Agrotechnological Strategies with Nanofertilizer Application to Enhance Secondary Metabolites Formation in *Cannabis* Species

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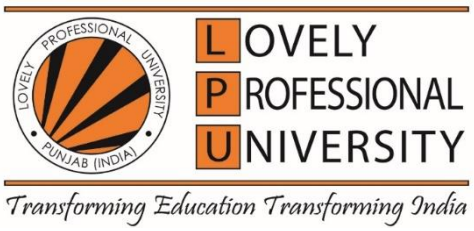

LOVELY PROFESSIONAL UNIVERSITY, PUNJAB

2023

DECLARATION

I, hereby declared that the presented work in the thesis entitled "**Agrotechnological strategies with Nanofertilizer application to enhance secondary metabolites formation in *Cannabis* species"** in fulfilment of degree of **Doctor of Philosophy (Ph.D.)** is outcome of research work carried out by me under the supervision Dr. Anand Mohan, working as Associate Professor, in the Department of Botany, 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.

(Signature of Scholar)

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CERTIFICATE

This is to certify that the work reported in the Ph. D. thesis entitled "Agrotechnological strategies with Nanofertilizer application to enhance secondary metabolites formation in *Cannabis* species" submitted in fulfillment of the requirement for the reward of degree of **Doctor of Philosophy (Ph.D.)** in the Department of Botany, School of Bioengineering and Biosciences, is a research work carried out Agrataben Vadhel, Registration No.11816263, 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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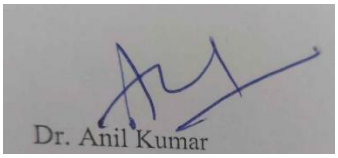

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ABSTRACT

Plant growth and health mainly depend on appropriate nutrients and suitable nutrient composition in the soil. Inadequate nutrient availability in soil systems negatively affects the overall development and growth of plant. Intensive cultivation and loss of essential nutrients result in the decrease in soil richness in agricultural systems. Agricultural systems usually require high chemical fertilizers to rejuvenate the nutrient pool in the soil and increase crop production. Urea is an effective chemical fertilizer and widely applied in soil globally due to its high nitrogen content and cost-effectiveness. However, urea fertilizer has some drawbacks, such as excess nitrogen leaching deep into groundwater leading to eutrophication and causing environmental pollution. The continuous use of chemical fertilizers deleteriously affects soil fertility and soil microflora. Similarly, Various types of conventional phosphorus fertilizers are available in the market but plant uptake phosphorus ions. The excessive use of phosphorus (P) fertilizers leads to environmental risks such as ground and surface water contamination, accumulation of toxic elements in the soil and soil fertility depletion. Nanofertilizer, a recent agricultural development, offer increased nutrient use efficiency by using nanoparticles to deliver nutrients slowly and steadily over a long period. Nanofertilizer are utilized when soil requires nutrients, as these nanostructured formulations effectively supply nutrients to plants.

Plants uptake nitrogen from urea with the help of soil microbes. Diverse microbial communities live in association with plants. It converts nitrogen in urea molecules into ammonium (NH_4^+) and then into nitrate (NO_3^-) ions and insoluble phosphorus into available phosphorus ion form to avail the plant, which increases the accessibility of vital macronutrients and influences plant growth. Various researches suggest that nitrogen and phosphorus supply affects the metabolic content such as cannabinoid and terpenoid profiles of *Cannabis sativa* L. *Cannabis sativa* L. also called “hemp” or “marijuana”, is a multipurpose and oldest plant that has been utilized for diverse purposes throughout history. Hemp is gaining more recognition in industries for producing textiles, rope, paper and other materials. The concentration of psychoactive compound delta-9-tetrahydrocannabinol (Δ^9THC) is the main distinguishing factor between hemp and marijuana. The cultivation of hemp in India is legal, but the plant must have a low THC (tetrahydrocannabinol) value. The Indian government regulates hemp cultivation through the Narcotic Drugs and Psychotropic Substances Act of 1985. *Cannabis sativa* L. grows easily and abundantly along roadsides in North India, including states like Punjab, Haryana and Himachal Pradesh. Despite this, limited research has been conducted to see if it could be a suitable crop for hemp biomass and its metabolic content. Irrespective of its potential uses and benefits, it is often considered a weed in north India due to its prolific growth and ability to conquer environmental stress.

This study delves into the synthesis and characterization of an environmentally friendly urea Nanofertilizer, aiming to revolutionize agricultural practices and enhance plant growth while mitigating environmental impacts. With a focus on improving soil fertility and promoting sustainable crop production, the research

investigates the efficacy of urea hydroxyapatite nanofertilizer (UHAPF) alongside Plant Growth Promoting Rhizobacteria (PGPR) in optimizing the bioactive compounds of wild Cannabis plants.

The primary objective of this research is to synthesize a novel urea-based nanofertilizer with enhanced nutrient delivery capabilities. To achieve this, a multi-step synthesis process is employed, beginning with the preparation of urea hydroxyapatite nanoparticles. The synthesis process is meticulously characterized using advanced analytical techniques. Following the synthesis and characterization of UHAPF, the study proceeds to investigate its release behavior and nutrient delivery kinetics. By monitoring the release of nitrogen from the nanofertilizer over time, aim to assess its efficiency in supplying essential nutrients to plants. The results demonstrate the controlled and sustained release of nutrients, indicating the potential of UHAPF to enhance soil fertility and promote healthy plant growth. In addition to assessing the efficacy of UHAPF, this study explores the synergistic effects of nanofertilizer and PGPR on the metabolite formation of Cannabis plants. Leveraging the beneficial properties of PGPR, such as biological nitrogen fixation and phosphate solubilization, our aim to maximize the bioavailability of nutrients and stimulate plant growth. The experimental setup involves growing Cannabis plants under various treatment conditions, including UHAPF alone, PGPR alone, and a combination of both. Growth parameters such as plant height, biomass accumulation, and leaf morphology are meticulously monitored throughout the experimental period. Furthermore, the impact of nanofertilizer and PGPR treatments on the metabolite profile of Cannabis plants is evaluated using Gas Chromatography-Mass Spectrometry (GC-MS). By analyzing the composition and abundance of bioactive compounds, including cannabinoids and terpenoids. The results reveal significant variations in metabolite profiles among different treatment groups, suggesting the influence of nanofertilizer and PGPR on the biosynthesis pathways of Cannabis metabolites.

Overall, our study contributes to the growing body of knowledge on sustainable agriculture and plant biotechnology by demonstrating the potential of urea nanofertilizer and PGPR in optimizing plant growth and metabolite formation. By harnessing the power of nanotechnology and microbial biotechnology, our aim to address the challenges of modern agriculture while promoting environmental sustainability and crop resilience. The findings of this research cover the way for future advancements in precision agriculture and tailored nutrient management strategies, ultimately leading to enhanced crop productivity and food security in a rapidly changing world.

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Abbreviations	Descriptions
NPK	Nitrogen-phosphorus-potassium
NH_3	Ammonia
NH_4^+	Ammonium ion
NO_2^-	Nitrite ion
NO_3^-	Nitrate ion
CO_2	Carbon dioxide
TCP	Tri Calcium Phosphate
HPO_4^{2-}	Orthophosphate
NUE	Nitrogen use efficiency
HAP	Hydroxyapatite
PSM	Phosphate Solubilizing Microorganisms
PGPB	Plant growth-promoting bacteria
PGPR	Plant growth-promoting rhizobacteria
THC	Tetrahydrocannabinol
Δ^9THC	Delta-9-tetrahydrocannabinol
CBG	Cannabigerol
CBC	Cannabichromene
CBDV	Cannabidivarine
HPLC	High-Performance Liquid Chromatography
UHAPF	Urea hydroxyapatite Nanofertilizer

CBDA	Cannabidiolic acid
DSC	Differential scanning calorimetry
SRFs	Slow release fertilizers
CRFs	Controlled release fertilizers
FT-IR	Fourier transforms infrared spectra analysis
PXRD	Powder X-Ray diffraction
FAO	Food and Agriculture Organization
ACC deaminase	(1-aminocy clopropane-1-carboxylic acid) deaminase
ROS	Reactive oxygen species

CHAPTER 1. INTRODUCTION

Soil serves as natural habitat for plant growth and is a mixture of elements and substances like, living organisms, minerals humus, etc. It makes the base which holds the plant root and delivers the nutrients and minerals. According to the criteria for essentiality for nutrition, the plant life cycle cannot be complete without essential nutrients required for metabolic activities (Arnon & Stout, 1939). Plants absorb nutrients mainly from the soil. The primary element such as nitrogen, phosphorus and potassium play crucial roles in plant development.

1.1 Essential Nutrients for Soil and Plants: Nitrogen and Phosphorus Explained

Nitrogen is the primary and essential macronutrient needed by the plant. It is part of a protein constituent and is fundamental to photosynthesis and an important element for plant growth in all plant cells, proteins, chlorophyll, etc. Also, it contributes to chemical component production to protect the plant from parasites and diseases and regulate plant growth processes. It also increases biomass, crop yield, agricultural product and food values (Sainju et al., 2020). The atmosphere contains an abundant amount of nitrogen (78%). However, Plants cannot utilize nitrogen present in the environment. The atmospheric nitrogen converts into nitrite (NO_2^-) and nitrate (NO_3^-) with the help of microorganisms known as nitrogen-fixing bacteria, including *Azotobactor, Bacillus, Clostridium, Nostoc, Rhizobium* and *Anabena* (Bhattacharjee et al., 2008), (Orr et al., 2011). The nitrogen circulation in the environment involves nitrogen fixation, nitrogen assimilation, nitrification, ammonification and denitrification.

Phosphorus is the second most crucial macronutrient after nitrogen. It plays an important function in plant metabolisms such as photosynthesis, cell division, development, formation of the nucleus, breakdown of metabolites and metabolic pathways regulation (Alori et al., 2017a), (Rafique et al., 2017). Phosphates can be categorized into three groups based on their solubility: soluble orthophosphate, insoluble inorganic and organic phosphate. Among these groups, orthophosphate (HPO_4^{2-} or $H_2PO_4^-$) refers to the form of phosphate that is readily soluble in water and available for plant uptake and utilization. However, because it reacts with many soil components, it becomes immobile and difficult for plants to take up. Usually, soil contains a small amount of available phosphorus.

There are a wide range of chemical fertilizer sources is accessible for commercial crop production. The characteristics inherent to each type of fertilizer determined whether its use is beneficial or detrimental to a farmer. The majority of commercial fertilizers fall under the category of water-soluble quick-release fertilizers, and become readily available to plants when placed in soil. Applying synthetic fertilizers contributes to global food security by substantially increasing crop yields, estimated at approximately 50% (Ibrahim et al., 2014).

Many types of nitrogenous fertilizers are available in the market, such as urea, calcium ammonium nitrate, ammonium nitrate, urea-ammonium nitrate, ammonium sulphate, etc. Urea ($CO(NH_2)_2$) is a widely applied fertilizer due to its high amount of nitrogen content, cost effectiveness and easy application and it is the primary plant nutrition source in agriculture. It is a solid and stable nitrogen-based granular fertilizer which provides a rapid and efficient application of nitrogen. Its nitrogen content is taken up by plants as nitrate (NO_3^-) and ammonium (NH_4^+) ions (Hirel et al.,2011).

Today, the nitrogen use efficiency (NUE) is only 30%; if a grower applies 10kg of nitrogen fertilizer to land, only 3kg of 10kg is utilized by crops and the rest of 70% is lost. By using fertilizers, growers fulfil nitrogen deficiency in the form of urea. In soil, urea easily dissolves in moist soil, and the urease enzyme transforms urea-nitrogen into ammonium ions (NH_4^+) through hydrolysis. Subsequently, ammonium ions (NH_4^+) convert into nitrite (NO_2^-) ions through the nitrification process, which is further catalysed by enzymes, followed by the nitrite (NO_2^-) to nitrate (NO_3^-) oxidation. However, plant uptake of nitrogen in urea is limited, ranging from 25% to 50% (Witte, 2011).

The rate of nitrogen conversion is depending on several factors including pH, soil temperature, soil moisture, presence of nitrifying bacteria and oxygen availability. During this process, the pH around urea granules tends to increase significantly, leading to the volatilization of ammonia, which is subsequently lost in the atmosphere. Rapid uptake of nitrate by plants is facilitated by the high mobility of nitrate particles in many plant species. As a result, plants generally exhibit a preference for nitrate over ammonium. Nitrogen is a mobile nutrient and taken up by plants through specialized membrane channels, allowing them to uptake both nitrate and ammonium forms (Krapp, 2015).

Natural phosphorus fertilizers such as rock phosphate, apatite, hydroxyapatite and oxy apatite are mineral phosphorus fertilizers in the soil. Soil microbes solubilizing the insoluble phosphorus in soil allows it to become accessible to plants. Plant roots absorb orthophosphate, the form of phosphorus that plants can take from the soil's water through root cells (Qureshi et al., 2012). Variety of phosphorus fertilizers are available in the market, such as Superphosphate, triple superphosphate, diammonium phosphate, mono ammonium phosphate etc. (Chien et al., 2016). Microbes play an important role in the global distribution and transformation of both insoluble inorganic and organic phosphate forms. These microorganisms possess the ability to solubilize insoluble phosphorus sources, such as insoluble organic and inorganic compounds commonly referred to phosphate-solubilizing microorganisms (PSM) (Baliah, 2018). Numerous bacterial strains, fungi and actinobacteria have been studied due to their capacity to solubilize phosphate. Bacterial strains such as *Pseudomonas, Bacillus, Burkholderia* utilizes organic acids and siderophores to solubilize phosphate in soil environments and highlights their potential in promoting nutrient availability and supporting plant growth in agricultural systems.

1.2 Microbial Role in Nutrient Availability and Uptake

Plants have a very close relationship with microorganisms. Diverse microbial communities live in association with plants. The efficient strains of microorganisms can enhance soil fertility by converting atmospheric nitrogen into nitrate ion form and insoluble phosphorus into available phosphorus ion form, which increases the availability of vital nutrients thus influence plant growth (Prakash et al., 2015), (Chen & Liu, 2019).

Efficient strains of microorganisms, also called Plant growth-promoting rhizobacteria (PGPR), have a favourable effect on plant growth (Zafar-ul-Hye et al., 2019). PGPR provides nutrients to the plant by fixing nitrogen, phosphorus and solubilizing mineral and producing phytohormones such as cytokinin, indole acetic acid and Gibberellins (Mhatre et al., 2019). It provides protection to the plants against diseases (Biotic stresses) by producing siderophore, HCN, enzymes and antibiotics (Islam et al., 2014). In addition, it also protects the plant from drought salinity & other abiotic stresses by modulating the level of plant stress markers like peroxidase, superoxide dismutase, L-proline, Polyphenol oxidase, etc., as well as producing ACC (1-aminocy clopropane-1-carboxylic acid) deaminase-containing deaminase (Barnawal et al., 2017). These microorganisms occur naturally, increase crop productivity and improve soil structure. Usually, growers apply these microorganisms as a biofertilizer to enhance soil fertility (Piromyou et al., 2011).

1.2.1 Exploring the Potential Applications of Plant Growth Promoting Strains

Bacillus megaterium is a gram-positive, rod-shaped, spore-forming and free-living bacteria. It multiplies in soil and secretes some metabolites such as organic acid and enzymes. *Bacillus megaterium* fixes nitrogen through the nitrogenase enzyme, which catalyses nitrogen present in soil into ammonium (NH_4^+) or nitrate (NO_3^-) (Liu et al., 2006). Moreover, it solubilizes fixed phosphorus to available phosphorus by producing a range of enzymes, phosphatase, and acid phosphate (do Carmo et al., 2019). It also makes siderophores that can chelate with metal ions that help to solubilize phosphorus. It is also a good plant growth-promoting rhizobacteria enabled by good bio-stimulant activity and good root and shoot development (Liu et al., 2020). It is an important microorganism for soil fertility that contributes to the nitrogen and phosphorus cycle and is considered a model organism for genetic and biochemical studies.

On the other hand, *Pseudomonas aeruginosa* is a gram-negative and rod-shaped bacterium that can grow in harsh conditions such as high salinity. It is known for the nitrogen cycle, but it can solubilize insoluble phosphorus by phosphatase and acid phosphate and contribute to the phosphorus cycle. Some strains of *Pseudomonas aeruginosa* are used to produce an enzyme, antibiotics, and other by-products (Wang et al., 2010). In addition, it is also used to bioremediate pollutants in the environment. It is also a model organism to study bacterial physiology, molecular biology, and biotechnology.

1.3 The Hidden Costs of Dependency on Chemical Fertilizers

The exponential population growth necessarily requires a subsequent proportional increase in food production. Growers are mainly depending on synthetic or chemical fertilizers for crop production. For crop production and soil improvement, fertilizer is an important component in modern agriculture and an essential global commodity (Hernandez & Torero, 2013). Chemical fertilizers are significant in increasing crop yields and sustaining global food production. The Food and Agriculture Organization (FAO) has stated that chemical fertilizers have been the primary factor in enhancing agricultural productivity worldwide over the last 30 years. Over the past 60 years, fertilizers have significantly increased global food production (Li et al., 2013). Global agricultural food production observed a significant increase, reaching double its previous levels by the 1990s. This remarkable growth can be partially attributed, in part, to 6.9 time upsurge in nitrogen fertilization and a 3.5 times in phosphorus fertilization (Rahman & Zhang, 2018). These substantial increases in the application of nitrogen and phosphorus fertilizers played a pivotal role in enhancing crop productivity and meet the increasing global demand for food (Rafie & Raj-Kumar, 2020).

Urea is an extensively used chemical fertilizer, although it is inherently limited because of high solubility, low thermal stability and low molecular weight. Since 2002, the production and utilization of urea fertilizer have steadily increased **(Figure 1.1)** (*FAO*) (Heffer & Prud'homme, 2016). The production of urea fertilizer worldwide was reported to be nearly 70 million metric tons year^{-1} in 2006 (Glibert et al., 2006). However, as of 2020, this production has increased significantly to over 187 million metric tons. According to FAO data, the total urea fertilizer production in India was 24,603,100 tonnes and the total urea used in agriculture was 35,042,500 tonnes in 2020.

The extensive use of chemical fertilizers leads to irreversible harm to the soil structure, mineral cycles and soil microbial flora, plants and other related aspects (Fu et al., 2023). Fertilizers are released faster than plants can absorb them, leading to wastage and environmental pollution. Approximately 50% of the applied urea fertilizer is lost through leaching with water due to low efficiency of nutrient utilization by crops (Davis et al., 2016).

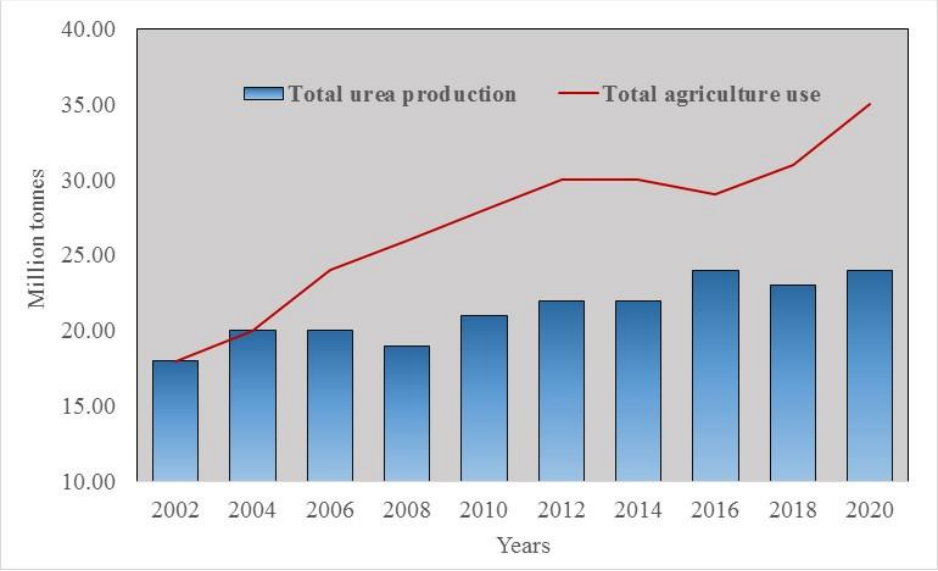


Figure 1.1 Urea Production and Application in Indian Agriculture (2000-2021) [Food and Agriculture Organisation of the United Nations. *FAO*]

The lower nitrogen use efficiency causes heavy fertilizer loss into water bodies and causes water and soil contamination. Some of the nitrogen-based fertilizers undergoes conversion into different inorganic ammonia forms, while some amount of fertilizer is lost through leaching, as it percolates down the soil and mixes with groundwater (da Costa et al., 2019). Additionally, some nitrogen compounds dissolve in surface water, leading to contamination of rivers, lakes and streams ultimately creating a major problem of present world **(Figure 1.2)**. Similarly, excessive use of phosphorus fertilizers leads to environmental hazards such as ground and surface water pollution, toxic elements accumulation like a high concentration of heavy metal in the soil and soil fertility depletion (Alori et al., 2017b).

The NUE from conventional fertilizers has plateaued and is alarmingly declining (Lateef et al., 2016). The average NUE of N, P, and K has dropped below 30-35%, 18-20%, and 35-40% respectively, over the years (Rai et al., 2015)(Burger & Venterea, 2011). Over-fertilization increases residual nitrate concentration in the soil. Leaching loss into the environment create many problems, such as eutrophication and NO_3^- deterioration (Ma et al., 2021). The use of synthetic nitrogen fertilizers in agriculture causes the release of greenhouse gases. A study found that in 2018, these fertilizers were responsible for emitting 1.13 billion tonnes of CO_2 equivalents globally, which is 2.1% of all greenhouse gas emissions. The production of fertilizers caused the highest emissions, followed by emissions from their use in fields and transportation. The top four emitters were China, India, USA, and EU28 (Menegat et al., 2022). Fertilizers generate water contamination by nitrates (NO_3^-) cause High levels of NO_3^- in drinking water and harm human health, as it can cause blue-baby syndrome (particularly in infants) and various diseases such as cancer, diabetes and liver damage.

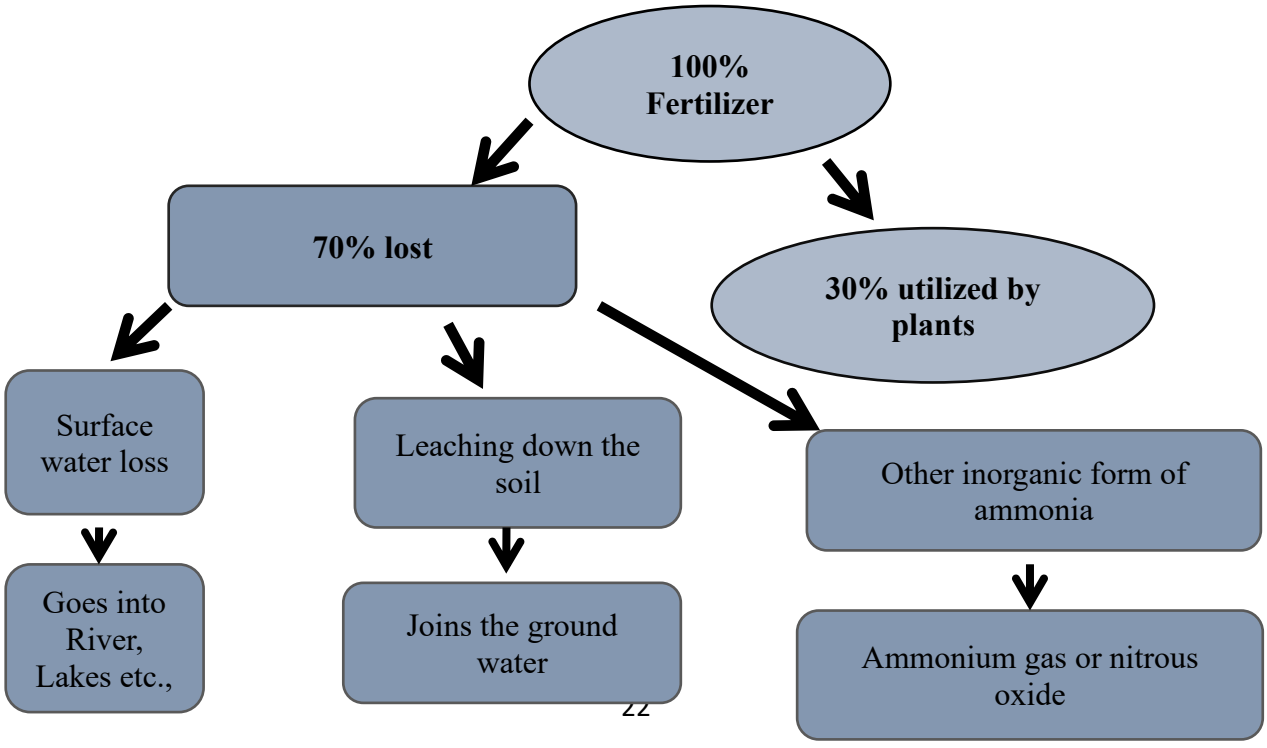


Figure 1.2 - Effect of Nitrogen Fertilizer Use on Water and Soil Contamination

Over the past few decades, the excessive use of agrochemicals has caused severe soil quality degradation, significant economic losses and is a primary environmental concern, including a severe decline in microbial, aquatic and insect populations (Good & Beatty, 2011), (Rahman & Zhang, 2018). Despite the adverse health effects, fertilizers are a fundamental input in agricultural production. Several strategies have been implemented to reduce the environmental impact of nitrogen fertilization, maintaining high crop yields and ensuring agroecosystem sustainability; such as improving nitrogen fertilizers, adopting precision agricultural techniques and applying sustainable agriculture practices (Torri, 2017). Developing slow-release fertilizers or controlled released fertilizers is a solution to this problem. It can improve fertilizer utilization efficiency and reduce the detrimental effects of excess nutrients on the environment and human health.

1.4 Role of Nanotechnology in Mitigating Environmental Challenges

The current utilization of nanotechnology in the agriculture and food industries involves developing and implementing various nanoscale materials and tools for multiple applications (Baker et al., 2017). One of the crucial applications of nanotechnology in agriculture is to enhance nutrient utilization efficiency and protect crops from pests (Vishwakarma et al., 2018), (Duhan et al., 2017). Various nanoscale tools, such as nanofertilizer, nano sensors and nano pesticides have demonstrated significant potential for promoting agricultural practices (Chhipa, 2019). In the field of agriculture, nanotechnologies are employed to enhance various aspects of crop production and protection. This encompasses the application of nanofertilizer, nano pesticides, nano growth regulators, nano-herbicides, as well as their smart delivery systems such as encapsulation. Additionally, nano sensors are utilized to monitor and optimize agricultural processes. Furthermore, nanotechnology is applied to develop Nano-based biofuels (He et al., 2019). These nano functionalized agents possess unique properties that contribute to increasing crop productivity and providing effective crop protection. In plants, nanoparticles also affect the bioactive compound concentration by modulating gene expression, reactive oxygen species and signalling pathways (Selvakesavan et al., 2023).

One potential application in agriculture involves the development of a fertilizer composed of nano-sized nitrogen molecules encapsulated within a polymer coating with a biosensor. This innovative method holds promising prospects; when soil requires the use of nanofertilizer, it delivers essential nutrients efficiently to the plants. The combination of nano composite nitrogen molecules, polymer coating, and biosensor technology offers the potential for improved nutrient uptake and enhanced plant growth. Nanofertilizer, a recent agricultural development, offers increased nutrient use efficiency due to their nanoscale size (Calabi-Floody et al., 2018). Studies have revealed that nanomaterials enhance root and shoot growth, plant biomass,

photosynthesis rate and seed germination at specific concentrations. The nanofertilizer concept raised interest in nano enabled and nanoscale bulk fertilizer (Maghsoodi et al., 2019).

