaned by removing other ligands and water molecules using AutoDockTools 1.5.6. Polar hydrogens and Kollmann charges were added, and Gasteiger charges were computed. The atomic type was set to assign AD4 type (Verma et al., 2023).

3.7.2. Ligand Preparation: The 3D canonical structures of the ligands—Cannabigerolic acid (CBGA), metribuzin, and glyphosate—were drawn using Marvin Sketch software. 2D and 3D cleaning was performed at each step, and the structures were verified for 3D geometrical conformation through visualization in Marvin View. Subsequently, the structures were optimized to the transition state, and energy minimization was carried out sequentially using Chem3D Pro 12.0.2, Avogadro 1.2, and Chem3D 22. The ligand with the most minimized energy was finally optimized using DFT via Gaussian 09 software. Here, we utilized RB3LYP functionals and a 6-311G basis set. These optimized structures were further used for docking purposes to determine the interactions of ligands with the enzyme THCA Synthase (Gulati et al., 2023).

3.7.3. Molecular Docking: The Rigid molecular docking experiment was performed with optimized ligands using Autodock 4.2. Grid box dimensions were set to cover the whole protein at a distance of 0.714 Å (Table 3.2). The Genetic Algorithm simulation program was used with a 300 population size, 100 genetic algorithm runs, and a medium number of evaluations for metribuzin and glyphosate due to a medium number of torsions, whereas a long number of evaluations were used for CBGA due to a higher number of torsions in CBGA, and the remaining parameters were set to their default values. The output file was saved as Lamarckian GA. Each ligand's top 10 conformations were saved based on the negative binding energy (ΔG). Conformations binding to the same active site and the most negative binding energy in all three were further analysed in Discovery Studio Visualizer v21.1 and ChimeraX 1.7 (Verma et al., 2023; Verma et al., 2022).

Table 3.2: Grid box values for THCA synthase enzyme.

Ligands	**Centre**			**Dimensions**		
	X	**Y**	**Z**	**X**	**Y**	**Z**
CBGA	40.12	39.992	-27.502	126	126	88
Metribuzin	41.44	42.887	-27.502	126	126	88
Glyphosate	40.29	43.683	-27.502	126	126	88

3.8. Fiber Extraction: Fiber was extracted according to the method given by Amaducci et al., (2015).

Reagents:

- NaOH

Procedure: Stems of control and treated plants were cut into roughly equal-length pieces (15cm). The stems were first treated by boiling them for 90 minutes in 0.35% NaOH solution to facilitate the separation of bark from the woody core. A second treatment was then carried out on the bark by boiling it in a 2% NaOH solution for 120 minutes. Later, the fiber was placed on a sieve and thoroughly rinsed with tap water. The obtained fiber was then dried at 105°C and weighed (Figure 3.2).

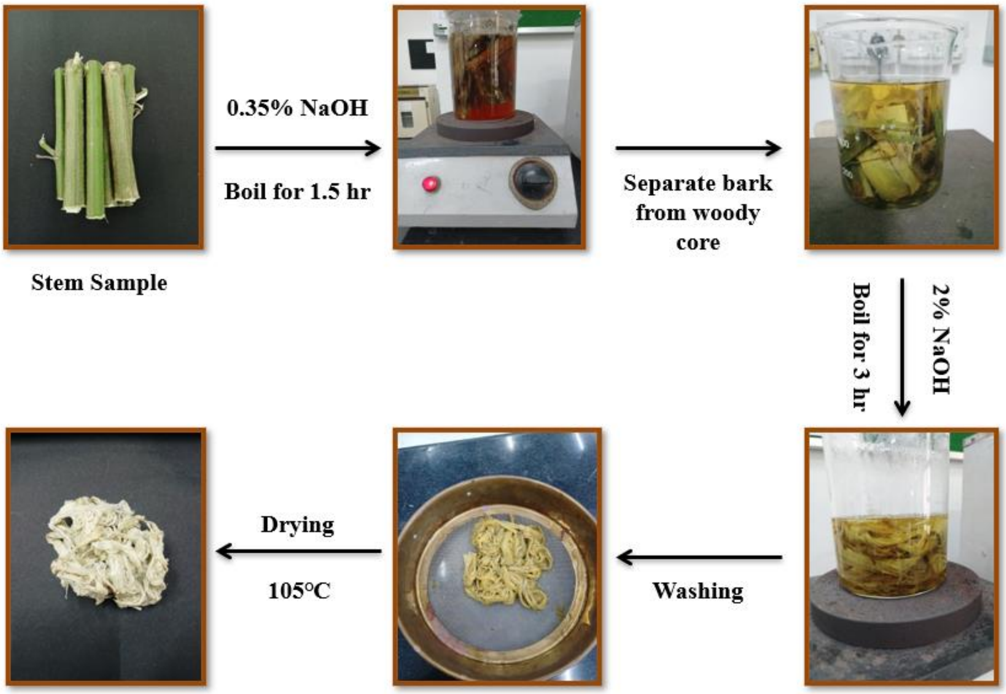


Figure 3.2: Stepwise methodology of fiber extraction.

3.9. Compositional Analysis

The Soxhlet extraction technique was utilized with 150 mL of acetone to determine the extractive content in the cannabis stem. The weight of the extractive-free sample was measured before and after extraction to calculate the constant weight of extractives (Ayeni et al., 2015; Shukla et al., 2023).

3.9.1. Hemicellulose Content: Hemicellulose quantification was then carried out following the protocol described by Ayeni et al., (2015).

Reagents:

- NaOH

Procedure: Extractive-free dried biomass (2g) was transferred to a 250mL Erlenmeyer flask, to which 150mL of 500 mol/m^3 NaOH solution was added. This mixture was boiled for 3.5 hours. After cooling, the mixture was vacuum-filtered and washed until a neutral pH was reached. The residue was then dried to a constant weight at 105°C in an oven. The hemicellulose content (% w/w) of the dry biomass was calculated using the following equation.

$$Hemicellulose\ content\ (\%) = \frac{\text{Initial Weight} - Final\ Weight}{\text{Initial Weight}} \times 100$$

3.9.2. Cellulose Content: The estimation of cellulose was performed as per method given by Sun et al., (2004).

Reagents:

- Acetic acid (80%)
- Nitric acid (70%)
- Ethanol (95%)

Procedure: A 2 g sample of extractive-free dried biomass was treated with an acid mixture containing 100 mL of 80% (v/v) acetic acid and 10 mL of 70% (v/v) nitric acid. The mixture was then incubated in a boiling water bath for 20 minutes. After incubation, the residue was alternately washed with distilled water and 95% (v/v) ethanol to eliminate reaction byproducts and residual acids until the pH was neutralized. The residue was then dried overnight at 60°C, and the cellulose content (% w/w) was determined using the following equation:

Cellulose content (%) = Final sample dry weight (g) / Initial sample dry weight (g) × 100

3.10. Bast Fiber Content

The bast fiber content was determined according to the method given by Sankari, (2000). The weight of the plant stems after removing the leaves was noted. The fiber extracted from each stem was then washed, dried, and weighed. The bast fiber was calculated by using the following formula.

Bast fiber content (%) = Dry weight of fiber/Dry weight of stem x 100

3.11. X-Ray Diffraction:

Sample Preparation: Dried fiber samples were first ground into a fine powder using a mortar and pestle to ensure uniform particle size and minimize preferred orientation effects. The powdered samples were then oven-dried at 60°C to remove residual moisture, as water content can interfere with diffraction patterns. The dried powders were carefully packed onto the XRD sample holder, ensuring a flat and even surface for accurate measurement.

Analysis: The cellulose crystallinity index (CrI) for all samples was analysed using the Bruker D8 Advance XRD system at the CIF Lab of Lovely Professional University. The analysis was performed using an X-pert Pro diffractometer, scanning within a 2θ range of 6–60° at a rate of 0.5°/minute, with Cu-Kα radiation X-ray.

The crystallinity index (CrI%) was calculated from PXRD analysis using the formula developed by Segal et al., (1959).

$$\text{CrI\%} = [(I_{002} - I\text{am}) / I_{002}] \times 100$$

Where,

CrI represents the crystallinity index,

I_{002} is the maximum intensity in the crystalline region of cellulose at the 002 planes,

I_{am} is the minimum intensity in the amorphous region of cellulose between its lattice planes Shukla et al., (2023).

3.12. Mechanical Properties

The mechanical properties of the fiber were tested at room temperature using a Zwick Roell universal testing machine with a 500N load cell. Tensile testing was conducted according to DIN EN ISO 1798 standards to determine the tensile behaviour of the flexible cellular materials when stretched at a constant rate until failure. The gauge length was set at 50mm between the clamps and the crosshead speed was 1mm/min to stretch the fiber to rupture.

3.13. Surface Morphology

FE-SEM was used to evaluate the surface morphology of the fiber. Fiber samples were cut into small pieces (~5–10 mm) and thoroughly dried to remove surface moisture. The dried samples were then mounted on aluminium stubs using double-sided carbon adhesive tape to ensure proper fixation. To prevent charging during imaging, a thin conductive coating of gold (~10 nm) was applied to the fiber surface using a sputter coater. The prepared samples were subsequently examined under a Field Emission

Scanning Electron Microscope (FE-SEM), and images were captured at different magnifications with accelerating voltages of 5 and 10 kV.

3.14. Cytochrome Studies

3.14.1. Cytochrome Isolation: Isolation of the cytochrome was performed according to the method proposed by He & Zhou, (2016).

Reagents:

- Homogenizing Buffer- Sucrose (0.25M), Tris-HCl (50Mm, pH-7.5), EDTA (1mM), DTT (1mM)
- Resuspension buffer- PBS with EDTA (1mg/mL)

Procedure: 10g of fresh plant tissue was weighed, cleaned and homogenized in homogenizing buffer. A 1:4 ratio for tissue and the homogenizing buffer was maintained. All steps were performed on ice to prevent protein degradation. The homogenate was filtered and centrifuged at 5000g for 10 minutes at 4°C to remove cell debris, nuclei, and unbroken cells. Then the supernatant was collected and centrifuged at 15000g for 30 minutes at 4°C. Again, the supernatant was collected and transferred to ultracentrifuge tubes and were spun at 150000g for 90 minutes at 4°C. After ultracentrifugation, the supernatant was carefully discarded, and the microsomal pellet was resuspended in a small volume of ice-cold resuspension buffer and kept on ice for further analysis.

3.14.2. Cytochrome P450 Content Estimation: The cytochrome P450 content was estimated by the method given by (He & Zhou, 2016).

Reagents:

- Potassium Phosphate Buffer (100mM, pH 7.4 - 7.7)
- EDTA (1.0mM)
- Glycerol (20% v/v)
- Sodium Cholate (0.5% w/v)
- Triton N (0.4% w/v)
- Sodium dithionite

Procedure:

In a small test tube, the microsome sample was diluted into 2mL of potassium phosphate buffer containing EDTA, glycerol, sodium cholate, and 0.4% Triton N. The contents were mixed, and sample was divided into two 1ml cuvettes. The two cuvettes were placed in the spectrophotometer and O.D was recorded at 400 and 500 nm. Then the sample cuvette was removed from the spectrophotometer and (in the fume hood) slowly CO gas was bubbled through this sample. Then ~1 mg of solid Sodium dithionite was added to both cuvettes. Finally, absorbance at 400 and 500 nm was recorded.

Calculation:

$(\Delta A450 - \Delta A490)/0.091$ = nmol of P450 per mg *pro*

3.14.3. Reduction Activity: The reduction activity of Cytochrome P450 was determined according to the protocol given by (Guengerich et al., 2009).

Reagents:

- Horse heart Cytochrome c (0.5mM), prepared in10 mM of potassium phosphate buffer (pH- 7.7)
- Potassium phosphate buffer (0.3M, pH- 7.7)
- NADPH (10mM)

Procedure:

80µl of horse heart cytochrome *c* was pipetted out in a cuvette, to which the microsomal sample was added. Potassium phosphate buffer was added to reduce the total volume in the cuvette to 0.99 ml. The contents were mixed, and the absorbance was recorded at 550nm. Then, 10µl of NADPH solution was added to the cuvettes. Again, absorbance at 550nm was recorded for about 3 minutes.

Calculation:

$(\Delta A_{550}/\text{min})/0.021$= nmol of cytochrome *c* reduced per min

3.15. Statistical Analysis

Statistical analysis was performed using one-way Analysis of Variance (ANOVA) with significance determined at $P < 0.05$, followed by Duncan's multiple comparison test for post-hoc analysis. Data represent mean values (± standard error) of three replicates (n=3). All analyses were conducted using IBM SPSS version 22. Graphs were plotted using OriginLab software. The chemical structures were drawn with ChemAxon Marvin Sketch software.

CHAPTER 4. RESULTS AND DISCUSSION

Objective 1: Pot culture experiment performance on Cannabis sativa with differential exposure of herbicides.

4.1. Pot Culture and Herbicide Treatment

The plants were cultivated in pots under controlled conditions until they attained the optimal growth stage, characterized by well-developed vegetative structures. Upon reaching this stage, herbicide treatment was given as per earlier mentioned concentrations, and an adequate quantity of leaf and stem samples were collected to ensure sufficient material for subsequent morphological, and biochemical analysis.

Figure 4.1: Pot culture experimentation, A: One week after germination, B: Three weeks after germination.

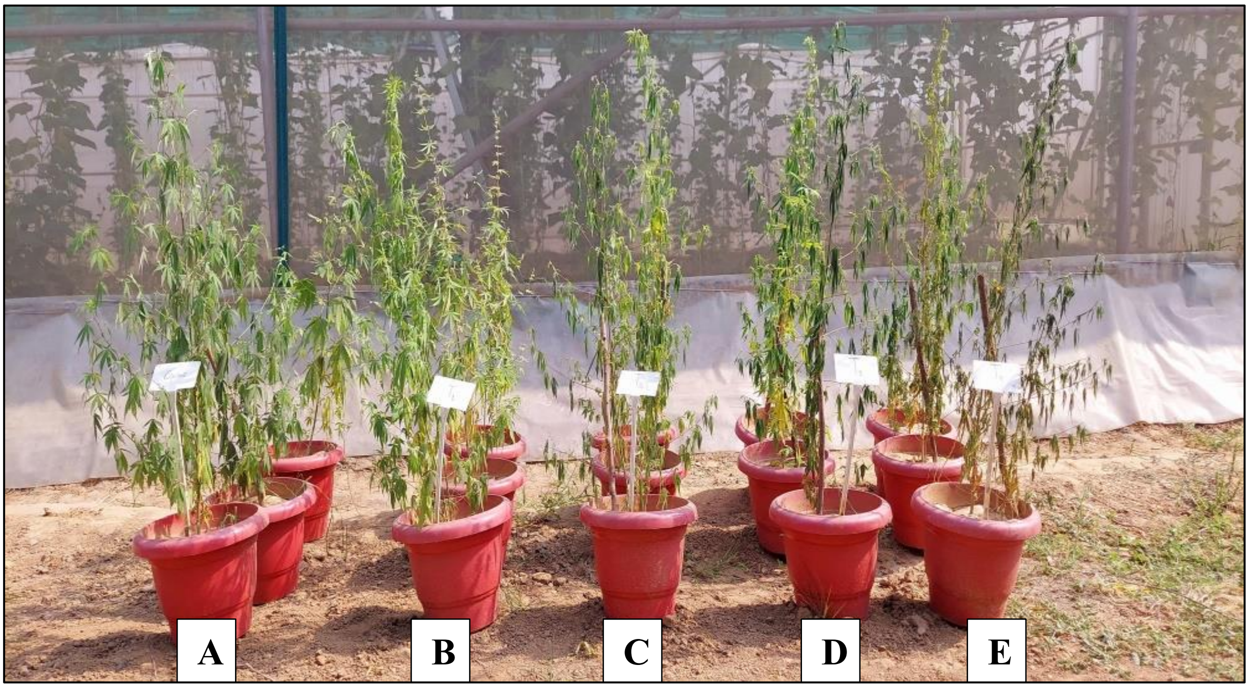


Figure 4.2: Plants after glyphosate treatment A: Control, B: T1, C: T2, D: T3, E: T4

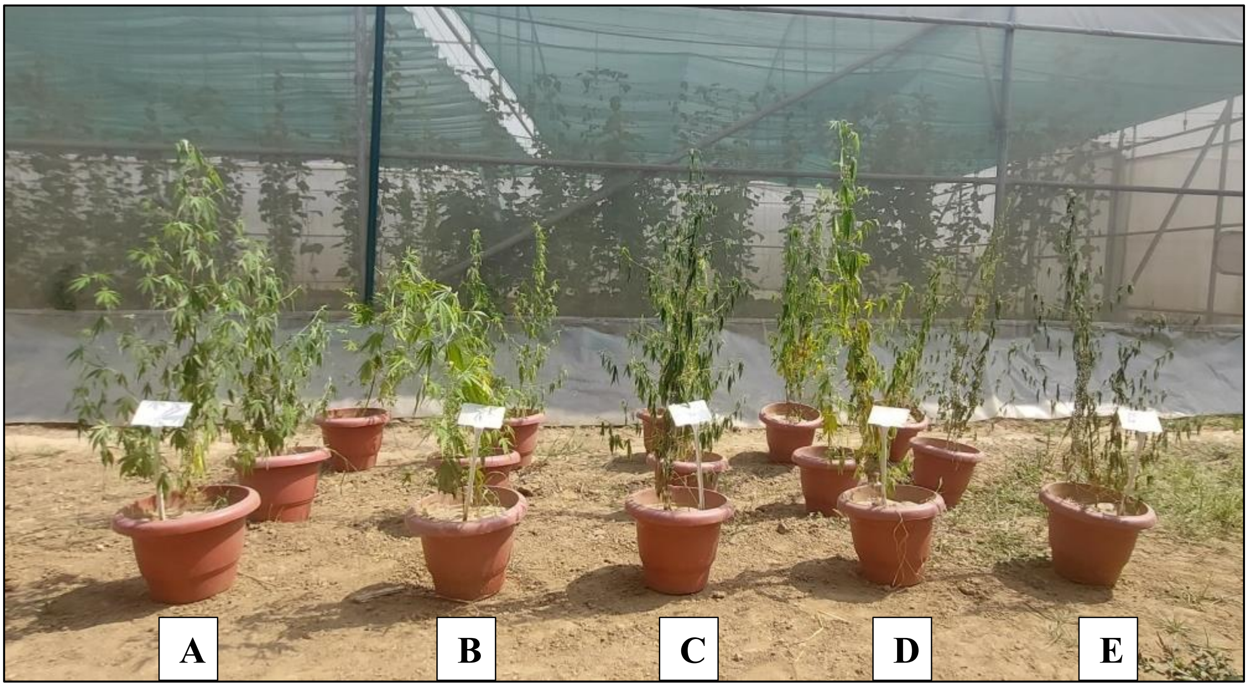


Figure 4.3: Plants after metribuzin treatment A: Control, B: T1, C: T2, D: T3, E: T4

Figure 4.4: (A) Industrial-grade glyphosate (B) Industrial-grade metribuzin

Objective 2: Analysis of morphological and biochemical parameters after exposure of herbicides.

