Plant-Microbe-based phytoremediation and bioremediation of Cd-contaminated soil

Tehreem Fatima

Registration No. 00000322510

Talha Farooq

Registration No. 00000323447

Rehana Yasmeen

Registration No. 00000322910

Supervisor: Dr. Rabia Amir

Atta-Ur-Rahman School of Applied Biosciences (ASAB)

National University of Sciences and Technology (NUST)

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Dr. Rabia Amir
Associate Professor
Atta-ur-Rahman School of Applied Biosciences (ASAB), NUST Islamabad

Signature _____

Name of Supervisor: **Dr. Rabia Amir**

Date 16/6/23

Dr. Sobia Manzoor
Tenured Professor
Head of Department
Deptt of Healthcare Biotechnology
Atta-ur-Rahman School of Applied Biosciences (ASAB), Islamabad

Signature (HOD) _____

Date 20.6.2023

Dr. Hussnain A. Janjua
Principal
Atta-ur-Rahman School of Applied Biosciences (ASAB)
NUST, Islamabad

Signature (Dean/Principal) _____

Date 22/6/23

BS Thesis Work

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Examination Committee Members

1. Name: Dr. Aneela Javed Signature: _____

2. Name: Dr. Rumeza Hanif Signature: _____

3. Name: _____ Signature: _____

4. Name of Supervisor: Dr. Rabia Amir Signature: _____
Dr. Rabia Amir
Associate Professor
Atta-ur-Rahman School of Applied Biosciences (ASAB), NUST Islamabad
Date: 16/6/23

5. HOD
Dr. Sobia Manzoor
Tenured Professor
Head of Department (HoD)
Deptt of Healthcare Biotechnology
Atta-ur-Rahman School of Applied Biosciences (ASAB), NUST-Islamabad
Signature: _____
Date: 20.6.2023

Countersigned

Dr. Hussnain A. Janjua
Principal
Atta-ur-Rahman School of Applied Biosciences (ASAB)
NUST, Islamabad

Dean/Principal

22/6/23
Date

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We, **Tehreem Fatima, Talha Farooq, and Rehana Yasmeen**, certify that this research work titled "Plant-Microbe-based phytoremediation and bioremediation of Cd contaminated soil" is our project. This project has not been submitted somewhere else for evaluation. The information obtained from another research has been correctly cited.

Signature of Student 1

Tehreem Fatima

Registration Number: 00000322510

BS Applied Biosciences

Signature of Student 2

Talha Farooq

Registration Number: 00000323447

BS Applied Biosciences

Signature of Student 3

Rehana Yasmeen

Registration Number: 00000322910

BS Applied Biosciences

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Dr. Rabia Amir
Associate Professor
Atta-ur-Rahman School of Applied Biosciences (ASAB), NUST Islamabad

Supervisor
Dr. Rabia Amir
Plant Biotechnology
ASAB, NUST

Dedication

We dedicate this thesis to our families, who have been our unwavering support system throughout our academic journey. Their love, encouragement, and sacrifices have been our guiding light, and we are forever grateful for their unwavering support.

We would also like to dedicate this work to our academic advisors and mentors, whose guidance, knowledge, and expertise have been invaluable to us. Their feedback and insights have challenged us to think critically and creatively and have helped us to develop as researchers and scholars.

Lastly, we dedicate this thesis to all the individuals who have inspired us, motivated us, and shaped us into the people we are today. Your kindness, wisdom, and encouragement have made an indelible impact on our lives and have helped us to pursue our dreams with passion and purpose.

Thank you all from the bottom of our hearts.

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Thank you all.

Tehreem Fatima

Talha Farooq

Rehana Yasmeen

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List of Abbreviations

°C	Degree Celsius
µL	Microliter
3D	3-Dimensional
AB-DTPA	Ammonium bicarbonate-diethylenetriaminepentaacetic acid
AmyE	Alpha-amylase - Bacillus *subtilis* (strain 168)
ANOVA	Analysis of Variance
AprE	*Subtilis* in E - Bacillus *subtilis* (strain 168)
ASA	Ascorbic acid.
ASAB	Atta-ur-Rahman School of Applied Biosciences, NUST, Islamabad, Pakistan.
B.*subtilis*	Bacillus *subtilis*.
BmtA	Borrelia metal transporter A.
Ca	Calcium.
Cas 9	CRISPR-associated protein 9.
CAT	Catalase.
Cd	Cadmium.
$CdCl_2$	Cadmium Chloride.
CO_2	Carbon dioxide.
Cr	Chromium.
Cpf 1	CRISPR-associated protein 12a.
CRISPR	Clustered regularly interspaced short palindromic repeats.