Nanofertilizer is prepared by binding nutrients alone or in combination with adsorbents containing Nano-sized particles. It improves nutrient use efficiency without any detrimental effect (Manikandan & Subramanian, 2013). These fertilizers can be made using physical or chemical approaches and carry cationic and anionic nutrients. The carrier material utilized for investigating the nanostructure fertilizer can be classified into six categories: nano clays, hydroxyapatite nanoparticles (HAP), carbon-based nanomaterials, polymeric nanoparticles, mesoporous silica, and other nanoparticles (Guo et al., 2018). Chandrasekaran et al. (2020) stated that the uptake of nano formulation promotes the free radicle production through various metabolic pathways. It also enhances the activation of gibberellins and facilitates the movement of stored proteins.

The utilization of nano-sized nitrogen particle with a polymer coating can be utilized to effectively manage soil nutrition balance. The application of a nano-coating offers multiple advantages, including cost reduction, improved crop productivity, enhanced moisture retention, promotion of soil aggregation and increased carbon build-up. Moreover, the use of nano-sized nitrogen particles can significantly increase the crop productivity per hectare as compared to organic fertilizers, thus making it a profitable choice for growers. Nano functionalized composite - an alternative to urea, has the potential to reduce urea requirements by up to 50 percent. Nanofertilizers are more beneficial than chemical fertilizers because they increase nutrient use efficiency by three times and achieve ten times more stress-tolerance by the crops (Kottegoda et al., 2015).

1.4.1 Nanofertilizers: A Revolutionary Approach to Boost Agricultural Production

With increasing concerns about food security and conventional fertilizer limitations, researchers have revolutionized nanotechnology-based fertilizers or nanofertilizers. The aim of synthesizing nanofertilizer is to improve nutrient utilization efficiency, raise agricultural yield and reduce environmental impact on agriculture. The application of nanofertilizer should be considered only when it can be ensured that it does not have any negative environmental impact. If the nanostructure used to make nanofertilizer, decreases long time fertility, harms the soil and increases soil erosion, then it should be used carefully. Hence, nanofertilizer can be considered with the advantage of "High yield by protecting environment". The ideal release system should be able to release nutrients as needed while protecting them from premature degradation. This is particularly important for nutrients like urea, which can easily break down in the soil. The perfect nanofertilizer should also help reduce the pollution of soil, water reservoirs and food products. It should also mitigate soil compaction, quality deterioration and reduce plant stress. These factors are crucial in developing efficient and sustainable nanofertilizer that can contribute positively to agricultural productivity and environmental conservation (Wanyika et al., 2012). Guo et al. (2018) implemented the key factors for evaluating the practical

viability of nanofertilizer in agriculture **(Figure 1.3)**. Among all the nanocarriers or coating-material, hydroxyapatite is the most suitable material to synthesize nanofertilizers as it is environment friendly as well as rich source of phosphorus.

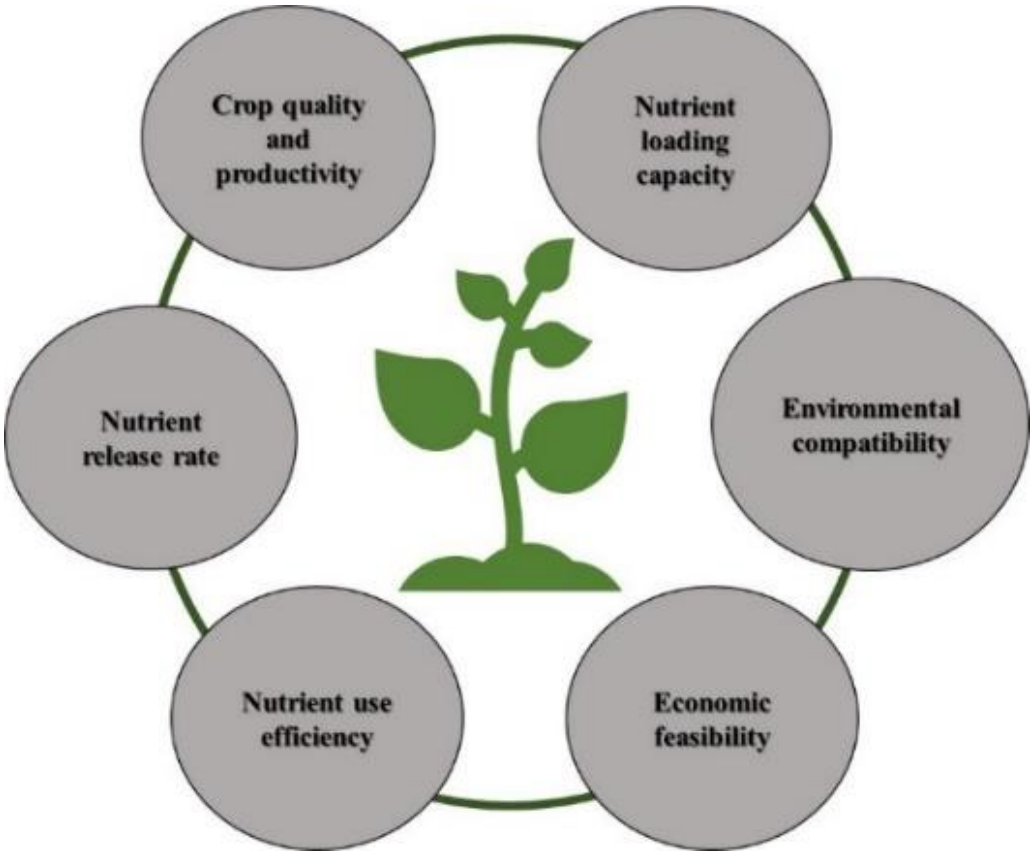


Figure 1.3 Key Factors for Assessing the Practical Viability of Nanofertilizer in Agriculture

1.4.2 Hydroxyapatite Nanoparticle as a Dual Solution for Eco friendly Coating and Phosphorus Enrichment

Hydroxyapatite (HAP) nanoparticles can be used as controlled-release fertilizers (CRFs) or coating material for providing nutrients to crops. However, the limited solubility of this compound in soils, as well as their tendency to form agglomerates, limit their mobility and uptake by plant. HAP nanoparticles can be modified to improve their bioavailability by coating their surfaces with soil-friendly and nontoxic materials, such as carboxymethyl cellulose, citric acid or urea (Yoon et al., 2020). Hydroxyapatite nanoparticles have the potential to be used as nanofertilizer. Marchiol et al. (2019) studied the effects of hydroxyapatite solutions stabilized with carboxy methyl cellulose assessed on *Solanum lycopersicum* L. The study found that increasing concentrations of hydroxyapatite nanoparticles did not affect germination percentage but strongly stimulated root elongation, with no phytotoxic effects observed in tomato plants grown in hydroponics. This suggests that hydroxyapatite nanoparticles is a safe and effective carrier of nutrients and could be used as a phosphorus supplier in agriculture. Also, it is a slow-release form of nutrient and provides phosphorus to the plant over a

more extended period. Usually, it is used in a soil amendment to improve soil fertility, structure and plant growth; in addition to reduce soil acidity.

1.5 *Cannabis sativa* L.: Exploring its Botanical, Chemical and Medicinal Properties

The current taxonomic classification of the *Cannabis sativa* L. species 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 sativa L. is an annual herbaceous plant. It is native to Central Asia, belongs to the Cannabaceae family and can be grown up to 5m tall in the wild (Farag & Kayser, 2017). It is characterized by long and narrow leaves with serrated leaf shapes, tall and thin stature and a small green flower cluster at the top of the plant (Chandra et al.,2017). The leaves are alternatively arranged on the stem and have distinct vein patterns with a pointed tip. The leaves comprise a central vein surrounded by smaller veins covered with outer epidermal cells. However, the stem is composed of a cylindrical and hollow central pith surrounded by xylem and phloem tissues, respectively, covered with outer epidermal cells ("Recomm. Methods Identif. Anal. *Cannabis* Prod.," 2013).

Cannabis sativa L. is a versatile plant with various bioactive compounds, including terpenoids, cannabinoids, flavonoids, and other phytochemicals (Andre et al., 2016). There are more than 100 different types of Phyto cannabinoids produced by the trichomes present in the inflorescences of *Cannabis* (Appendino et al., 2011). The most well-known cannabinoid in hemp is cannabidiol (CBD), which has non-psychoactive properties and diverse therapeutic effects, such as reducing anxiety and inflammation (Rehman et al., 2021). Other cannabinoids in hemp include cannabigerol (CBG), cannabichromene (CBC) and tetrahydrocannabinol (THC), which are known to be psychoactive. Terpenes such as myrcene, pinene, and limonene found in hemp are responsible for the plant's aroma and have therapeutic effects, including reducing inflammation and promoting relaxation (Zhou et al., 2019). Flavonoids in hemp have antioxidant and anti-inflammatory properties (Calzolari et al., 2017).

Additionally, hemp seeds are abundant in essential fatty acids, amino acids, minerals and vitamins, making them a great source of nutrition (Ascrizzi et al., 2019). However, the composition of secondary metabolite in

hemp can change depending on the strain and growing environments, with some strains containing higher levels of certain compounds such as CBD and others containing more THC (Liu et al., 2015). Different studies suggested that various agronomic practices can affect the production of pharmacologically helpful plant compounds, which may lead to improved health benefits. However, research suggests that these compounds synergistically produce a more significant effect than any compound alone.

1.5.1 Understanding the Overlapping Features and Contrasting Aspects of Hemp and Marijuana

Cannabis sativa L. is recognised as “Marijuana” and “Hemp”. Both “Marijuana” and “Hemp” varieties are a member of the *Cannabis sativa* L. species and are not similar with a taxonomic or evolutionary categorization of the species but rather than it indicates the variances in the phytochemical profile of the plant. Recently, *Cannabis* species have gained attention globally due to their extensive use in textiles, pharmaceutical and food industries. It is one of the valuable biomass crops in the textile industry due to its high tensile strength fibres (De Vos et al., 2023). It possesses low narcotic values due to very low tetrahydrocannabinol (THC) content (less than 0.3%) (ElSohly et al., 2017). Conversely, marijuana has a high THC content and is mainly used for recreational and medicinal purposes. Due to the THC content variation, hemp is legal in various countries, whereas marijuana remains illegal (Zuardi, 2006).

1.5.2 The Economic Advantages and Opportunities in *Cannabis* Cultivation

Hemp is a sustainable crop that grows quickly and can be grown locally, making it a viable alternative to synthetic fibres and cotton. It also has many benefits in terms of cultivation. The stiffness and roughness of hemp fabric, once a drawback, have been improved through advancements in fibre treatment and spinning techniques. This has made hemp fabric's attractive quality similar to cotton (Schumacher et al, 2020). Furthermore, the by-products of the hemp plant, such as the shives, also have multiple applications. For instance, it can be used to create insulation panels eco-friendly construction purposes, animal bedding in stables and for production of bioenergy (Rehman et al., 2021). The versatility of hemp by-products supports its value in various industries and promotes sustainable resource utilization.

Hemp has been grown in India for centuries and is known for multiple uses, including the production of fibres for textiles, paper, and building materials and its medicinal properties. It is mostly cultivated in the northern states of Uttar Pradesh, Uttarakhand and Himachal Pradesh. The Indian government regulates hemp cultivation through the Narcotics Act of 1985, classifying it as a "narcotic drug" and requiring farmers to obtain licenses to grow it. The Indian hemp market is driven by the increasing demand for its fibre, which produces textiles, cordage, paper and building materials. Industrial hemp has a low THC level (less than 0.3%) and it is used for various industrial purposes.

Although hemp cultivation has potential benefits, it still faces challenges in India, including difficulties in obtaining licenses to grow hemp due to government regulations and a lack of infrastructure and processing

facilities that limit the industry's growth (Malabadi & Chalannavar, 2023). Recently there has been revived interest in hemp cultivation in India as a sustainable alternative to traditional crops and the Indian government has started to relax regulations on hemp cultivation, raising hope for the growth of the industry in the future. Despite this, more research and development are needed, as well as the building of infrastructure and processing facilities to support the industry's growth.

1.5.3. The Potential of Hemp for Soil Remediation and Environmental Sustainability

As a fibre plant, hemp produces bioenergy, timber fibre, pulp and fodder. Hemp is a multipurpose crop that has gained renewed interest, and nitrogen significantly impacts the plant's ability to produce various valuable compounds, including cannabinoids, terpenes and flavonoids (Landi et al., 2019). Industrial hemp is a source of biomass for energy production. Hemp's short growing cycle reduces the need for pesticides, its low maintenance requirements makes it a promising energy crop (Kołodziej et al., 2023). *Cannabis* also has phyto attenuation potential and can remove pollutants from the soil and water. Studies have shown that hemp can effectively reduce the levels of heavy metals, polycyclic aromatic hydrocarbons and other organic compounds in contaminated soil (Mohan et al., 2015). Moreover, the researchers suggest that the lignocellulosic hemp biomass harvested from contaminated sites can be transformed into bioethanol through safe distillation processes (Premjet, 2019).

1.6 Significance and Identification of Secondary Metabolites in Plants

Secondary metabolites derived from plants are of great significance to human beings as they serve as important sources of food and medicine. In both developing and industrialized countries, ensuring the quality, safety, and efficacy of medicinal plants and drugs has become a crucial concern. For centuries, plants have been employed for preservation, flavour enhancement, treatment, and prevention of various ailments. The biological properties of plants are mainly associated with secondary metabolites synthesized through secondary metabolism, which have been globally utilized for diverse purposes, particularly in the treatment of diseases (Teoh, 2016). Pharmaceutically important bioactive compounds are extracted and isolated from medicinal plants for the synthesis of drugs. Plant-derived compounds exhibit various pharmaceutical activities based on their chemical nature (Gad et al., 2013). For instance, terpenoids and flavonoids possess anticancer, antibacterial, anti-inflammatory, antimalarial, and antiviral properties, while alkaloids predominantly exhibit anaesthetic activities. Phenolic bioactive compounds have a significant impact on neutralizing free radicals and thus serve as abundant sources of antioxidants. They have contributed significantly to the development of modern drugs targeting ailments such as cancer, tumours, arthritis and hepatic diseases. In addition to medicinal applications, phytochemicals are extensively utilized in cosmetics, fragrances, and flavouring agents, with many of them already available commercially as both medicines and dietary supplements. As a result, bioactive compounds have emerged as an alternative system for addressing health issues on a global scale in the present era (Yeshi et al., 2022).

The qualitative and quantitative analysis of bioactive compounds heavily relies on the appropriate extraction techniques. The crude extract of plants comprises a mixture of compounds such as alkaloids, terpenoids, and saponins. However, plant materials contain a relatively small quantity of bioactive compounds, and their extraction, purification, and characterization are challenges in drug discovery (Justin et al., 2014). Therefore, selecting suitable extraction procedures and analytical techniques is crucial for achieving optimal extraction, isolation, and purification of the desired bioactive compound (Reddy et al., 2019). Modern spectrometric and chromatographic techniques have analysed secondary metabolites more accurately as compared to earlier methods. However, the success of these techniques still depends on the extraction methods, input factors, and the specific characteristics of different plant parts. Key parameters influencing the extraction process include the choice of solvent, extraction time, temperature, plant matrix, and pressure (Citti et al., 2020).

Numerous extraction methods can be employed to extract bioactive compounds. In addition to conventional methods like percolation, maceration, heat reflux, Soxhlet extraction, and infusion, non-conventional techniques have been recently established (Martinez et al., 2023). These non-conventional methods are considered eco-friendlier due to their reduced use of synthetic and organic solvents, shorter extraction times, higher yield, and improved extract quality. Non-conventional techniques that have shown promise for the selectivity of phytochemical compounds and increased overall yield include microwave-assisted extraction, ultrasound-assisted extraction, supercritical fluid extraction, etc. (Rudaz, 2005). In the process of identifying and characterizing bioactive compounds, the separation of these phytochemicals from plant parts remains an important challenge due to the presence of numerous compounds. Various techniques have been employed for the identification and quantification of these phytochemicals.

1.6.1 Bioactive compound extraction from *Cannabis sativa* L.

Several methods can be used to extract and analyse the bioactive compounds found in hemp, such as Solvent extraction, supercritical CO_2 extraction, steam distillation, chromatography, spectroscopy, High-Performance Liquid Chromatography (HPLC) and Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) (Zivovinovic et al., 2018). Solvent extraction uses ethanol, methanol, or hexane to extract the bioactive compounds. In contrast, Supercritical CO_2 extraction uses CO_2 as a solvent to extract the compounds and is considered an environment friendly method. To extract essential oils and volatile compounds Steam distillation is used. Chromatography is used to purify and isolate specific compounds, Spectroscopy uses light to analyse the chemical composition and HPLC is used to identify and quantify a diverse bioactive compound. Other techniques, such as microscopy, mass spectrometry and X-ray diffraction, may also be used. The selection of technique will rely on the specific bioactive compound or compounds of interest and the level of purity and quantification required.

Cannabis sativa L. comprises of several bioactive compounds including cannabinoids, terpenoids, sterols, flavonoids etc. in various plant parts such as flowers, stem barks, roots and leaves. The complete profile of

bioactive compounds provides a valuable baseline clinical study (Jin et al., 2020). In *Cannabis* CBD is in the form of cannabidiolic acid (CBDA) which loses carboxyl group in the process of decarboxylation within the plant itself and the process can be accelerated by heat. CBDA can effectively prevent vomiting and nausea-induced behaviour at low doses. Still, due to low thermal stability, a stable analogue of CBDA known as CBDA methyl ester or HU-580 was studied (Pertwee et al., 2018). Murillo-Rodríguez et al. 2021 *In vivo* studied the effect of HU-580 and found that NeuN and c-Fos immunoreactivity observed in hypothalamus nuclei implies its potential role in modulating the sleep-wake cycle by interacting with the hypothalamus.

Cannabis sativa L. is a highly adaptive and fast-growing plant species that grows widely along the roadside in various agro-climatic regions of North Indian states such as Punjab, Haryana and Himachal Pradesh. Despite this, less research has been conducted to assess the feasibility of utilizing these wild plants as a potential hemp crop or to determine the variability of their metabolic content. It is considered a roadside weed in north India due to its adaptive and prolific growth habit and ability to conquer environmental stress.

Our research aimed to synthesize an environment-friendly nanofertlizer using hydroxyapatite as a carrier. Hydroxyapatite generates massive interest and is naturally found in bones and teeth (Ferraz et al., 2004). Calcium phosphates are utilized in bone regeneration due to biocompatibility and resemblance to natural bone. Hydroxyapatite, the key inorganic constituent of bone tissue, is particularly interesting as a grafting material (Fiume et al., 2021). It is a calcium carbonate compound with good biocompatibility and a rich phosphorus source. Many synthetic routes have been developed over the last decade to produce HAP nanoparticles with various processing parameters (Sadat-Shojai et al., 2013).

A GC-MS analysis has been conducted to study comprehensive and crucial insights into the chemical composition of hemp plants. Furthermore, its releasing behaviour was investigated by using the kinetic release model. The synthesized fertilizer was then applied to the hemp crop to check its efficiency and variation in metabolic formation. To understand the combination with PGPB, a selected PSB strain was also used. The selected strain was *Bacillus megaterium* and *Pseudomonas aeruginosa*. The genus *Bacillus* has the largest number of reported species with the ability to solubilize phosphorus, followed by *Pseudomonas* (Prabhu et al., 2019).

CHAPTER 2. REVIEW OF LITERATURE

This thesis intends to synthesize environmentally benign urea hydroxyapatite nanofertilizer (UHAPF) and study its impact on the development and yield of *Cannabis sativa* L. plants. This chapter reviews the literature about various nanofertilizer formulations - synthesis, materials characterization and application. Also, the review of the combined effect of nanofertilizer and plant growth-promoting bacteria (PGPB) on the growth and yield of various crops highlights their synergistic impact. This chapter provides a strong database to gain insight into innovation and advance research in new vistas for agro-nanotechnology.

Table 2.1. Description of different urea nanofertilizers and their effects

Nanofertilizer	Comparison	Plant	Effect on plant /Agronomic findings	References
Nano chitosan-NPK fertilizer	NPK	*Triticum aestivum*	Accelerating growth and productivity in plant	(Abdel-Aziz et al., 2016)
Urea-HAP Nanohybrid	Granular urea	*Oryza sativa*	Increase rice yield and Nitrogen and potassium content	(Kottegoda, et al., 2017)
Urea−Montmorillonite-Extruded Nanocomposites	Conventional Urea	N/A	Slowed N release rate	(Pereira et al., 2012)
Urea-kaolinite controlled release fertilizer	Conventional Urea	*Oryza sativa*	Grain yield and yield components of the rice crop responded more to coated CRF than to two other fertilizers.	(Roshanravan et al., 2014)
Urea loading-Mesoporous Silica NPs	N/A	N/A	Slow-release behaviour	(Wanyika et al., 2012)
Urea/APTMS-modified zeolite	Urea without zeolite	N/A	Control release of nitrogen from urea	(Rahmat et al., 2015)
Urea-nano ZnO and nano RP	Conventional urea	N/A	Reduce nitrate emission from conventional urea	(Kundu et al., 2016)

Urea nanoclay polymer composite	Conventional urea	N/A	Reduction N_2O emission from urea and slow Nitrogen release rate	(Yamamoto et al., 2016)
Urea–HA-Mt Nanohybrid composites	Conventional urea	*Oryza sativa*	Increase in yield, low nutrient utilization efficiency	(Madusanka et al., 2017)
U-ACP - calcium phosphate nanoparticles doped with urea	Conventional urea	*Triticum durum*	Nitrogen uptake by durum wheat increase	(Ramírez-Rodríguez et al., 2020)

2.1 Slow and controlled-release fertilizers (CRFs) Development and Efficiency

Slow-release fertilizers (SRF) and controlled-release fertilizers (CRF) both are designed to provide a controlled release of nutrients to plants, but they differ in their mechanisms of nutrient release. Slow release fertilizers rely on a coating to slow the release rate of nutrients, while slow-release fertilizers release nutrients slowly over time through various mechanisms. CRFs are important for increasing agricultural productivity; yet, their use is limited due to high costing. Many researches have been conducted over the last 20 years to examine the characteristics of slow-release urea about its impact on agricultural output and environmental protection. Several manufacturing routes exist for CRFs, such as complex fertilizers, coated fertilizers, encapsulated fertilizers, matrix-based CRFs, super-granules and briquettes. Among these, coated fertilizers are the most common type of CRF. These fertilizers have a protective coating that controls the release of nutrients through diffusion. In contrast, complex fertilizers have chemically controlled nutrient release, while matrix-based CRFs incorporate nutrients into a matrix that may be coated or uncoated. Encapsulated fertilizers release nutrients through a membrane and other types of CRFs include super-granules and briquettes (Ali & Danafar, 2015).

The coating materials significantly reduce the nitrogen release rate from the urea fertilizer. Coating materials are classified into two types: polymer and inorganic. Polymer coatings are usually made from petroleum-based derivatives like polypropylene, polyvinyl chloride, polyvinyl alcohol, polyethylene, polyacrylamide and alkyd resin; natural polymers like resin, vegetable oils, gum arabic, starches, lignin, gelatin, sodium alginate, cellulose etc. The inorganic coating includes sulphur, gypsum, phosphate, bentonite, silicate and other materials.

2.1.1 Sulphur-coated Nitrogen Fertilizer for Growth and Soil Health

The urea granules coated with a layer of elemental sulphur slow down the release of nitrogen into the soil. Ayub et al., (2001) studied sulphur coated urea and evaluate the quality and rate of urea dissolution of coated particles. The study examined the effects of the flow rates of sulphur, atomized air and temperature of the spouted bed air on the coated particle surface quality. The study showed that the spouting air temperature affected the nutrient release rate but was not significantly impacted by varying the atomizing air flow rate. The sulphur coating effectively prolonged nutrient release, but its brittleness resulted in easy cracking. The slow nitrogen release reduces the nitrogen leaching loss. Another research related to sulphur was conducted by Ibrahim et al., (2014) who used sulphur as a coating material and mixed it with four different materials: gypsum, cement, sulphur and zeolite. The experimental findings indicated that the application of a thicker coating material resulted in improved strength of the coated urea granules. Additionally, incorporating wax and sieving the coating materials demonstrated enhanced release characteristics of the coated urea.

The impact of sulphur-coated urea on plants in a field experiment was studied by Gao et al. (2015) by developing two types of CRFs using polymer and polymer sulphur-coated urea and applied in potato fields (*Solanum tuberosum* L.). The controlled release of urea significantly increased nitrogen use efficiency, quality of potato crop and tuber yield. Furthermore, it increased the vitamin C, soluble protein and starch content. The outcome reported the released urea on potato, the manufacturing process and the nitrogen use efficiency. The polymer-coated urea usage rate was 150 $kgNha^{-1}$. However, there are also some drawbacks for using sulphur-coated urea. It is generally more expensive than traditional urea fertilizer, which can be a limiting factor for farmers.

2.1.2 Layered double hydroxides (LDHs)-based Slow Release Fertilizers

LDHs are a synthetic inorganic compound, which can be exchanged with other anions. LDHs have a wide range of potential applications due to their unique structure and anion exchange capacity, including catalysis, adsorption, drug delivery, flame retardants and energy storage. LDHs are also known as hydrotalcite-like compounds, as their structure is similar to natural mineral hydrotalcite. Ureña-Amate et al. (2011) synthesized nitrate-hydrotalcite hydrotalcite-like-LDHs to decrease the environmental pollution caused by nitrogen fertilizers and created two systems: granules and tablets. Release experiments were conducted in both water and a simulated soil solution to determine the release rate of nitrate. The results indicated that the shape of the formulations significantly impacted the release rate of nitrate, with tablets being more effective than granules. Another LDHs study was conducted by Kottegoda et al. (2015) to develop nanohybrid formulations. The urea intercalation with LDH was confirmed using various characterization techniques. The urea molecules bound to the clay nanolayers through different interaction modes slow their hydrolysis and decomposition. The release behaviour of urea showed slow release behaviour compared to pure urea. Layered double hydroxides (LDHs) have many advantages and potential applications and some limitations that need to be considered, including cost, synthesis conditions, limited anion exchange capacity, structural stability etc.

2.1.3 Mineral Clay-based Nanofertilizer for Slow-release Nutrient

Clay minerals are composed of layered silicate minerals. Nano clays, such as montmorillonite and kaolinite have been widely used as carriers for material synthesis due to their high surface area and cation exchange capacity that can interact with fertilizer elements. Various clay was used to synthesize urea nanofertilizer such as bentonite, montmorillonite, kaolinite, halloysite etc.

Montmorillonite (MMT) is a natural clay mineral known for its high cation exchange capacity, which allows it to hold onto positively charged ions, such as nutrients, heavy metals, and organic compounds. In agriculture, montmorillonite clay is used as a soil conditioner and for soil fertility improvement, water retention, and plant growth. Pereira et al. (2012) prepared the urea MMT clay intercalated nanocomposites and further confirmed characterization and analysed through XRD, SEM-EDX and DTA. The nanocomposites were deformable and showed slow-release behaviour. Yamamoto et al. (2016) created SRFs using a mixture of MMT and a urea/urea-formaldehyde polymer matrix. The mixture was made using a process called cold plastic extrusion. The resulting nanocomposites had good release of urea and mechanical resistance and was controlled by the degree of polymerization.

Kaolinite clay has high plasticity and is easily moulded into various shapes. Kaolinite clay mineral was used by Roshanravan et al. (2014) to develop CRFs. Kaolinite clay-coated urea containing 20 weight% urea with water-based epoxy resin was used. The study suggested that intercalating urea into kaolinite causes a three times decrease in nitrogen release behaviour compared to non-coated samples. A trial with rice plants showed that coated urea significantly increased yield and yield components compared to non-coated and conventional urea fertilizers. Another clay bentonite is an aluminium phyllosilicate incorporated into urea by Hermida & Agustian, (2019) using different proportions of corn starch and hydroxypropyl methylcellulose as a binder and forming pellets. Further, analysis of the material desorption mechanism and structural properties was carried out. Urea was ultimately released in 50 min in a static release experiment. However, hydroxypropyl methylcellulose (HPMC) was a less effective binder and had a faster release of urea than corn starch due to its hydrophilic nature.