4.2. Visible Plant Injury

The visible injury of glyphosate and metribuzin damage, such as necrosis and chlorosis, were observed on the surface of the leaves of all plants, which increased with increasing concentrations of herbicide stress.

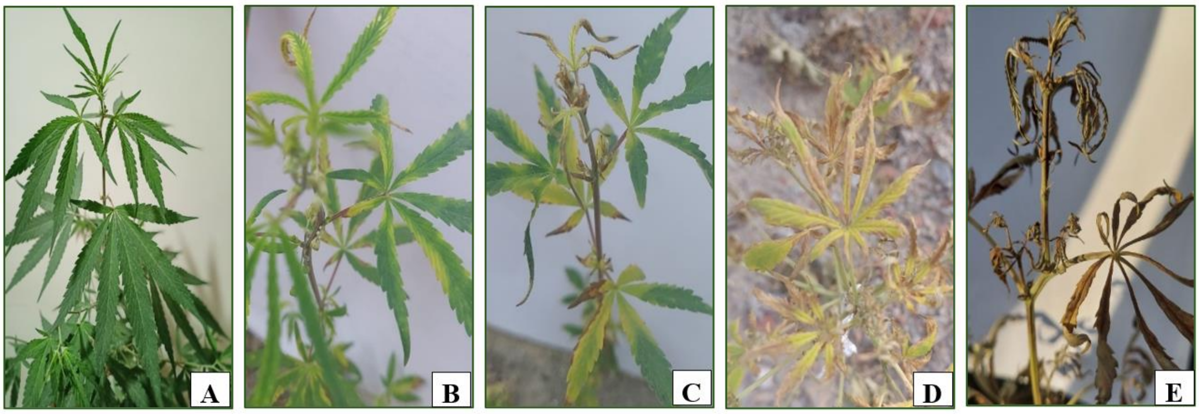


Figure 4.5: Visible plant injury on the whole plant after glyphosate treatment; A: Control, B: T1, C: T2, D: T3, E: T4

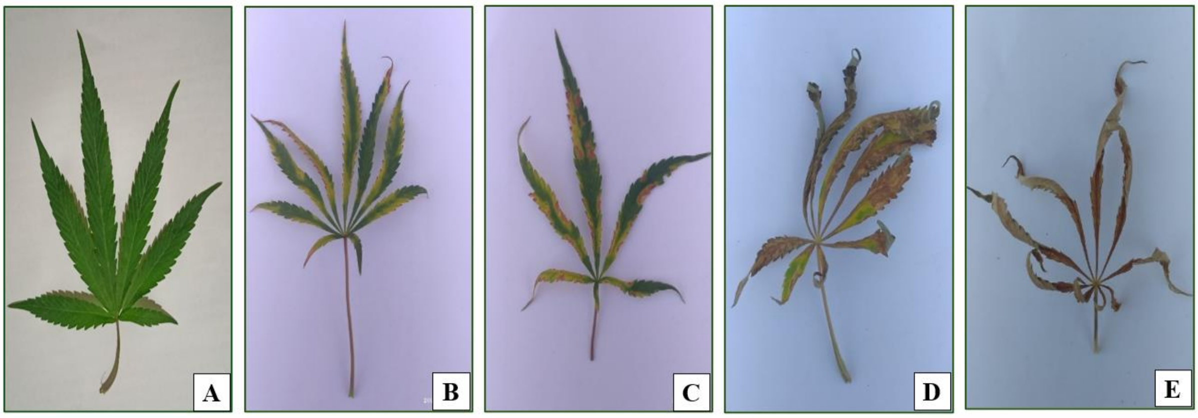


Figure 4.6: Visible plant injury on individual leaf after glyphosate treatment; A: Control, B: T1, C: T2, D: T3, E: T4

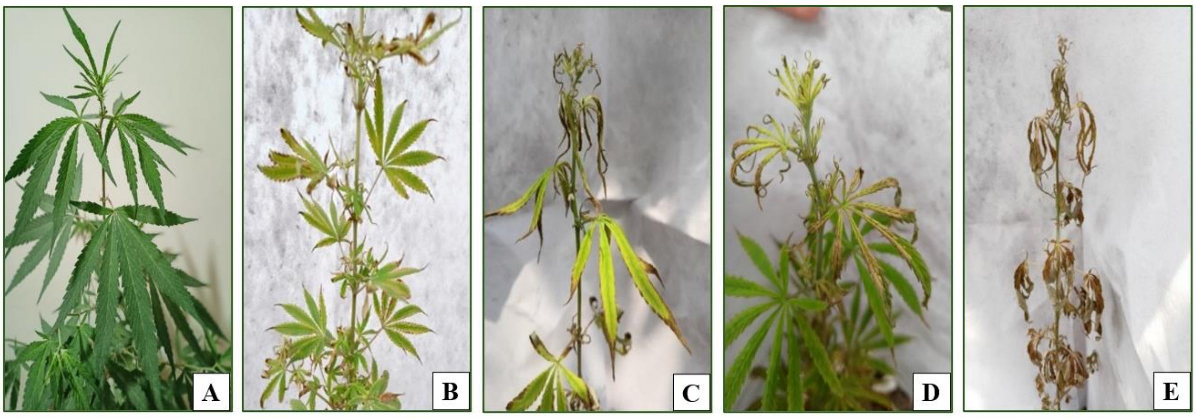


Figure 4.7: Visible plant injury on the whole plant after metribuzin treatment; A: Control, B: T1, C: T2, D: T3, E: T4

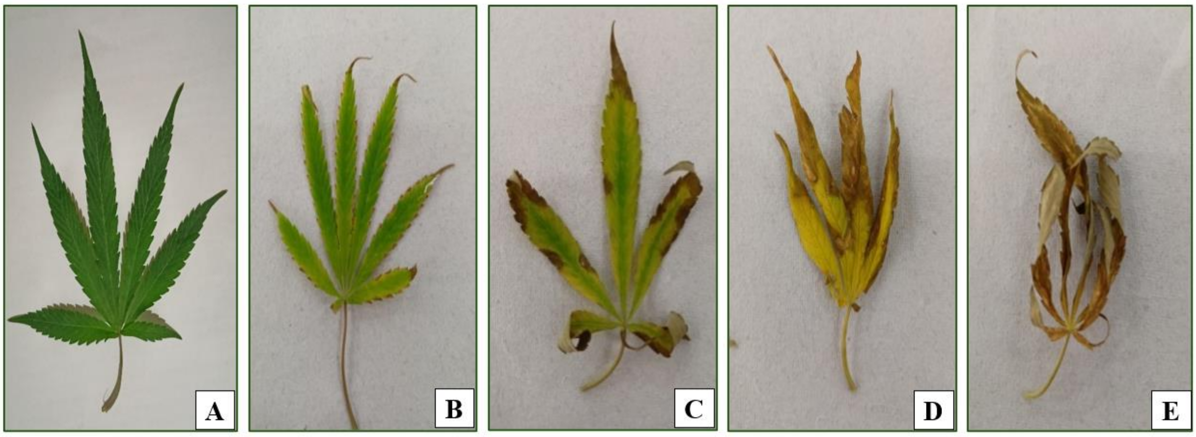


Figure 4.8: Visible plant injury on individual leaf after metribuzin treatment; A: Control, B: T1, C: T2, D: T3, E: T4

4.3. Fresh Weight, Dry Weight, and RWC

Increased herbicide stress led to a significant reduction in fresh and dry weights, with glyphosate and metribuzin compared to the control at the highest concentration (T4). The RWC of leaves markedly decreased in both herbicide treatments, reaching its lowest value at T4 (30.3% for glyphosate and 28.6% for metribuzin) (Figure 4.9).

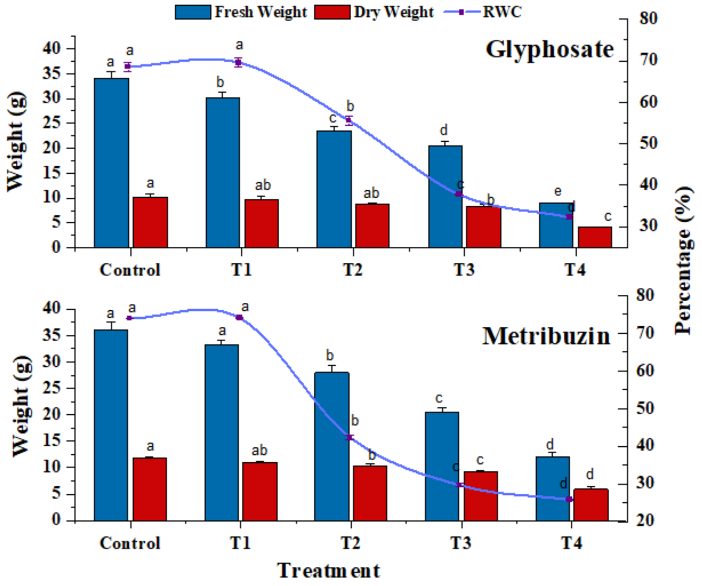


Figure 4.9: Change in fresh weight (g), dry weight (g), and RWC (%) in control and treated plants.

Another study also recorded similar results (Issaad et al., 2022), where RWC decreased with glyphosate and other herbicide applications with elevation observed at the lowest concentration. The RWC indicates the extent of dehydration of leaves. Due to the treated plant's lower water content, the overall weight shift was less pronounced even though fresh weight and water content both fell, suggesting increased cellular concentration.

4.4. Photosynthetic Pigments

The photosynthetic pigments showed a significant fold reduction in levels of Chlorophyll *a* (2.51, 3.34), Chlorophyll *b* (5.2, 1.89), total Chlorophyll (2.95, 2.69), and carotenoids (1.77, 1.64) under highest glyphosate and metribuzin herbicide stress (T4) as compared to the control group. However, at the lowest concentration (T1), a notable increase was observed in the levels of pigments. Anthocyanin content also declined with increasing stress of 1.8-fold in glyphosate and 1.12-fold in metribuzin at T4 (Figure 4.10). Similar results were observed in a study by (Gomes et al., 2016), where different concentrations of glyphosate on willow trees led to a reduction of photosynthetic pigments and ultimately reduction in photosynthesis. Another study (Volova et al., 2020) showed a reduction in photosynthetic pigments while studying the biological effects of metribuzin on various weed species.

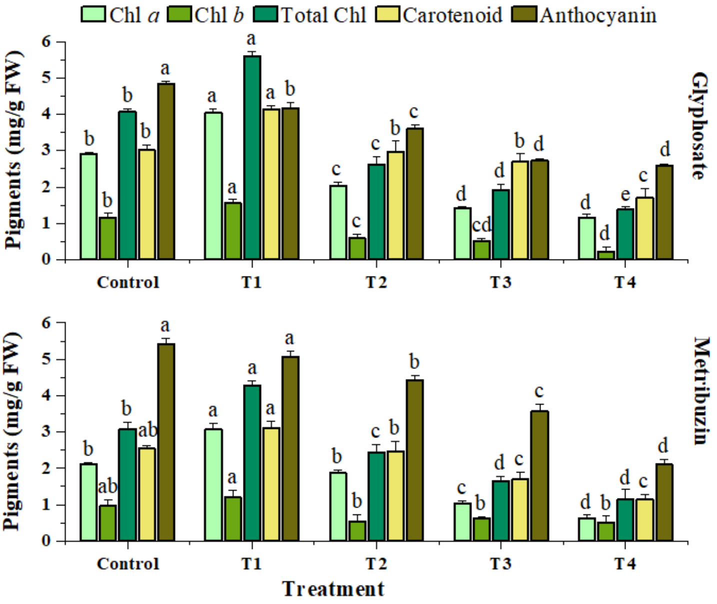


Figure 4.10: Effect of glyphosate and metribuzin on photosynthetic pigments of *Cannabis sativa* L.

The reduction in the chlorophyll content in the case of glyphosate can be attributed to an upsurge in chlorophyll degradation, as documented in the study by (Gomes et al., 2016). Similarly, the decline in carotenoids can result from the suppression of the shikimate pathway, which lowers the concentration of plastoquinone (PQ). The decrease in PQ content is significant because it functions as a cofactor for two essential enzymes in the carotenoid biosynthesis pathway: phytoene desaturase and ζ-carotene desaturase. Plastoquinone's declining concentration therefore exerts a direct effect on carotenoid production (Gomes et al., 2017). Furthermore, blocking EPSPS inhibits chorismate, which in turn is responsible for phenylalanine synthesis and ultimately anthocyanin via the phenylpropanoid pathway. This helps to explain the decrease in anthocyanin concentration with increasing glyphosate stress (Fuchs et al., 2021).

Quinone A (QA) to Quinone B (QB) electron transport is impeded by metribuzin's binding to the D1 protein on the plastoquinone binding site. Pigment production is hindered by this obstruction of electron transport, which results in electron buildup and ultimately leads to an increase in radiation. As plastoquinone remains unreduced, it causes triplet chlorophyll to build up. The triplet chlorophyll can produce singlet oxygen in the absence of efficient quenchers (Krieger-Liszkay, 2004). The accumulation of singlet oxygen and triplet chlorophyll can result in lipid peroxidation, which alters the fluidity of the membrane, causing oxidative damage, and consequently declining the production of chlorophyll, carotenoid, and anthocyanin (Kruk & Szymańska, 2021).

4.5. Protein Content

The protein content declined in correlation with an increase in concentrations of both glyphosate and metribuzin. The maximum reduction in protein content reaches 2.04-fold at T4, the highest glyphosate stress level. Similarly, with the increase in metribuzin concentration, the protein content was observed to decrease, culminating in a maximum reduction of 2.29-fold at the most intensive metribuzin stress point− T4. This indicates a consistent pattern of decreasing protein content with heightened herbicide concentrations except at the lowest doses where a 1.12-fold and 1.11-fold increase in protein content was observed for glyphosate and metribuzin respectively in comparison to the untreated group (Figure 4.11). Reduction in protein content was also observed in a study (Nemat et al., 2008b) while studying the effect of field dose application of metribuzin on 10-day-old wheat and maize seedlings. Another study (Radwan & Fayez, 2016) showed that glyphosate reduced the protein content and showed other morphological symptoms in *Arachis hypogaea* leaves.

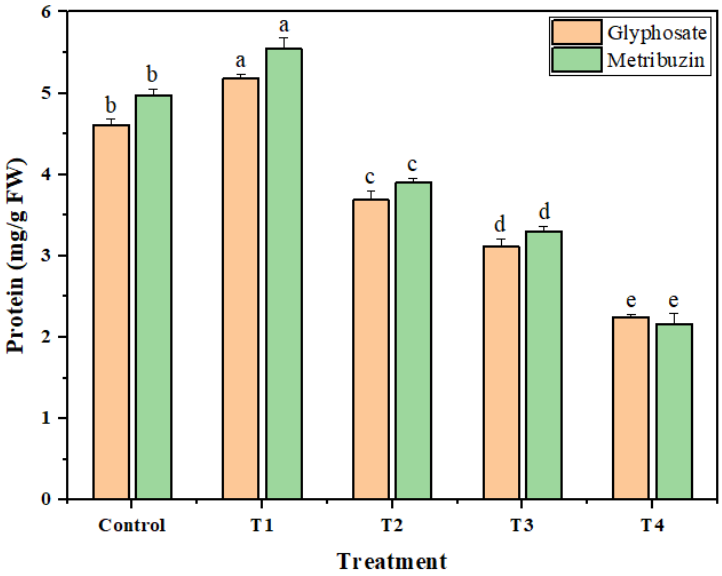


Figure 4.11: Change in protein content of *Cannabis sativa* L. after glyphosate and metribuzin treatment.