List of Abbreviations

DNA	Deoxyribonucleic acid.
dS/m	Deci Siemens per meter.
EDTA	Ethylene Diamine Tetra acetic acid.
Fe	Iron.
G	Grams.
GFP	Green fluorescent protein.
GI	Gastrointestinal.
H_2O_2	Hydrogen Peroxide.
K	Potassium.
Kg	Kilogram.
Mg	Magnesium.
mg/kg	Milligram per kilogram.
mg/ml	Milligram per milliliter.
Min	Minutes.
Ml	Milliliter.
mM/cm	Millimolar per centimeter.
mM	Millimolar.
Mn	Manganese.
NARC	National Agricultural Research Centre, Islamabad, Pakistan.
NBT	Nitro blue tetrazolium.
Nm	Nanometer.
OD	Optical Density.
P	Phosphorus.

List of Abbreviations

P-value	Probability Value.
Pb	Lead.
PBS	Phosphate buffered saline.
PCs	Phytochelatins.
PGPR	Plant growth-promoting rhizobacteria.
pH	The potential of Hydrogen.
PMC	PubMed Central.
POD	Peroxidase.
PAHs	Polycyclic Aromatic Hydrocarbons.
Psi	Pound per square inch.
RNA	Ribonucleic acid.
ROS	Reactive oxygen species.
Rpm	Revolutions Per Minute.
RWC	Relative Water Content.
SPAD	Soil Plant Analysis Development.
TBA	Tertiary Butyl alcohol.
Tukey HSD	Tukey’s honestly significant difference.
U	Uranium.
U/ml	Units per milliliter.
Zn	Zinc.

Abstract

Cadmium (Cd) is a heavy metal known to be harmful to both the environment and human health. It is released into the environment through various human activities, such as industrial processes, sewage, plastic, Ni-Cd batteries, and phosphate fertilizers. This metal can accumulate in soil and air, posing a threat to crops, and food security, and causing economic losses. To address this issue, it is crucial to develop sustainable and cost-effective methods for removing Cd from contaminated soil.

Phytoremediation and bioremediation are two environmentally friendly and cost-effective techniques that can be used to remediate Cd-contaminated soil. Sunflower is a plant known for its ability to absorb and reduce the toxicity of heavy metals like Cd from polluted soil. Additionally, Bacillus subtilis, a type of bacteria, can enhance plant growth and biomass production, aiding in Cd remediation.

Cadmium pollution is a significant problem that requires immediate attention to safeguard agricultural land and human health. Traditional remediation methods, such as excavation and soil washing, are expensive and can cause soil erosion. Hence, there is a need to explore sustainable and cost-effective approaches for Cd remediation.

To investigate the combined effect of Sunflower's hyperaccumulation property and Bacillus subtilis' resistance to Cd transport in plants, pot experiments were conducted. These experiments compared the impact of Cd stress on plant growth, yield quality, and quantity, with and without the presence of the bacteria. Morphological, physiological, and biochemical analyses of the plants were performed, along with comparisons of root and shoot structures.

The results revealed that plant growth and development were more adversely affected by increasing concentrations of Cd. However, the presence of Bacillus subtilis significantly improved the plants' morphological and physiological traits. This effect was particularly notable at high Cd concentrations, indicating that the bacteria reduced Cd toxicity. The activity of antioxidant enzymes increased, indicating the presence of reactive oxygen species (ROS) under stress conditions. Nevertheless, the presence of bacteria further activated the plants' defense mechanisms, preventing damage to plant structures.