Zeolite is a type of mineral that is frequently found in volcanic rocks and sedimentary rocks. Zeolites are aluminosilicates with a three-dimensional crystal structure with cavities and channels that can trap other molecules. Khan et al. (2021) incorporated macronutrients using nano-zeolite as a carrier and synthesized nano-zeolite composite fertilizer and further characterized it by using different techniques such as FT-IR, powder XRD, TG/DTA and SEM. Applying the prepared nanofertilizer showed a significant enhancement in soil quality and water retention capacity compared to commercial fertilizer. Slow-release studies revealed that

nanofertilizer promotes plant growth. To eliminate NO_3^- from water, Bhardwaj et al. (2012) prepared SRFs known as "surfactant modified silicates" using natural zeolite and montmorillonite for slowly releasing nutrients over time. The material was further confirmed by various analytical techniques. The results indicate that it releases nitrate even after 15–20 days of leaching study. There are some limitations to the use of mineral clay-based urea fertilizers, such as cost production, environmental concerns and limited availability of high-quality mineral clay deposits in some regions, making the production and use of mineral clay-based urea fertilizers difficult or impractical.

2.1.4 Biochar-based slow release fertilizers

Biochar-based fertilizer has been developed to enhance the sustained release of nutrients. It is a carbon-rich material originated by biomass pyrolysis in a low-oxygen environment and improves the slow nutrient release in soil. Biochar-based fertilizers can improve soil properties by reducing the rate of nutrient release (Sim et al., 2021). Biochar-based SRFs were prepared by González et al. (2015) and investigated for the efficiency of diverse polymeric materials to control nitrogen leaching. The study concluded the potential for leaching nitrogen compounds in soil columns with and without plants, including nitrate, nitrite, ammonium and urea. Urea encapsulated in cellulose acetate, sodium alginate and ethyl cellulose. The findings revealed that the highest concentration of ammonium leachates was observed on the 22nd day of the experiment and the predominant form of nitrogen in the leachates was nitrate.

2.1.5 Wax and Resin-based Urea Nanofertilizers

Resins and wax are popular for coating fertilizers because they repel water, are inexpensive, easy to melt and break down naturally. Mukerabigwi et al. (2015) coated urea fertilizer granules using biopolymers like xanthan, tamarind and guar gums with diatomite. Results showed that xanthan urea diatomite had good nutrient slow release properties and water retention capacity after 28 days. Natural or synthetic waxes were used in fertilizer synthesis which included paraffin wax, beeswax, microcrystalline wax, etc. According to Mehmood et al. (2019), incorporating sulphur, gypsum, starch and bentonite as a coating material with paraffin wax as a binder can decrease the rate of urea release. The research revealed that the release rate of urea was lowest for sulphur/gypsum-coated urea, compared to sulphur/bentonite and sulphur/starch-coated urea. Among all the coating materials, the mixture of sulphur and gypsum resulted in the lowest nitrogen release rate from the urea fertilizer (37%), making it a promising candidate for developing new products.

2.1.6 Superabsorbent-based Slow Release Fertilizers

Superabsorbent materials are substances that absorb and hold large amounts of liquid relative to their own weight. Xiao et al. (2017) developed a slow-release urea fertilizer in a one-step process known as reactive melt mixing. The process involves the creation of starch-based superabsorbent polymers. The fertilizers were

developed from high-amylopectin starch or high-amylose starch. The study showed that urea embedded in the starch-based superabsorbent polymer gel network, controlled the fertilizer release rate in the water.

Superabsorbent materials function by forming a network of hydrophilic polymer chains that can absorb water through a process called osmosis. When the polymer chains come into contact with water, it swells and form a gel-like substance. Rashidzadeh & Olad, (2014) created a slow-release NPK fertilizer using a superabsorbent material to control nutrient release. Superabsorbent nanocomposite was made by mixing different materials together such as acrylic acid, sodium alginate, montmorillonite and acrylamide. Result showed that the montmorillonite in the prepared material resulted in a more controlled release of nutrients compared to the neat superabsorbent. A good controlled release fertilizer property and high-water holding capacity of the formulation, make it potentially suitable for use in agriculture as a fertilizer.

2.1.7 Chitosan based Slow Release Fertilizers

Polymeric nanoparticles, such as poly(lactic-co-glycolic acid) (PLGA) and chitosan, have been investigated as carriers for pesticides and fertilizers due to their biodegradability and biocompatibility. Chitosan is a type of natural polymer derived from the deacetylation of chitin. Ha et al. (2019) prepared NPK-loaded chitosan nanofertlizer through the tripolyphosphate ionic gelation and chitosan solution. The prepared material was then analysed by TEM, SEM, size distribution and zeta potential. Furthermore, the release kinetics of nanofertilizer were studied for 240 hours. In addition, the research investigated its effects on coffee plant growth and concluded that nanofertilizer enhanced nutrient uptake, photosynthesis and development of coffee plants in greenhouse conditions. The total chlorophyll content and net photosynthesis rate also increased. Also, the application of the nanofertlizer improved the plant height, leaf number and leaf area of the coffee seedlings. To study the effectiveness of NPK-loaded chitosan on crop plant Abdel-Aziz et al. (2016) evaluated chitosan-NPK nanoparticles on wheat plants through foliar uptake. The study showed that the nanoparticles was successfully taken up by the wheat plant and transported through phloem tissues, resulting in significant increase in wheat yield variables such as crop index, harvest index and mobilization index. Moreover, using nanofertilizer led to a shorter life cycle for wheat plants, accelerating plant growth and productivity.

2.1.8 Polyurethane Coated Controlled Release Fertilizers

Polyurethane also is a thermosetting polymer, which means that it does not soften or melt when heated. Feng et al. (2019) designed polyurethane-coated urea with low viscosity and hydroxyl value. It was formed using polyols derived entirely from vegetable oils and polymethylene polyphenylene isocyanate. Using oleic acid and epoxidized soybean oil as the primary raw materials, vegetable oil-based polyols were synthesized via one-pot synthesis. The study concluded that prepared oil-based polymer-coated urea has excellent degradability and great potential for better crop production. Depending on the specific formulation and

processing conditions polyurethane can be made into a wide range of materials with different properties, such as flexible or rigid foams, elastomers, coatings and adhesives. Tian et al. (2019) developed a new coating method for SRFs using biobased materials like polyurethane and polyolefin wax. The coating method involved using polyolefin wax as an inner coating to improve the fertilizer's surface and reduce urea surface roughness. A degradable biobased polyurethane film was used as an outer coating and epoxy resin was used as a protective layer to control the nutrient release. The coated materials extended the release period of nutrients threefold. A nitrogen fertilizer coated with polyurethane - a bio-based material derived from liquefied locust sawdust was prepared by Zhang et al. (2016) for controlled nitrogen release. Epoxy resin was also used to synthesize an interpenetrating network with the bio-based polyurethane to create epoxy resin-modified polymer-coated urea with enhanced slow-release properties. A corn growth study was carried out to assess the effectiveness of field application of coated fertilizer. The results showed that it increases total dry matter accumulation and corn yield compared to conventional urea fertilizer.

2.1.9 Poly(acrylic acid) (PAA) Made Slow Release Fertilizers

Poly(acrylic acid) (PAA) is a synthetic polymer made from acrylic acid monomers. The polymer is water-soluble and form hydrogen bonds with water molecules. It has a high-water absorbing capacity, which makes it useful as a superabsorbent material. A slow-release nitrogen fertilizer with water holding capacity has been developed by Zhou et al. (2018) utilizing leftover rice and crosslinking methods. Urea was combined with leftover rice-g-poly(acrylic acid)/montmorillonite network. This composite acts as a loss control agent for water and nutrients when applied to the soil. It exhibits the ability to retain water and nutrients and reduce leaching. Experimental findings revealed that the prepared composite exhibited a lower nitrogen leaching loss (19.7%) than pure urea (52.3%).

PAA can be synthesized using various methods such as solution polymerization, emulsion polymerization and inverse emulsion polymerization. The resulting polymer can have different properties depending on the reaction conditions and the molecular weight of the polymer. PAA is biodegradable and non-toxic, making it a safer alternative to some other types of synthetic polymers. Ni et al. (2009) developed environment-friendly slow-release urea fertilizer with water-retention properties using two different materials, crosslinked poly(acrylic acid-co-acrylamide) and ethyl cellulose, to create an outer and inner coating, respectively. The prepared material contained 21.1% nitrogen. The biodegradability of the ethylcellulose coating in soil was tested using differential scanning calorimetry measurements, which showed that the glass transition temperature of the ethylcellulose coating decreased over time, indicating biodegradation in soil.

2.1.10 Hydroxyapatite based Nanofertilizer

Calcium phosphate nanocomposite have been broadly used in biomedicine because of its high biocompatibility and biodegradability. Ramírez-Rodríguez et al. (2020) incorporated potassium (K) and nitrogen (N) in the

form of nitrate and urea into calcium phosphate nanoparticles to deliver potassium (K) and nitrogen (N) to plants in a controlled manner. The nanofertilizer showed promising results with a slow-release mechanism that contained calcium, phosphorus, potassium, nitrate and urea. Furthermore, it enhanced the nitrogen uptake efficiency in durum wheat plants by 40%.

Hydroxyapatite (HAP) is a calcium phosphate compound, specifically a calcium hydroxide phosphate, which is a significant component of the mineralized tissues such as bones and teeth in vertebrates. HAP nanoparticles have been investigated as potential carriers for fertilizers. Giroto et al. (2015) developed nanocomposite fertilizers by incorporating hydroxyapatite (HAP) into urea and thermoplastic starch at nanoscale. These fertilizers were designed to release urea slowly, facilitating the faster release of poorly soluble phosphate phases while ensuring a controlled release of highly soluble nitrogen sources. This approach aimed to enhance fertilization efficiency and reduce environmental pollution associated with excessive fertilizer use. Similarly, Yoon et al. (2020) also explored the synthesis of a phosphorus nanofertilizer by combining HAP nanoparticles with synthetic or natural humic substances through a simple dipping process. Pot experiments with *Zea mays* (corn) demonstrated significant improvements in corn productivity, microbial consortia and resistance to abiotic stresses induced by NaCl.

HAP is a rich P source, which contains insoluble phosphorus solubilised by PSB making it environment-friendly material. There are various soil microorganisms that can solubilize/mineralize insoluble soil phosphate to release soluble phosphorus ion. To produce an environment friendly fertilizer Kottegoda et al. (2017) synthesized urea hydroxyapatite nanofertilizer, which can slowly release nitrogen over time. Urea incorporated into rock phosphate, has excellent biocompatibility. Similarly, Gunaratne et al. (2016) studied the plant *Gliricidia sepium* and examined the application of a nanocomposite comprised of urea-coated hydroxyapatite and potassium enclosed within montmorillonite. The resulting nanocomposites exhibited a slow-release behaviour, gradually releasing the nutrients over time. The study concluded that the utilization of nanoscale urea has a potential to serve as a basis for developing efficient fertilizers. The impact of using coated urea on N_2O emission was investigated by Kundu et al. (2016). The coated urea was prepared by incorporating nanoparticles of zinc oxide and rock phosphate with diameter less than 48.6 nm, and coating agent called pine oleoresin (POR). Pine oleoresin-coated urea significantly reduced N_2O emissions by 20.26% when compared to uncoated urea. The findings suggested that coating urea with two percent nano zinc oxide and thirty five percent nano rock phosphate particles could be a potential strategy to minimize N_2O emissions resulting from the use of nitrogen fertilizers.

2.1.11 Diverse Material for Development of Slow Release Fertilizers

There are various synthesis methods for slow release urea fertilizers, including coating, encapsulation, intercalation, reaction with formaldehyde, physical blending etc. These synthesis methods can be tailored to produce slow release urea fertilizers with different properties, such as release rate, nutrient content, and

physical form. Carvalho et al. (2019) examined the effect of slow release urea microencapsulation in ruminant diet due to its ability to inhibit the hygroscopy of urea. To develop an environment friendly technology Lan et al. (2011) developed a slow release urea fertilizer by using polymer latex. The study focused on investigating the influence of various factors in the coating process, such as latex spray rate, atomizing gas flow rate, film permeability, and gas temperature, on the structure of the film. The findings highlighted the significant role of water transfer in the spray coating process.

Mesoporous silica nanocomposite has been explored as carriers for agrochemicals due to their tunable pore size and biocompatibility. Wanyika et al. (2012)investigated mesoporous silica nanoparticles as a delivery system with specific particle, pore sizes loaded with urea. The study found that highly concentrated urea solution was more effective for loading the nanoparticles and the release process showed a sustained slow-release profile in both water and soil. Another compound aminopropyltrimethoxysilane is a chemical compound that belongs to the family of organosilicon compounds. It is also known as (3-Aminopropyl)trimethoxysilane(APTMS). Rahmat et al. (2015)studied the effectiveness of a nanofertilizer made from urea/ APTMS -modified zeolite as a slow release nitrogen fertilizer. The modified zeolite had a maximum adsorption capacity of urea and was able to release urea slowly over time. The experiment showed that urea without clay was quickly released, while urea incorporated with APTMS-modified zeolite was released slowly over a period of 120 minutes in 1 M NaCl media.

Yang et al. (2017) investigated the effects of matrix-based urea on maize production. The results showed that matrix-based urea increased agronomic efficiency, biomass and grain yield and apparent recovery efficiency compared to common urea. The improved plant development was attributed to greater available nitrogen in the top soil layers due to reduced ammonia emission and nitrogen leaching. Moreover, the profitability of maize production with matrix-based urea was greater than that of common urea. Rop et al. (2018) formulated a slow-release fertilizer composite using a polymer hydrogel made from water hyacinth cellulose-graft-poly(acrylamide), which incorporated nano-hydroxyapatite and urea fertilizer. The data suggested that the slow-release fertilizer composite was found to lower the risk of nutrient loss through leaching and prevent toxic effects on plant roots. Additionally, it provided a consistent release of nutrients that met the requirements of the crops.

2.2 Urea Releasing Behaviour Study using Kinetic Models

Various SRFs studies were done using kinetic models to analyse the release profile data and determine the urea release mechanism and associated kinetics. Wei et al. (2019) created a water-retention and slow-release fertilizer through copolymerization of free radical of acrylic acid, potato starch, modified β-cyclodextrin and acrylamide with the addition of halloysite nanotubes loaded with urea. The study on urea release kinetics

showed that the release behaviour of urea in water was influenced by its concentration, while in soil, it followed the Fickian diffusion mechanism. The findings suggested that the produced fertilizer had effective water retention and urea release properties and including halloysite nanotubes enhanced its release properties. To understand nitrogen release pattern, Maghsoodi et al. (2020) prepared different urea fertilizers and analyzed the nitrogen release behaviour in water and waterlogged calcareous soil. Urea was incorporated into biochar, hydrochar, zeolite and hydroxyapatite nanorods as carriers and the interactions between the urea and the carriers/nanorods controlled the release of urea. The fertilizers with carriers/hydroxyapatite nanorods and other carriers released urea much slower than urea alone, reducing the urea release by 4.5-11.5 times. The kinetics of urea release from these fertilizers followed the Korsmeyer-Peppas model based on Fickian diffusion law.

2.3 Phosphorus Fertilizer Development and Solubilization by Microorganism

In order to enhance the effectiveness of urea utilization, Yu & Li (2019) prepared a slow and sustained release nitrogen fertilizer using phosphogypsum as a carrier and granulating agent for urea, which was then coated with paraffin wax. To improve the adhesion and wetting properties between the fertilizer core and the paraffin coating, a biodegradable surfactant was added to the paraffin. The researchers compared the urea release behavior of prepared urea material with that of paraffin-coated urea particles. The findings demonstrated that the paraffin-coated phosphogypsum-granulated urea exhibited a significantly slower urea release rate compared to paraffin-coated urea. Over a period of 28 days, less than 35% of the urea was released from the paraffin-coated urea.

Phosphogypsum is source of P fertilizer, solubilized by PSM. To understand the effect of rock phosphate and phosphogypsum in the soil and plant growth, Amri et al. (2022) investigated the effects of a microbial consortium of PSM on the solubilization using ryegrass as a model plant. The results indicated that the addition of *Pseudomonas fluorescens* with phosphogypsum resulted in significant root proliferation and increased plant biomass dry weight, as well as improved total P uptake. Conversely, the addition of *Pseudomonas fluorescens, Stenotrophomonas maltophilia* and *Pantoea agglomerans* resulted in small amount of total phosphorus assimilation. The study suggested that co-inoculation of *Pseudomonas* species with phosphogypsum may be a promising substitute to chemical fertilizers for providing a source of P fertilizer to plants while maintaining a high level of nutrients in the soil.

A study was conducted by Mikhak et al. (2017) in a greenhouse to examine the effectiveness of synthetic nano zeolite (nCp)/nanohydroxyapatite (nHA) as a fertilizer for chamomile compared to traditional phosphorus (P) fertilizers. The experiment involved various treatments which were applied to the plants. The results indicated that using nCp/nHA (nanohydroxyapatite) as a novel form phosphorus fertilizer has a potential to increase agronomical yield while reducing the risks of water eutrophication. The nanohydroxyapatite particles used in

the study were produced through a wet chemical process with diameters ranging from 25-50 nm and were compared to the P solubility of traditional fertilizers such as triple superphosphate and rock phosphate.

2.4 Plant Growth Promoting Bacteria (PGPB) Role in Soil health and Plant Growth

PGPB play an essential role in soil processes and are becoming popular as eco-friendly biopesticides for plant-parasitic nematode control (Mhatre et al., 2019). PGPB inhabit plant roots and interacts with the plant in various ways, such as producing hormones, fixing nitrogen, solubilizing phosphorus and secreting enzymes that break down organic matter. These activities can significantly improve crop yield by increasing nutrient availability, improving soil structure and suppressing plant pathogens. Plant Growth Promoting Rhizobacteria (PGPR), are specific groups of PGPB that colonize the rhizosphere and the soil surrounding the plant roots and promote plant growth through similar mechanisms. PGPR is a type of PGPB that specifically colonizes the rhizosphere.

Effective plant PGPR were chosen for forage corn cultivation in a study by Piromyou et al. (2011). The research explored the PGPR inoculation effect on indigenous microbial community structure. *Brevibacillus sp.* and *Pseudomonas sp*. were chosen for evaluation of their efficiency in promoting growth in pot experiment as well as in a field experiment. The results showed that selected strain of PGPR inoculated with compost promoted forage corn yield better than commercial strains, and therefore could be used as inoculants. The PGPR likely promoted growth through factors such as ACC-deaminase and P-solubilization. The study also found that the inoculated PGPR did not significantly impact the structure of the microbial population in the rhizosphere. A study conducted by Sagar et al. (2022) obtained a bacterial strain named PR19, isolated from soil and examined its potential to enhance maize growth individually and in combination with inorganic fertilizer (NPK). The PR19 strain was identified as *Azotobacter nigricans* and confirmed through submission to NCBI. The strain showed several beneficial traits for plant growth-promotion, including production of siderophore, indole-3-acetic acid (IAA), ammonia and ACC Deaminase. Moreover, PR19 demonstrated tolerance to high salt levels, heavy metals, wide pH ranges and resistance to multiple antibiotics. Under abiotic stress conditions, PR19 also showed significant increase in antioxidant enzymes activity. The combined application of PR19 and NPK resulted in notable enhancements across several growth and yield parameters in maize.

2.4.1 PGPR as a Biofertilizer in Soil Health Improvement

Biofertilizers have emerged as more environment friendly fertilizers for enhancing plant health and promoting sustainable agricultural practices. He et al. (2015) developed efficient slow-release biofertilizer formulations using microbial strain encapsulated with alginate and sodium bentonite composites. The release patterns of bacteria from the microcapsules made of sodium bentonite and alginate exhibited an initial burst, followed by a gradual increase, conforming to a first-order release pattern. These findings indicate that sodium bentonite

and alginate composites have the potential to be a cost-effective encapsulation material for slow-release bacterial fertilizers in agricultural applications. Furthermore, to improve soil microflora Kaur et al. (2017) investigated the effect of different fertilizers, including organic, inorganic and biofertilizers, on pea plants at different stages of growth. The study concluded that the combined use of inorganic and organic fertilizers improved soil microflora, leading to higher enzyme activities, which were further improved by adding biofertilizers.

2.4.2 PGPR Improved Metal Uptake under Stressful Conditions

The impact and regulatory mechanisms of co-inoculating rhizobium and PGPRs in plant-soil systems concerning alleviating metal toxicity are poorly understood. To explore this, Ju et al. (2019) examined the effects of co-inoculating the metal-resistant rhizobium *Sinorhizobium meliloti* in copper (Cu) and the PGPR *Paenibacillus mucilaginosus* in contaminated soil. The co-inoculation of beneficial microorganisms greatly enhanced plant growth, elevated nutrient levels in plant tissues and alleviated the harmful effects of copper-induced reactive oxygen species (ROS) and lipid peroxidation. This improvement was achieved by boosting the activities of antioxidant enzymes, thus reducing oxidative stress. Co-inoculation also increased Cu uptake in plant tissues, improved soil fertility and biological activity by increasing soil microbial biomass, total nitrogen, soil organic matter contents, enzymatic activities and available phosphorus. Furthermore, the combination of PGPR along with rhizobium had positive effect on the microbial community in the rhizosphere and led to a slight increase in microbial diversity. These results suggest that the simultaneous inoculation of PGPR and rhizobium can have positive effects on alfalfa plants grown in soils contaminated with heavy metals. It can help alleviate copper (Cu) stress in plants and improve the biochemical properties of the soil.

2.4.3 PGPR Role in Ammonia Emission Mitigation

PGPR have been found to play a vital role in mitigating ammonia emissions from agricultural activities. Excessive NH_3 emissions from urea fertilizer application caused significant disturbances to the global environment. In this regard, biofertilizers containing PGPR have been proposed as an effective strategy for reducing NH_3 emissions and improving soil health. To assess the potential of *Bacillus subtilis* biofertilizer in diminising NH_3 volatilization, a field experiment was conducted by Sun et al. (2020). The results demonstrated that incorporating biofertilizer derived *Bacillus subtilis* led to reduction upto 44% in NH_3 volatilization compared to organic fertilizer. Moreover, *Bacillus subtilis* biofertilizer application decreased in the abundance of ureC gene, which is associated with urea hydrolysis. These changes contributed to a decrease in the conversion of fertilizer nitrogen to NH_4^+ and an increase in the nitrification process. In addition, PGPRs reduce N_2O emissions by improving nutrient utilization efficiency in plants and reducing the amount of excess nitrogen in the soil that can lead to N_2O production (Florio et al., 2019).

2.4.4 Phosphate Solubilizing Bacteria for Insoluble Phosphorus Solubilization

Phosphorus is an essential element for plant growth, but it often exists in insoluble forms in soil, making it unavailable for plants. However, certain microorganisms can solubilize these forms of phosphorus and enhance plant nutrition. These microorganisms use various mechanisms to solubilize fixed or unavailable phosphorus, including acidification through organic and inorganic acid production, exopolysaccharide and siderophore production, as well as enzymes such as phosphatase, C-P lyase and phytase. As a result, potential phosphate-solubilizing microorganisms have been developed as biofertilizers and are widely used in agriculture to promote plant growth. Additionally, the property of phosphate solubilization has found applications in phytoremediation (Prabhu et al., 2019).

Phosphate solubilizing microorganisms (PSM) usage in seeds, crops and soil inoculation is a novel method to enhance food production while minimizing negative environmental impacts. Adesemoye & Ugoji, (2009) evaluated the ability of *Pseudomonas aeruginosa* as a PGPR in three crops commonly grown in West Africa. The study examined whether the inoculation method affects the PGPR's effectiveness. Seeds were inoculated with the bacterium by soaking in a bacterial suspension. A coating was created using starch solution as a seed adhesive, which was combined with a bacterial suspension of the same concentration. Another treatment involved soaking the seeds in distilled water followed by the application of NPK 15:15:15 fertilizer. The findings suggested that different inoculation methods produced similar outcomes to plants treated with fertilizer. This highlights the significant potential of *Pseudomonas aeruginosa* as a plant growth-promoting rhizobacteria (PGPR).

Using P-solubilizing microorganisms to inoculate seeds is a promising technique for addressing phosphorus deficiencies. An experiment was led by Qureshi et al. (2012) to estimate the efficacy of phosphate solubilizing rhizobacteria in enhancing the yield and growth of cotton. The results indicated that the microbial inoculum significantly increased seed cotton yield from 1511 to 1630 kgPha^{-1}. The treatment with the highest fertilizer level, combined with inoculum, resulted in the highest seed cotton yield of 1733 kg ha^{-1}. Similarly, Oteino et al. (2015) investigated the endophytic bacterial ability to produce gluconic acid and solubilize insoluble phosphate, promoting growth in *Pisum sativum* L. plants. The study examined that several endophytic strains secrete gluconic acid and had limited phosphate solubilization capacities.

2.5 Exploring the Benefits of PGPR, Fertilizers and Phytoremediation in *Cannabis* cultivation

Cannabis cultivation has potential benefits and has shown the ability to remediate contaminated site due to its ability to absorb and accumulate heavy metals and other environmental toxins. Various factors such as genetics, growing condition, and fertilizers are crucial in plant health and potency. Adequate fertilizer management is essential for obtaining a high yield and potency of *Cannabis* plants.

2.5.1 PGPR in *Cannabis sativa* L. (Hemp) Growth and Development

The endophytes microbial diversity in industrial hemp biological control and growth promotion experiments was carried out by Scott et al. (2018). The study investigated the abundance, diversity and biochemical traits of endophytes present in different plant parts of 3 industrial hemp cultivars. Total 134 bacterial and 53 fungal strains were identified, grouped into eighteen bacterial and thirteen fungal taxa, respectively. The endophytes exhibited biochemical traits such as siderophore production, phosphorus solubilization and cellulase production.

Conversely, to encourage the growth of the *cannabis* industry, Balthazar et al. (2022) utilised the beneficial characteristics of inoculants *Pseudomonas* bacteria to improve yield and sustainability while reducing production costs in the emerging *Cannabis* market. The presence and variety of *Pseudomonas* strains in the *cannabis* microbiome and their capacity to enhance plant development and tolerance to stress were highlighted, focusing on hemp and marijuana crops. *Pseudomonas spp.* can help improve the yield and quality of marijuana and hemp crops. They have different ways of working that can be used to target specific issues faced by these crops.

2.5.2 Fertilizer Impact on Bioactive Compounds of *Cannabis sativa* L.

Nitrogen is a crucial component in leading plant development and structure. Nitrogen affects various aspects, such as productivity, yield and the content of bioactive molecules such as alkaloids and glucosinolates. Bernstein et al. (2019) explored the impact of nutritional supplements, including, inorganic elements (N, P and K) and humic acid effect on cannabinoid profile of *Cannabis* plants. The study found that the macronutrient supplements influence cannabinoid content in the plants. Specifically, the supplementation of NPK led to a significant increase of 71% in CBG (cannabigerol) levels in flowers. Moreover, it resulted in a decrease of 38% in CBN (cannabinol) levels in flowers and a decrease of 36% in CBN levels in inflorescence leaves.