Although glyphosate does not directly affect the production of proteins in plants, it indirectly reduces protein levels by blocking the activity of the vital enzyme EPSPS. The production of phenylalanine, tyrosine, and tryptophan-essential aromatic amino acids needed for protein synthesis are curtailed as a result of this suppression (Ahsan et al., 2008). On the contrary, metribuzin-induced decline in protein content doesn't stem from ammonia scarcity but rather from a probable insufficiency in its conversion into organic forms. Metribuzin inhibits the enzyme system involving glutamine synthetase (GS) and glutamine-2-oxoglutarate aminotransferase (GOGAT), which are pivotal for ammonia conversion, causing accumulation of unconverted ammonia and impacting protein synthesis (Nemat et al., 2008b).

4.6. Carbohydrate and Sucrose

The levels of carbohydrate and sucrose mirrored a similar trend with the behaviour of photosynthetic pigments when subjected to varying herbicide concentrations. Both compounds displayed a consistent decrease as the herbicide concentration increased, aligning with the anticipated impact on these plant sugars. An interesting deviation from this trend emerged at the lowest concentration (T1), where a slight increase of 1.4-fold and 1.2-fold (glyphosate) and 1.12-fold and 1.07-fold (metribuzin) was observed in both carbohydrate and sucrose levels respectively. Remarkably, when exposed to glyphosate, the most significant reduction percentages were noted at the T4, measuring 2.3-fold for carbohydrates and 2.5-fold for sucrose. Comparatively, with metribuzin application, the reduction percentages were recorded at 2.3-fold for carbohydrates and 3.9-fold for sucrose at the same T4 concentration (Figure 4.12). Analogous results were observed in a study (Razieh, 2012a), where the foliar application of metribuzin resulted in carbohydrate reduction. Another study (Salman et al., 2016) was conducted, where the effect of glyphosate on biochemical features of *Oscillatoria limnetica* resulted in a marked decrease in carbohydrates. Various other studies (Beker et al., 2018; Vital et al., 2017), also noticed a reduction in sugar levels in glyphosate-treated sunflower and maize plants.

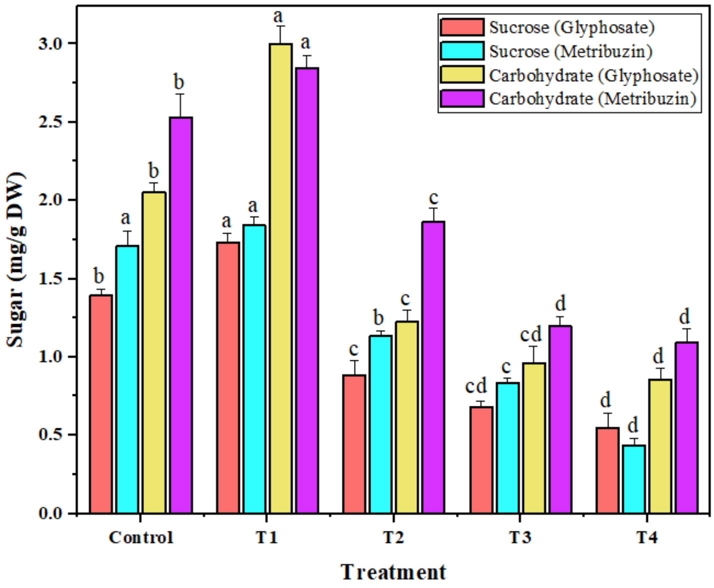


Figure 4.12: Variation in sucrose and carbohydrate in *Cannabis sativa* L. under glyphosate and metribuzin herbicide stress.

Distinct in their methods, glyphosate and metribuzin cause a fall in the quantities of carbohydrates and sucrose through targeted interference with basic metabolic processes. The inhibition of EPSPS by glyphosate and inhibition of photosystem II by metribuzin results in the reduction of photosynthesis, which explains the reduction of carbohydrate and sucrose levels under herbicide stress (Pline et al., 2003). Change in the photosynthetic process directly impacts the concentration of carbohydrate and sucrose levels (Vital et al., 2017).

4.7. Antioxidant Enzymes

The observed data indicates a dose-dependent escalation in peroxidase and catalase activities for both glyphosate and metribuzin treatments. The most substantial enhancement in catalase and peroxidase activities, reaching 3.6-fold and 3.7-fold for glyphosate, and 3.4-fold and 5.2-fold for metribuzin compared to the control, was notably evident at T4, signifying the highest response to oxidative stress (Figure 4.13). Simultaneously, the Malondialdehyde (MDA) levels, serving as an index of oxidative damage, consistently rose with increasing concentrations of the herbicides. At T4, MDA also exhibited its highest content, displaying an increase of 3.0-fold and 2.4-fold compared to the control, underlining the culmination of oxidative stress at the highest concentration levels of both glyphosate and metribuzin respectively (Figure 4.14). Similar results have been observed by (Eceiza et al., 2022), where glyphosate-resistant and glyphosate-sensitive *Amaranthus palmeri* were analysed to determine glyphosate-induced oxidative stress and found that an increase in antioxidant enzymes is associated with EPSPS inhibition. Another study (Karimmojeni et al., 2022a) analysed that at high metribuzin dose, *Echinacea purpurea* leaves showed an increase in MDA levels. Similarly, while studying the toxicity assessment of metribuzin on *Vigna radiata*, it was found that metribuzin-treated plants induced antioxidant enzyme activities (Kumar et al., 2023).

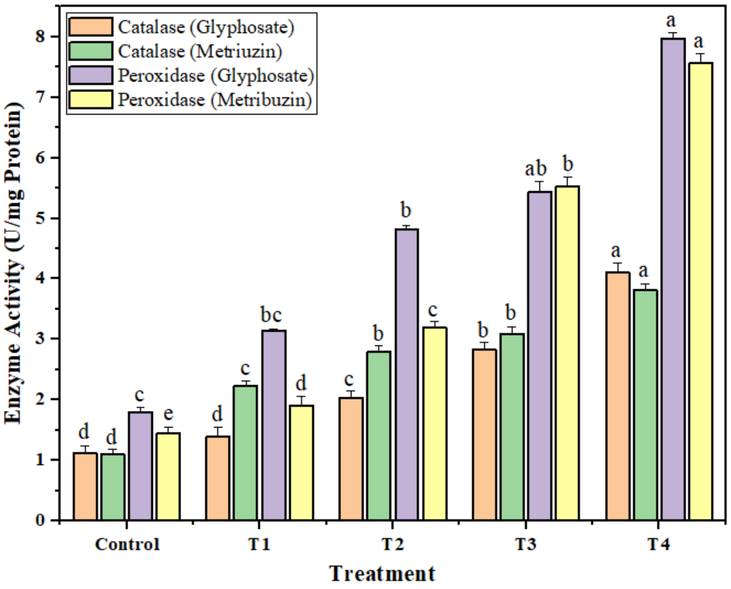


Figure 4.13: Variation in enzyme activity of *Cannabis sativa* L. under glyphosate and metribuzin herbicide stress.

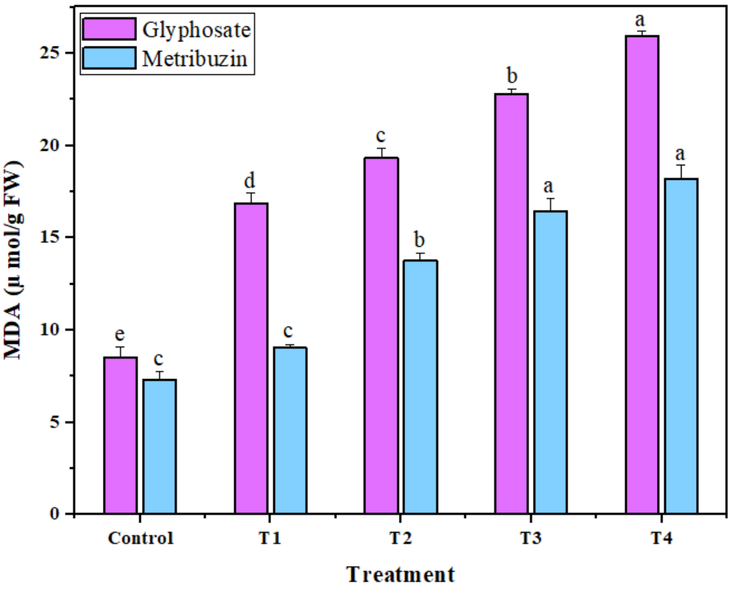


Figure 4.14: Increase in MDA after application of glyphosate and metribuzin.

The principal action mechanism of glyphosate is to inhibit EPSPS, a key component in the shikimate pathway, for the synthesis of aromatic amino acids. The accumulation of shikimate and its derivatives because of the disturbed shikimate pathway puts the plant under oxidative stress (Yokoyama et al., 2021). Metribuzin similarly obstructs photosynthesis by suppressing photosystem II (PSII), preventing electron transfer, and causing an excess of reactive oxygen species (ROS) to be produced in plant cells (Kostopoulou et al., 2020). Antioxidant enzymes like peroxidase and catalase are upregulated as a result of the stress that triggers the plant's defence mechanisms. The higher amounts of reactive oxygen species (ROS) brought on by the disruption in the metabolic pathways are neutralized and detoxified by these enzymes. Nevertheless, the imbalance brought on by herbicide stress can outweigh the plant's defence mechanisms despite the antioxidant response, which can lead to the building up of malondialdehyde (MDA), a sign of lipid peroxidation and cellular damage (Khaleghnezhad et al., 2021). Therefore, the simultaneous increase in MDA concentration indicates oxidative damage and disruption by herbicide application, while the increase in antioxidant enzymes demonstrates the plant's attempt to fight oxidative stress (Karimmojeni et al., 2022a).

4.8. Hormesis

The unexpected results observed at the lowest herbicide concentration can be explained through hormesis, a dose-response phenomenon wherein low doses promote biological responses while high doses lead to their inhibition. Various herbicides like glyphosate at low concentrations can induce plant growth regulation through a variety of mechanisms like auxin production, antioxidant defence, and anatomical modifications, affecting rhizosphere cation transporters (Islam et al., 2017). Lower glyphosate levels hinder the shikimic acid pathway, which lowers lignin and promotes better plant growth (Nguyen et al., 2016). In the current study, the hormesis effect due to metribuzin has been documented for the first time. Herbicide sub-doses promote physiological and enzymatic processes as well as plant growth (Brito et al., 2018). Plant growth and function are ultimately enhanced by this adaptive mechanism, which is triggered by exposure to low-stress levels and activation of cellular defence mechanisms (Jalal et al., 2021).

4.9. Bioactive Compound Identification

The methanolic leaf extract of the cannabis plant comprised various compounds identified as shown in Table 4.1. Among the identified compounds, the most abundant compounds were hexadecenoic acid, methyl stearate, 2,4-Di-tert-butylphenol (2,4-DTBP), and eicosane. The most important phytocannabinoids included delta.9-

tetrahydrocannabivarin (THCV), dronabinol (THC), cannabinol (CBN), Cannabidivarol (CBDV), and cannabidiol (CBD). Dronabinol is the synthetic active enantiomer of Δ9 -tetrahydocannabinol (THC) which is a plant-derived cannabinoid (Devinsky et al., 2015). Therefore, in this thesis, the word THC has been used in place of dronabinol. Other compounds including heptadecane, hexadecane, heneicosane, docosane, and tetracosane were also found in the extract in trace amounts (Figure 4.15). Similar compounds have been observed in different studies (Tayyab & Shahwar, 2015; Vadhel et al., 2023;Wishart et al., 2024).

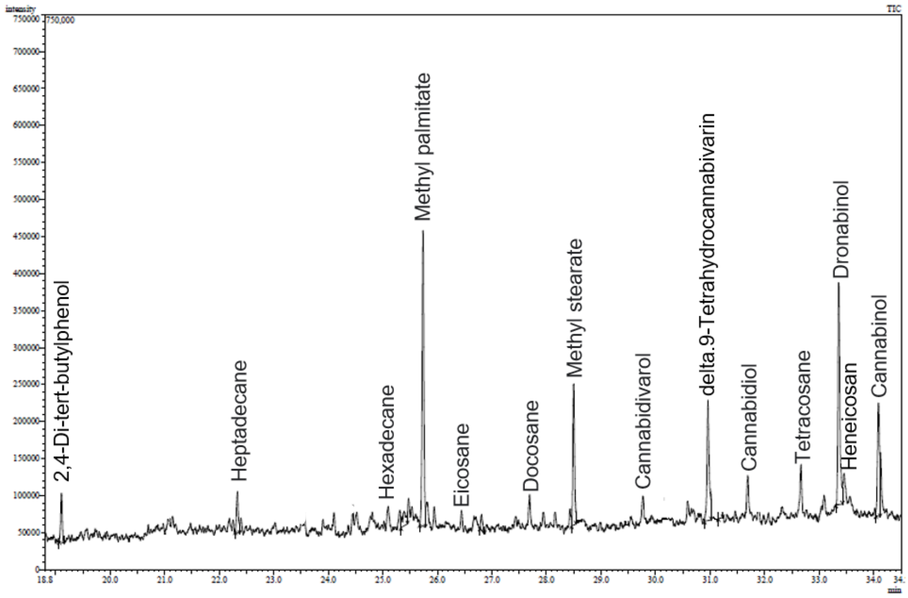
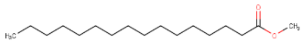
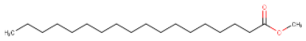
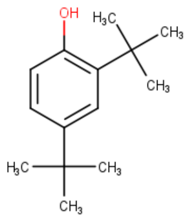
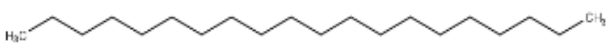
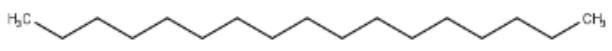
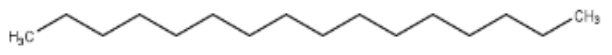
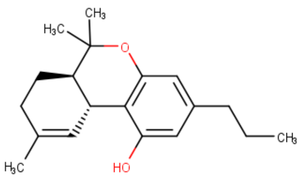
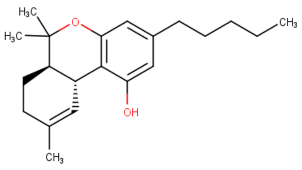
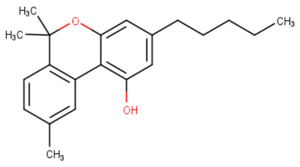
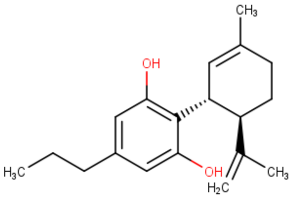
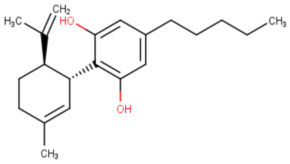
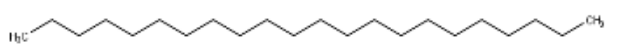
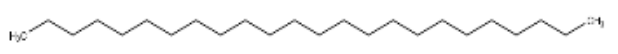


Figure 4.15: Secondary metabolites identification in *Cannabis sativa* L. extract through GC-MS technique.

Table 4.1: IUPAC, chemical formula, structure, and similarity index of identified *Cannabis sativa* L. compounds.

Sr.No.	Identified compound	IUPAC	Chemical Formula	Chemical Structure	Similarity Index
1.	Methyl palmitate	Methyl hexadecanoate	$C_{17}H_{34}O_2$		95%

2.	Methyl stearate	Methyl octadecanoate	$C_{19}H_{38}O_2$		95%
3.	2,4-DTBP	2,4-Di-tert-butylphenol	$C_{14}H_{22}O$		93%
4.	Eicosane	Icosane	$C_{20}H_{42}$		91%
5.	Heptadecane	Heptadecane	$C_{17}H_{36}$		90%
6.	Cetane	Hexadecane	$C_{16}H_{34}$		90%
7.	delta.9-Tetrahydrocannabivarin	(6aR,10aR)-6,6,9-trimethyl-3-propyl-6a,7,8,10a-tetrahydrobenzo[c]chromen-1-ol	$C_{19}H_{26}O_2$		92%
8.	Dronabinol (THC)	(6aR,10aR)-6,6,9-trimethyl-3-pentyl-6a,7,8,10a-tetrahydrobenzo[c]chromen-1-ol	$C_{21}H_{30}O_2$		91%
9.	Cannabinol	6,6,9-trimethyl-3-pentylbenzo[c]chromen-1-ol	$C_{21}H_{26}O_2$		94%

10.	Cannabidivarol	2-[(1R,6R)-3-methyl-6-prop-1-en-2-ylcyclohex-2-en-1-yl]-5-propylbenzene-1,3-diol	$C_{19}H_{26}O_2$		92%
11.	Cannabidiol	2-[(1R,6R)-3-methyl-6-prop-1-en-2-ylcyclohex-2-en-1-yl]-5-pentylbenzene-1,3-diol	$C_{21}H_{30}O_2$		90%
12.	Heneicosane	Henicosane	$C_{21}H_{44}$		90%
13.	Docosane	Docosane	$C_{22}H_{46}$		90%
14.	Tetracosane	Tetracosane	$C_{24}H_{50}$		91%

4.10. Semi-Quantitative Analysis of Bioactive Compounds from *Cannabis sativa*

2,4-Di-tert-butylphenol (2,4-DTBP), an allelopathic component identified in cannabis, has shown significant variation when exposed to different concentrations of glyphosate and metribuzin herbicides. Interestingly, an increase in area percent of 2,4-DTBP was noted at lower concentrations of both herbicides, which gradually decreased as the herbicide stress increased, indicating a direct link between herbicide stress and 2, 4-DTBP biosynthesis (Figure 4.16).