This research has significant implications for the development of environmentally friendly and economically viable approaches to remediate Cd-contaminated soil. Moreover, this approach can be extended to address other heavy metal contaminants, contributing to environmental protection and human health. It can be applied to restore contaminated industrial sites, leading to cleaner environments, and reducing Cd exposure in food, thereby improving public health.

Keywords:

Antioxidants, *Bacillus subtilis* assisted bioremediation, Cadmium toxicity, Heavy metals contamination, Hyperaccumulators; Plant growth-promoting rhizobacteria (PGPR); Reactive Oxygen species; Sunflower assisted phytoremediation; Sustainability.

Chapter 1: Introduction

1.1 Helianthus annus

The Asteraceae family and the genus Helianthus include more than 70 species of sunflowers, which are short-season plants. The name "sunflower" originated from the plant's shape and size, which resemble the sun, and its tendency to revolve around the sun. Sunflowers have broad, rough, and coarsely hairy leaves, as well as extensive taproots and hairy stalks. They are also characterized by their large, round, yellow inflorescent flower head that faces the sun (Vilvert, 2018b). **Figure 1** shows the morphology of sunflower plants.

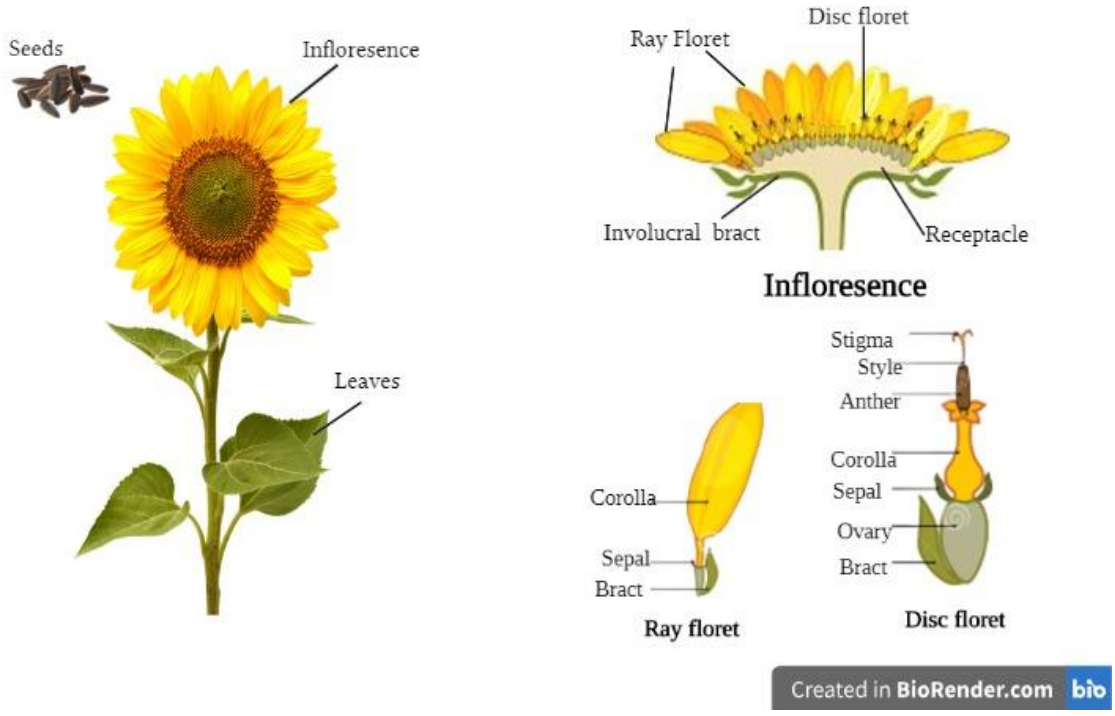

Figure 1: Morphology of the Sunflower plant.

Sunflower is recognized as one of the most valuable and economically viable oilseed crops globally, ranking fourth behind soybeans, rapeseed, and safflower. Plant scientists primarily develop high-oil varieties under favorable circumstances or maximum yield and productivity that require rich soil, appropriate rainfall, and proper climatic conditions. Sunflowers can be cultivated successfully over a broader range of latitudes than other oilseed crops, along with

having the shortest lifecycle. Additionally, sunflowers can flourish farther north than soybeans.