Cannabis sativa L. is a multipurpose crop that has gained interest in recent years. Mineral nutrition in terms of nitrogen, significantly impacts the crop ability to produce various valuable compounds, including cannabinoids, terpenes and flavonoids. Saloner & Bernstein (2020) investigated that how nitrogen supply affects the physiology and development of *Cannabis* plants during the vegetative growth phase under long photoperiod. The study revealed that plant morphology and physiological functions were optimal at the optimal nitrogen level, while deficiency symptoms were observed at a lower nitrogen supply. The nitrogen use efficiency decreased with increasing nitrogen supply and nutrient accumulation was found to be very low. The study suggested that growth delay observed under lower nitrogen supply was caused by limited photosynthetic pigment availability, impaired water relations and reduced carbon fixation. In contrast, excessive nitrogen uptake under higher supply resulted in developmental restrictions constrains due to ion-specific toxicity or indirectly induced restriction on energy availability and carbon fixation. In addition, to understand how nitrogen supply affects terpenoid and cannabinoid profiles in *Cannabis*, Saloner & Bernstein

(2021) conducted another study on the impacts of various nitrogen treatments on the chemical and physiological characteristics of *Cannabis* at the flowering stage. The results showed that nitrogen supply impacts the metabolism of cannabinoids and terpenoids. Inflorescence yield increased with increasing nitrogen supply, but was not affected by additional increase in nitrogen supply. The findings suggested that a high supply of nitrogen has a negative impact on bioactive compound production in *Cannabis* but it promotes overall production. Nitrogen supply may regulate terpenoid and cannabinoid profiles or increase in plant yield depending on production amount. The optimal nitrogen level for achieving high yield and comparatively high secondary metabolites content was 160 mg/L.

The impact of fertilization and planting density on *Cannabis* stem and seed content was examined in various European locations by Tang et al. (2017). Stem yield was increased by 29% with an increase in planting density and by 32% with increase in nitrogen fertilization. However, further increase in fertilizer amount did not significantly affect stem yield. Seed yield content was not affected by planting density but showed a trend towards increase with increase in nitrogen fertilization. The use of *Cannabis sativa* L. in the bio-economy is promising due to its high biomass yield and low resource requirements. Tang et al., (2017) also studied photosynthesis in hemp leaves exposed to different nitrogen and temperature levels to understand its high yield potential. The research found that the total photosynthesis rate improved with leaf temperature up to 25-35°C but decreased at higher temperatures. Using a photosynthesis model, they estimated various parameters and found that hemp has higher photosynthetic capacity than cotton plant and kenaf when leaf nitrogen is <2.0 g N m^{-2}. The findings suggested that hemp can be cultivated sustainably for bioenergy under diverse climatic and agronomic conditions.

The recent attention given to industrial hemp is due to its various applications and ability to be cultivated in various agro-climatic conditions. The selection of the appropriate genotype is essential for the multiple output. Campiglia et al. (2017) assessed the effect of plant density, genotype and nitrogen fertilization on hemp yield. The experiment was carried out over two years and showed high nitrogen fertilization levels beneficial to stem yield but not inflorescence and seed yields. The appropriate fertilization techniques are necessary for the optimal production of high-yielding cannabidiol hemp varieties under field conditions in the expanding hemp cannabidiol industry. Atoloye et al. (2022) examined the influence of nitrogen (N) fertilization on the biomass of bud and yield of cannabidiol in two high-yielding cannabidiol hemp varieties grown in field conditions. The results indicated that the dry and fresh bud biomass and cannabidiol content per plant exhibited a quadratic increase with increasing N fertilization rates. The optimal fertilization rates were found to be between 140 and 190 kg N per hectare, maximizing the production of buds and CBD yield.

2.5.3 Phytoremediation Potential of *Cannabis sativa* L.

Industrial hemp has a high degree of tolerance to heavy metals and great potential for using it in phytoremediation in contaminated soil. Citterio et al. (2003) conducted experiments to study hemp plant

tolerance to heavy metals and its ability to accumulate cadmium, nickel and chromium in two different soils. The plants did not show any significant changes in growth or appearance, but it activates different molecular mechanisms to avoid cell damage caused by heavy metal stress.

Heavy metals tend to accumulate predominantly in *Cannabis sativa* L. roots, with only a partial transfer to above-ground tissues. Husain et al. (2019) explored the potential of hemp in remediating unrestricted coal mine soils in Pennsylvania through phytoremediation. Different hemp varieties were grown in contaminated and commercial soils under different environmental conditions (outdoors and in the greenhouse). The study found no substantial differences in germinated seed and stem height among the hemp varieties cultivated in different soils. However, plants grown in the greenhouse exhibited greater height compared to those grown outdoors. Analysis of leaf samples showed a higher concentration of nickel in hemp leaves in outdoors, although no significant differences were observed in the heavy metal transporter genes expression. Notably, the analysis of floral buds revealed a significant increase in the total cannabidiol content in plants grown on mine land soil. The use of qRT-PCR for molecular analysis showed that cannabidiolic acid synthase gene expression was 18 times higher in plants cultivated on soil from mine land.

In the context of *Cannabis* cultivation, phytoattenuation can be used to improve the quality of contaminated soils by using the plant to produce biomass, such as fibre or biofuel. Phytoattenuation is a phytoremediation technique that involves the use of plants to remove, degrade or immobilize environmental pollutants from contaminated soils. This approach can be particularly useful in areas where the soil has been contaminated by heavy metals or other pollutants from previous industrial activities. De Vos et al. (2023) examined the potential use of industrial hemp fibres grown on soil contaminated with heavy metals as a safe material for the textile industry. The study utilized phytoattenuation to improve soil quality by producing non-food biomass. Two different hemp variety were grown on a contaminated and clean site, and their stem height and diameter, fibre yield and heavy metal concentrations in the fibres were analysed. The results showed that although both cultivars had decreased stem yields when grown on the contaminated site, hemp crop production on contaminated soil may still be economically feasible. Moreover, Pb, Cd and Zn concentration in the fibres were well below the toxicity thresholds for textile production, indicating that the use of hemp fibres produced on heavy metal-contaminated soil is a promising strategy for valorizing contaminated land and developing a non-food value chain.

The potential of hemp as a renewable resource for decontaminating heavy metal polluted soils was investigated by Linger et al. (2002), with a focus on its impact on fibre quality. The heavy metal concentration in seeds, leaves, fibres, and hurds was measured using atomic absorption spectroscopy, and it was found that all parts of the plant contain heavy metals, with the highest concentrations found in the leaves. The fibre quality was assessed by measuring fibre properties and pure fibre content, after mechanical separation, fibre fineness and strength. The results were compared between hemp grown on polluted and non-polluted soil.

2.5.4 The Bioactive Compounds of *Cannabis sativa* L.: Identification and Analysis

Cannabis sativa L. is a versatile plant species used for centuries for medicinal and industrial purposes. Its abundance of phytochemicals and fibres makes it an important resource for various sectors. More than 500 compounds have been reported in *Cannabis sativa* L., with 125 identified as cannabinoids, which are unique C_{21} terpenophenolic compounds. Non-cannabinoid constituents include flavonoids, phenols, alkaloids, terpenes and others (Radwan et al., 2021). Further, more than 565 bioactive compounds have been identified and isolated from different parts of the *Cannabis sativa* plant, offering opportunities for the exploration of potential therapeutic effects and the development of new medications to benefit individuals (Chaachouaya et al., 2023).

Various methods were used to comprehensively analyse the bioactive compound of *Cannabis sativa* L. (Hemp and Marijuana) plant material. Pellati et al. (2018) analysed the secondary metabolite composition present in various fibre-type *Cannabis sativa* L. (hemp) inflorescences using HPLC and GC methods. The cannabinoids, flavones and terpenes were the main phytochemicals found in hemp. The non-psychoactive cannabinoids were profiled using high performance liquid chromatography using various detectors. The content of prenylated flavones, specifically cannflavins A and B, was determined using High performance liquid chromatography. Additionally, the volatile compounds present in *Cannabis* were analysed by a method that combines headspace solid-phase microextraction coupled with gas chromatography-mass spectrometry and gas chromatography with flame ionization detection. The study found that CBDA and CBD were the high amount cannabinoids in hemp samples, β-caryophyllene and β-myrcene were the major terpenes. Cannflavin A was found to be the primary compound in most of the plant materials. To quantify major terpenes in different chemovars, *Cannabis sativa* L. Ibrahim et al. (2022) developed and validated a GC-FID method. The terpenes analyzed linalool, β-pinene, β-caryophyllene, β-myrcene, α-terpineol caryophyllene oxide, α-pinene, limonene, terpinolene and α-humulene.

HPLC is a widely used chromatographic technique for cannabinoid analysis from plant. Brighenti et al. (2017) developed a new and reliable technique to analyse non-psychoactive cannabinoids in hemp plants and their products, focusing on identifying high-quality samples. The newly developed analytical method was utilized to characterize 9 hemp samples and 6 pharmaceutical products derived from hemp. On the other hand, Rashid et al. (2021) used GC-MS to conduct an untargeted metabolomic analysis of seeds from two accessions of *Cannabis sativa* L. grown in different environments. In the study, the researchers identified a total of 236 metabolites, with 43 metabolites showing variation between the two accessions. The high-altitude variety exhibited advantages over the low altitude variety in terms of accumulation of significant cannabinoids, fatty acids, alkaloids, and amino acids. The seed oil derived from the high altitude temperate Himalayan variety demonstrated a lower presence of α-linolenic acid and linoleic acid. The methanolic extracts from low altitude

subtropical variety also showed higher antioxidant and nutraceutical potential. The study suggested that environmental factors can affect the antioxidant and nutraceutical value of seeds.

Decarboxylation is a significant step in cannabidiol (CBD) from cannabidiolic acid (CBDA) production in *Cannabis* plants. During this thermal process, carbon dioxide removed from the acidic cannabinoids, resulted in the formation of neutral cannabinoids. In a recent investigation conducted by Lee et al. (2022), researchers examined the impact of heat on CBDA and acidic cannabinoids found in *Cannabis* extract. The extraction method employed ultrasonication extraction and two-step column chromatography on the *Cannabis* inflorescence. The results indicated that subjecting CBDA to a temperature of 130℃ for 20 minutes predominantly converted it into CBD. Moreover, CBD underwent a partial transformation, leading to the generation of psychoactive THC isomers via a process known as cyclization. The study also observed the presence of minor byproducts resulting from oxidation, such as cannabielsoin acid and cannabielsoin..

Hemp roots have a diverse chemical composition than the rest of the plant, with abundant triterpenes and phytosterols and no significant levels of cannabinoids. To explore the potential industrial uses of hemp roots, Kornpointner et al. (2021) analysed the bioactive composition and antioxidant activity of three different chemovars using both *in vivo* and *in vitro* methods. GC-MS analysis identified several triterpenes, phytosterols and aliphatic compounds, including some that were previously unknown in *Cannabis* root extracts. Friedelin and epifriedelinol were the predominant triterpenoids found, and their levels varied based on chemovar, harvest times, drying conditions and extraction methods.

2.6 Hypothesis

The development of environment friendly nanofertilizer synthesis is necessary for sustainable agriculture. Although the use of nanofertilizer has the potential to reform agriculture, we still have a limited understanding of its impact of fertilizer on human health and environment, which highlights the need for further research in this field. The current methods of synthesis often involve the toxic chemicals use, which can be harmful. There is a lack of research on the development of environmentally friendly nanofertilizer synthesis methods, which would reduce environmental pollution and provide a more sustainable option for agriculture. The effectiveness of nanofertilizers in enhancing plant growth and increasing yield has been demonstrated in several studies. Still, more research is needed to determine their long-term effects on soil health and biodiversity.

Punjab is located in Northwestern India and is primarily an agricultural region. The soil in Punjab is fertile and well-suited for agriculture. The outgrowth of *Cannabis* along the roadside in the North Indian state can have valuable and risky effects on the soil. *Cannabis* plants have deep roots that help stabilize the soil and prevent erosion. Additionally, they are effective in phytoremediation. However, on the other hand, *Cannabis* plants can also have adverse effects on the soil by competing with other plants for nutrients and water and possibly reducing biodiversity. Despite the wild *Cannabis* species being a potential hemp crop, there is a lack

of research on their feasibility and metabolic content variability. The potential industrial use of wild *Cannabis* in India is being explored, and there is limited research on its bioactive compounds and the effects of mineral nutrition on them. Some Indian states have started allowing hemp cultivation under strict regulations, but there is a lack of research on the impact of fertilizer and PGPR on hemp growth. Wild *Cannabis* can potentially be used for industrial purposes, but it is important first to understand the various bioactive compounds it contains and how mineral nutrition can affect them. Currently, there is a limited *Cannabis* research and development in India; however, there has been It is currently limited to research and development in India. However, there has been some movement towards exploring the potential of industrial hemp in India, particularly in the textile and construction industries. Some states have also started to allow hemp cultivation under strict regulations. This study focused on examining the benefit and drawbacks of the proliferation of hemp plants in the Punjab area. Additionally, the research investigated the effect of fertilizer and plant growth Promoting microorganism on hemp growth.

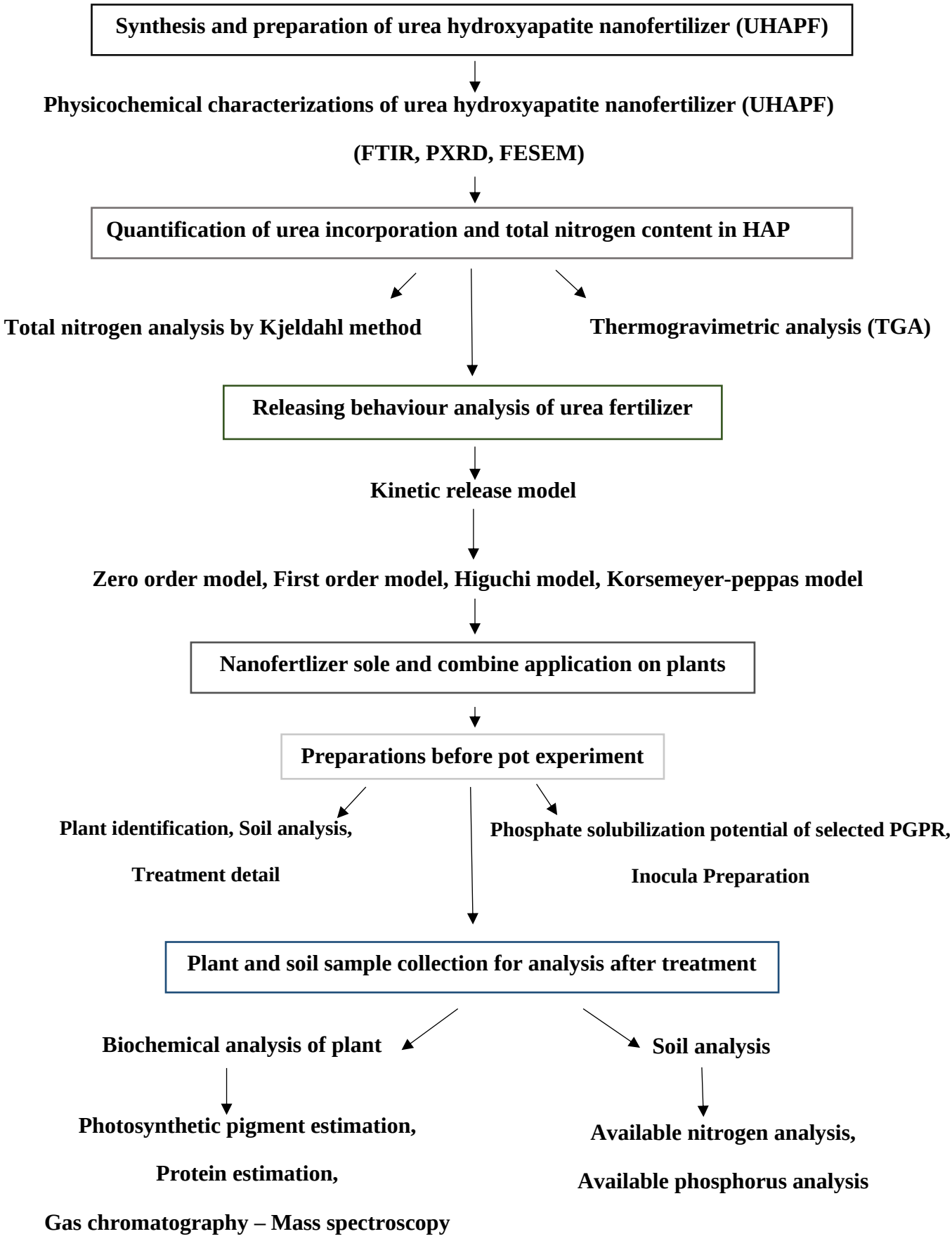
2.7 Objectives of the proposed work

1. Synthesis and characterization of urea based Nanofertilizer for efficient fertility

2. Analysis of release behaviour and kinetics study of urea based Nanofertilizer

3. In vivo study of utilization of Nanofertilizer and Phosphate solubilizing bacteria for efficient metabolite formation in *Cannabis sativa* L.

2.8 Schematic representation of the present study



CHAPTER 3. MATERIALS AND METHODS

3.1 Chemicals and reagents

All the chemicals, reagents, and media of analytical grade were obtained from Hi Media Laboratories Private Limited, Sigma Aldrich-Merck Specialties Private Limited and Loba Chemie, India. CTAB (Cetyltrimethylammonium bromide), $Ca(NO_3)_2.4H_2O$, and $(NH_4)_2HPO_4$ were purchased from Loba Chemie. The Microbial culture media such as nutrient media and nutrient broth were obtained from Hi Media Laboratories Private Limited, India. All aqueous solutions were prepared using distilled water.

3.2 Synthesis and Preparation of Urea Hydroxyapatite Nanofertilizer (UHAPF)

The adapted method developed by Elhassani et al., (2019) was used for synthesizing Urea-Hydroxyapatite (UHAPF) nanohybrids. This method comprises of two stages: the first stage is the synthesis of hydroxyapatite, and the second stage is the impregnation of the hydroxyapatite with urea **(Figure 3.1)**.

In the first stage, HAP was synthesized by combining two solutions. The first solution was prepared by dissolving 7.92 g of $(NH_4)_2HPO_4$ in distilled water and adding 1.82 g of Cetyltrimethylammonium bromide (CTAB), stirring until a clear solution was formed. The pH was then adjusted to 11 using ammonia. The second solution was prepared by dissolving 23.615 g of $Ca(NO_3)_2.4H_2O$ in distilled water. The second solution was added to the first solution while vigorously stirring to obtain a milky suspension. The suspension was heated for 5 hours at 80°C. After 24 hours, the precipitate was obtained from the solution through centrifugation with a water wash, followed by oven drying at 80°C to produce a dry solid powder.

In the second stage, the prepared HAP powder was sonicated in distilled water until a homogeneous mixture was obtained. The HAP mixture was then impregnated with urea solution and stirred for 15 hours. Following centrifugation, the mixture was dried and water washed to remove unreacted urea. The resulting UHAPF nanohybrids were obtained.

3.3 Physicochemical Characterizations of Urea Hydroxyapatite Nanofertilizer (UHAPF)

All the characterization was carried out at the Central Instrument Facility of Lovely Professional University (Punjab, India).

3.3.1 Fourier Transforms Infrared Spectra analysis (FTIR)

FTIR spectroscopy is a commonly used method for detecting and characterizing functional groups in pure compounds and mixtures. This type of infrared study relies on the measurement of the vibrational motion of atoms or molecules. It helps elucidate the structure of both organic and inorganic compounds. When compounds absorb energy, they exhibit specific infrared spectrum regions due to quantized vibrations. The

position of a particular absorption band is determined by its wave number. To determine the chemical bonding of synthesized samples, a PerkinElmer Spectrum IR Version 10.6.1 with Diamond ATR was used. The sample was pelletized using ten tons of pressure, and the spectra were recorded with a detector set at a resolution of 400 to 4000 cm^{-1}.

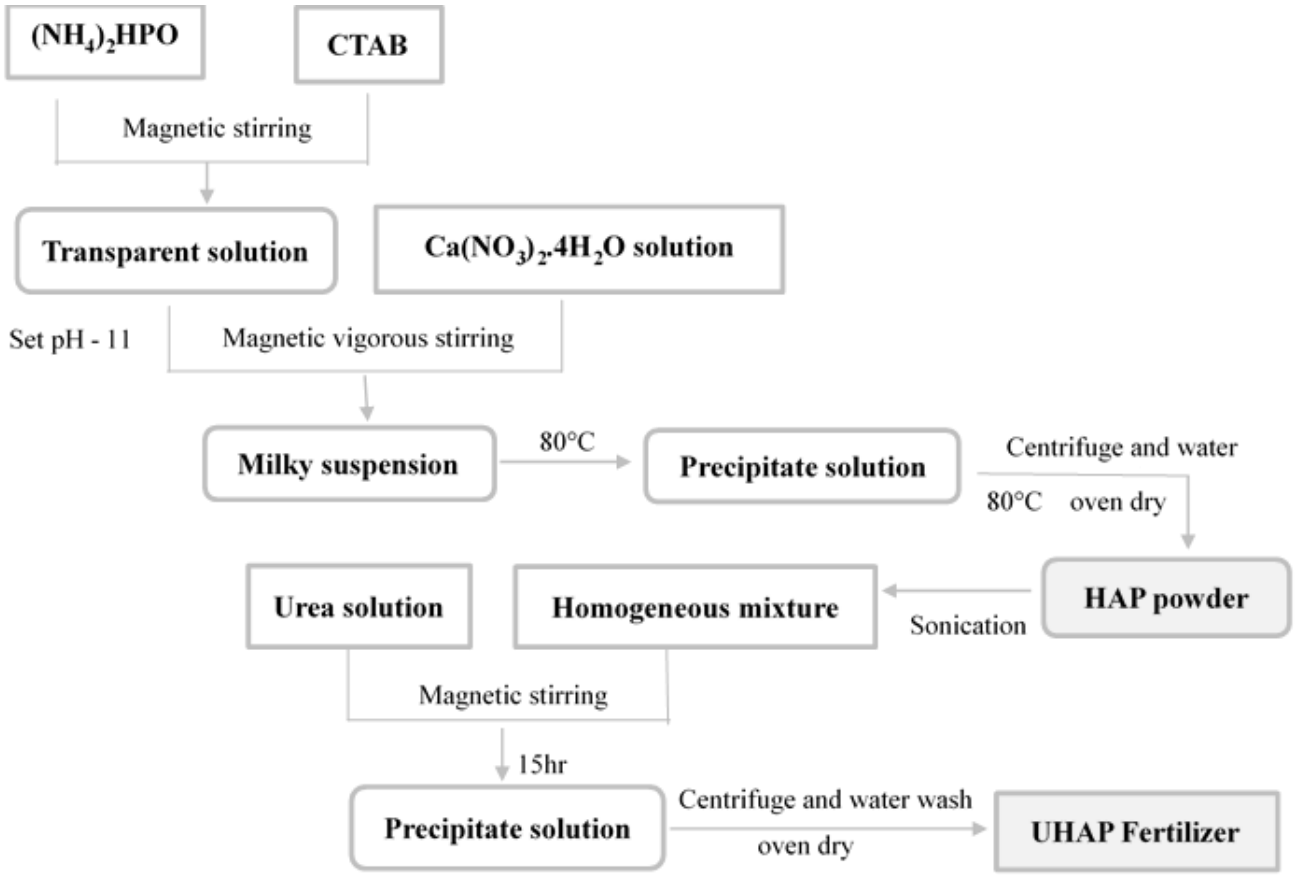


Figure 3.1. Schematic flow chart of the UHAP Fertilizer synthesis.

3.3.2 Powder X-Ray Diffraction (PXRD)

XRD is a non-destructive, multipurpose and highly precise analytical method used to detect and identify the crystal structure of a sample. The diffraction patterns produced when the waves interact with a structure with repeating distances are approximately the same as the wavelength. The crystallinity of the nanofertilizer was analysed using an X-ray diffractogram. Bruker D8 Advance XRD with CuKα radiation (k = 1.54060 Å) as an X-ray source operated at a voltage of 30 kV and current of 30 mA. The diffraction pattern was recorded in the range of 2θ = 10°-70°.

3.3.3. Field Emission Scanning Electron Microscopy (FESEM)

Field emission scanning electron microscopy has been performed to examine the morphological aspects. It provides a high-resolution image by scanning the sample surface with an electron beam. The high-energy electron beam strikes the sample surface atoms, generating secondary electrons, backscattered electrons, and

characteristic X-rays that reveal information on the sample surface topography and composition. The SEM studies were carried out using a JEOL Field Emission Scanning Electron Microscope (FESEM). The images were recorded in the secondary electron mode, and the sample was coated with high-purity gold.

3.4 Quantification of Urea Incorporation and Total Nitrogen Content in HAP

3.4.1 Total Nitrogen Analysis by Kjeldalh Method

To determine the total nitrogen content in synthesized urea hydroxyapatite fertilizer Kjeldahl method was carried out by the Kjeldahl apparatus (Kjeldahl, 1883). The method is widely used to determine protein content in foodstuff fertilizer and feed samples. Total Nitrogen estimation was done in the School of Agriculture at Lovely Professional University. The Kjeldahl method involves a three-step process: digestion, distillation, and titration.

Digestion- To digest the sample into its constituent components, the sample (0.3 g) was mixed with sulfuric acid (10 ml) and a catalyst (0.3 g) ($CuSO_4$ + potassium sulphate). The sulphuric acid helps to digest the sample to reduce nitrogen to ammonia. Afterwards, the tubes were placed in the digestion apparatus and covered with a scrubber. Temperature was then increased up to 420℃ for the digestion process. The greenish colour appearance indicates the completion of the digestion process.

Distillation- After digestion, 20 ml water was added to each tube and fit into the distillation unit. The boric acid (4%) in a 250 mL conical flask was placed under a condenser then sodium hydroxide (40%) was added to the digested sample to run the distillation process. The ammonia was penetrated in the boric acid solution. The mixture was then distilled to convert nitrogen to ammonium sulphate.

Titration- The ammonium sulphate is then quantified using a colorimetric method to determine the amount of nitrogen in the sample. 1 ml of an indicator (Methyl red + bromocresol) was added into the ammonia distillate. The sample was then titrated with a standard 0.1N sulfuric acid until a change in colour from green to pinkish occurred. The volume of acid used during the titration was recorded.

$$\text{Nitrogen percentage} = \frac{14.01 * 0.1N * (TV - BV) * 100}{W * 1000}$$

Where, 14.01- Molecular weight of ammonia,

0.1N- Titration solutions Normality,

TV- Titer value,

BV- Blank value,

W- sample weight.

3.4.2 Thermogravimetric analysis (TGA)

To study the composition, thermal stability and degradation behaviour of the material thermogravimetric analysis was conducted. The sample was heated in a controlled atmosphere to measure the changes in the weight of the sample. Thermal stability analysis was carried out on Perkin Elmer TGA 400 apparatus. A 6mg sample was heated from upto 700°C at a rate of 10°C/min with an airflow, to perform the analysis. The difference in weight of the sample was continuously recorded.

3.5 Releasing Behaviour Analysis of Urea Fertilizer

The study of the nitrogen release behavior of UHAPF in water, the experiment was conducted using a vertical column set up over 30 day's period. The vertical column setup consisted of a column with a cotton-covered tap at the end. 1 g of Urea and UHAPF sample was placed in the column and 50 mL of water was added. Subsequently, the elute was collected at regular time interval, further elute was centrifuged at 5000 rpm for 15 minutes. The supernatant was then analyzed by colorimetric para-dimethyl-amino-benzaldehyde method to estimate the total amount of nitrogen released from the nanofertilizer.

The para-dimethyl-amino-benzaldehyde method is a widely used colorimetric method for the analysis of nitrogen-containing compounds, including urea (Knorst et al., 1997). For nitrogen release analysis, stock solution was prepared with 20 g/L para-dimethyl-amino-benzaldehyde and 4 mL of 2 mol/L H_2SO_4. From stock solution, 2 mL was added to 0.2 mL of urea solution (dm^{-3}). After 15 minutes, the absorbance was measured at 422 nm and the concentration of urea was calculated using a standard curve. A standard curve was generated using known concentration of urea by calculating the unknown concentration of urea in the solution. UV–vis spectra of synthesized urea were recorded by diluting the samples with double distilled water (DDW), using a Perkin Elmer spectrophotometer (model- LI-2800 Ex).

3.5.1 Kinetic Release Models for Evaluating the Urea Release Behaviour

Kinetic release model is a common method used in pharmacological study to understand the drug releasing pattern. Various studies have been examined to understand the in vitro release kinetic of several drug loaded on carriers to investigate compound entrapment efficacy. In drug release studies, different models are used to analyze the mechanism of drug release, similar studies have been carried out in the present work for analysis of nanofertilizer.