A dynamic profile of fatty acid methyl esters, including methyl stearate, hexadecanoic acid, hexadecane, heptadecane, eicosane, etc. was also observed in GC-MS analysis of leaf extract of cannabis. The glyphosate-induced stress led to an increase in fatty acids area percentage (Figure 4.16). Consequently, stress induced by metribuzin had an inverse effect, resulting in a decline of the fatty acids.

The presence of cannabinoids including THCV, THC, cannabinol, Cannabidivarol, cannabidiol, etc. was also observed. The present data revealed an increase in area percent of THCV and THC under glyphosate stress while metribuzin stress exhibited a negative correlation (Figure 4.16). However, the results of THC show some inconsistencies as in the case of metribuzin treatment, the THC area percent in T1 is higher which can be explained by hormesis. On the other hand, glyphosate resulted in a decrease in THC at the lowest concentration. The cannabinol content was observed exclusively in the control group and the lowest concentration of metribuzin. In contrast, Cannabidivarol and cannabidiol were absent in the control group but were present at the lowest concentration of glyphosate and the highest concentration of metribuzin.

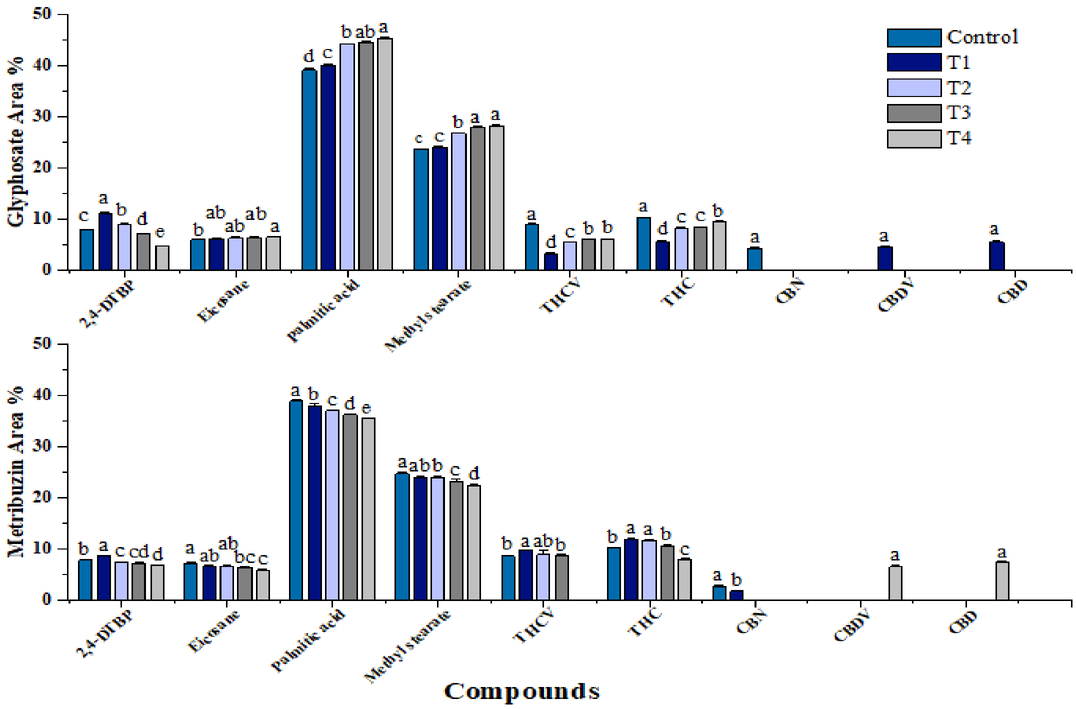


Figure 4.16: Percent relative peak area value of compounds found in *Cannabis sativa* L. under different glyphosate and metribuzin herbicide stress concentrations.

2,4-DTBP, despite being identified in at least 169 species of organisms, there is yet no explanation as to why organisms produce 2,4-DTBP, a toxic lipophilic phenol. A recent study revealed that healthy rice plants show similar levels of 2,4-DTBP as shown by viral infection and insect herbivory samples (Zhao et al., 2020). Nevertheless, more experimentation is required to confirm the correlation between stress and 2,4-DTBP production.

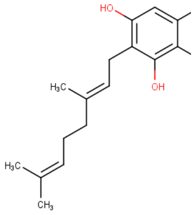
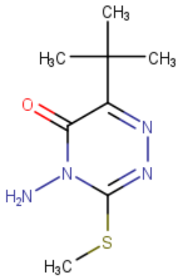
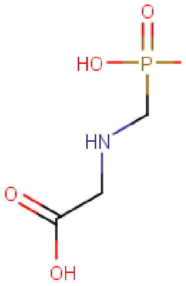
Plants, being sessile organisms, strategically utilize fatty acid production as a crucial part of their adaptive stress response to minimize the negative impact (He & Ding, 2020). Many aliphatic chemicals, such as membrane glycerolipids, TAG, cutin/suberin, jasmonates, and nitroalkenes (NO_2-FAs), are produced by plants using fatty acids as raw material. All these compounds including fatty acids contribute to plant defence against different biotic and abiotic stressors (He and Ding, 2020; de Oliveira et al., 2022). There are reports of herbicides having ecotoxicological and biochemical effects on a variety of marine plankton species, leading to an increase in the proportion of fatty acids in response to toxicity (Filimonova et al., 2018). The mechanisms may include alteration in the *de novo* synthesis of fatty acids through the fatty acid synthetase enzyme and/or via modification of existing lipids via desaturase enzyme action. In a study, an increase in ~17% lipid content was observed in glyphosate-treated *Chlorella sorokiniana* as compared to the control (Jaiswal et al., 2020). The effects of metribuzin on fatty acid synthesis are not well documented yet, but fatty acid synthesis is an energy-expensive process that requires ATP and NADPH at different steps; thus, it can be hypothesized that blocking of electron transport chain by metribuzin reduces energy production which in turn affects production of fatty acids. In addition, delay in electron transport from QA to QB results in over-reduction on the PSII acceptor side, which raises the chances of producing reactive oxygen species as explained in the photosynthetic pigments section, leading to lipid peroxidation in thylakoid membranes, resulting in a decrease in fatty acids (Roncel et al., 2007). This complex interaction highlights the diverse ways in which plants react to herbicidal stresses and advances our knowledge of the complex mechanisms underlying plant adaptability.

The impact of herbicides on cannabinoids can be plausibly explained by the observed relationship between herbicide treatments and fatty acid synthesis as the cannabinoids are synthesized from Geranyl pyrophosphate (GPP), a precursor derived from Acetyl-CoA via the fatty acid synthesis pathway (Macherone, 2020). On the other hand, the decrease in THC at the lowest concentration of glyphosate is due to the biosynthesis of another compound CBD at the same treatment since THC and CBD are synthesized from the same precursor (CBGA); so even if the precursor were produced in greater concentrations as can be correlated from higher synthesis of fatty acids, the precursor would be utilized by the plant in the production of CBD in addition to THC. According to various studies, the synthesis of cannabinoids in plants varies under different environmental conditions. Drought stress, for example, was associated with an increase in cannabinoid production (Caplan et al., 2019). Similarly, a study reported that the active compound THC increased when the plants were exposed to UVB, whereas identical conditions did not cause any changes in the plant's cannabinoids (Lydon et al., 1987). Copper was also found to cause a significant temporal increase in the production of THC and CBD (Cahill et al., 2024). Abscisic acid has been implicated in affecting THC concentration in plants, although its effect remains controversial (Mansouri & Asrar, 2012). Contrastingly, cannabinoid levels were observed to decline under herbicide stress (Toth et al., 2021). From these observations, it can be concluded that there is a complex relationship between environmental factors and upsurge and decline in cannabinoid levels which has not yet been studied in detail and needs further investigation.

4.11. Ligand Structural Comparison and Optimization

This study conducted a comparative analysis of the physicochemical characteristics of CBGA, metribuzin, and glyphosate as shown in Table 4.2. The aromatic ring (AR) is responsible for π−π interactions with amino acid residues, which are present in both CBGA and metribuzin, leading to an increase in binding affinity and influencing biological activity. Glyphosate has the highest hydrogen bond acceptors (HBA) and hydrogen bond donors (HBD) which positively influence the number of hydrogen bonds between the ligand and the enzyme THCA Synthase; however, the number of rotatable bonds (RB) in metribuzin is lower than in glyphosate. Furthermore, metribuzin has a slightly greater molecular weight (MW) compared to glyphosate.

Table 4.2: Physicochemical properties comparison of CBGA, metribuzin, and glyphosate.

Name	Chemical formula	Structure	MW (g/mol)	HBD	HBA	RB	AR	TPSA	PubChem CID Reference
CBGA	$C_{22}H_{32}O_4$		360.5	3	4	13	1	77.8	6449999
Metribuzin	$C_8H_{14}N_4OS$		214.29	1	5	3	1	96.4	30479
Glyphosate	$C_3H_8NO_5P$		169.07	4	6	7	0	107	3496

After initial energy minimization using several software programs, CBGA, and metribuzin still exhibited positive ΔG energy, while glyphosate demonstrated negative ΔG energy (Table 4.3). Upon Gaussian optimization, all three ligands exhibited minimal negative ΔG energy (Figures 4.17, 4.18, 4.19). CBGA had the lowest energy, followed by metribuzin and glyphosate (Table 4.4). Upon optimization, the bond length and angle of CBGA and metribuzin exhibited minor modifications; however, in the case of glyphosate, considerable alterations were observed (Table 4.5).

Table 4.3: Start and minimized energy (kcal/mol) after optimizing sequentially in the given order using different softwares. The underline shows the most minimized energies of the ligands.

Software (Force field)	Chem3D Pro (MM2)		Chem3D (MM2)		Avogadro (MMFF94)		Chem3D (MMFF94)	
Energy (kcal/mol)	**Start**	**Minimized**	**Start**	**Minimized**	**Start**	**Minimized**	**Start**	**Minimized**
CBGA	1203.416	14.5137	17.446	14.5	72.0069	39.4885	39.462	39.462
Metribuzin	383.008	11.131	102.9129	15.75	161.3568	112.6683	39.602	69.732
Glyphosate	31.276	13.6115	18.4667	13.61	-7.3735	-23.961	-23.945	-23.9493

Table 4.4: Ligand parameters obtained from Gaussian optimization.

Ligands	Start energy (kcal/mol)	Minimized energy (a.u.)	Dipole Moment (Debye)
CBGA	14.5137	-1158.837799	2.1327
Metribuzin	11.131	-1005.813539	9.2666
Glyphosate	-23.961	-891.6263467	2.9354

Table 4.5: Ground state configurations of CBGA, metribuzin, and glyphosate with every atom's spatial arrangement: bond length and angle.

CBGA					
Bond Length (Å)	**Unoptimized**	**Optimized**	**Bond Angle (°)**	**Unoptimized**	**Optimized**
C-C (17)	1.54	1.54	H-C-H (19)	109.47125	109.47116
C=C (5)	1.3552	1.3552	C-C-H (43)	109.47122	109.47113
C=O	1.2584	1.2584	C-C-C (9)	109.47122	109.47122
C-O (3)	1.43	1.43	C=C-C (12)	120	120
C-H (29)	1.07	1.07	C-C-O (2)	120	120
O-H (3)	0.96	0.96	C-O-H (3)	109.471	109.471
			O=C-O	120	120

			C=C-H (3)	119.99986	118.04065
			C=C-O (2)	120.00007	113.89094
			C-C=O	120.00008	128.23227

Metribuzin					
Bond Length (Å)	**Unoptimized**	**Optimized**	**Bond Angle (°)**	**Unoptimized**	**Optimized**
C-C (5)	1.54	1.54	H-C-H (12)	109.47122	109.47122
C=N (2)	1.30531	1.30531	H-S-C (3)	109.47122	109.47122
C=O	1.2584	1.2584	C-C-C (7)	109.47122	109.47122
C-H (12)	1.07	1.07	C-C=O	120.00009	120.00009
N-N (2)	1.40701	1.40701	C-O-H	109.47127	109.47127
C-N (1)	1.47526	1.37124	C-S-C	109.47122	99.02128
N-C (1)	1.46380	1.40647	S-C=N	119.04398	116.21160
C-S (2)	1.78000	1.76828	C=N-N (2)	123.07735	118.50765
N-H (2)	1.00000	1.01775	C-C=N (2)	118.92519	112.36916
			C-N-N (2)	123.07735	109.38199
			C-N-C	110.31853	110.31856
			N-C-S	119.03777	119.03777
			O=C-N	120.6129	120.6129
			N-N-H (2)	109.47122	106.98, 107.67

Glyphosate					
Bond Length (Å)	**Unoptimized**	**Optimized**	**Bond Angle (°)**	**Unoptimized**	**Optimized**
P=O	1.5048	1.47966	O-P-O	109.4712	104.6909
P-OH	1.71	1.62665			113.3118
		1.62035			114.4972

P-C	1.82	1.82095	O-P-C	109.4712	120.4209
C-N	1.47	1.45318			99.88669
		1.45504	O=P-C	109.47122	120.42088
C-H	1.07	1.10725	P-C-N	109.4712	109.93572
		1.10285	C-N-C	109.4712	113.29973
		1.09412	N-C-C	109.4712	112.89148
		1.09165	C-C-O	120	106.20121
C-C	1.54	1.51562	C-C=O	120	124.92402
C=O	1.2584	1.20155	O=C-O	120.0	123.07
C-OH	1.43	1.35432			
O-H (3)	0.96000	0.96476			

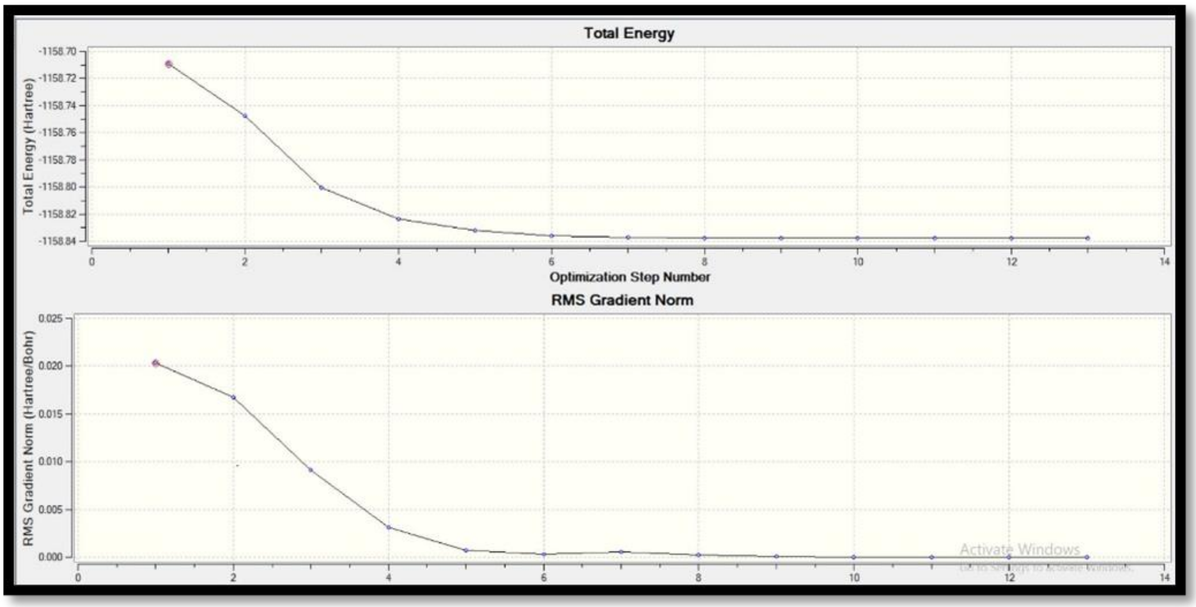


Figure 4.17: The energy minimization and RMS gradient normalization for CBGA with optimization stem number.