However, the yield of sunflower products, such as seeds, oil content, and other products, may be reduced due to nutrient-limiting environmental circumstances, such as climate and management variables. Synthetic fertilizer and organic manure can impact the productivity and quality of sunflowers. The availability of crucial micronutrients like potassium can boost agricultural output and enhance the crops' resilience to environmental stressors such as drought. (Bartholomew Saanu Adeleke, September, 2020).

Sunflowers are the main oilseed crop, generating over 87% of all vegetable oil (Doglas Bassegio a, 2016). Sunflower is a promising and economically viable crop that offers several benefits, including increasing marketable commodities, acting as a source of income, and reducing poverty. However, due to harsh weather conditions and a lack of quality seeds, farmers have not been able to fully utilize this technology along the food value chains. If used correctly, the yield of sunflower could be explored as a potential replacement for current oil crops, such as soybean, rapeseed, peanut, soybean kernel, and palm oil.

Sunflowers are generally classified into two groups: oil and non-oil types. The selection criteria for oil-type sunflowers are based on their unique oil and meal properties, such as oil yield, composition, and meal protein content. The seeds of oil-type sunflowers are black and have flimsy hulls that cling to the kernels. These seeds contain between 38 to 50 percent oil and 20 percent crude protein. (Carter, 1987).

The sunflower crushing industry primarily grows oil-type seeds to produce sunflower seed oil and meal. There has been a growing demand for oil-type sunflowers due to their use as an ingredient in bird food blends. The main non-oil type of sunflower seeds is known as confection sunflower seeds. These seeds are thick and large with striped hulls that are weakly attached to the kernels. Confectionary sunflower seeds are in higher demand both domestically and internationally. A portion of these seeds are dehulled and sold as confectionary "nuts," while some are roasted like peanuts. In addition to oil-type seeds and smaller confection seeds, novel types of sunflowers have been created specifically for the bird food industry to serve as food for small animals, wild birds, and pet birds.

1.1.1 Composition of Sunflower Oil

Both vegetable and animal oils and fats contain saturated and unsaturated fatty acids. Vegetable oils with a high content of unsaturated fatty acids are considered essential fatty acids because they cannot be produced by the body and play a crucial role in maintaining human health. Essential fatty acids must be obtained through a balanced diet. Standard sunflower oil has about 15% saturated fatty acids and 85% unsaturated fatty acids, including oleic acid and linoleic acid, which make up around 14-43% and 44-75% of the unsaturated fatty acids, respectively. Sunflower oil is one of the most important vegetable oils for human nutrition due to its high-quality fatty acid composition. Sunflowers provide around 50% of the vegetable oil output, with oil content ranging from 22-50%.(Akkaya, October 2018).

Table 1 shows the content of different fatty acids in sunflower oils.

Table 1: Fatty acid content of sunflower oil.

Fatty Acid	Sunflower oil (%) (standard)	Sunflower oil (%) (mid-oleic acid)	Sunflower oil (%) (High-oleic acid)
Palmitic acid	5.0-7.6	4.0-5.5	2.6-5.0
Stearic acid	2.7-6.5	2.1-5.0	2.9-6.2
Oleic acid	14.0-39.4	43.1-71.8	75-90.7
Linoleic acid	48.3-74	18.7-45.3	2.1-17
Linolenic acid	0-0.3	0-0.5	0-0.3

1.1.2 Nutritional Attributes and Health Importance

Sunflower is a globally grown oilseed crop that provides high-quality oil and dietary fiber, both of which offer significant health benefits. Sunflowers also contain other nutritious elements such as cake and meals. According to (Malik, 2018) Sunflower meal, also known as sunflower cake, is a by-product produced from harvested and processed sunflower seeds, comprising about 36% of the total mass. It has a protein content ranging from 45% to 50%. Sunflower meal is considered a valuable nutritional source for humans as well as composite meals for livestock, as it contains essential amino acids, vitamin B, minerals, and significant antioxidant properties. (Wanjari, 2015).