Different kinetic release models, such as the zero-order, first-order, higuchi and Korsmeyer-Peppas models, were used to understand urea releasing behavior over time (**Table.3.1)**. The zero-order model describes the

release rate as independent of concentration, while the first-order model relates the release rate to concentration. Higuchi's model, based on Fickian diffusion, explains drug release from insoluble matrices as a square root of time. The Korsmeyer-Peppas model, developed by Korsmeyer and Peppas, is commonly used to study the release kinetics of drug-loaded polymeric nanoparticles.

Table.3.1 Mathematical model used to study urea releasing behavior

S.N.	Kinetic models	Mathematical Expression	Parameters
1	Zero order model	$\mathbf{Q_t = Q_0 + K_0 t}$	Q_t: the amount of urea dissolved in time t Q_0: initial concentration of urea in the solution K_0: zero-order release constant
2	First order model	$\mathbf{Q_t = Q_0 e^{K_1 t}}$	K_1: first-order release constant
3	Higuchi model	$\mathbf{Q_t = k_H t^{½}}$	K_H: Higuchi dissolution constant
4	Korsmeyer Peppas model	$\mathbf{M_t/M_\infty = k_{KP} t^n}$	M_t/M_∞: the fraction of release of time k_{KP}: the release rate constant n: the diffusion exponent related to mechanism of drug release

3.6 Plant Growth Promoting Rhizobacteria Strain: Phosphate Solubilization Potential

In this study, two bacterial strains, *Bacillus megaterium* MTCC1684 and *Pseudomonas aeruginosa* MTCC7453 were selected for evaluation of their ability to solubilize insoluble phosphorus. These specific strains were acquired from the Microbial Type Culture Collection and Gene Bank (MTCC) located in Chandigarh, India. The bacterial strains were cultured on a nutrient media. The composition of the medium included peptone (5), HM peptone B (1.5), yeast extract (1.5), sodium chloride (5) and agar (15) g L^{-1}. The composition was dissolved in 1 litre distilled water with 7.4±0.2 pH to provide a suitable growth medium for bacteria.

The NBRIP media was then used to screen the solubilizing capacity of the bacterial strains from insoluble phosphate, using tricalcium phosphates as the phosphorus source (Gupta et al., 1994). The NBRIP media contained glucose (10), calcium phosphates (5), potassium chloride (1), ammonium sulphate (0.5), magnesium

sulphate (0.1), manganese sulphate (0.01), yeast extract (0.5), bromocresol purple (0.1) and agar (20) in g L^{-1}. Tricalcium phosphates were used as the source of insoluble phosphorus. The bromocresol purple served as pH indicator. The indicator changed color from purple to yellow as the pH decreased. The indicator was yellow below pH 3.8 and purple above pH 5.4, providing a visual representation of the growth and acid production of the bacteria.

3.7 Plant Material and Growing Condition

The plant was collected from the roadside and identification was carried out at CSIR-IIIM Jammu (Council of Scientific and Industrial Research - Indian Institute of Integrative Medicine, Jammu) **(Figure.3.2)**. The herbarium was then submitted to Janaki Ammal Herbarium, a national referral facility and was given accession number - 26832.

Figure.3.2 Herbarium of *Cannabis sativa* L. (Accession number – 26832)

Soil analysis before a pot experiment is an important step in the field experiment. The soil texture, pH, electrical conductivity, essential plant nutrients, soil moisture content and pH affect the nutrient availability in the soil. The soil was collected after treatment from the surface (0-15 cm) and analysis was carried out at Punjab Agriculture University, Ludhiana. The analysis estimated the pH at 8.1 with the mineral contents in their available form (kg $acre^{-1}$): P;7.8, potash;92, Zn;0.83, Fe;7.51, Mg;6.71, and Cu;0.39. Pots were filled with 5 kg of unsterilized field soil.

Treatments	**Symbol**
Control	**C**
Urea Fertilizer (UF)	**T_1**

Synthesized Urea Hydroxyapatite fertilizer (UHAPF)	**T_2**
B1 - *Bacillus megaterium*	**T_3**
B2 – *Pseudomonas aeruginosa*	**T_4**
UHAPF+B1 (Synthesized Urea Hydroxyapatite fertilizer + *Bacillus megaterium*)	**T_5**
UHAPF+B2 (Synthesized Urea Hydroxyapatite fertilizer + *Pseudomonas aeruginosa*)	**T_6**

Seed collection-

The *Cannabis sativa* L. seed was obtained in the summer season of 2020, specifically in the month of July. At the time of collection, it possessed the potential to grow into a healthy *Cannabis sativa* L. plant. The seed was stored under optimal conditions to preserve its viability until the next growth cycle. In January 2021, it was planned to initiate the germination process to allow the seed to sprout.

Seed germination -

Cannabis sativa seeds were collected from a single plant that was suitable for germination. To initiate the germination process, several layers of sterile paper towels were moistened with distilled water to create a suitable substrate for the seeds. The moistened paper towels were placed on a clean and sterile Petri dish, providing an optimal environment for seed germination. The *Cannabis sativa* L. seeds were evenly distributed on the moistened paper towels, ensuring adequate spacing between them. The paper towels were then folded over the seeds, creating a dark and moist environment. The prepared setup was transferred to a temperature-controlled environment set to a consistent temperature range of 70-85°F (21-29°C). This temperature range is known to promote optimal germination conditions for *Cannabis sativa* L. seeds. Additionally, the location was kept dark to prevent any potential negative effects of light exposure on the germination process. Over the course of several days, the seeds were monitored for signs of germination. Once the radicle (root) became visible, indicating successful germination, the seedlings were carefully transferred to a suitable soil medium. During this process, the radicle was positioned downward, and the seedlings were lightly covered with the medium to facilitate further growth while providing necessary protection. To ensure healthy and vigorous seedling development, appropriate growing conditions were provided, including adequate light intensity, temperature and moisture. The seedlings were gradually acclimated to their desired growing environment to minimize any potential stress and promote successful adaptation (Moon et al., 2020).





The pot experiment was conducted at Lovely Professional University. The site selected for the treatment of plants was the agriculture farms at Lovely Professional University Phagwara (Punjab, India), located at 31˚ 14 ˙N and 75˚ 42 ˙E longitude and an altitude of 249 meters above the MSL. Cold and extremely hot conditions determine the climate. The experiment was conducted under control conditions at a mean temperature of 24°-28℃. The microbial strains were cultured in a sterilized nutrient broth medium at 30℃ for 48 hours at 120 rpm on a shaker to prepare the inoculum. The composition of the broth was same as the nutrient medium without agar g L^{-1}(peptone (5), HM peptone (1.5), Yeast extract (1.5), sodium chloride (5)) dissolved in 1 litre distilled water with pH of the broth adjusted to 7.4±0.2. The bacterial culture was harvested at a concentration of 10^8 CFU/mL to ensure sufficient bacteria. The peat was used as a carrier to enhance the viability and effectiveness of the microbial culture (Novinscak & Filion, 2020). Hemp crop is a fast-growing annual plant with a growing season of 4-6 months. The buds were fully matured 14 weeks after flowering began. The crop was grown in a warm, temperate climate, green shade with moderate humidity.

3.8 Biochemical Analysis

3.8.1 Photosynthetic Pigment Quantification

The photosynthetic pigment quantification in leaves (chlorophyll *a*, chlorophyll *b*, Total chlorophyll and carotenoids) was performed by the method given by Arnon (Arnon, 1949) and Lichenthaler (Lichtenthaler & Wellburn, 1983), respectively. Fresh leaves were collected from both treated and control plants, cleaned, and weighed for analysis. 0.1 gram of fresh leaves were homogenized in 80% acetone and centrifuged at 6000 rpm for 15 min. The supernatant of centrifuged extract sample was measured for its optical density (OD) at different sample wavelengths 663 nm, 645 nm for chlorophyll, and 480 nm, and 510 nm for carotenoid, using UV-vis spectrophotometer (model- LI-2800 Ex). The photosynthetic pigment estimation was calculated using the given formula

$$\textit{Chlorophyll a}\ (\text{mg/g FW}) = \left[(Abs663 * 12.7) - (Abs645 * 2.69) * \frac{V}{1000 * W}\right]$$

$$\textit{Chlorophyll b}\ (\text{mg/g FW}) = \left[(Abs645 * 22.9) - (Abs663 * 4.68) * \frac{V}{1000*W}\right]$$

$$\text{Total chlorophyll content (mg/g FW)} = \left[(Abs645 * 22.2) + (Abs663 * 8.03) * \frac{V}{1000*W}\right]$$

$$\text{Total carotenoid content (mg/g FW)} = \left[(7.6 * Abs480) - (1.49 * Abs510) * \frac{V}{d*W*1000}\right]$$

3.8.2 Protein Estimation

The protein of *Cannabis* leaves was determined using the method given by Lowry et al. (1951). The Lowry protein estimation uses the biuret reaction and Folin-Ciocalteu reagent to detect proteins. The presence of proteins in a sample was detected based on the copper ions reaction with peptide bonds in proteins, resulting in the formation of a violet-coloured complex. Bovine serum albumin (BSA) used as a standard and its absorbance is measured at 660 nm to estimate the amount of protein present in a sample. The fresh *Cannabis* leaves were collected and cleaned by water to remove all the dust. Then, 0.1 gram of leaves was homogenised in a pre-chilled mortar and pestle using 3 ml of 100mM potassium buffer with a pH of 7.0. The material was subjected to centrifugation using a cooling centrifuge at 13,000 rpm for 20 minutes at 4℃. The resulting supernatant was collected and utilized for protein analysis.

3.9 Soil Analysis after Treatment

Available nitrogen and available phosphorus analysis experiment was conducted at School of Agriculture, Soil science Laboratory, Lovely Professional University, Punjab.

3.9.1 Available Nitrogen ($KgNha^{-1}$)

The alkaline potassium permanganate (KMnO4) method was used to estimate available nitrogen in the soil (Asija & Subbiah, 1956). Alkaline $KMnO_4$ oxidizes the organic matter present in soil and ammonia evolved from organic matter, which is then trapped in boric acid mixed indicator solution. 20gm of air-dried soil was taken in a distillation tube with 20 ml Distilled water, 100 ml $KMNO_4$ (0.35%) and 100 ml NaOH (2.5%). Instantly the sample was distilled in Kjel plus auto distillation unit. The distillate was collected in a flask containing 20 ml of boric acid. Due to the presence of nitrogen, the solution turns green in colour. Further, the sample was titrated with 0.1N sulphuric acid (H_2SO_4). The burette reading was recorded after titration. The amount of ammonia trapped is calculated by given formula.

$$\text{Nitrogen percentage} = \frac{14.01*0.1N*(TV-BV)*100}{W*1000}$$

Where, 14.01- Molecular weight of ammonia,

0.1N- Titration solutions Normality,

TV- Titer value,

BV- Blank value,

W- sample weight.

3.9.2 Available Phosphorus ($KgPha^{-1}$)

The available phosphorus content of the soil was determined by the method described by Olsen's method (Olsen, 1954). A sample containing 2.5 g of dried soil and 0.5 g of Darco G-60 activated carbon was extracted with 50 mL of 0.5 M $NaHCO_3$ by adjusting the pH to 8.5 with 10% NaOH and shaking the sample on a mechanical shaker for 30 minutes. The filtrate was collected after filtering the sample and adding 2-3 drops of nitrophenol indicator, resulting in a yellow color. To further decolorize the yellow color, the solution was acidified with 5N H_2SO_4 until the pH reached 5. Further ascorbic acid (4mL) solution was added to the flask and the volume was made up to a certain level. The presence of phosphate was indicated by the development of a blue color, the color intensity is directly proportional to the quantity of phosphate present in the sample.

Available phosphorus (kg/ha) = ppm of P calculated from standard curve × dilution factor × 2.24

3.10 Identification of Bioactive Compounds through GC-MS Analysis

3.10.1 Extraction Techniques

The hemp leaves were collected after 14 weeks, washed thoroughly, air dried and ground to fine powder, which were stored for further extraction and analysis. To analyse the bioactive compounds in hemp leaves traditional maceration extraction technique was used. In the maceration extraction, methanol was used as a solvent. 1 g of dried leaf powder was mixed with 100 ml of methanol in a 250 ml Erlenmeyer flask and agitated on the shaker at room temperature for 24 hours. The resulting solution was filtered and then centrifuged for 10 minutes at 5000 rpm, after which the supernatant was collected and the solvent was evaporated. The resulting extract was subsequently stored in a closed container at a temperature of 4℃ (Pande & Chanda, 2020).

3.10.2 Instrument Condition

The extracted compound was further analysed by gas chromatograph-mass spectrometer (GC-MS). It is a highly sensitive and standard technique for the separation and identification of individual bioactive compounds. The GCMS-TQ8040 NX gas chromatograph coupled with a mass spectra detector was used. The compound was separated using an SH-RXi-5Sil MS (30 meters × 0.32 mm × 0.25 µm df) capillary column. The initial column temperature was set to 40℃, which was gradually increased to 250℃ at a rate of 7℃/min

and held at this temperature for 3 minutes. Helium gas was used a carrier gas (flow rate of 1 mL/min). The Q3 scan acquisition mode was applied in the mass-to-charge (m/z) range of 40 to 500. Compound identification was conducted using the National Institute of Standards and Technology (NIST) Web Book spectral database. The outcomes were stated as the relative percentage of peak area compared to the total peak percentage (Pellati et al., 2018).

3.11 Statistical Analysis

The data collected were analysed using a statistical method one-way Analysis of Variance (ANOVA) with a significant level of $P < 0.05$. Duncan's multiple comparison test was used for further analysis. The statistical analysis was conducted using the IBM SPSS version 22 software. Regression equations and coefficients were calculated to determine the release longevity. Origin Lab software was employed to interpret the characterization data. Figures and tables present the mean values of three replicates. ChemAxon Marvin Sketch software was utilized to draw the chemical structures.

CHAPTER 4. RESULTS AND DISCUSSION

The environment-friendly slow-release fertilizer was synthesized by encapsulation of urea-modified hydroxyapatite composite called urea hydroxyapatite fertilizer (UHAPF) **(Figure 4.1)**. It was characterized by various techniques such as FTIR, XRD, TGA and FESEM, along with its investigation of urea release behaviour. The Plant Growth Promoting Bacteria (PGPB) *Bacillus megaterium* and *Pseudomonas aeruginosa* were selected based on their efficiency as a phosphate solubilizer and further exerting their synergistic effect with UHAPF on *Cannabis sativa* L. plant for simultaneous nitrogen and phosphorous availability.

Figure 4.1 Synthesized urea hydroxyapatite (UHAPF) material

4.1 Physicochemical Characterization

4.1.1 Fourier Transforms Infrared Spectra Analysis (FTIR)

The functional group of UHAPF was investigated using FTIR analysis. The FTIR spectra of both UF and Urea-Coated Hydroxyapatite UHAPF are depicted in **Figure No. 4.2**. The O=C-NH_2 functional group, including N-H, C=O and -CN, exhibited spectral absorption peaks at 3428.82 cm^{-1}, 1673.66 cm^{-1} and 1148.75 cm^{-1} for conventional urea fertilizers. Furthermore, detecting two absorption bands at 3428.82 cm^{-1} and 1588.20 cm^{-1} corresponds to the deformation and stretching vibrations of the N-H bond in pure urea, respectively (Roshanravan et al., 2014).

The interface interaction between urea and hydroxyapatite (HAP) was examined by observing the spectral band shifting or changes in the spectral band. The detection of two sharp and intense absorption bands at 1088.00 cm^{-1} and 1020.73 cm^{-1} indicated the phosphate group PO_4^{3-} stretching. The P-O deformation vibration

was detected at 598.59 cm^{-1}, while the 962.38 cm^{-1} indicated P-O asymmetrical and symmetrical stretching vibrations. The peak at around 3403.20 cm^{-1} in the spectrum of HAP indicates the stretching vibrations of hydroxyl groups.

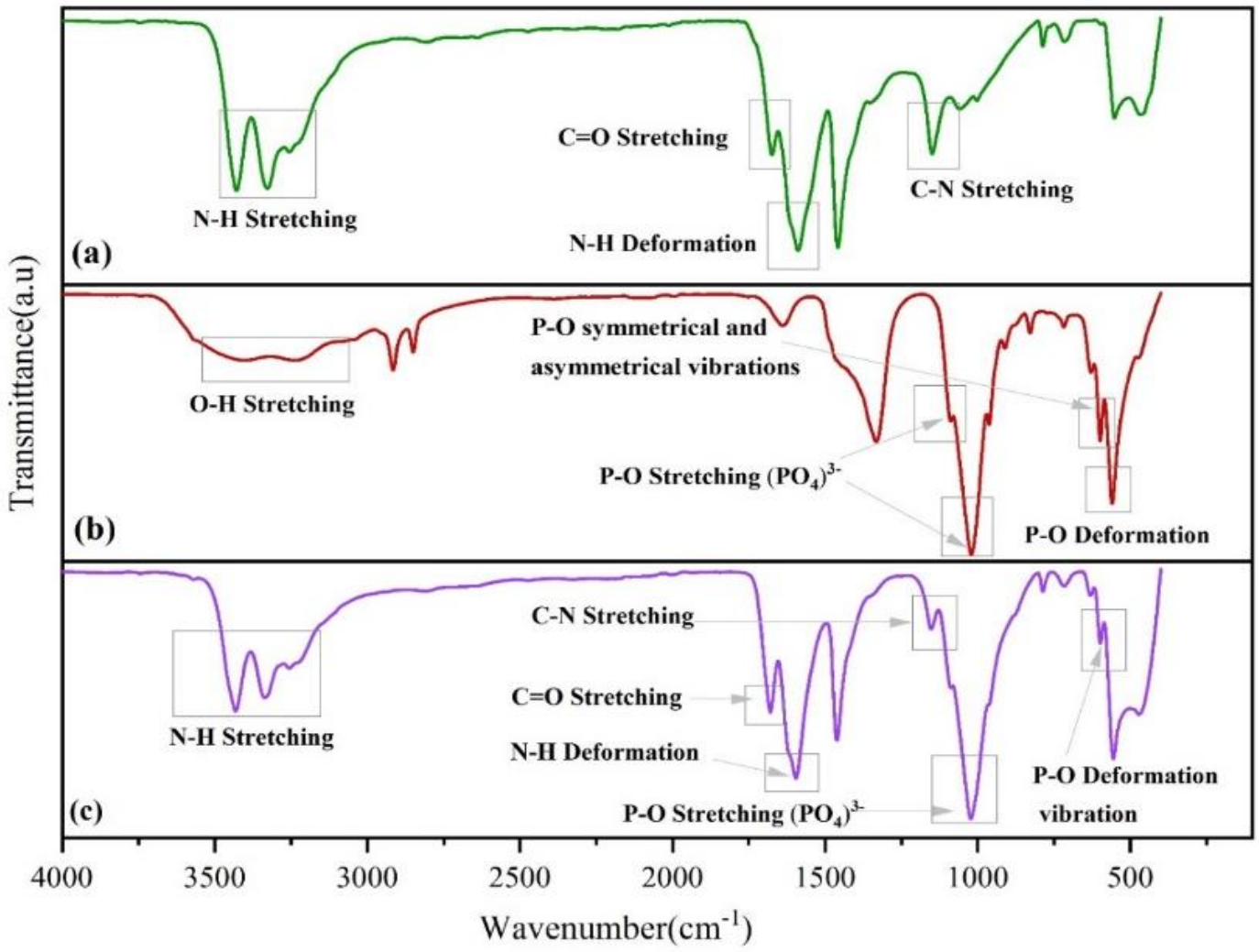


Figure No. 4.2 FTIR spectra of (a) UF, (b) HAP and (c) UHAPF.

Campos et al. (2011) explored the biological properties of 3-D Hydroxyapatite/Collagen Scaffolds and observed phosphate bands (PO_4^{3-}) at 473, 560, 604, 961and 1030 (cm^{-1}). Further, Gheisari et al., (2015) confirmed the functional group of the HAP bond, specifying that the PO_4^{3-} group produces strong bands at 560 and 600 cm^{-1} as well as at 1000-1100 cm^{-1}.

After urea incorporation in hydroxyapatite, new spectral absorption bands were observed at 3428.82 cm^{-1} and 1588.20 cm^{-1} in the spectra, which correspond to the N-H bond stretching and deformation vibrations in pure urea, which was then shifted to higher frequencies in the spectrum of UHAPF at 3431.64 cm^{-1} and 1595.14 cm^{-1} absorption peak **(Table 4.1)**. This shift results from the strong hydrogen bonding between the O-H groups of HAP and N-H of urea, suggesting a strong interaction between the two materials (Madusanka et al., 2017).

Table-4.1 FTIR spectra of Urea (UF), Hydroxyapatite (HAP) and UHAPF

Wavenumber cm^{-1}	**Urea (UF)**	**Wavenumber cm^{-1}**	**Hydroxyapatite (HAP)**	**Wavenumber cm^{-1}**	**Urea-hydroxyapatite Nanofertilizer (UHAPF)**
3428.82, 1588.20	N-H stretching/ N-H deformation			3431.64, 1595.14	N-H stretching/ N-H deformation
1673.66	C=O stretching			1678.90	C=O stretching
1148.75	C-N stretching			1151.76	C-N stretching
		1088.00, 1020.73	P-O stretching of PO_4^{3-}	1022.38	P-O stretching of PO_4^{3-} bond
		598.59	P-O deformation of PO_4^{3-}	598.18	P-O deformation of PO_4^{3-} bond
		3403.20	O-H group	3335.56	O-H group

4.1.2 Powder X-Ray diffraction (PXRD)

The powder X-ray diffraction (PXRD) patterns of synthesized HAP and UHAPF are shown in **Figure No.4.3**. The XRD pattern of HAP shows a well-defined hexagonal crystalline structure, which matches with the pattern reported in the JCPDS (Joint Committee on Powder Diffraction Standards) card number 01-076-694 with a monoclinic crystal system. The XRD pattern of the immobilized urea is the same as that of pure urea, suggesting that the immobilization process of urea on the HAP did not change the crystal structure of urea and no peak shifting was observed. Urea immobilized on the HAP support without disrupting the crystal structure of urea, indicating the successful incorporation of urea on the HAP.

Table No. 4.2 X-Ray Diffraction Analysis of UF and UHAPF Lattices

2θ HAP	**2θ UHAPF**	**Lattice (*hkl*)**
10.8	10.8	100
16.8	16.8	101
21.7	21.7	200
25.8	25.8	002
32.8	32.9	300
35.4	35.4	301

The characteristic reflections of urea indexed with 2θ values of 22.2, 24.6, 29.3, 35.5, 41.5, and 31.6 degrees, which are attributed to crystal planes (110), (101), (111), (210), (102), and (200), respectively, as reported in the JCPDS file N°00-031-1979 **(Table 4.2)**. The peak with the highest intensity and most prominent located at a 2θ value of 22.2° corresponds to the (110) plane of the tetragonal phase and matches with the JCPDS file N°00-031-1979 (Bakshi et al., 2021).

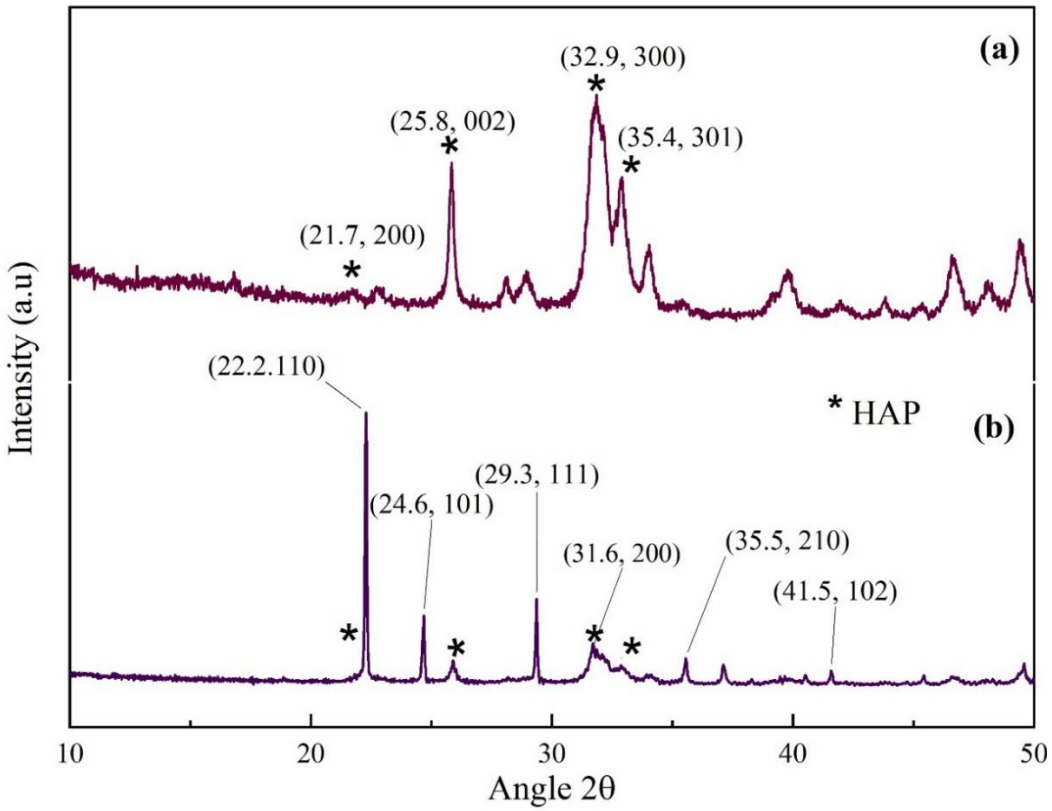


Figure No.4.3 PXRD patterns of (a) HAP and (b) UHAPF. Here * is Hydroxyapatite

Notably, no significant shift was observed in the peak positions of HAP before or after the impregnation with urea in the synthesis of UHAPF. Based on Bramhe et al. (2015) study on the hydroxyapatite synthesis, the result indicates the similar card numbers (01-076-694) or XRD pattern when hydroxyapatite synthesized by the hydrothermal and solvent combustion methods. The study suggested that adding ammonia solution in the solution combustion method could be explored as a potential approach for synthesizing hydroxyapatite for coating applications. The XRD result confirms the effective immobilization of urea on the HAP support without disrupting the crystal structure of HAP.

4.1.3 Field Emission Scanning Electron Microscopy (FESEM)

The field-emission scanning electron microscopy (FESEM) imaging of the synthesized urea-hydroxyapatite nanohybrid fertilizer (UHAPF) showed its morphological aspect as shown in **Figure No. 4.4.** The image showed hydroxyapatite revealed a rod-shaped structure and hexagonal crystal form. The rod shape provides a huge surface area, which can enhance the reactivity of the material and increase its effectiveness as a fertilizer.

The rod-shaped morphology of the hydroxyapatite component in UHAPF is an important factor in its potential application as a fertilizer.