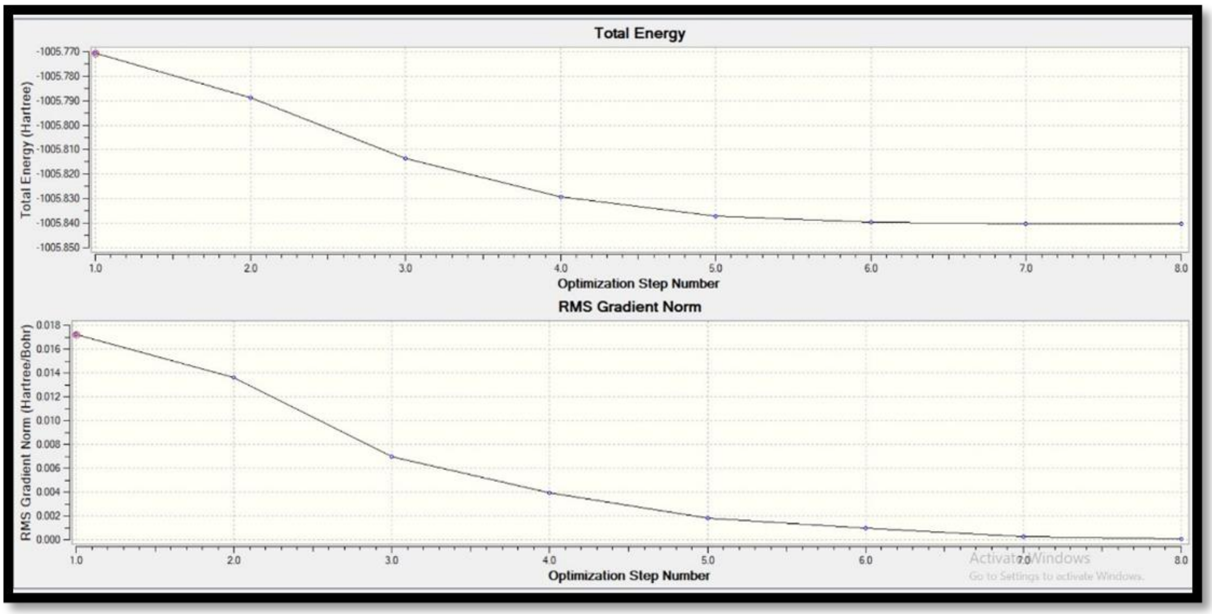


Figure 4.18: The energy minimization and RMS gradient normalization for metribuzin with optimization stem numbers.

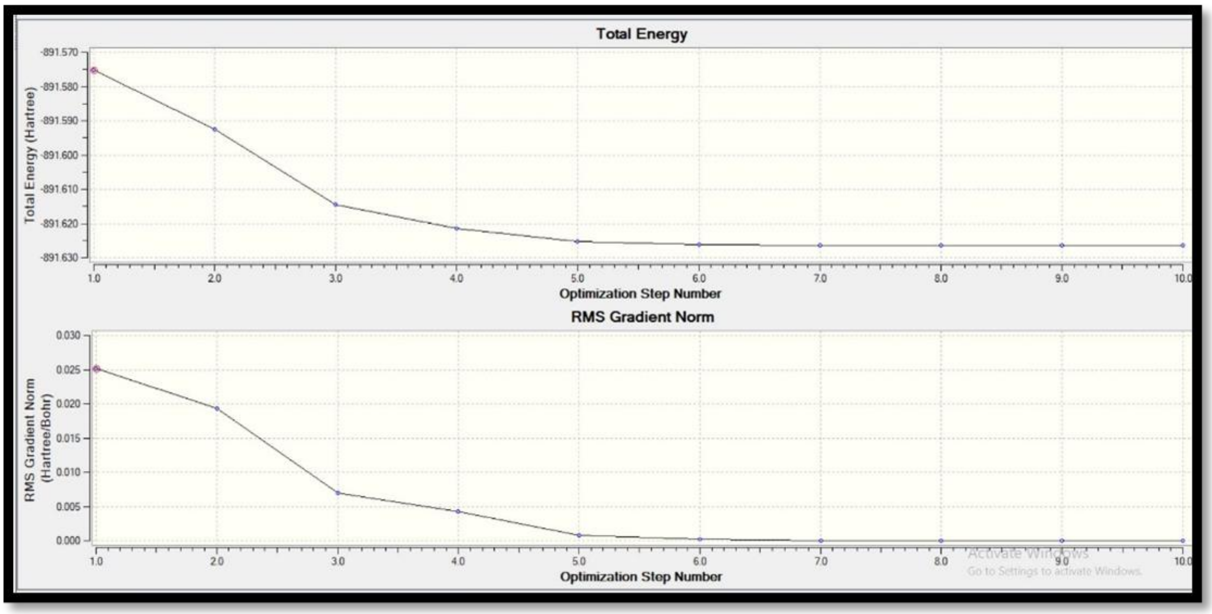


Figure 4.19: The energy minimization and RMS gradient normalization for glyphosate with optimization stem numbers.

4.12. Molecular Docking

Molecular docking studies were performed to understand the impact of varying glyphosate and metribuzin herbicide concentrations on the biosynthesis of THC by analysing their possible interactions with the active site of THCA synthase. THCA synthase is the enzyme that converts the CBGA precursor into THC, hence CBGA was also docked with THCA synthase, and the docking model is shown in (Figure 4.20). (Figure 4.21) shows the docking model of metribuzin and glyphosate at the same site respectively.

Table 4.6: Docking results of CBGA, metribuzin, and glyphosate with THCA Synthase along with interacting amino acids.

Ligand	Interacting amino acids (H-bond)	Hydrophobic interaction	van der Waal's interaction	Number of hydrogen bonds	Binding Affinity (Kcal/mol)
CBGA	Thr 379, Ser 448	FAD, His 114, Lys 378, Phe 381, Cys 176, Pro 177, Ile 383	Gln 69, Thr 446, Thr 178, Gly 356, Ala 375, Gly 376, Lys 377, Met 413, Tyr 484,	3	-6.06
Metribuzin	Thr 379, Lys 377	FAD, His 114, Lys 378, Phe 381	Gln 69, Thr 446, Ser 448, Ile 383	3	-5.79
Glyphosate	Lys 261, Tyr 312	NA	NA	4	-2.87

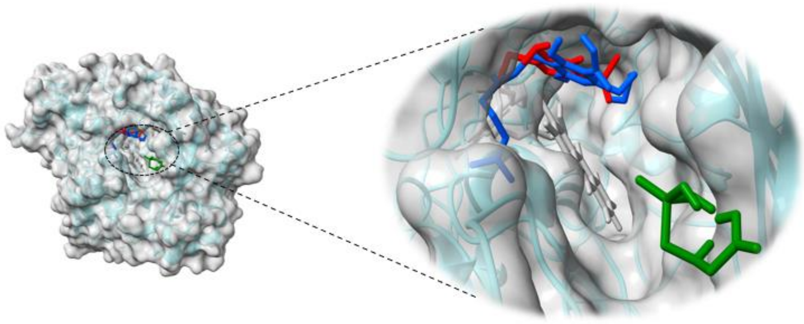


Figure 4.20: Three-dimensional surface view of THCA synthase and enlarged view of docked ligands CBGA (blue), Metribuzin (red), and Glyphosate (green) at the active channel containing FAD (grey).

Metribuzin was completely embedded in the active site and showed interactions with the same amino acids as CBGA. Both metribuzin and CBGA formed conventional hydrogen bonds, alkyl, and π-alkyl interactions with Thr 379, Lys 378, and His 114, respectively. Phe 381 interacted with CBGA and metribuzin with π–π stacked interactions and an additional π-Alkyl interaction with metribuzin. FAD, responsible for the core catalytic activity of enzyme THCA synthase, formed alkyl interaction with CBGA and metribuzin. On top of that, it also formed π-alkyl interaction with CBGA and π-π interaction with metribuzin. Moreover, both CBGA and metribuzin interacted with Thr 446, Ile 383, Ser448, Lys 377, and Gln 69 to form alkyl, conventional hydrogen bond, and van der Waals interactions. Therefore, metribuzin and CBGA bind at the same active site with similar kinds of interactions with binding energies of -5.79Kcal/mol and -6.06Kcal/mol respectively. From the docking result, it is clear that the interaction of metribuzin is slightly lower than the CBGA; but both are bound deep inside the active site as there are slight differences in their binding energies. However, glyphosate was observed to be present at the entrance of the active site as its binding energy is less (-2.87Kcal/mol) as compared to the former two i.e., metribizin and CBGA. Glyphosate interacted with Tyr 312 and Lys 261 amino acid residues of THCA synthase by forming conventional hydrogen bonds as shown in Table 4.6. The same observation was obtained from the GC-MS analysis that supports our *in-silico* result.

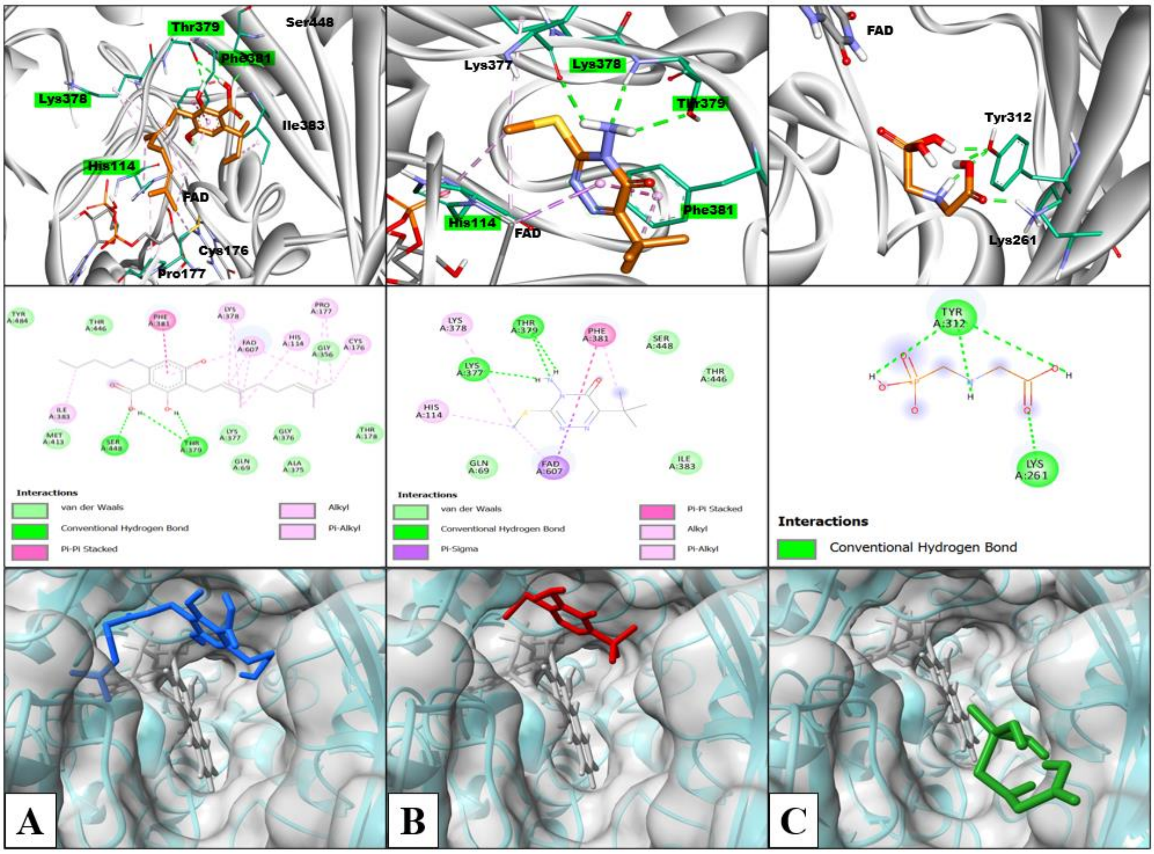


Figure 4.21: Visualization of docking results and interacting residues A) CBGA, B) Metribuzin, and C) Glyphosate. The top row shows the 3D interactions of ligands with amino acid residues (green) and FAD (grey); Carbon atoms: rust, nitrogen atoms: light purple, oxygen atoms: red, and sulfur atoms: yellow; common amino acid residues among CBGA and metribuzin are highlighted in green, the middle row shows types of interactions, and the bottom row shows the surface view of the active site with the docked ligands.

The *in-silico* studies provided valuable insights into the interactions of metribuzin and glyphosate with the target- THCA synthase enzyme. The interaction between the target enzyme and the ligand is influenced by the physicochemical properties and molecular structure of the ligands. The enzymatic activity of THCA synthase occurs in a hydrophobic environment (Rodziewicz et al., 2019), which is consistent with the minimal topological polar surface area (TPSA) of its substrate CBGA. Compared to glyphosate, metribuzin possesses a lower TPSA value, suggesting metribuzin may more freely access and bind to THCA synthase as compared to glyphosate. Based on the physicochemical and optimization analysis, it was observed that metribuzin is more similar to CBGA than glyphosate. Molecular docking studies elucidated the impact of glyphosate and metribuzin on the biosynthesis of THC by analysing their possible interactions with the active site of THCA synthase (Shoyama et al., 2012). The comparative analysis of the three docking models provides valuable insights into the mechanisms of metribuzin and glyphosate in THC production. The models demonstrate that metribuzin directly competes with CBGA for binding to the active site of THCA synthase. Hence, it might be possible that metribuzin hinders the activity of CBGA by preventing CBGA from binding at the active site of THCA synthase and consequently reduces THC biosynthesis with an increase in metribuzin stress. Moreover, at higher metribuzin concentrations, there might be a reduction in CBGA production through inhibition of the fatty acid synthesis pathway that supplies CBGA. This dual impact of metribuzin, reducing both CBGA production and binding, leads to low activity of THCA synthase and the lowest area percent of THC at the highest concentration (T4) as compared to other treatment groups. In contrast, glyphosate partially blocks the entry to the active channel of THCA synthase, hindering CBGA binding to the active site, and thus resulting in a significant reduction in THC synthesis. The increase in fatty acids from glyphosate treatment produces a slight upward trend in THC levels due to the increased availability of CBGA. From our *in-silico* investigation, it can be concluded that while glyphosate partially blocks CBGA entry, still causing an increase in THC production due to precursor quantity enhancement, metribuzin leads to a downward trend in THC synthesis by binding to the THCA synthase active site and decreasing CBGA substrate availability through fatty acid pathway inhibition. Thus, at the highest herbicide concentration, metribuzin has more impact on the cannabis plant's THC production as compared to glyphosate as evident from the experimental data and supported by the *in-silico* data analysis.

Objective 3: Extraction and analysis of plant fiber after treatment.

4.13. Fiber Extraction

The boiling of stem pieces in 0.35% NaOH solution for 90 minutes facilitated the separation of the bark from the woody core. The bark still had some chlorophyll content present in it. The fiber was successfully extracted during the second treatment, and it was yellowish. The extracted fiber was then washed and oven-dried, which gave the fiber its characteristic color and texture (Figure 4.22).

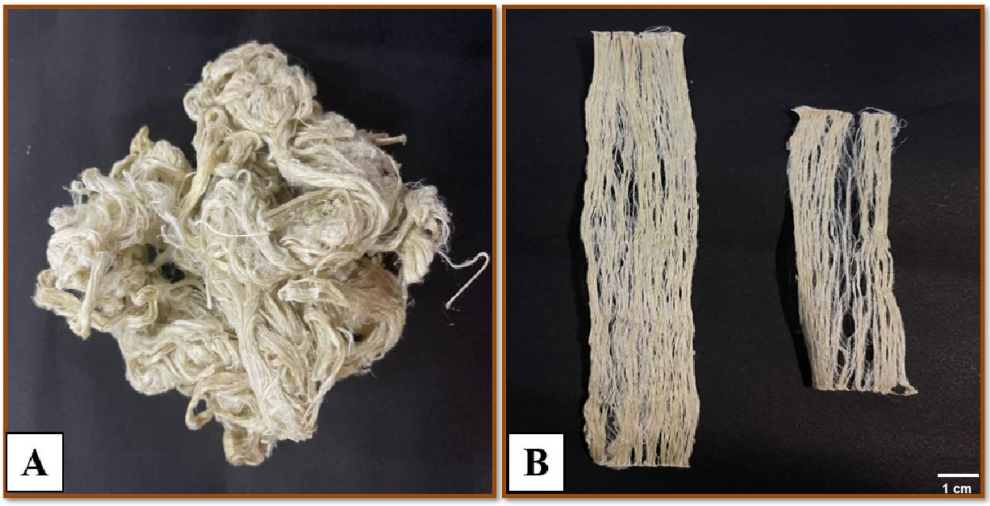


Figure 4.22: Extracted fiber after NaOH treatment, A: Raw fiber, B: Straightened fiber.