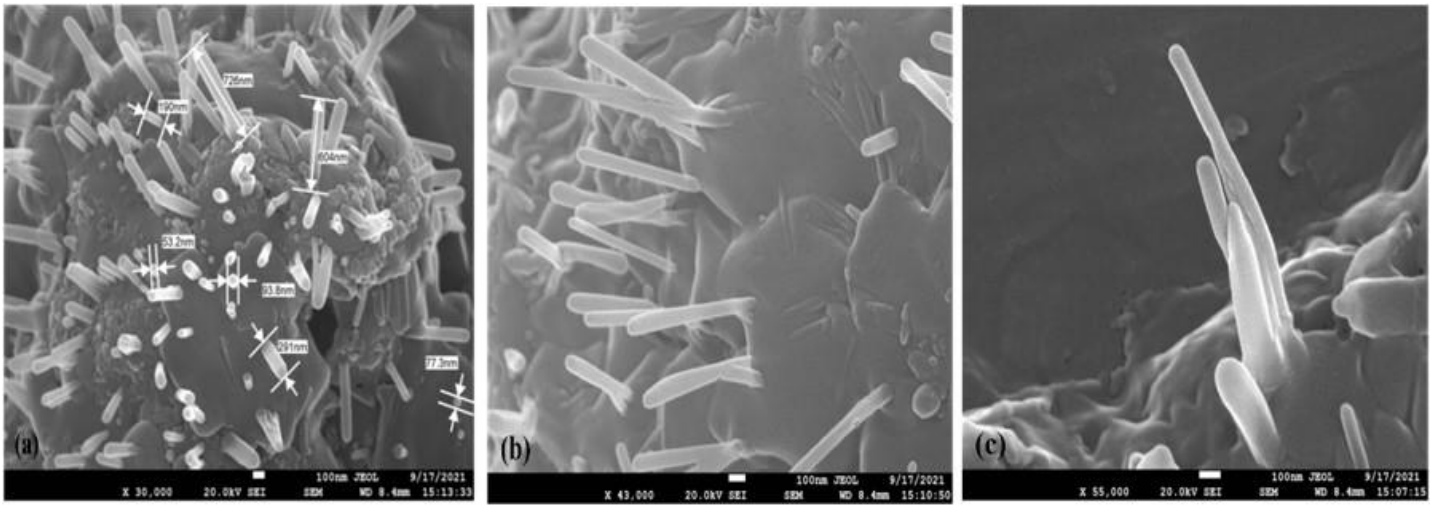


Figure No.4.4. FESEM images of Nano synthesized UHAP fertilizer. Here (a) is representing the particle size of urea fertilizer incorporated into HAP (b and c) representing formation of rod shape synthesized UHAP fertilizer

The observed hexagonal crystal form of the hydroxyapatite component is also important in the context of its stability and durability and also reported in literature (Kottegoda et al., 2017). Aghayan & Rodríguez, (2012) researched bi-phasic calcium phosphates and observed that rod-shaped hydroxyapatite nanoparticles formed when the ratio of urea to HNO_3 is close to 1. This morphology is attributed to the complete oxidation of urea, which generates more energy during the process. The hexagonal structure provides mechanical stability to the material, crucial for its long-term effectiveness as a fertilizer. The results obtained from the FESEM imaging of UHAPF provide important information on the morphology of the hydroxyapatite component, which is a key factor in its potential application as a fertilizer.

4.2 Investigation of Total Amount of Urea (Nitrogen) Incorporated in HAP

4.2.1 Total Nitrogen Estimation (Kjeldahl Analysis)

Total nitrogen was calculated using Kjeldahl method. The total nitrogen content of pure urea is 32.72%; upon analysis, it was found that UHAPF contains 13.54% nitrogen, which concludes that nearly half of urea is incorporated in the HAP.

4.2.2 Thermogravimetric Analysis (TGA)

The thermogravimetric analysis of the UF and UHAPF is depicted in **Figure No. 4.5**. The thermogravimetric analysis of the synthesized UHAPF was conducted to examine the total amount of urea incorporation in HAP. Urea has a complicated thermal behaviour or, in other words, is a thermally unstable compound (Chen et al.,

2018). It undergoes decomposition before reaching its melting point of 132.5℃, ultimately leading to complete oxidation at 400℃. The sample was heated at a constant temperature until it reached 700℃, after being maintained at this temperature for 1 minute. Approximately 52% of the UHAPF remained after the thermal treatment indicating that 48% of the urea had been efficiently incorporated in HAP. Additionally, the thermal degradation of urea was increased when supported by HAP, it suggests that HAP serves not only as a support but also as a catalyst for the thermal degradation of urea.

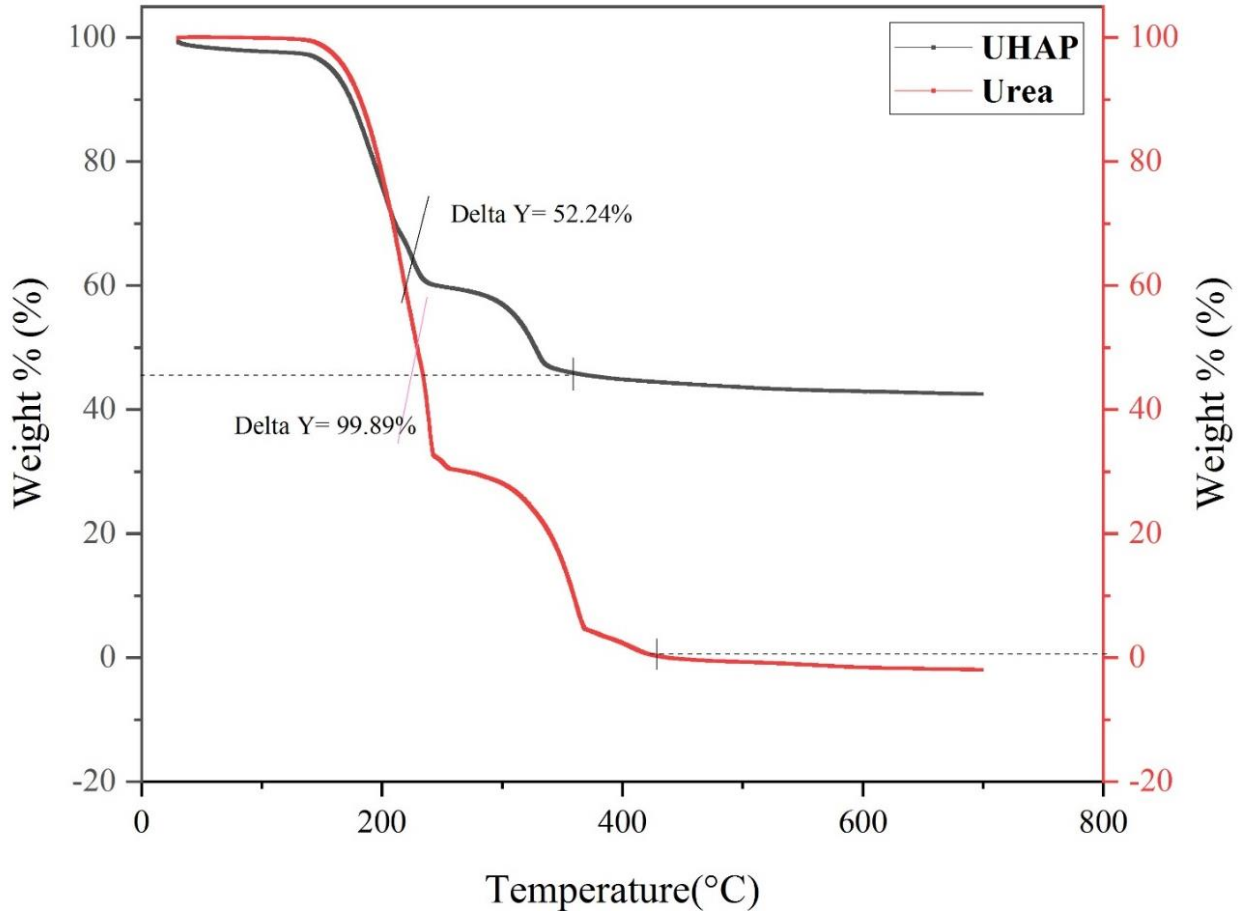


Figure No. 4.5 Thermogravimetric curve of Urea fertilizer and synthesized UHAP fertilizer.

4.3 Releasing Behaviour Result

The nitrogen leaching estimation over 30 days provided a preliminary indication of a novel slow-release formulation of synthesized UHAPF compared to pure urea. UHAPF nitrogen release rate strongly depends on the coating material and formulation. The UHAPF in an aqueous medium showed a much slower release rate as compared to pure urea depicted in **Figure No. 4.6** The moderately strong bond of HAP and urea formed through its amine and carbonyl groups confirms that UHAPF meets the plant nitrogen demand (Gunaratne et al., 2016).

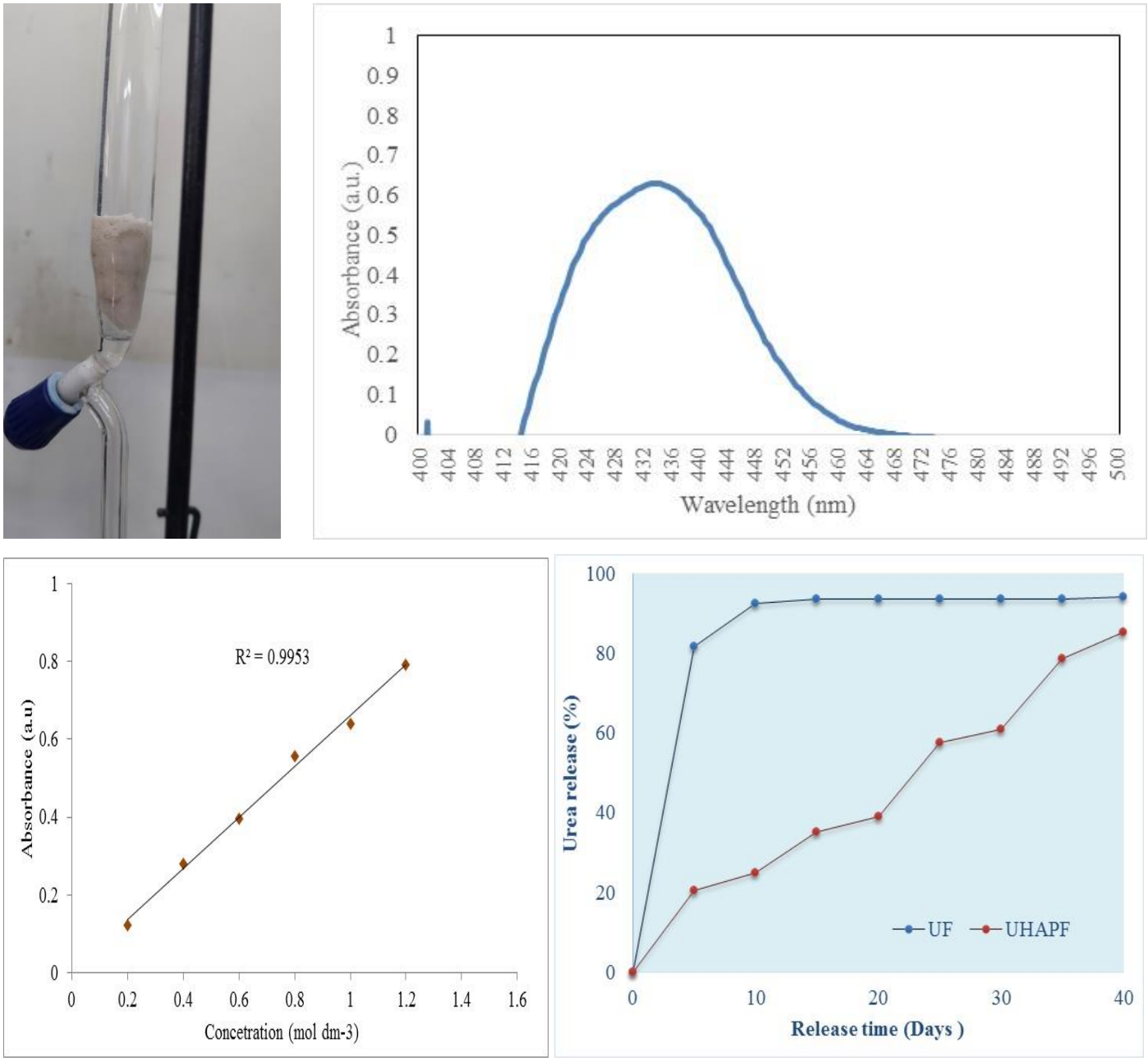


Figure No. 4.6. Urea releasing behaviour (a)Vertical column Setup, (b) UV–vis absorption spectrum (c) Standard curve of the Urea using para dimethyl aminobezaldehyde method and (d) The release rate of urea fertilizer (UF) and UHAP fertilizer (UHAPF) as a function of time at 28℃.

4.3.1 Kinetic Release Models

The release profile of urea hydroxyapatite nanofertilizer (UHAPF) were analysed by using kinetic release models based on the best fitting including zero order release model, first order release model, Higuchi model and Korsmeyer- Peppas model. The mean square error (MSC) values represent the average squared difference between the predicted and actual values of the release rate. A lower MSC value indicates a better fit between the model and the experimental data. A negative MSC value indicates that the model is worse than predicting the average value of the dependent variable. The akaike information criterion (AIC) measures relative quality

of model, with lower AIC values indicating a better model fit. The AIC and MSC are statistical measures used to compare and select the best-fit model from a set of candidate models. A good model should have a low AIC and MSC value **(Table 4.3)**.

Table 4.3 -Table showing the results of different kinetic release models.

Kinetics Model	Constant			R^2		MSC		AIC	
		UF	UHAPF	UF	UHAPF	UF	UHAPF	UF	UHAPF
Zero order	k_0	3.293	2.171	-0.6383	**0.9729**	-2.7911	3.0068	86.9204	48.2646
First order	k_1	0.315	0.034	0.9679	0.9400	1.1400	2.2123	51.5407	55.4150
Higuchi	k_H	18.803	11.435	0.4529	0.8933	-1.6943	1.6364	77.0493	60.5985
Korsmeyer-Peppas	k_{KP}	78.703	3.228	**0.9945**	**0.9785**	2.6754	3.0139	37.7220	48.2008
	N	0.053	0.884						

The table includes the constants (k_0, k_1, k_H, k_{KP}) and their corresponding values for UF and UHAPF, as well as the R-squared (R2), mean square error (MSC), and akaike information criterion (AIC) for each model.

In the zero-order kinetic release model, the constant values "k_0" for UF and UHAPF are 3.293 and 2.171, respectively. The R^2 values for UF and UHAPF are -0.63 and 0.97, respectively. The negative R^2 value for UF suggests that the model may not fit this system well. This model assumes that the rate of drug release is constant over time. On the other hand, the high R^2 value for UHAPF indicates that the zero-order kinetic model is a good fit for this system. The MSC values for UF and UHAPF are -2.79 and 3.00, respectively. The AIC values for UF and UHAPF are 86.92 and 48.26, respectively **Figure 4.7 (a)**. The results show that the R^2 value for UHAPF is higher than UF, indicating a better fit for UHAPF. However, the AIC value for UHAPF is lower than UF, indicating a worse fit for UHAPF. Overall, this model is not the best fit for the data.

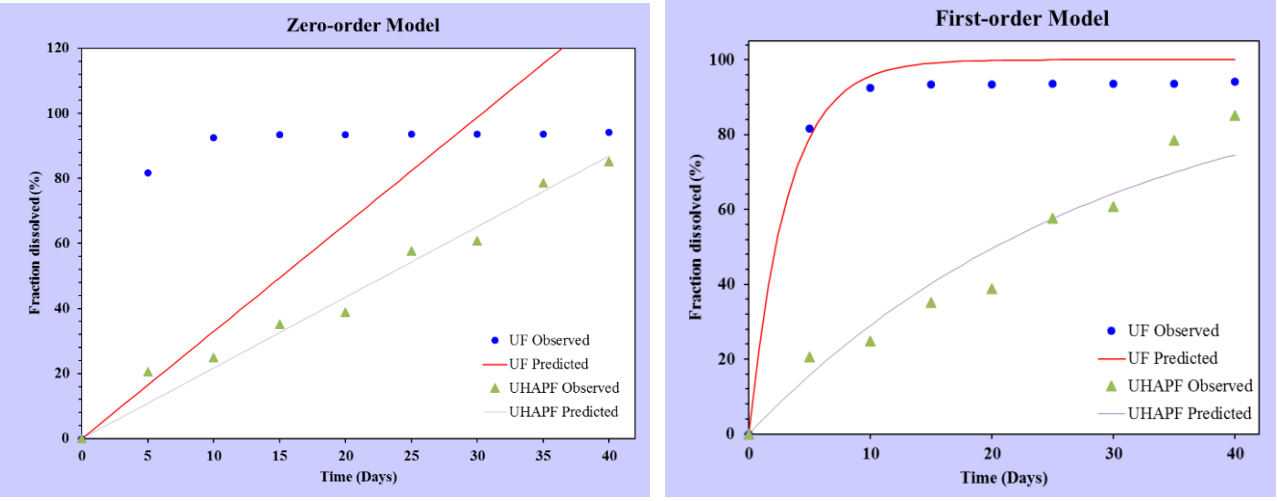


Figure 4.7 kinetic model of nitrogen release **a)** Zero-order Model **b)** First-order Model

In the first-order kinetic model, the constant values "k_1" for UF and UHAPF are 0.315 and 0.034, respectively, representing the first-order rate constant. The R^2 values for UF and UHAPF are 0.9679 and 0.9400, respectively. These values indicate that the first-order kinetic model fits both UF and UHAPF systems well. The MSC values for UF and UHAPF are 1.1400 and 2.2123, respectively. The AIC values for UF and UHAPF are 51.5407 and 55.4150, respectively. The first-order kinetic model fits UF and UHAPF systems based on the high R^2 and low MSC and AIC values **Figure 4.7 (b)**. This model assumes that the drug release rate decreases over time. The data indicate that UF and UHAPF have high R^2 values, indicating a good fit for both. The AIC value is also lower than the other models, indicating the best fit for this model.

In the Higuchi model, the constant values "k_H" for UF and UHAPF are 18.803 and 11.435, respectively, representing the release rate constants. The R^2 values for UF and UHAPF are 0.4529 and 0.8933, respectively. These values indicate that the Higuchi model is a better fit for the UHAPF nitrogen release than the UF nitrogen release, as the R^2 value for UHAPF is closer to 1. The MSC values for UF and UHAPF are -1.6943 and 1.6364, respectively. The negative MSC value for UF indicates that the Higuchi model is not a good fit for the UF system. This model assumes that drug releasing pattern depends on the square root of time. The results show that UHAPF has a higher R^2 value than UF, indicating a better fit for UHAPF. However, the AIC value for UHAPF is higher than UF, indicating a worse fit for UHAPF **(Figure 4.8 (a))**. Overall, this model is not the best fit for the data.

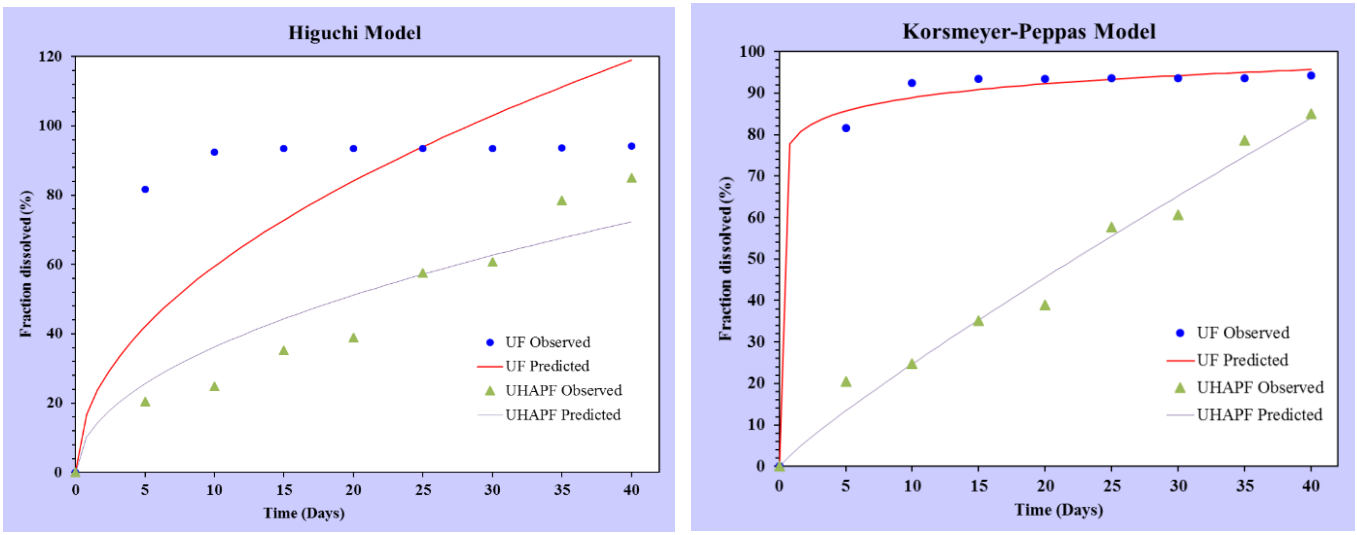


Figure 4.8 Kinetic model of nitrogen release **a)** Higuchi Model **b)** Korsmeyer-Peppas Model

In the Korsmeyer-Peppas model, the constant values "k_{KP}" for UF and UHAPF are 78.703 and 3.228, respectively, representing release rate constants. The R^2 values for UF and UHAPF are 0.9945 and 0.9785, respectively. The MSC value for UF is 2.6754, and for UHAPF, it is 3.0139. The AIC value for UF is 37.7220, and for UHAPF it is 48.2008. The value of "n" for the Korsmeyer-Peppas model is 0.053 for UF and 0.884 for UHAPF. Based on the R-squared values, the Korsmeyer-Peppas model appears to be the best-fit model for both the UF and UHAPF systems, as it has the highest R-squared values. This model assumes that urea release

is controlled by both diffusion and polymer relaxation. The results show that UF has a higher R^2 value than UHAPF and lowest AIC value, indicating the best fit for this model **(Figure 4.8 (b))**. Additionally, the n-value for UHAPF is close to 1, indicating Fickian diffusion, while the n-value for UF is much smaller, indicating anomalous diffusion.

Based on these kinetic release model results, the Korsmeyer-Peppas model is a good fit for the UF and UHAPF systems. The high R^2 values indicate that the model explains a noteworthy proportion of the variation in drug release data. The low mean square errors and AIC values further support the model's suitability. The exponent (n) values also suggest that the drug release mechanism is non-fickian or anomalous in both fertilizers. This indicates the similarity with the water-soluble drug molecules from a homogeneous matrix. In slow-release urea formulations, the release is controlled by diffusion. Similarly, Xiaoyu et al. (2013) developed a controlled release urea by adding organic polymer and bentonite to make a structure around the urea that releases slowly. The double-exponent equations and peppas were used to analyse releasing behaviour and it was found that it primarily affected the dissolution and erosion of the lattice structure surrounding the urea molecules.

4.4 Phosphate Solubilization Potential of *Bacillus megaterium* and *Pseudomonas aeruginosa*

The solubilization of fixed inorganic phosphorus is crucial for improving crop yield, and phosphate-solubilizing bacteria (PSB) have been shown to be effective in releasing more P into the soil. The qualitative solubilization test was performed by bacteria inoculation in NBRIP media plates containing precipitated tricalcium phosphate with bromocresol purple dye shown in **Figure 4.9**. The plates were incubated for six days (Room temperature - 28±2°C), then colour change and halo formation were observed. The results of the phosphate-solubilization test showed that both bacterial strains were capable of solubilizing tricalcium phosphate, as indicated by the yellow coloration and clear halo formation around the bacterial colonies.

However, the *Bacillus megaterium* MTCC1684 strain showed higher phosphate-solubilizing activity than the *Pseudomonas aeruginosa* MTCC7453 strain. The yellow coloration around the colonies of *Bacillus megaterium* MTCC1684 was more pronounced and had a larger halo size compared to *Pseudomonas aeruginosa* MTCC7453. Tricalcium phosphate solubilization is primarily attributed to the secretion of organic acids, which lowers the pH and enhances the solubility of phosphate.

The present study outcome revealed a clear yellow zone formation near the bacterial colonies, indicating organic acid production, and thereby the solubilization of insoluble phosphorus. The results demonstrate the significant role played by the two bacterial strains in solubilizing tricalcium phosphate and making it more readily accessible to plants. The study highlights the significant phosphate-solubilizing potential of *Bacillus megaterium* MTCC1684 and *Pseudomonas aeruginosa* MTCC7453. The two bacterial strains were able to solubilize tricalcium phosphate through organic acid production, indicating their potential as natural sources of phosphorus for plants to enhance soil fertility.

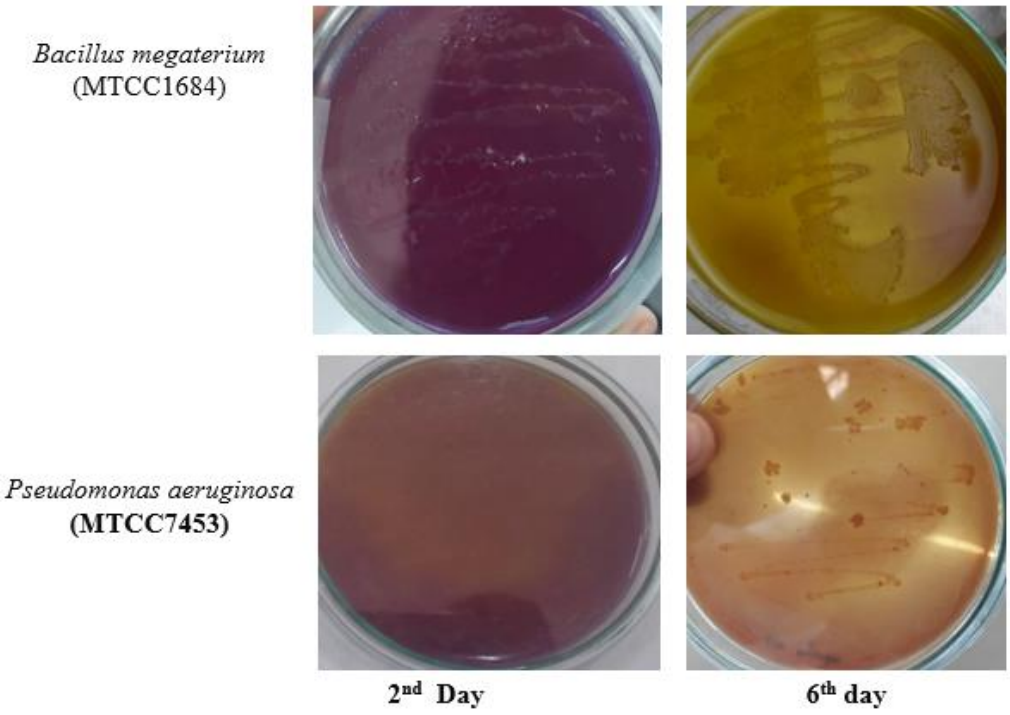


Figure 4.9. Solubilization of Tri-calcium phosphate on NBRIP media with bromocresol purple dye.

Zheng et al. (2018) used a 96-well microplate method to screen PSB strains for their solubilizing ability, which allowed for the simultaneous isolation of PSB strains from two agricultural soils. Phylogenetic analysis revealed that *Bacillus megaterium* strain was the most prevalent PSB strain, and four representative strains were selected for further analysis of Phosphorus solubilization. The main organic acid produced by *Bacillus megaterium* was determined to be succinic acid, which exhibited a strong correlation with the increase in soluble phosphorus (P) concentration during a 168-hour incubation period. The pH showed a negative correlation with the amount of soluble P, while the quantity of succinic acid displayed a linear correlation with the amount of released phosphorus. These findings suggest that organic acid production may play a vital role in facilitating the mobilization of microbial phosphorus. The PSB community had a capability to solubilize inorganic phosphorus and show organic anions compared to individual strains.

4.5 Root and Shoot Length of Plant after Treatment

The impact of nitrogen and phosphorus supplementation on the growth of *Cannabis sativa* L. were studied. The group treated with nanofertilizer and plant growth-promoting microorganism showed an increase in both shoot and root length. The maximum growth was observed in the shoot, which recorded a length of 150cm, while the root length increased to 70cm. These findings suggest that nanofertilizer and plant growth-promoting bacteria use promote the overall development of *Cannabis sativa* L. and a promising strategy for enhancing crop yield **(Figure 4.10)**.

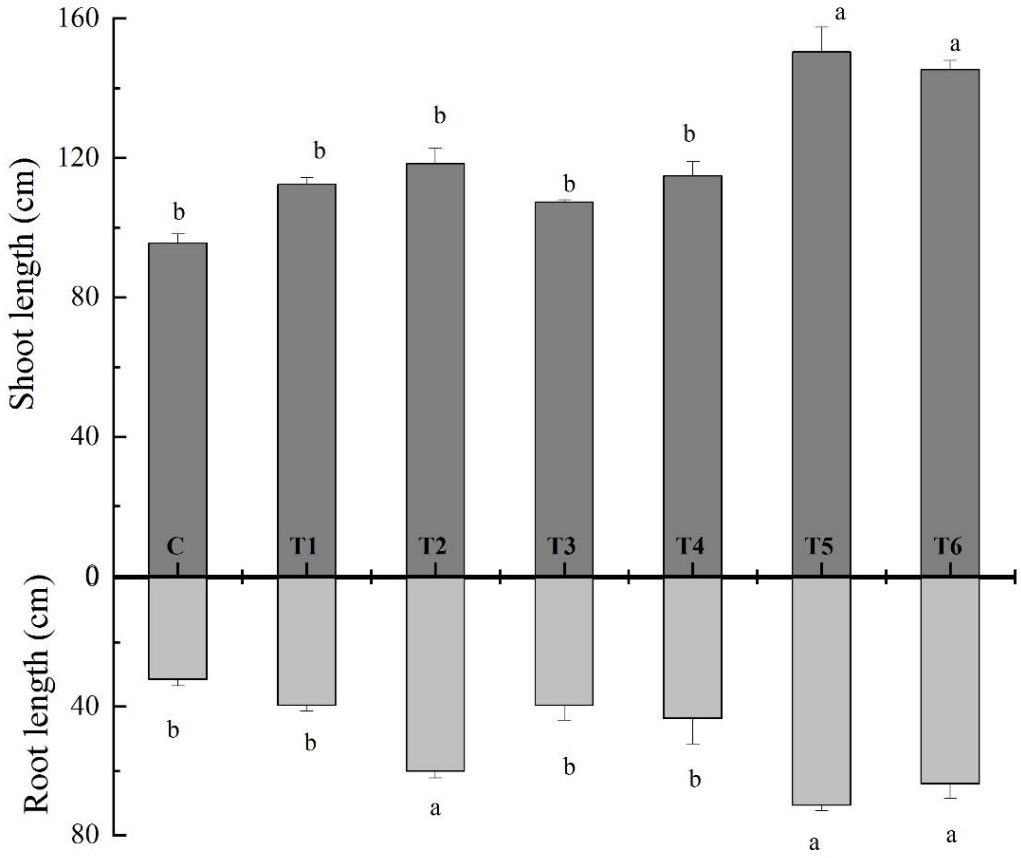


Figure 4.10. Individual and combined treatment of synthesized fertilizer and Biofertilizer on root and shoot length of *Cannabis sativa* L. crop. Data are presented as mean± S.E (n=3). At the P<0.05 level, different letters on each error bar are statistically significant. Treatments:1) C- Control, 2) T1- Urea, 3) T2-Synthesized Urea Hydroxyapatite Nanofertilizer (UHAPF), 4) T3-*Bacillus megaterium* (B1), 5) T4-*Pseudomonas aeruginosa* (B2), 6) T5-B1+UHAPF, 7) T6- B2+UHAPF.

In terms of stem length, the control group exhibited a length of 104 cm, which was exceeded by T1, T2, T3, T4, T5 and T6 groups. Among them, the T5 group recorded the highest stem length of 150 cm, followed by T6 with a length of 145 cm. The increase in stem length in the treated groups suggests that the supplementation of nitrogen and phosphorus through nanofertilizer and plant growth-promoting bacteria can positively impact stem growth in *Cannabis sativa* L. **(Figure 4.11)**. Moreover, the root length of the control group was recorded as 31 cm. The T1 and T4 groups showed a minor increase in root length, while T2 exhibited a significant increase to 60 cm. T3 and T5 showed a similar trend to the control group. T6 showed the second-highest root length of 64 cm, indicating the combine treatment of nanofertilizer and plant growth-promoting bacteria can significantly impact root growth in *Cannabis sativa* L. Overall, the study highlights the potential of nitrogen and phosphorus supplementation using nanofertilizer and plant growth-promoting microbes to promote both stem and root growth in *Cannabis sativa* L.

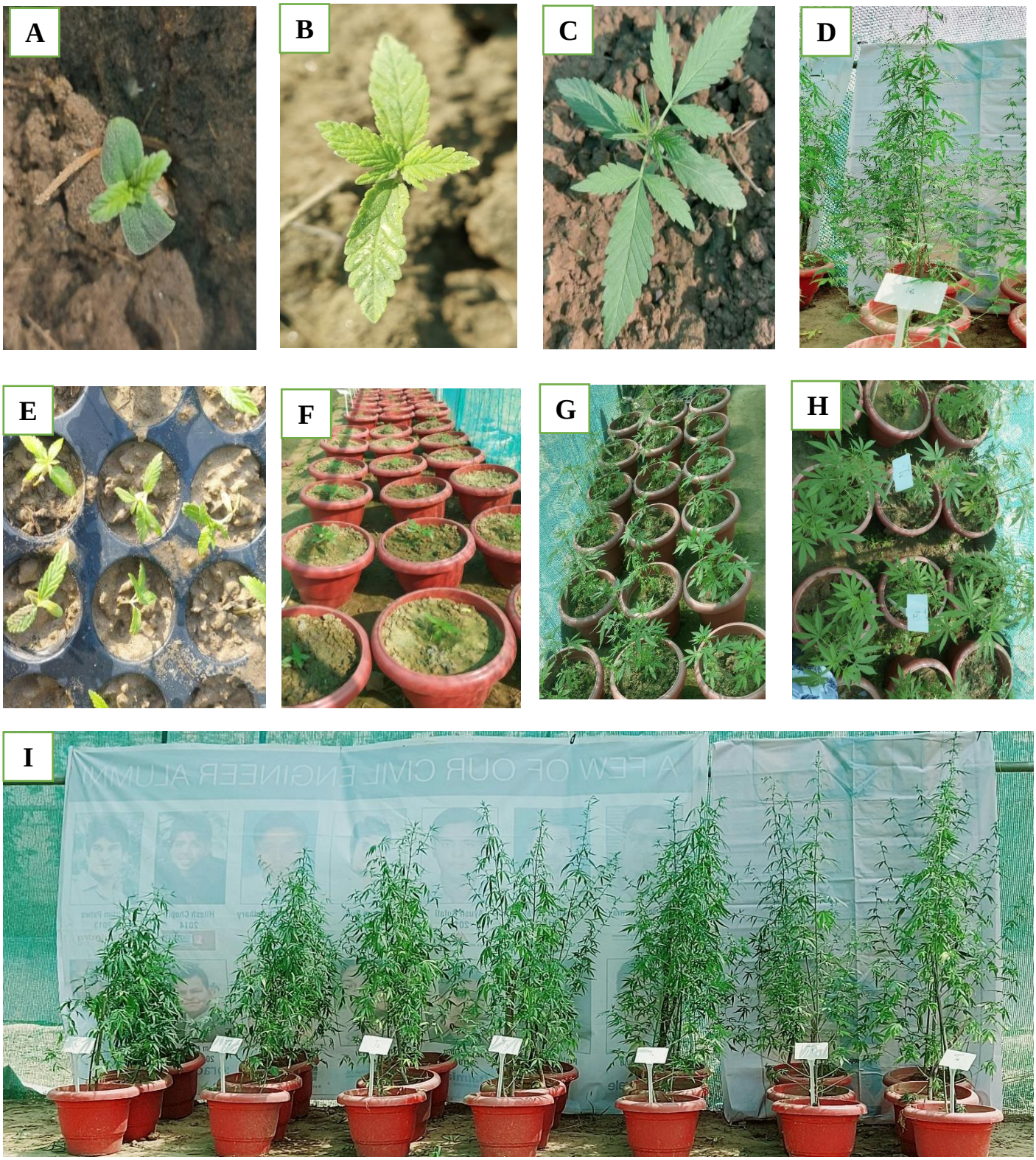


Figure 4.11 Field experiment **(a)** Developmental stages of *Cannabis sativa* L. under Green Shade Condition (A to H), **(b)** Impact of treatment on *Cannabis sativa* L.: Observing Changes in Growth and Morphology. Treatments:1) C- Control, 2) T1- Urea, 3) T2-Synthesized Urea Hydroxyapatite Nanofertilizer (UHAPF), 4) T3-*Bacillus megaterium* (B1), 5) T4-*Pseudomonas aeruginosa* (B2), 6) T5-B1+UHAPF, 7) T6- B2+UHAPF.

Landi et al. (2019) reviewed nitrogen nutrition impact on the *Cannabis sativa* L. plant and suggested that nutrient availability influences the growth, biomass, and fibre yield. Marchiol et al. (2019) carried out an experiment on effects of various hydroxyapatite nanoparticle solutions on the growth and metabolism of *Solanum lycopersicum* L. were evaluated and suggested that increasing hydroxyapatite nanoparticle concentrations did not affect germination, but strongly stimulated root elongation without causing any phytotoxic effects. Therefore, hydroxyapatite has the potential to be used as a phosphorus supplier and carrier of other elements and molecules without harming plant growth.

4.6 Treatment Effect on Photosynthetic Pigments and Protein Content of *Cannabis sativa* L.

A study found that the photosynthetic pigment content of *Cannabis sativa* L. leaves, including chlorophyll *a*, chlorophyll *b*, total chlorophyll and carotenoids, was remarkably increased by combine exposure to UHAPF and *Bacillus megaterium* compared to sole application or control. Chlorophyll *a* is essential for photosynthesis and serves as an indicator of plant health. The higher levels of chlorophyll *a* in T5 suggest that the combined application of B1 and UHAPF positively increases chlorophyll synthesis. Chlorophyll *b* is involved in light absorption and energy transfer within the plant. The higher levels of chlorophyll *b* in T1 and T6 indicate that urea application and the combined treatment of B2 and UHAPF might have enhanced photosynthetic activity and energy utilization in *Cannabis sativa* L. plants.

Total chlorophyll content reflects the overall photosynthetic capacity of plants. The increased total chlorophyll content in T5 suggests that the combined treatment of B1 and UHAPF had a synergistic effect on enhancing photosynthetic pigments in *Cannabis sativa* L. Carotenoids play an important role in photoprotection and contribute in defence system. The higher levels of total carotenoids in T5 indicate that the combined treatment of B1 and UHAPF might have promoted antioxidant activity and protection against oxidative stress. The high amount of carotenoid and chlorophyll contents 3.10 mg/g FW and 3.39 mg/g FW respectively were observed in the combined treatment **(Figure 4.12)**.

The results suggest that the slow release of nitrogen from UHAPF leads to increased nitrogen uptake by plant and reduce fertilizer leaching with water. The co-inoculation of PGPB and UHAPF synergistically affected plant growth and increased the yield compared to the sole application. Furthermore, the sole application of PGPB produced a higher yield than the control, possibly because of its phosphate solubilization and organic acid production.

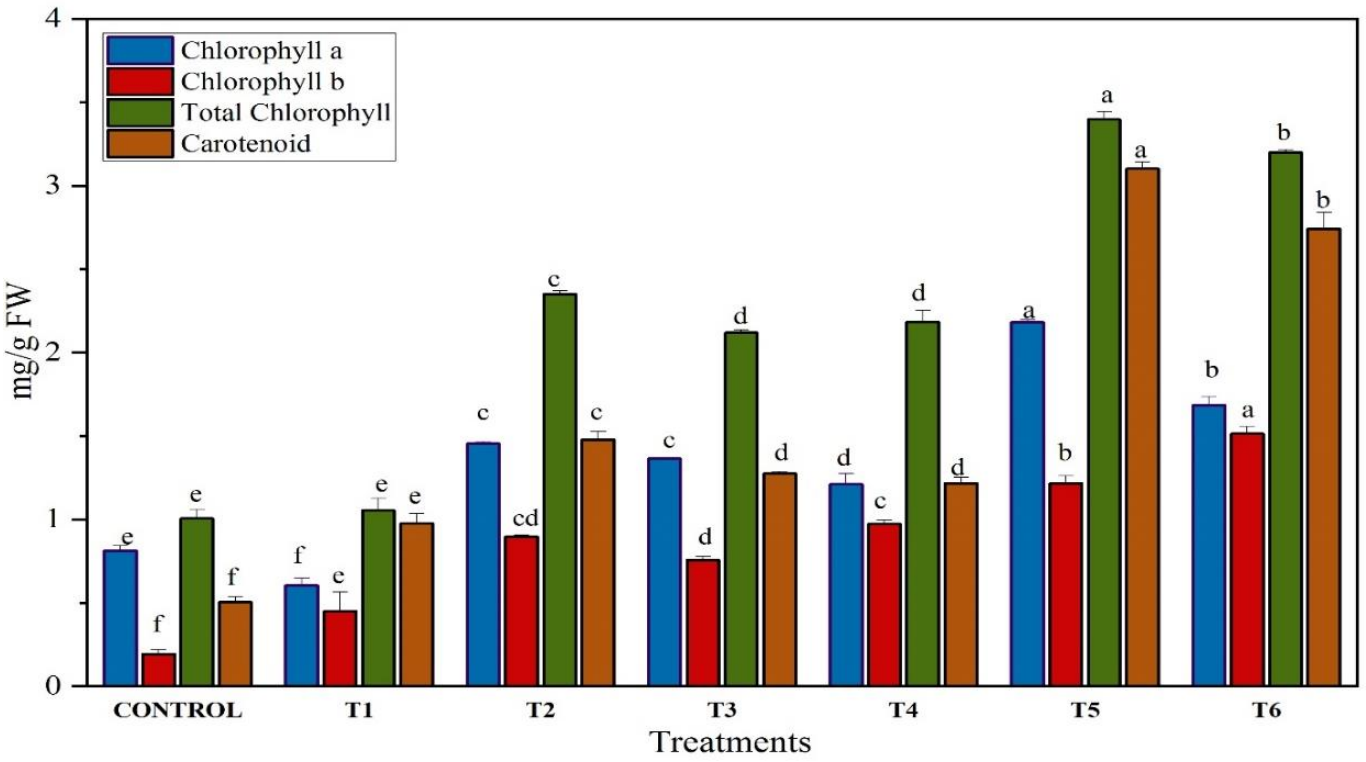


Figure 4.12 Effect on photosynthetic pigments of *Cannabis sativa* after individual and combine treatment of synthesized fertilizer and Biofertilizer. Data are presented as mean ± S.E (n=3). At the P<0.05 level, different letters on each error bar are statistically significant. Treatments:1) C- Control, 2) T1- Urea, 3) T2-Synthesized Urea Hydroxyapatite Nanofertilizer (UHAPF), 4) T3-*Bacillus megaterium* (B1), 5) T4-*Pseudomonas aeruginosa* (B2), 6) T5-B1+UHAPF, 7) T6- B2+UHAPF

Similarly, the protein content was also increased by combined treatment administered to the plants **(Figure 4.13)**. Both microbial strain interaction helps in plant and soil health improvement. For protein quantification, the linearity plot was finalized by graphing the peak area against the standard protein concentration of bovine serum albumin. By performing linear regression analysis on the obtained data, the linearity of the plot was assessed and quantified. Proteins are essential for various plant functions, including growth, development, and defence responses. The significantly higher protein content in T5 suggests that the combination of B1 and UHAPF positively influenced protein synthesis in *Cannabis sativa* L. plants. Overall, the results demonstrate that the combined treatment of B1 and UHAPF (T5) had notable effects on the physiological parameters of *Cannabis sativa* L. plants, including increased chlorophyll content, enhanced antioxidant activity and improved protein synthesis.

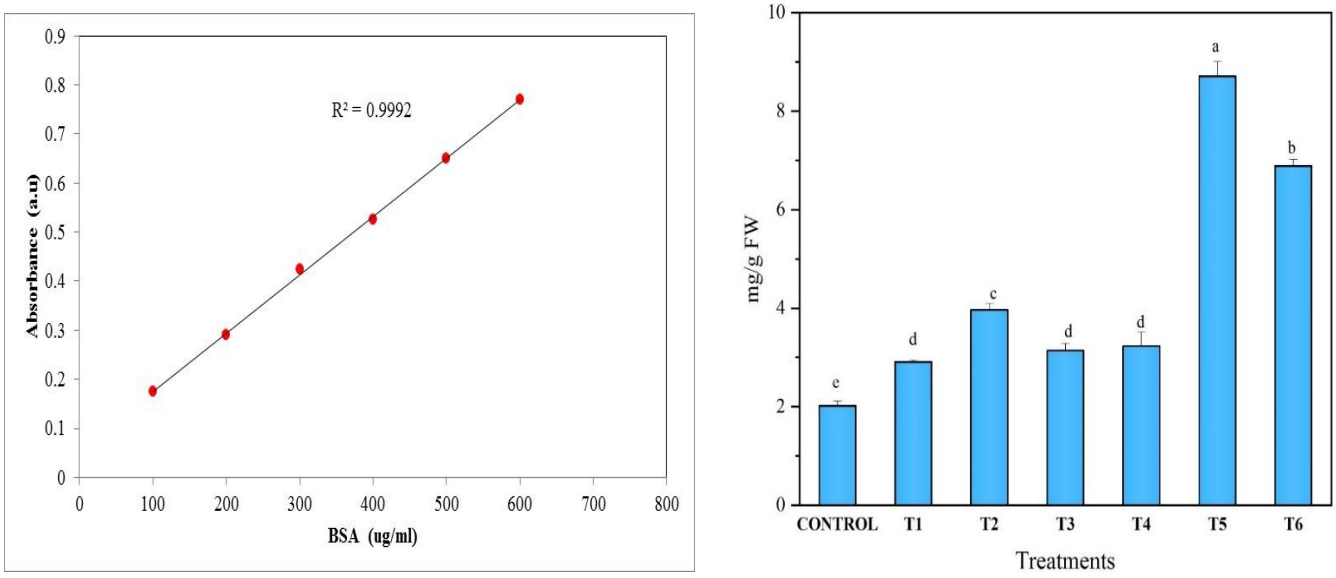


Figure 4.13. Protein estimation a) The linearity graph of the BSA standard b) Effect on protein content of *Cannabis sativa* after individual and combine treatment of synthesized fertilizer and Biofertilizer. Data are presented as mean ± S.E (n=3). At the P<0.05 level, different letters on each error bar are statistically significant. Treatments:1) C- Control, 2) T1- Urea, 3) T2-Synthesized Urea Hydroxyapatite Nanofertilizer (UHAPF), 4) T3-*Bacillus megaterium* (B1), 5) T4-*Pseudomonas aeruginosa* (B2), 6) T5-B1+UHAPF, 7) T6-B2+UHAPF.

Previous studies have shown that *Bacillius megaterium* have a positive effect on mustard plants and increases root length, shoot length and fresh weight (Kang et al., 2014). PSB enhances total phosphorus absorption by solubilizing HAP in soil and increases plant biomass, root proliferation, etc. (Amri et al., 2022). In a comparative analysis of PGPR and chemical fertilizer, Sedri et al. (2022) found that PGPR can be considered a significant alternative to improve production and reduces the need for chemical fertilization as it helps to improve crop yield.

4.7 Soil Analysis after Treatment

The significant interaction of treatment was found to be T5 and T6. The concentration of available nitrogen in soil after treatment in T5 and T6 are 454.72 $KgNha^{-1}$ and 546.18 $KgNha^{-1}$. Notably, the PGPR not only solubilizes insoluble forms of phosphorus in the soil but also improves soil fertility after the plant is taken out of the soil respectively. **(Table 4.4)**. Gagnon et al. (2012) conducted a comparison research of various types of urea-based fertilizer effect on corn yield, plant nitrogen accumulation and soil nitrate level. The result showed that controlled release urea such as polymer coated urea increases corn yield, nitrogen accumulation in plant and minimize the risk of excessive nitrate loss.

Table. 4.4 Available nitrogen and available phosphorus in the soil after individual and combined treatment of synthesized fertilizer and biofertilizer

Treatments	Control	T1 UF	T2 UHAPF	T3 B1	T4 B2	T5 UHAPF+B1	T6 UHAPF+B2
Available Nitrogen ($KgNha^{-1}$)	245.65± 13.066^{f}	284.85±13.66^{d,e}	297.92±0^{d}	363.25±13.066^{c}	324.05±13.06^{c,d}	454.72± 22.63^{b}	546.18± 13.066^{a}
Available Phosphorus ($KgPha^{-1}$)	6.56± 0.245^{e}	6.535± 0.155^{e}	8.68±0.097^{d}	11.75±0.5914^{c}	12.13±1.1303^{c}	28.72± 0.1297^{a}	26.90±0.5205^{b}

Data are presented as mean± S.E (n=3). At the P<0.05 level, different letters on each error bar are statistically significant. Treatments:1) C- Control, 2) T1- Urea, 3) T2-Synthesized Urea Hydroxyapatite Nanofertilizer (UHAPF), 4) T3-*Bacillus megaterium* (B1), 5) T4-*Pseudomonas aeruginosa* (B2), 6) T5-B1+UHAPF, 7) T6-B2+UHAPF.

Similarly, the concentration of available phosphorus is gradually increase 28.72 $KgPha^{-1}$ (T5) and 26.90 $KgPha^{-1}$ respectively. The lowest phosphorus value was recorded in control and T1 due to lack of phosphorus source. The increase in available phosphorus observed in treatments T2 to T6 may be due to release of phosphorus through microbial solubilization of HAP. The solubilization of HAP in soil is affected by microbial consortia of phosphate-solubilizing bacteria which also improve total P absorption. Previous investigation has suggested that native microbial consortia have the ability to solubilize phosphate source like apatite. Among these microorganisms, *Pseudomonas* have been identified in the soil, which can hydrolyse insoluble Phosphorus though various mechanisms, such as chelating mineral ions, secreting low molecular mass organic acids, or reducing the pH (Alori et al., 2017a).

4.8 Treatment Effect on Phytoconstituent of *Cannabis sativa* L.

The secondary metabolite content of *Cannabis sativa* L. extracted by maceration was analysed by GC-MS and identified from MS spectral library depicted in **Figure No. 4.14**. Cannabinoid compounds are not classified as alkaloid due to their lack of nitrogen atom in its structure, and are considered as terpenophenolic compounds. Some identified compounds are specific to the *Cannabis* plant, such as cannabidiol, Δ^9 tetrahydrocannabivarin, cannabichromene, cannabispiran and dronabinol. Fatty acid methyl esters such as palmitic acid, methyl ester, linoleic acid, methyl ester, methyl elaidate, methyl stearate, cis-methyl 11-eicosenoate, methyl erucate, glycidyl oleate, methyl nervonate were also detected in *Cannabis sativa* L.

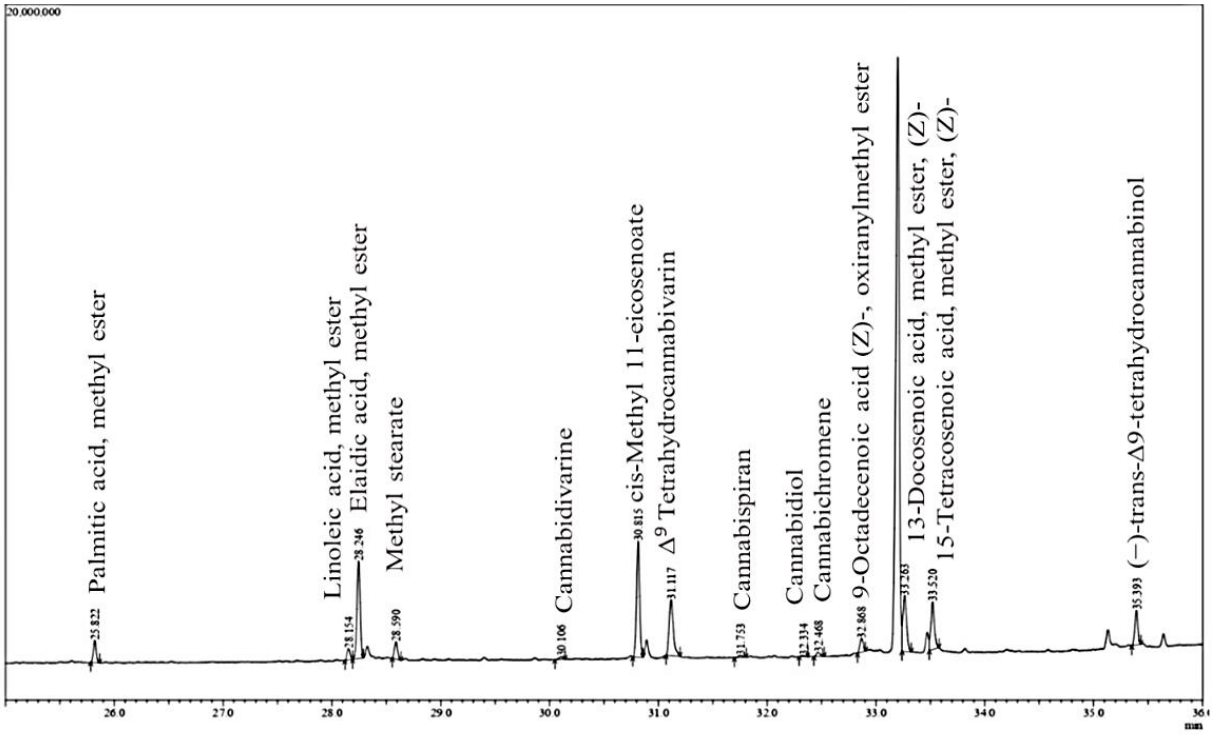
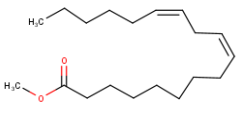
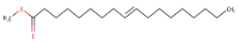
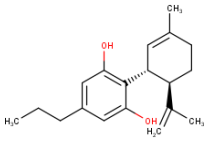
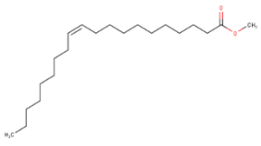
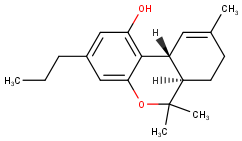


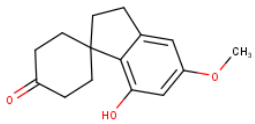
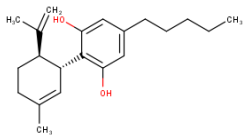
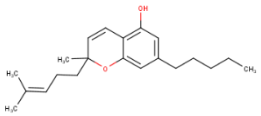
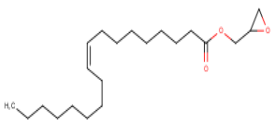
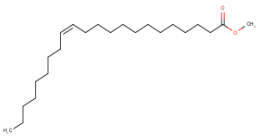
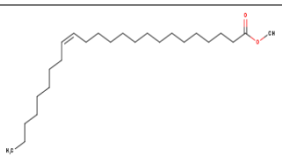
Figure No. 4.14 Identification of Compounds in Treated *Cannabis* Plants using GC-MS Chromatography

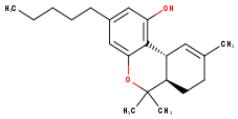
Among all phytocannabinoids, tetrahydro cannabinoid is the main psychoactive compound and is present in relatively low amounts in hemp. The molecular weight, molecular formula, retention time, IUPAC name and similarity index of identified compounds are given in **Table No. 4.5**. Palmitic acid and methyl stearate are saturated fatty acids commonly found in plants. Its methyl ester form is a derivative, often used in producing soaps, detergents, and cosmetics due to its emollient and moisturising properties. Methyl stearate is commonly used as a lubricant and a precursor in the production of surfactants and detergents and is also used as a solvent and a carrier for active ingredients in cosmetic formulations. In *Cannabis*, palmitic acid methyl ester may be extracted from hemp oil and used in formulations for topical applications or as a carrier oil for other cannabinoids.