Natural fiber is a composite material made from renewable plant and animal sources, making them environmentally sustainable with well-understood structures (Thakur et al., 2014). The main component of plant fiber is the polymer cellulose. Lignocellulosic or plant fiber can be obtained from various parts of plants like bark, wood, leaves, seeds, fruits, vegetables, straw, bagasse, and roots (Thakur et al., 2014). Alongside cellulose, the plant fiber contains other constituents such as hemicellulose, lignin, pectin, and waxes. The exact composition of each component varies based on the plant source, its age, and any preprocessing steps applied (John & Thomas, 2008a). Hemp fiber is a prominent type of bast natural fiber, commonly extracted from the hemp plant of the Cannabis genus. The inherent mechanical, thermal, and acoustic properties of hemp fiber, combined with the general characteristics of natural fiber, make it beneficial for a wide range of applications, including use as reinforcements in polymer composite materials (Dhakal et al., 2007). In our study, we used sodium hydroxide for the fiber extraction process. A study tested effects of different levels of alkali concentrations on the hemp fibers' mechanical properties (like length, strength, and weight) and physical properties (like diameter and surface texture). The treatment produced textile-grade hemp fiber that can be spun using a cotton spinning system. Also, the alkali treatment significantly improved the quality of the hemp fiber, making them suitable for textile applications (Sahi et al., 2022).

4.14. Compositional Analysis

The analysis of cellulose and hemicellulose content in fiber after treatment with varying concentrations of glyphosate and metribuzin herbicides revealed significant differences (Figure 4.23). In the untreated control fiber, the cellulose content was 63.3%, while the hemicellulose content was 7.8%. When fiber was treated with a low concentration of glyphosate (G1), a decrease in both cellulose and hemicellulose content was observed, with values of 49.5% and 6.417%, respectively. This reduction becomes more pronounced with high concentration of glyphosate (G2), where the cellulose content dropped to 37.15%, and the hemicellulose content decreased to 5.21%.

In contrast, treatment with a low concentration of metribuzin (M1) led to an increase in cellulose content to 64.1%, showing no significant difference with the control, although the hemicellulose content showed a minor reduction to 6.98%. However, fiber treated with a high concentration of metribuzin (M2) exhibited a cellulose content of 50.2%, lower than the control but higher than both glyphosate treatments. Notably, the hemicellulose content in this fiber was the lowest at 4.81%.

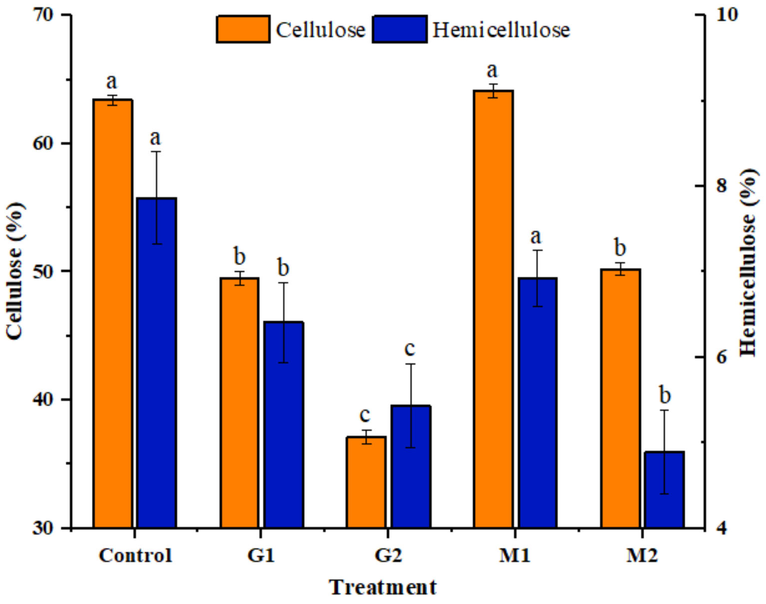


Figure 4.23: Change in cellulose and hemicellulose content with varying herbicide concentrations.

Cellulose exhibits a structural configuration comprising long, linear polymeric chains of glucose monomers linked together by robust hydrogen bonds (Kaushik et al., 2010). These hydrogen bonding interactions confer a highly stable and rigid geometry upon cellulose, endowing it with remarkable tensile strength properties. The length of the cellulosic fiber and its micronaire value (quantitative measure of fiber fineness and maturity), are significantly influenced by agronomic and climatic factors, as the growth and development of the fiber are susceptible to most factors that impact overall plant growth. Since cellulose constitutes the primary component of the plant fiber, any factor modulating the plant's photosynthetic capacity and carbohydrate biosynthesis will exert a commensurate effect on fiber growth (Torres-Salinas et al., 2013). Cell expansion during the growth phase is strongly driven by turgor pressure, the hydrostatic pressure exerted by fluids within the plant cell, hence the plant's water relations will likewise modulate fiber elongation. Sucrose, a non-reducing disaccharide and the primary product of photosynthesis in plants serves as the fundamental precursor compound in cellulose biosynthesis (Roberts et al., 1992); consequently, any perturbation in sucrose concentration would directly impact cellulose synthesis. Our findings suggest that while low metribuzin concentration (M1) positively impacted cellulose content, glyphosate treatments negatively affected both cellulose and hemicellulose levels, with higher concentrations causing more severe reductions. Furthermore, high metribuzin concentration also decreased cellulose content compared to the control, but its effect on hemicellulose content was more substantial.

4.15. Cellulose Crystallinity Index Analysis

The X-ray diffraction (XRD) analysis revealed varying crystallinity indices (CrI) among hemp fiber samples subjected to different herbicide treatments (Table 4.7, Figure 4.24). The untreated control fiber exhibited an intensity at Iam of 5.873 and I002 of 52.897, resulting in a CrI of 88.90%. Glyphosate treatment caused a concentration-dependent decrease in CrI. Specifically, low-concentration glyphosate treatment (G1) reduced the intensity at Iam to 3.805 and at I002 to 29.948, resulting in a CrI of 87.29%. High-concentration glyphosate treatment (G2) showed the most substantial reduction, with Iam at 3.560 and I002 at 19.686, leading to a CrI of 81.92%.

In contrast, metribuzin treatment displayed divergent effects. Low concentration metribuzin treatment (M1) resulted in the highest CrI (90.83%), with Iam at 4.983 and I002 at 54.354, surpassing the control. However, high concentration metribuzin treatment (M2) led to a reduction in CrI to 85.55%, with Iam remaining at 4.983 but I002 decreasing to 34.494.

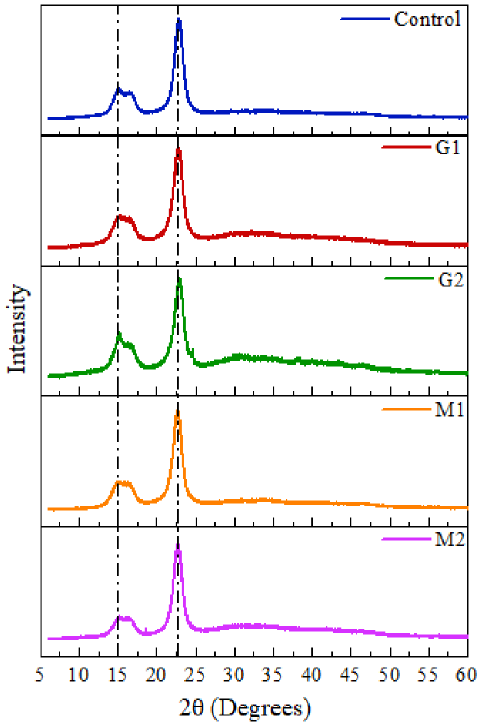


Figure 4.24: X-ray diffraction pattern of fiber under varying herbicide stress.

Table 4.7: Determined Cellulose crystallinity Index from XRD analysis.

Treatment	**Intensity at *I*am**	**Intensity at I_{002}**	**CrI (%)**
Control	5.873	52.897	88.89729
G1	3.805	29.948	87.29464
G2	3.56	19.686	81.91608
M1	4.983	54.354	90.83232
M2	4.983	34.494	85.55401

The crystallinity index of fiber can change under stress. A study found that drought stress can significantly influence the crystallinity of hemp fiber (Kwiatkowska et al., 2024). The crystallinity index (CrI) and its contributing parameters, Iam and I002, offer insights into the structural changes in hemp fibers subjected to different herbicide treatments. Our results also showed a variation in crystallinity index under different herbicide stress. The untreated control exhibited a high CrI (88.90%), characterized by significant I002 intensity (52.897) and a moderate Iam intensity (5.873), indicative of a well-ordered crystalline structure. The values of CI obtained through XRD were similar to values of other studies (Hakamy et al., 2016; Kwiatkowska et al., 2024; Marrot et al., 2013). The crystallinity index significantly influences the mechanical properties of natural fibers (Khan et al., 2021), as it's the crystalline region that gives a fiber its strength (Halbeisen, 2005). Fibers with high crystallinity have tightly packed molecular chains, making them less accessible to water molecules. This restricts the fiber's ability to swell, as fewer spaces are available for water to penetrate. Conversely, fibers with a lower CI have more amorphous regions, which allow for greater water absorption and swelling (Lawrence, 2015). Our results of the crystallinity index of different fiber samples were found to be in a similar trend to the breaking strength results. Also, cellulose content showed a similar pattern. Our findings suggest that while low metribuzin concentration enhances the breaking strength of the fiber, glyphosate treatments, particularly at high concentrations, negatively impact fiber strength. Conversely, high metribuzin concentration also led to a reduction in breaking strength compared to the control, albeit to a lesser extent than the glyphosate treatments.

4.16. Mechanical Properties

The extracted fiber's mechanical properties were analysed and the following results were obtained.

4.16.1. Strain at Maximum Load: The tensile strain of untreated hemp fiber showed a maximum strain of 1.81% before breaking. Fiber treated with low glyphosate concentration showed a decreased strain of 0.55%, while those treated with low metribuzin concentration had an increased strain of 4.17% as compared to control. However, the fiber treated with high concentrations of both herbicides showed reduced strain at maximum load i.e., 0.78% for glyphosate and 1.17% for metribuzin (Figure 4.25).

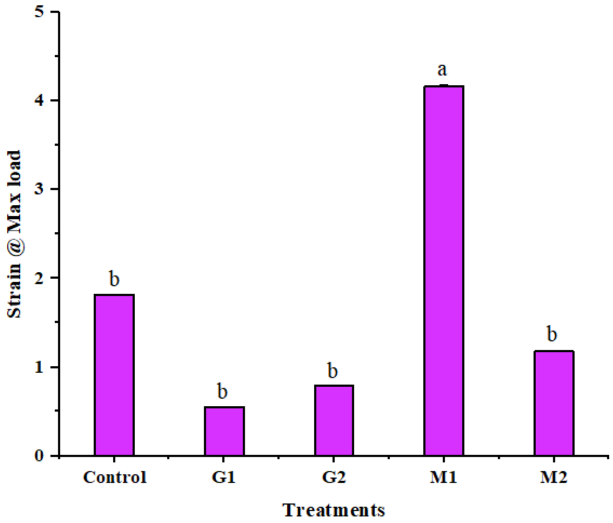


Figure 4.25: Variation in strain at maximum load of fiber under herbicide stress.

4.16.2. Young's Modulus: Young’s modulus quantifies a material's ability to resist deformation under lengthwise tension or compression, indicating its stiffness and elasticity. Fiber treated with the lowest glyphosate concentration exhibited the highest Young's modulus value of 75695.87 MPa. In contrast, fiber treated with the lowest metribuzin concentration showed the lowest Young's modulus of 7533.04 MPa. Untreated fiber had an intermediate Young's modulus value of 15641.05 MPa. At the highest glyphosate and metribuzin concentrations, Young's modulus was 48056.24 MPa and 37458.04 MPa respectively (Figure 4.26).

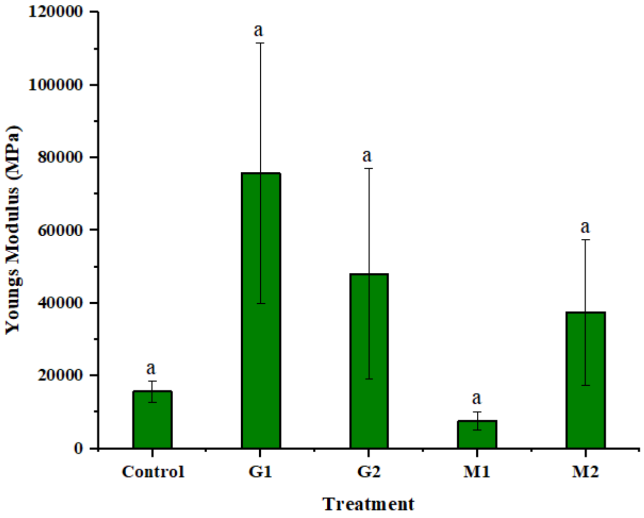


Figure 4.26: Variation in Youngs modulus under varying herbicide stress.

4.16.3. Maximum Extension: The results showed that low metribuzin concentration (M1) resulted in the greatest fiber extension of 74.56 mm, indicating an increase in fiber extensibility. High glyphosate concentration (G2) gave the next highest extension of 74.066 mm, also increasing extension compared to the untreated fiber which extended up to 73.55 mm. In contrast, low glyphosate concentration gave the lowest extension of 72.26 mm, decreasing fiber extensibility. High metribuzin concentration resulted in a fiber extension of 74.03 mm, having little effect on extensibility compared to the control (Figure 4.27).

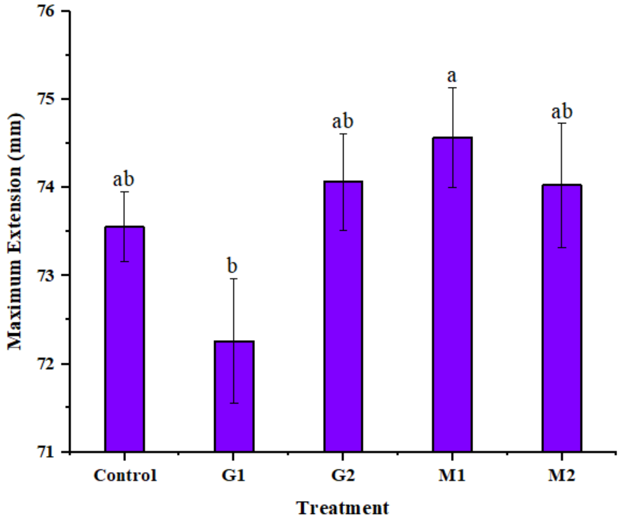


Figure 4.27: Maximum extension of fiber obtained from herbicide-stressed plants.

4.16.4. Elongation at Break: Fiber extracted from the lowest metribuzin concentration treated plants showed the highest elongation of 6.52%. The highest glyphosate concentration resulted in the next highest elongation of 5.8%. Untreated fiber had 5.08% elongation. The lowest elongation of 3.22% occurred with the lowest glyphosate concentration, followed by 5.75% elongation with the second-highest metribuzin concentration (Figure 4.28).

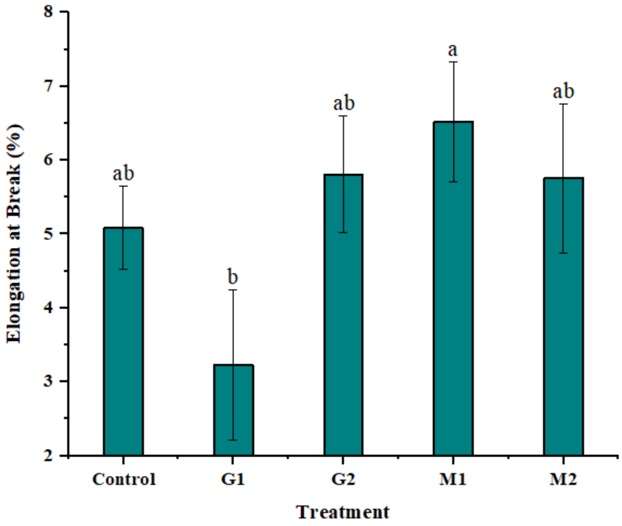


Figure 4.28: Maximum elongation of fiber before breaking.

4.16.5. Stress-Strain Curve: The stress-strain curve was drawn for the fiber extracted from varying concentrations of glyphosate- and metribuzin-treated pants (Figure 4.29). The breaking strength of hemp fiber was estimated using a stress-strain curve after herbicide treatment (Figure 4.30). The untreated fiber exhibited a breaking strength of 260.141 MPa. Fiber treated with a low concentration of glyphosate (G1) showed a decreased breaking strength of 237.151 MPa compared to the control. This reduction in strength was more pronounced for fiber treated with a high concentration of glyphosate (G2), which had a breaking strength of 190.817 MPa, the lowest among all treatments. In contrast, fiber treated with a low concentration of metribuzin (M1) displayed a breaking strength of 264.089 MPa, higher than the control, indicating an increase in strength. However, fiber treated with a high concentration of metribuzin (M2) had a breaking strength of 239.942 MPa, lower than the control but higher than both glyphosate treatments.

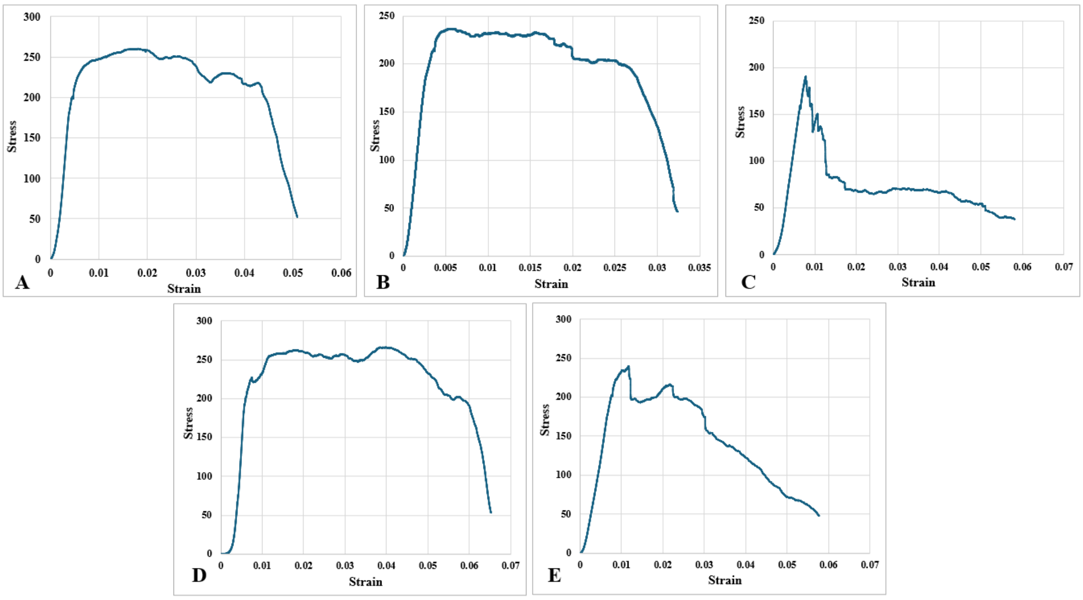


Figure 4.29: Stress-strain curve of fiber under different herbicide stress

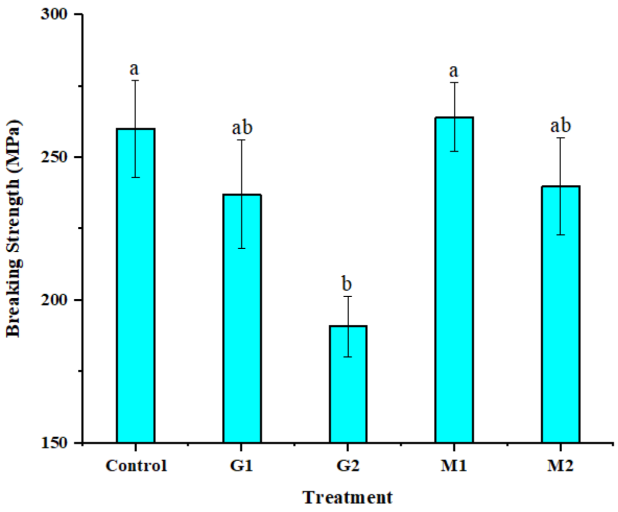


Figure 4.30: Breaking strength of fiber obtained from stress-strain curve.

The cellulose filaments exhibit a parallel alignment along the longitudinal axis of the fiber, conferring maximal tensile and flexural strengths, imparting structural rigidity. The mechanical properties of the fiber is primarily governed by factors such as the cellulose content, the degree of polymerization (DP), and the fibrillar angle. Notably, a high cellulose content and a low fibrillar angle are desirable characteristics for fiber, intended for use as reinforcements in biological composite materials (John & Thomas, 2008b). The parallel orientation of the cellulose filaments along the fiber length, facilitates the efficient translation of the cellulose polymer's inherent strength and rigidity to the fiber's overall mechanical performance. Furthermore, higher cellulose content and a smaller fibrillar angle, representing the acute angle between the cellulose microfibrils and the longitudinal fiber axis, are advantageous as they optimize the fiber's stress transfer and load-bearing capabilities when incorporated as reinforcing elements within composite matrices (Wattanakornsiri & Tongnunui, 2014). A study on flax seeds concluded that applying glyphosate to flax stems initiates a predictable retting process, where fiber bundles start separating due to the drying and eventual microbial action. Mechanical tests showed that as the stems dry, the work needed to separate fibers initially increases and then decreases as microbial retting progresses (Goodman et al., 2002).

Studies have demonstrated that the highest mechanical qualities, such as a greater Young's modulus, are displayed by bio-composites made of natural fiber that have a high combined percentage of cellulose and hemicellulose. Cellulose, hemicellulose, and lignin make up the fiber. Hydrophilic crystalline cellulose influences the material's elastic limit. The ductility of a material is significantly influenced by hydrophobic lignin, whereas hemicellulose and cellulose also have an impact (Tanguy et al., 2018). In our study, low levels of glyphosate increased fiber stiffness as indicated by the high Young's modulus, while low metribuzin levels decreased fiber stiffness. High concentrations of both herbicides led to intermediate Young's modulus values compared to the low concentration and control groups. The Young's modulus and tensile strength of natural fiber, particularly hemp fiber, are well-documented properties in the literature, with reported values ranging from 58 GPa to 70 GPa (Ariati et al., 2021; Cruz & Fangueiro, 2016) and 550 MPa to 1110 MPa (Nobrega et al., 2022; Singh et al., 2021; Thamarai et al., 2023), respectively. These variations in mechanical properties and Young's modulus are attributed to factors such as fiber diameter, fiber length, and test speed employed during characterization. Notably, the findings from the present study indicate that low metribuzin concentrations and high glyphosate concentrations resulted in increased fiber extensibility compared to the control sample, whereas low glyphosate concentrations led to a decrease in extensibility, and high metribuzin concentrations had a minimal impact on fiber extension. Additionally, it was observed that both tensile strength and elongation at break exhibited an inverse relationship with fiber length, diminishing as the fiber length increased. Conversely, Young's modulus demonstrated a positive correlation with fiber length, increasing with an augmentation in fiber length. Furthermore, an escalation in the test speed during mechanical characterization was found to elicit a concomitant increase in the fiber's Young's modulus (Ribeiro et al., 2023a).

The elongation at break, a critical parameter characterizing the ductility of fiber, has been extensively studied, with literature values reported to be around 1.6% (Ribeiro et al., 2023b). Although fiber length adjustment under water deficit conditions may seem directly associated with cell expansion, the influence of water availability on flowering duration, timing, and fiber elongation creates complex physiological interactions between water stress and fiber characteristics (Bradow & Davidonis, 2010). The results obtained in the present study indicate that low concentrations of glyphosate herbicide compromised the fiber strength, manifesting as a reduction in strain at failure. Conversely, low levels of metribuzin herbicide fortified the fiber, enhancing their strain capacity. However, high concentrations of both herbicides elicited a decrease in the tensile strain of the fiber at the maximum load, compared to the untreated control samples. These findings underscore the intricate interplay between herbicide exposure and fiber mechanical properties, with the specific herbicide type and concentration playing a pivotal role in modulating the ductility and strain response of the fiber.

4.17. Bast Fiber Content

Bast fiber may be defined as those obtained from the outer cell layers of the stems of various plants (Jones et al., 2017). In the control plants with no herbicide treatment, the bast fiber content was 3.07%. At low concentrations, both herbicides increased bast fiber content compared to the control, with glyphosate treatment resulting in a maximum increase of 4.53% and metribuzin treatment resulting in 3.91% increase. This suggests low levels of these herbicides may have a positive effect on hemp fiber production. However, as the concentration of the herbicides was increased, the bast fiber content declined, indicating higher levels of herbicide stress, decreased fiber content (Figure 4.31).

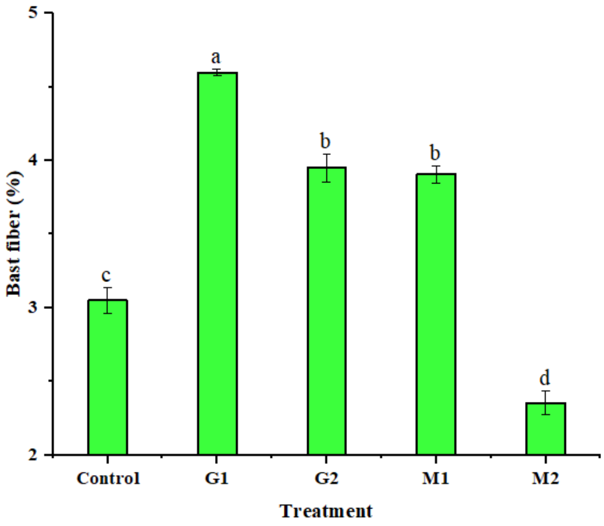


Figure 4.31: Bast fiber (%) content in fiber after herbicide treatment.

4.18. Surface Morphology

The scanning electron microscope (SEM) micrographs show the untreated fiber's natural state, characterized by a smooth and uniform surface topography (Figure 4.32 A). Upon exposure to glyphosate herbicide at the lowest concentration (Figure 4.32 C), the fiber exhibits initial signs of surface disruption and textural alterations compared to the control. At the highest concentration, surface irregularities and roughening escalations were observed, indicating structural damage (Figure 4.32 E).

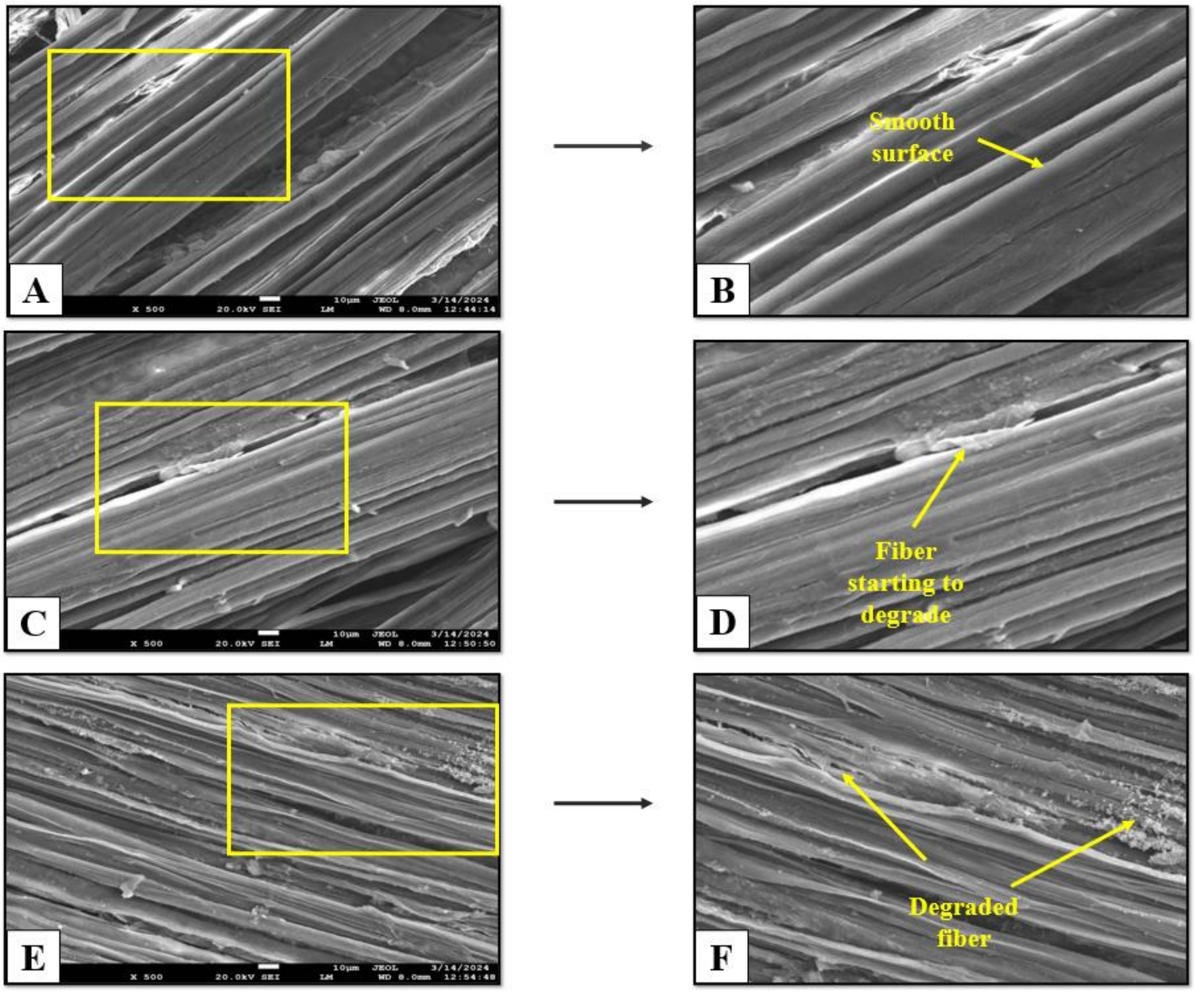


Figure 4.32: SEM image (×500) of fiber under varying glyphosate stress. A: Control, B: Zoomed in Control, C: G1, D: Zoomed in G1, E: G2, F: Zoomed in G2; (B, D, F: Manually zoomed approximately two times at the degraded spot for enhanced visualization).

In metribuzin-treated samples, the SEM image reveals extensive surface erosion and significant degradation, characterized by substantial loss of the original smooth fiber morphology (Figure 4.33 C and Figure 4.33 E). At the highest concentration, bubbling can also be seen indicating the highest form of degradation among all herbicide concentrations ultimately impacting the fiber quality.

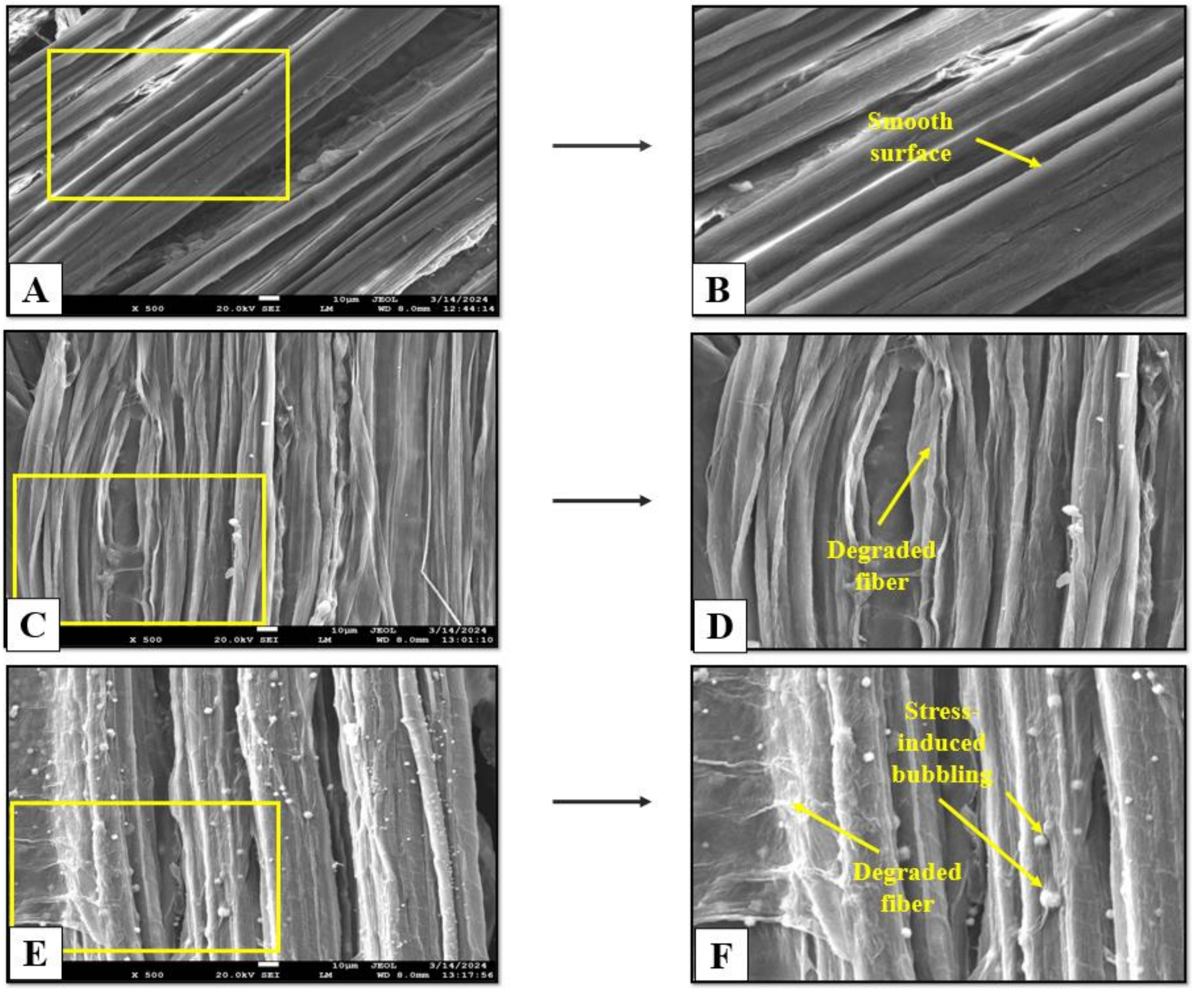


Figure 4.33: SEM image (×500) of fiber under varying metribuzin stress. A: Control, B: Zoomed in Control, C: M1, D: Zoomed in M1, E: M2, F: Zoomed in M2; (B, D, F: Manually zoomed approximately two times at the degraded spot for enhanced visualization).