Linoleic acid, methyl elaidate, cis-methyl 11-eicosenoate, glycidyl oleate, methyl erucate and methyl nervonate are unsaturated fatty acids. These are used in producing cosmetics, soaps, detergents, surfactants and emulsifiers, and in synthesising other organic compounds. Linoleic acid methyl ester may also have the potential as a carrier oil for other cannabinoids due to its emollient and moisturising properties and ability to penetrate the skin. Methyl elaidate is commonly found in vegetable oils such as sunflower and soybean. It has been used as a precursor in the synthesis of other organic compounds, including fragrances and flavours. It is also an important component of the lipid profile of hemp oil and may contribute to its shelf life and stability. Methyl esters can be extracted from hemp oil using various methods, including solvent extraction and supercritical CO_2 extraction.

Table No 4.5. The molecular weight, molecular formula, retention time, IUPAC name and similarity index of identified compounds are given in table

Sr. No.	Identified compound	IUPAC name	Chemical Formula	Chemical Structure	RT	CAS number	SI
1	Palmitic acid, methyl ester	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$		25.822	112-39-0	96%
2	Linoleic acid, methyl ester	9,12-Octadecadienoic acid (Z, Z)-, methyl ester	$C_{19}H_{34}O_2$		28.154	112-63-8	94%
3	Methyl Elaidate	9-Octadecenoic acid, methyl ester, (E)-	$C_{19}H_{36}O_2$		28.246	1937-62-8	96%
4	Methyl stearate	Octadecanoic acid, methyl ester	$C_{19}H_{38}O_2$		28.590	112-61-8	95%
5	Cannabidivarine	2-((1S,6S)-3-methyl-6-(prop-1-en-2-yl) cyclohex-2-enyl)-5-propylbenzene-1,3-diol	$C_{19}H_{26}O_2$		30.106	24274-48-4	95%
6	cis-Methyl 11-eicosenoate	cis-Methyl 11-eicosenoate	$C_{21}H_{40}O_2$		30.815	2390-09-2	95%
7	Δ^9Tetrahydrocannabivarin	(6aR,10aR)-6,6,9-trimethyl-3-propyl-6a,7,8,10a-	$C_{19}H_{26}O_2$		31.117	31262-37-0	90%

		tetrahydrobenzo [c]chromen-1-ol					
8	Cannabispiran	4-hydroxy-6-methoxyspiro[1, 2-dihydroindene-3,4'-cyclohexane]-1'-one	$C_{15}H_{18}O_3$		31.753	61262-81-5	90%
9	Cannabidiol	2-[(1R,6R)-6-Isopropenyl-3-methylcyclohex-2-en-1-yl]-5-pentylbenzene-1,3-diol	$C_{21}H_{30}O_2$		32.334	13956-29-1	90%
10	Cannabichromene	2-Methyl-2-(4-methylpent-3-enyl)-7-pentyl-5-chromenol	$C_{21}H_{30}O_2$		32.468	20675-51-8	95%
11	Glycidyl Oleate	9-Octadecenoic acid (Z)-, oxiranylmethyl ester	$C_{21}H_{38}O_3$		32.868	5431-33-4	91%
12	Methyl erucate	13-Docosenoic acid, methyl ester, (Z)-	$C_{23}H_{44}O_2$		33.263	1120-34-9	91%
13	Methyl nervonate	15-Tetracosenoic acid, methyl ester, (Z)-	$C_{25}H_{48}O_2$		35.393	2733-88-2	93%

14	Dronabinol	((−)-*trans*-Δ^9-tetrahydrocannabinol)	$C_{21}H_{30}O_2$		33.520	1972-08-3	93%

RT- Retention time, SI- Similarity index

Cannabidiol (CBD), cannabidivarine (CBDV), and cannabichromene (CBC) are non-psychoactive cannabinoid with potential therapeutic properties, anti-inflammatory analgesic, anxiolytic, and anti-epileptic effects. CBDV, CBD and CBC are in relatively low concentrations of hemp plants. Cannabidiol (CBD) have potential therapeutic properties, including anti-inflammatory, analgesic, and anxiolytic effects. CBD is one of the most abundant cannabinoids found in hemp plants and is often extracted and used in the production of *cannabis*-based medications and supplements **(Figure 4.15)**.

Δ^9Tetrahydrocannabivarin (THCV) have potential therapeutic properties, including appetite suppression and antipsychotic effects. THCV is present in hemp plants in relatively low concentrations. Cannabispiran is a bicyclic compound, and its presence is not well documented. It is a cyclic compound whose biological activity is not well understood, but it may have potential as a precursor or intermediate in synthesising other organic compounds. The presence of cannabispiran in hemp may be related to its role in plant metabolism or as a byproduct of other biosynthetic pathways.

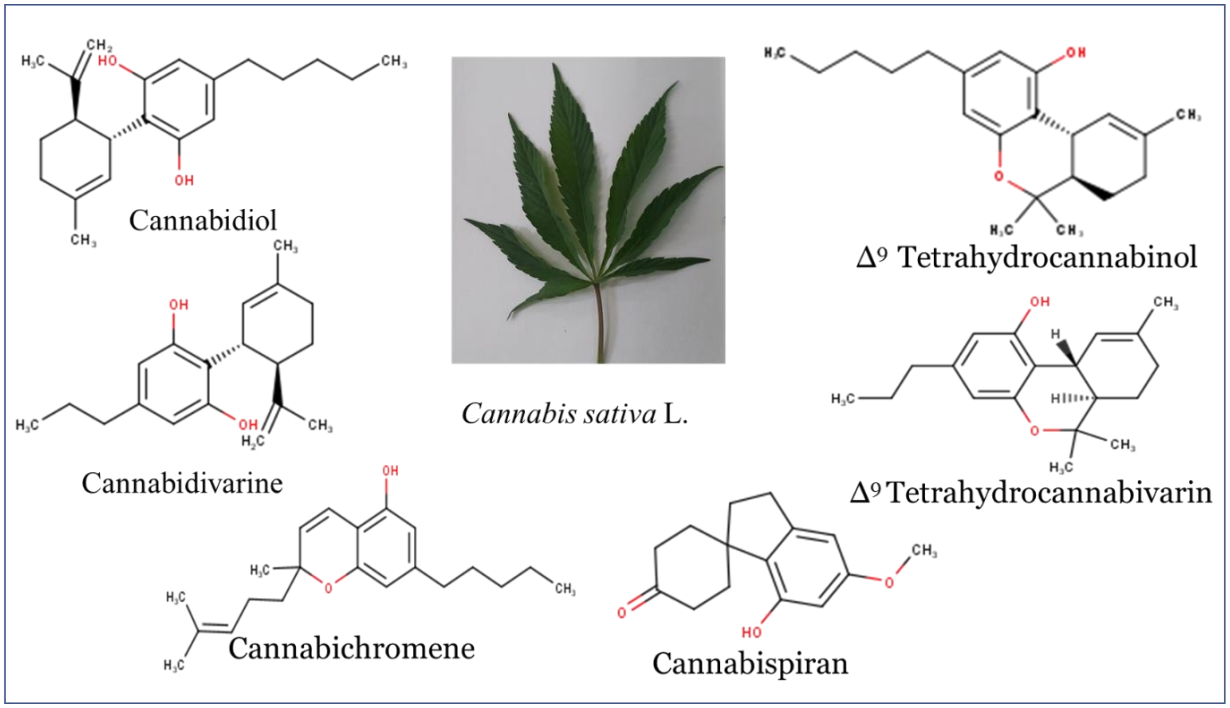


Figure 4.15 Chemical Structure of the Primary Cannabinoid in *Cannabis sativa* L.

Dronabinol is a synthetic form of Δ^9 tetrahydrocannabinol (THC), the main psychoactive compound in *cannabis* and hemp plants. The FDA approves its usage in the treatment of chemotherapy-induced nausea and vomiting, as well as for stimulating appetite in patients with AIDS-related wasting syndrome. Dronabinol is not naturally present in hemp plants. Still, its development and approval for medical use highlight the potential benefits of cannabinoids and the importance of continued research in this field (Brafford May & Glode, 2016).

4.9 Semi- quantitative Data for Bioactive Compound from *Cannabis sativa* L.

From GC-MS analysis of *Cannabis*, the identified compounds are divided into three categories based on their chemical nature: fatty acid methyl esters, *Cannabis*-specific compounds, and others. Fatty acid methyl esters were found in all treatments, such as palmitic acid, methyl ester and linoleic acid. The concentration of these compounds decreased with fertilizer treatments (T1 to T6), possibly due to reduced fatty acid biosynthesis. This is due to nutrient deficiency which may result in an increase in fatty acid production as a defence mechanism against the stressor. *Cannabis*-specific compounds such as cannabidiol (CBD), Δ^9-tetrahydrocannabinol (THC), Δ^9-tetrahydrocannabivarin (THCV), cannabichromene (CBC) and cannabidivarine (CBDV) were also detected in the samples.

Table 4.6. Semi- quantitative data (% Relative peak area value) of the Phyto cannabinoid in Hemp extracts[a].

Sr. No.	Compound Name	% Relative peak area						
		Control	T1	T2	T3	T4	T5	T6
1.	**Hexadecanoic acid, methyl ester**	2.77 ±0.02[c]	2.1±0.06[a]	1.69 ±0.01[b]	1.99 ±0.005[b]	3.4 ±0.05[d]	1.98±0.3[b]	3.98 ±0.01[e]
2.	**(9,12-Octadecadienoic acid (Z,Z)-, methyl ester)**	1.54±0.005[b]	1.16 ±0.04[a]	1.f19±0.1[a]	1.11 ±0.05[a]	2.23 ±0.04[c]	1.02±0.14[a]	2.19±0.18[c]
3.	**(9-Octadecenoic acid, methyl ester, (E)-)**	13.11 ±0.02[d]	9.78 ±0.01[c]	9.17 ±0.02[b]	8.84±0.19[a]	17.09±0.09[e]	9.04±0.06[b]	19.28±0.09[f]
4.	**Methyl stearate**	2.06 ±0.02[d]	1.53 ±0.02[c]	1.22 ±0.02[a]	1.42 ±0.017[b]	2.31±0.1[e]	1.44±0.09[b c]	3.17±0.02[f]
5.	**cis-Methyl 11-eicosenoate**	14.56±0.02[d]	1.59±0.02[a]	1.57 ±0.03[a]	9.57±0.02[c]	2.98±0.1[b]	1.66±0.03[a]	22.15±0.13[e]
6.	**9-Octadecenoic acid (Z)-, oxiranylmethyl ester**	48.7±0.17[f]	8.32±0.03[d]	0.97±0.01[a]	0.96±0.03[a]	16.63±0.03[e]	1.28±0.01[b]	2.84±0.11[c]
7.	**13-Docosenoic acid, methyl ester, (Z)-**	7.87±0.02[a]	57.7±0.2[f]	54.7±0.2[e]	52.63±0.09[d]	9.18±0.02[b]	58.07±0.01[g]	12.31±0.18[c]

8.	**15-Tetracosenoic acid, methyl ester, (Z)-**	9±0.09^{f}	2.88±0.02^{bc}	2.76±0.04^{ab}	2.7±0.09^{a}	5.49±0.02^{d}	3.01±0.07^{c}	6.65±0.17^{e}
9.	**Cannabispiran**	0	0	0	0.47±0.01^{a}	0.68±0.06^{d}	0.54±0.04^{b}	0.6±0.02^{c}
10.	**Cannabichromene**	0	0.9±0.07^{a}	2.21±0.01^{e}	1.2±0.05^{c}	2.25±0.04^{e}	1.35±0.04^{d}	0.980.01^{b}
11.	**Cannabidivarine**	0	0.6±0.03^{b}	0.25±0.02^{a}	0.74±0.02^{c}	3.67±0.1^{e}	1.97±0.14^{d}	0.56±0.11^{b}
12.	**Δ^{9}-Tetrahydrocannabivarin**	9.26±0.02^{e}	2.39±0.02^{a}	4.7±0.2^{c}	4.87±0.02^{c}	7.65±0.2^{d}	3.83±0.15^{b}	14.33±0.06^{f}
13.	**Cannabidiol**	0	3.29± 0.02^{c}	0.05c0.02^{a}	3.7±0.2^{d}	13.1±0.01^{f}	7±0.02^{e}	0.27±0.02^{b}
14.	**Δ^{9}-Tetrahydrocannabidiol**	9.23±0.01^{b}	7.6±0.02^{a}	19.34±0.2^{f}	9.63±0.03^{c}	13.06±0.05^{e}	7.63±0.19^{a}	10.33±0.11^{d}

Data are presented as mean± SD (n=3). SD<0.05 level. Treatments 1) Control, 2) T1- Urea. 3) T2- Synthesized Urea Hydroxyapatite Nanofertilizer (UHAPF), 4) T3- *Bacillus megaterium* (B1), 5) T4- *Pseudomonas aeruginosa* (B2), 5) T4- B1+UHAPF, 7) B2+UHAPF

Treatment T1 and T2 the area percent decreased in most of the identified compounds compared to the other treatment. The application of bacterial inoculants (T3 and T4) also change the chemical profile of the *Cannabis* plant. The most significant increase in T3 were observed for CBS, CBC, and CBDV and T4 were observed for 15-tetracosenoic acid, methyl ester, (Z)-, 13-docosenoic acid, methyl ester, (Z)-, and Δ9-tetrahydrocannabinol (THC). The increase in % relative peak area of these compounds was due to the positive impact of *Pseudomonas aeruginosa* and *Bacillus megaterium* bacteria in the soil fertility and plant growth, which can affect the biosynthesis of these compounds. In addition, the application of synthesized urea fertilizer (T2, T4, T5) showed a significant increase compared to control in some compounds.

The analysis indicated that the CBD, THC, and THCV concentration increased with increasing treatments, while CBC and CBDV showed inconsistent changes. Other compounds such as methyl stearate, cis-methyl 11-eicosenoate, and glycidyl oleate were also found in the samples. The concentration of these compounds showed varying trends with increasing treatments. The increase in CBD, THC and THCV in combine treatments could be due to the activation of cannabinoid biosynthesis pathways. Cannabinoid biosynthesis in hemp plants is influenced by a variety of factors, such as genetics, environmental conditions, and cultivation practices. One of the factors that can influence cannabinoid content include temperature, humidity and nutrient availability. On the other hand, the decrease in fatty acid methyl esters could indicate a reduction in lipid

biosynthesis, possibly as an adaptive response to the treatments. Overall, the results suggest that the conditions used in T3 and T5 could promote the synthesis of beneficial compounds in the *Cannabis* plant. The application of different treatments to *Cannabis* plants can affect the chemical composition of the plant. Cockson et al. (2020) explored the impacts of different concentrations of phosphorus on the growth, development, and quality of *Cannabis sativa* L. and monitored plant height, leaf tissue mineral nutrient concentrations, diameter and final fresh flower bud weight. They also examined the cannabinoid and terpene levels in the flowers to assess the impact of P fertility on floral quality. The analysis suggested that P concentrations had a substantial effect on the growth and development of *Cannabis*, applying different treatments can significantly change the chemical profile of the *Cannabis* plant. The increase in the percentage of certain compounds, such as cannabinoids and fatty acid esters, may have implications for the medicinal and industrial use of the plant. The results also suggest that using bacterial inoculants and synthesized fertilizers may be a viable method for increasing the specific compound production in the *Cannabis* plant.

Atoloye et al. (2022) studied different hemp variety to examine the effect of nitrogen fertilizer on cannabinoid yield and bud biomass in the field conditions. Their finding suggests that cannabidiol content was influenced by nitrogen fertilizer. Likewise, Caplan et al. (2017) tested four different concentration of organic fertilizer and the result showed that the highest cannabinoid content were observed at organic fertilizer rate of 389 mg N/L. Both bacterial strains significantly enhance the root length and shoot length of the plant. Hemp is an annual herbaceous plant that demands high nitrogen (N) supply for increasing plant yield and serves for the regulation of terpenoid and cannabinoid profiles (Saloner & Bernstein, 2020).

CHAPTER 5. SUMMARY

The global demand for food is continuously increasing due to population growth and the shrinking availability of agricultural land. Urbanization and land constraints pose significant threats to food security, while factors such as climate change and irrigation practices further impact soil fertility and agricultural productivity. In this context, microorganisms play crucial roles in nutrient cycling and soil health maintenance, highlighting the importance of sustainable agricultural practices. Conventional fertilizers, particularly urea and urea-based fertilizers, have long been essential for ensuring agricultural success. However, the low assimilation efficiency of urea by plants contributes to environmental issues such as nitrate leaching, surface runoff, soil denitrification, and volatilization, leading to groundwater contamination and global warming.

To address these challenges, nanotechnology has emerged as a promising solution, offering innovative approaches to enhance nutrient use efficiency and reduce environmental impacts. Nano-synthesized fertilizers, designed with various nutrient carriers, aim to deliver nutrients to plants in a controlled and targeted manner, thereby minimizing nutrient losses and maximizing crop yields. Among these nanofertilizers, hydroxyapatite (HAP) nanoparticles have garnered significant attention due to their biocompatibility, biodegradability, and potential for controlled nutrient release. By complexing urea with HAP nanoparticles, our aim to protect urea from rapid release and decomposition, thereby improving its assimilation by plants and reducing environmental pollution.

In addition to technological innovations in nanofertilizers, the study also explores the role of plant growth-promoting rhizobacteria (PGPR) as biofertilizers. PGPR strains such as Bacillus megaterium and Pseudomonas aeruginosa have demonstrated positive effects on plant growth and nutrient uptake by facilitating processes such as mineralization, nutrient mobilization, denitrification, and decomposition. The combination of nanofertilizers with PGPR holds immense potential for improving soil health, enhancing plant growth, and increasing crop yields in a sustainable manner.

The focus of this study extends to the agricultural application of Cannabis sativa L., a versatile crop with historical uses for food, fiber, and medicine. Cannabis, belonging to the Cannabaceae family, has experienced a resurgence in interest, particularly in its hemp form, due to its potential for textile production, biomass generation, and medicinal applications. Furthermore, Cannabis plants exhibit unique phytoremediation capabilities, making them valuable for soil and water detoxification.

Our study introduces an environmentally benign urea-hydroxyapatite nanofertilizer (UHAPF) synthesized to minimize nutrient runoff and enhance nutrient uptake efficiency, with a particular interest in its application to

hemp crops. The research investigates the impact of natural and controlled conditions on the phytochemical profile of hemp crops, with a focus on cannabinoid production and other bioactive compounds.

The experimental methodology involves the preparation and characterization of urea-hydroxyapatite nanofertilizer (UHAPF) using advanced techniques such as Fourier-transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), and field emission scanning electron microscopy (FESEM). The thermal stability of the synthesized nanofertilizer is also assessed through thermogravimetric analysis (TGA), providing insights into its structural integrity and suitability for agricultural applications. Furthermore, the nitrogen-release behavior of UHAPF is investigated over a 30-day period using a vertical column setup, with different kinetic models employed to understand the time-dependent release of urea from the nanofertilizer. The results reveal a controlled and sustained release of nitrogen from UHAPF, indicating its potential for improving nutrient availability to plants over an extended period.

In addition to nanofertilizer characterization and nitrogen-release studies, two plant growth-promoting rhizobacteria (PGPR) strains, Bacillus megaterium and Pseudomonas aeruginosa, are evaluated for their phosphate solubilization potential. These strains are cultured on nutrient agar and screened for their ability to solubilize tricalcium phosphate in NBRIP medium. Inocula are prepared from nutrient broth cultures, and their viability in peat samples is confirmed after incubation. The PGPR strains are then used in a pot-culture experiment with Cannabis sativa L. to observe the synergistic effects with UHAPF on plant growth and yield.

The pot-culture experiment is conducted under controlled conditions, with soil analyses performed to determine the initial soil characteristics. Seven treatment groups, including a control, urea, UHAPF, individual PGPR strains, and combinations of PGPR with UHAPF, are compared. After 30 days of treatment, various analyses are conducted on plant samples, including the estimation of photosynthetic pigments, total protein content, and available nitrogen and phosphorus levels. Additionally, the study explores the volatile compounds in hemp leaves using traditional maceration extraction, followed by gas chromatography-mass spectrometry (GC-MS) analysis to determine the relative concentrations of individual components.

The findings of the study suggest that the synergistic application of UHAPF and PGPR can significantly enhance the growth, nutrient uptake, and bioactive compound profiles of Cannabis sativa L. Combining Bacillus megaterium and Pseudomonas aeruginosa with UHAPF proves to be the most effective treatment, reducing nitrogen loss and improving plant health and yield. The controlled-release properties of UHAPF contribute to sustained nutrient availability, while the phosphate solubilization and nitrogen-fixation abilities of PGPR strains enhance soil fertility and plant nutrition. Overall, the study highlights the potential of

nanofertilizers and biofertilizers in sustainable agriculture, with specific implications for crops with unique nutrient requirements and economic importance, such as Cannabis sativa L.

In conclusion, this comprehensive study addresses the global challenges in agriculture, emphasizing the role of nanotechnology and biofertilizers in sustainable and efficient nutrient management. The application of environmentally friendly urea-hydroxyapatite nanofertilizer (UHAPF), particularly in the cultivation of Cannabis, offers promising avenues for both ecological and economic benefits..

CHAPTER 6 CONCLUSION

The synthesis of urea nanofertilizer using hydroxyapatite as a carrier, and its application in combination with plant growth-promoting microorganisms (PGPB), represents a significant advancement in sustainable agriculture practices. The primary objective of current crop fertilization practices is to enhance nutrient use efficiency and minimize environmental impact. This study has successfully demonstrated the effectiveness of a slow-release functionalized urea hydroxyapatite fertilizer (UHAPF) in meeting these objectives, particularly in the cultivation of hemp crops. Analytical techniques such as FTIR, PXRD, FESEM, TGA, and Kjeldahl analysis have confirmed the successful incorporation of urea into hydroxyapatite, resulting in a slow-release fertilizer that sustains nitrogen release over a 30-day period. The strong bond formed between urea and hydroxyapatite is a key factor contributing to the slow-release properties of UHAPF. This novel nanofertilizer holds promise in providing crops with a consistent and prolonged supply of essential nutrients, addressing the demand for effective and easily processable nitrogen fertilizers.

The combined application of UHAPF and PGPR has demonstrated superior benefits compared to individual treatments in hemp crops. The symbiotic relationship established between PGPR and plants promote nutrient availability and enhances plant defence mechanisms. UHAPF provides a nutrient-rich environment for PGPR, nurturing their colonization and activity in the rhizosphere. This synergistic effect leads to improved plant growth in hemp crops. Gas chromatography-mass spectrometry (GC-MS) analysis has been instrumental in evaluating the impact of UHAPF treatment on bioactive compounds in hemp crops. The positive effects of UHAPF are evident in the increased protein content, essential for metabolic processes and overall plant growth. Additionally, UHAPF enhances photosynthetic pigment levels, such as chlorophyll, thereby improving photosynthesis and energy production in hemp plants. The supplementation of UHAPF also positively influences soil nutrient levels, particularly nitrogen and phosphorus, creating a more fertile soil environment for sustained plant growth. The application of nanofertilizers, such as UHAPF, in agriculture offers environment friendly properties, making them an attractive option for crop cultivation. However, further exploration is needed to understand the penetration, accumulation, and transport mechanisms of nanoparticles within plants.

Hemp plants, known for synthesizing various organic compounds, including cannabinoids, terpenes, and fatty acids, play a crucial role in natural defence mechanisms. The exploration of hemp's potential in phytoattenuation aligns with the broader goal of sustainable and environment friendly agricultural practices. Understanding the relationship between hemp plants, nanofertilizers, and PGPR in contaminated environments could provide valuable insights into optimizing phytoremediation strategies for diverse pollutants. As we investigate deeper into the intersection of nanotechnology, plant-microbe interactions, and sustainable agriculture, it becomes imperative to continue research efforts. Investigating the long-term effects of UHAPF and microbial consortia on soil health, crop yield, and environmental impact will contribute to the development

of innovative and sustainable agricultural practices. Additionally, addressing knowledge gaps related to nanoparticle behaviour in plant systems will further inform guidelines for their responsible use in agriculture.

In conclusion, the synthesis of UHAPF represents a significant advancement in fertilizer technology, offering a sustainable solution for nutrient management in crop cultivation. The positive impact observed in hemp crops, particularly when combined with PGPR, underscores the potential for integrated approaches to enhance agricultural sustainability. As we navigate the challenges of feeding a growing global population while minimizing environmental impact, the exploration of nanofertilizers and plant-microbe interactions holds promise for shaping the future of agriculture.

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DETAIL OF RESEARCH PUBLICATIONS

- "Synergistic and non-synergistic impact of HAP-based Nano fertilizer and PGPR for improvement of Nitrogen and Phosphorus utilization in Hemp crop" *Journal – Environmental Science* **Impact factor – 9.473. https://doi.org/10.1039/D3EN00380A**
- "Opium alkaloids, biosynthesis, pharmacology and association with cancer occurrence" *Journal- open biology* **Impact factor – 7.12. https://doi.org/10.1098/rsob.220355**
- "Noscapine: An Effective Anti-Tumor Drug", International Journal of Emerging Technologies and Innovative Research (www.jetir.org), ISSN: 2349-5162, Vol.6, Issue 1, page no. 323-329, January 2019, Available: https://ssrn.com/abstract=3691152.
- "Polyhydroxybutyrate-Based Nanocomposites for Bone Tissue Engineering", Pharmaceuticals Volume-14, Page No. 1163, **Impact factor - 5.8** https://doi.org/10.3390/ph14111163
- "Effect of Lead Toxicity on Wild Cannabis Species of Punjab Region" https://doi.org/10.18311/ti/2023/v30i4/31022
- "Assessment of Phytoremediation Potential of Three Weed Plant Species in Soil Contaminated with Lead and Chromium" https://doi.org/10.1155/2023/2271039
- "Innovative development of Nitrogen (Urea) and Zinc-based Nanofertilizers and their applications" *ICFMMP conference paper* (Accepted)

- **CONFERENCES**

- International congress organic agriculture 2021 by IAAS world, Lebens, capacitaciones, Instituto Azteca and CARGO on 11th-13th June 2021.
- 5th International conference on advancement in agriculture technolgy and allied sciences (ICATAAS 2022) on June 4-5, 2022 held at Odisha. Topic-Synergistic effect of ure hydroxyapatite Nanofertilizer & pplant groth promoting bacteria on Hemp (Oral presentation).
- 4th International conference on global efforts of agriculture, forestry, environment and Food secutiy (GAFEF-2022) ON 17-19 September 2022 at Nepal. Topic- Synthesis and application of Urea Hydroxyapatite Nanofertilizer for sustainable agriculture (Poster Presentation)
- National e-conference on "Recent advances and future trends in Biological, physical and chemical science - 2021 organized by President Science College, Ahmadabad, affiliated to Gujarat University on 30th & 31st July Topic- Urea loaded Hydroxyapatite: A novel slow-release fertilizer.Category- Research scholar
- International conference of Pharmacy (ICP-2019) on theme of Pharmacy: realigning the focus on health on 13- 14 september 2019. Topic- Extraction and analysis of morphine, codeine and related alkloids in Papaver somniferum (Oral presentation).

Workshops

- one-day online workshop on **Creating Lab-to-Land Ecosystem: Challenges & Opportunities** jointly organized by Incubation Centre - Raja Ramanna Centre for Advanced Technology (RRCAT) and Indian Institute of Technology (IIT) , Indore on August 14, 2021 (Saturday).
- Online training programme on "**Comprehensive Landslide Risk Management**" on 28 to 30 July 2021 organised by National Institute of Disaster Management, Ministry of Home Affairs, Govt of India in collaboration with Indian Institute of Technology Mandi**.**
- Short term course on **Material Characterization: Analysis and Interpretation** organised by Central instrument Facility (CIF), Lovely Professional University (Date 23rd – 28th August, 2021)

Awards

- Society of Agriculture Research and Social Development (SARSD), New Delhi Best Research Scholar Award 2023, for outstanding contribution and recognition in the field of "Botany" on the occasion of 6th International conference on Advances in agriculture technology an allied sciences (ICAATAS 2023) at Loyola academy, Secundarabad (Date-19th -21st June 2023)