Concerning the changes in the morphological features of the fiber, the SEM results indicate that while both herbicides demonstrated the capacity to degrade the fiber structure, with higher concentrations yielding more severe effects, the data suggests that metribuzin, particularly at its highest concentration, exhibited the most detrimental impact on the fiber's surface topography. The extensive erosion and degradation observed suggest a critical compromise in the fiber's structural integrity, potentially rendering it unsuitable for applications that demand robust mechanical properties and surface uniformity. A study analysed the effects of different thermal stresses on the morphology of natural fibers like palm and coir and SEM micrographs showed significant structural degradation of the fibers after thermal treatments. Also, they concluded that heat treatments led to the breakdown of external cell walls and secondary layers of internal cells, indicating that these regions are more susceptible to thermal degradation (Thomason & Rudeiros-Fernández, 2021). SEM analysis by another study revealed that excessive or improperly controlled chemical and mechanical treatments can negatively impact natural fibers by causing surface cracks, roughness, and structural degradation. Alkali treatment often leads to over-fibrillation and void formation, while benzoylation and silane treatments can result in embrittlement and brittle surface layers. Peroxide, plasma, and ozone treatments introduce oxidative damage and micro-cracks, while acetylation can make fibers overly stiff and brittle. Additionally, thermal degradation during curing or drying causes shrinkage, cracking, and scorching. These effects highlight the need for optimized treatment conditions to preserve fiber integrity while enhancing their properties (Ali et al., 2018).

Objective 4: Analysis of herbicide interaction with cytochrome P450 in treated plants.

4.19. Cytochrome P450 Content

The cytochrome P450 content was measured following treatment with increasing concentrations of glyphosate and metribuzin. The untreated control group exhibited a Cytochrome P450 content of 0.448µg/g Protein. The fold change in Cytochrome P450 content was calculated relative to the control to assess the effect of these herbicides.

Upon glyphosate exposure, there was a dose-dependent increase in Cytochrome P450 content. The lowest glyphosate treatment resulted in a 1.23-fold increase, whereas T2 and T3 exhibited 1.64-fold and 2.01-fold increases, respectively. The highest glyphosate concentration (T4) induced the most significant response, showing a 2.05-fold increase compared to the control.

Similarly, metribuzin treatment also led to a dose-dependent enhancement in Cytochrome P450 levels. The lowest metribuzin treatment (T1) resulted in a 1.07-fold increase, while T2, T3, and T4 showed 1.11-fold, 1.32-fold, and 1.35-fold increases, respectively (Figure 4.34). Notably, while both herbicides increased Cytochrome P450 content, glyphosate induced a more pronounced response compared to metribuzin at equivalent concentrations. These findings suggest that glyphosate has a stronger effect on Cytochrome P450 induction.

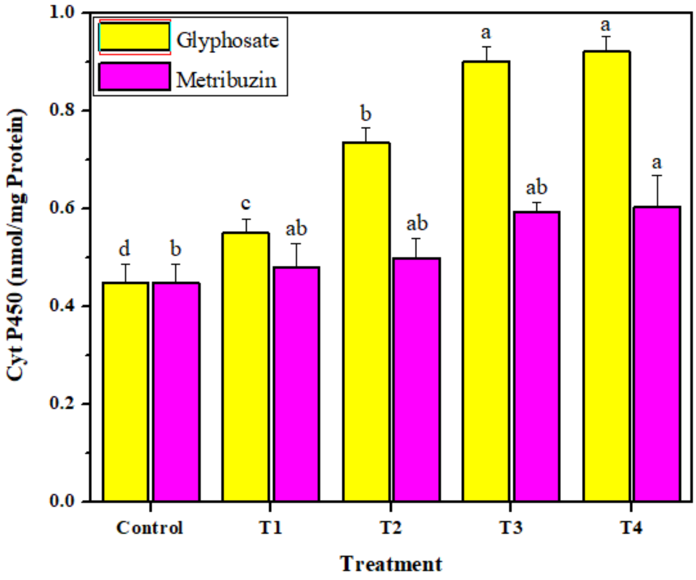


Figure 4.34: Change in the Cytochrome P450 content under glyphosate and metribuzin herbicide stress.

Cytochrome P450 enzymes play a significant role in plant responses to environmental stress by regulating hormone biosynthesis, secondary metabolism, and antioxidant activity. They are also involved in herbicide metabolism, enabling crop selectivity and weed resistance. In rice, CYP81A6 confers resistance to the ALS-inhibitor herbicide bentazon, as demonstrated by genetic transformation studies (Nelson et al., 2008). Similarly, CYP71C6v1 in wheat metabolizes various sulfonylurea herbicides, enhancing detoxification (Li et al., 2010). The allelochemical sorgoleone, synthesized via CYP71AM1 in Sorghum bicolor, inhibits weed growth, demonstrating CYPs' role in plant-plant interactions (Pan et al., 2018).

Overall, cytochrome P450 enzymes are essential for plant adaptation to environmental stress through hormone signalling, secondary metabolism, and detoxification pathways.

4.20. NADPH-P450 Reductase Assay

The reduction activity of cytochrome P450 was assessed following exposure to increasing concentrations of glyphosate and metribuzin. The untreated control group exhibited a baseline Cytochrome P450 reduction activity of 0.238.

Following glyphosate treatment, there was a progressive increase in Cytochrome P450 reduction activity. The lowest glyphosate concentration (T1) resulted in a 1.47-fold increase, while T2 and T3 demonstrated 1.67-fold and 2.00-fold increases, respectively. The highest glyphosate treatment (T4) exhibited the most significant enhancement, with a 2.07-fold increase compared to the control.

Similarly, metribuzin treatment also induced an increase in Cytochrome P450 reduction activity in a dose-dependent manner. The lowest metribuzin treatment (T1) showed a 1.33-fold increase, while T2, T3, and T4 exhibited 1.60-fold, 2.07-fold, and 2.13-fold increases, respectively (Figure 4.35). Notably, both glyphosate and metribuzin treatments enhanced the reduction activity of Cytochrome P450, with the highest levels observed at T4 treatments. These findings indicate that the induction of Cytochrome P450 reduction activity is an adaptive response, potentially aiding in herbicide detoxification and stress mitigation.

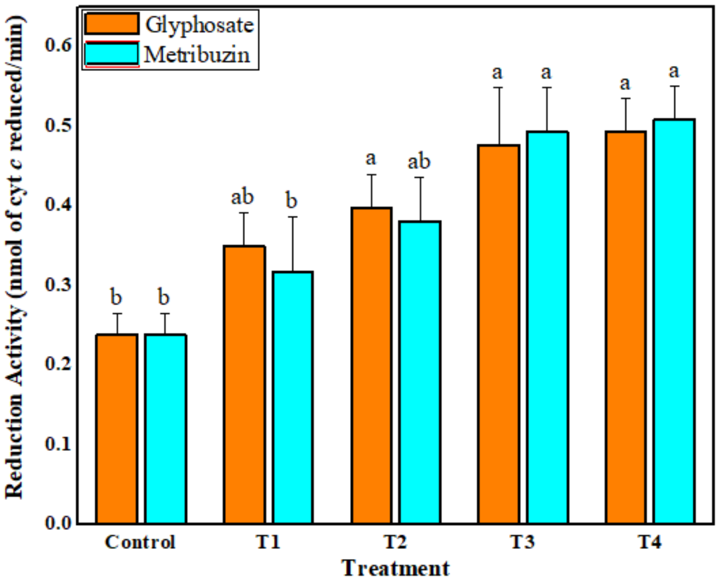


Figure 4.35: Reduction activity of Cytochrome P450 under varying concentrations of glyphosate and metribuzin herbicide.

Similar results were observed by another study where the Cytochrome P450 enzyme activity showed a significant increase of 5.9-fold in atrazine-exposed rice plants (Rong et al., 2015). When a plant is under herbicide stress, the activity of cytochrome P450 enzymes, particularly their reduction activity, often increases as a defence mechanism to metabolize and detoxify the herbicide, effectively making the plant more resistant to the herbicide's effects (Chakraborty et al., 2023).

CHAPTER 5. SUMMARY AND CONCLUSION

The current research investigated the effects of glyphosate and metribuzin herbicides on *Cannabis sativa* plants, with a specific focus on metabolite production, fiber properties, and cytochrome P450 activity. *Cannabis sativa* represents a significant agricultural crop with diverse applications spanning industrial fiber production, medicinal compounds, and emerging nutraceutical markets. The increasing agricultural importance of this plant necessitates a thorough understanding of how common herbicides affect its physiology and biochemical composition. Our study employed multiple analytical approaches, including biochemical assays, morphological examination, mechanical testing, and *in silico* molecular docking to provide a holistic understanding of cannabis responses to herbicidal stress. This research addresses critical knowledge gaps regarding herbicide impacts on specialized metabolite synthesis pathways and fiber development in this economically important plant species.

Both herbicides significantly altered the production of primary and secondary metabolites in cannabis plants. We observed substantial changes in pigment composition and sugar content, with differential responses depending on herbicide type and concentration. The plants exhibited enhanced antioxidant activities with increasing herbicide concentrations, suggesting the activation of stress response mechanisms to counteract herbicide-induced oxidative damage. This antioxidant upregulation represents a critical adaptive response that helps maintain cellular homeostasis under chemical stress conditions.

One of the most significant outcomes was the **differential impact of herbicides on cannabinoid biosynthesis**. Both herbicides induced the synthesis of cannabidivarol (CBDV) and cannabidiol (CBD), which were absent in untreated controls. However, glyphosate and metribuzin showed **contrasting effects on THC production**, mechanistically explained through molecular docking. Metribuzin directly occupied the active channel of THCA synthase, competing with CBGA, while glyphosate obstructed access by binding at the channel entrance, highlighting distinct inhibitory pathways.

In terms of **fiber properties**, our results demonstrate that **low doses of metribuzin improved fiber strength, crystallinity, and cellulose content**, likely through stress-induced cell wall fortification. Conversely, **glyphosate negatively affected fiber parameters**, particularly at higher concentrations, with SEM imaging revealing pronounced surface degradation. These findings suggest that mild metribuzin exposure could potentially enhance hemp fiber for industrial use, while glyphosate may compromise fiber integrity.

Herbicide exposure also significantly reprogrammed **primary and secondary metabolism**. Hormetic effects were evident at low concentrations, leading to increased plant biomass and metabolite accumulation. Glyphosate promoted **fatty acid synthesis**, likely via compensatory metabolic pathways, whereas metribuzin reduced it, indicating interference with lipid biosynthesis. Pigment alterations, antioxidant enzyme upregulation, and sugar fluctuations further validated the stress response.

A critical adaptive response observed was the **upregulation of Cytochrome P450 content and activity**. Glyphosate caused a 2.05-fold increase in P450 content and a 2.07-fold rise in reductase activity, compared to 1.35- and 2.13-fold, respectively, for metribuzin. These results highlight the pivotal role of the cytochrome system in **detoxification and oxidative stress mitigation**, with glyphosate triggering a more pronounced enzymatic activation.

Overall, this study underscores the **herbicide-specific modulation of biochemical pathways and structural integrity in Cannabis sativa**, with direct implications for medicinal, industrial, and agricultural practices.

In conclusion, this comprehensive analysis of glyphosate and metribuzin effects on *Cannabis sativa* highlights the multifaceted impact of herbicides on plant biochemistry and physiology. The study contributes valuable insights into plant resilience against herbicides and provides a scientific foundation for optimizing cannabis cultivation practices in the face of environmental stressors. These findings may ultimately inform the development of more targeted cultivation strategies that maximize desirable plant traits while minimizing negative herbicide impacts in modern agricultural systems.

While the present study provides significant insights, certain considerations highlight directions for future research, it was designed under controlled pot-based conditions to ensure precision in monitoring morphological and biochemical responses of *Cannabis* *sativa* to glyphosate and metribuzin stress. While this approach offered valuable clarity, it naturally cannot capture every complexity of field environments, such as variable soil properties, climatic fluctuations, or crop–weed interactions. Similarly, the focus on morphological and biochemical parameters provided a comprehensive overview, though gene-level and molecular insights were beyond the present scope. These aspects open exciting avenues for future research, where integrating molecular tools and field-scale experiments could complement the findings of this study and further strengthen the understanding of herbicide–cannabis interactions.

Future Prospectus:

The present study has laid a foundational understanding of how industrial herbicides such as glyphosate and metribuzin modulate the biochemical, morphological, and molecular characteristics of *Cannabis sativa*. These findings open multiple avenues for future exploration to advance both scientific knowledge and practical applications in cannabis agronomy, pharmacognosy, and sustainable fiber production. Future research should focus on the long-term and transgenerational effects of glyphosate and metribuzin on *Cannabis sativa*, particularly investigating epigenetic changes and inheritance of altered biochemical traits. Molecular studies should further examine the modulation of THC, CBD, and CBDV synthesis by identifying gene expression patterns and enzyme activity under herbicide stress. The differential binding behavior of glyphosate and metribuzin to THCA synthase suggests the potential to use herbicide analogs as targeted tools for regulating specific cannabinoid biosynthesis. Engineering herbicide-tolerant cannabis varieties through manipulation of cytochrome P450 genes could enhance resistance without compromising fiber or metabolite quality. Additionally, field trials should optimize sub-lethal herbicide doses to harness observed hormetic effects for improved growth and fiber strength. Evaluating safer herbicide alternatives and assessing their impact on metabolite profiles and fiber properties will be crucial. Finally, environmental impact assessments are necessary to ensure responsible herbicide use in sustainable cannabis cultivation systems.

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DETAIL OF RESEARCH PUBLICATIONS

Unravelling herbicide stress and its impact on metabolite profiling in Cannabis sativa: an investigative study

Bashir, S., Kaur, N., Vadhel, A., Verma, A. K., Girdhar, M., Malik, T., ... & Mohan, A. (2025). Unravelling herbicide stress and its impact on metabolite profiling in Cannabis sativa: an investigative study. Journal of Cannabis Research, 7(1), 40. DOI: https://doi.org/10.1186/s42238-025-00300-z

Herbicide-induced alterations in hemp fiber: A comparative analysis of strength and morphology

Bashir, S., Siddiqui, M. A., Al-Khedhairy, A. A., Girdhar, M., Malik, T., Kumar, A., & Mohan, A. (2025). Herbicide-induced alterations in hemp fiber: A comparative analysis of strength and morphology. Journal of Engineered Fibers and Fabrics. DOI: 10.1177/15589250251319321

Innovative development of nitrogen (urea) and zinc-based nanofertilizers and their applications

Vadhel, A., Bashir, S., Kumar, D., & Mohan, A. (2024, February). Innovative development of nitrogen (urea) and zinc-based nanofertilizers and their applications. In *AIP Conference Proceedings* (Vol. 2986, No. 1). AIP Publishing.

Effect of Lead Toxicity on Wild Cannabis Species of Punjab Region

Balgotra, D., Bashir, S., Vadhel, A., & Mohan, M. G. A. Effect of Lead Toxicity on Wild Cannabis Species of Punjab Region. DOI: 10.18311/ti/2023/v30i4/31022

Synergistic and non-synergistic impact of HAP-based Nano fertilizer and PGPR for improved nutrient utilization and metabolite variation in Hemp crop

Vadhel, A., Kumar, A., Bashir, S., Malik, T., & Mohan, A. (2023). Synergistic and non-synergistic impact of HAP-based nano fertilizer and PGPR for improved nutrient utilization and metabolite variation in hemp crops. Environmental Science: Nano, 10(11), 3101-3110. DOI: 10.1039/D3EN00380A

Assessment of Phytoremediation Potential of Three Weed Plant Species in Soil Contaminated with Lead and Chromium

Kaur, N., Bashir, S., Vadhel, A., Girdhar, M., Malik, T., & Mohan, A. (2023). Assessment of Phytoremediation Potential of Three Weed Plant Species in Soil Contaminated with Lead and Chromium. International Journal of Agronomy, 2023, 1-5. DOI: 10.1155/2023/2271039

Opium alkaloids, biosynthesis, pharmacology and association with cancer occurrence

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3. International conference on Sustainability: Life on Earth 2021(ICS-LOE 2021) held on 17- 18 December 2021, organized by Department of Botany and Zoology, School of Bioengineering and Biosciences, and Institute of Forest Productivity, Ranchi, Jharkhand at Lovely Professional University, Punjab.
4. International Conference on Recent Advances in Biotechnology (icRAB-2022) held on December 02-04, 2022, organized by Dr B R Ambedkar National Institute of Technology, Jalandhar, Punjab.
5. International Conference on Plant Physiology and Biotechnology (ICPPB-2023) held on April 20-21, 2023 organized by School of Bioengineering and Biosciences, at Lovely Professional University, Punjab.

Workshops:

1. Workshop on 'Idea, Research, Innovation, Intellectual Property Rights and Technology Transfer' held at Sardar Swaran Singh National Institute of Bio-Energy, Kapurthala, Punjab on 30 April, 2024.