Sunflowers are a rich source of nutrients and phytochemicals that are beneficial for human health. These include flavonols, phenolic acids, procyanidins, amino acids, phytosterols, antioxidants, dietary fiber, potassium, arginine, and monounsaturated and polyunsaturated fatty acids. Sunflower seeds and oil are not only consumed as a nutrient-rich dietary source, but refined oil is also widely used in the food industry to produce margarine, butter, bread, and snacks. Sunflower products have functional qualities that can help prevent or manage human ailments such as diabetes, cancer, hypertension, hypercholesterolemia, and coronary heart disease, due to their nutritional composition. Moreover, products derived from sunflowers can be combined with other ingredients to create a diverse range of diets with complex nutritional profiles that promote human health (Katsarou, 2015). Compared to other oilseed crops, sunflower seeds are considered to have a low level of sugar and glycemic index. They are an excellent source of essential minerals such as copper, phosphorus, manganese, zinc, and iron, as well as amino acids, unsaturated fats (which are cholesterol-free), fiber, vitamins, proteins, and folate. (Poulsen, 2017). The biological effects of different constituents of sunflower seeds are shown in **Table 2**.

Table 2: Biological Effects and Constituents of sunflower seeds (Guo, 2017).

Biological effect	Biological constituents
Antioxidant	Enzymes (catalase, glutathione reductase, guaiacol peroxidase, glutathione dehydrogenase), carotenoids, l-ascorbic acid, peptides, phenolic compounds (flavonoids, phenolic acids, and tocopherols),
Anti-inflammatory	α-tocopherol, helianthosides, triterpene glycosides
Antihypertensive	11S globulin peptide
Antimicrobial	Saponins, alkaloids, phenolic compounds, tannins
Antidiabetic	Quinic acid, glycosides, chlorogenic acid, caffeic acid, phytosterols

1.1.3 World Production of Sunflower Oil

Sunflowers are an important crop worldwide, with top producers including Nigeria, Tanzania, South Africa, Brazil, India, Argentina, Canada, China, France, Russia, Spain, and Australia. Argentina and Eastern European countries produce over 10% of the global sunflower seed crop. China, the fifth largest sunflower producer, produced 2.7 million tons in 2021. From 2013 to 2017, the world oilseed crop increased by nearly 15%. In 2017, the United States produced approximately 132 million metric tons of oilseeds, followed by other significant producers such as Brazil, Argentina, and China. (Cheng, 2017). With the global population projected to reach 10 billion by 2050 and an increase in food demand, crop production needs to increase from 133 million tons to 282 million tons. While South Africa, Malaysia, and Indonesia have lower oilseed production, two major producers account for 60% of the world's oilseed supply. France is the top sunflower oil producer in Europe, surpassing countries like Spain (482,000 tons), Hungary (390,000 tons), and Romania (339,000 tons) with a production rate of 550,000 tons. Ukraine, Argentina, the United Arab Emirates, and Russia account for 82% of the world's edible oil production (Vilvert, 2018a).

1.1.4 Sunflower Meal

Among the other high-protein ingredients in animal feed, one is sunflower seed meal. Soybean, cottonseed, com gluten, canola, linseed, and fish meals are also suitable alternatives. Linseed and canola meals made locally are the most likely replacements as most sunflower seed is produced in the Northern United States. The remaining meals can all be utilized as sources of protein for animal feed (Tangendjaja, 2022).

The amount of protein in sunflower seed meal depends on how many hulls are kept. The crude protein and fiber in sunflower seed meal made from whole sunflower seeds ranges from 25 to 28 percent. Sunflower seeds can be partially or fully dehulled to obtain a meal with different nutritional profiles. Partially dehulled seeds will yield a meal containing 32-38% protein and 18% fiber, while fully dehulled seeds will produce a meal with 40-42% protein and 12-14% fiber. Currently, meals with 28–32% protein content serve as the baseline for price. Even when entirely dehulled, sunflower seed meal contains more fiber and less total digestible nutrients than soybean meal. Sunflower seed meal has less net metabolizable energy than soybean meal, making it less suitable for breastfeeding, gaining weight, or

maintenance. Lysine is the scarcest amino acid in sunflower seed meal. This restriction is not significant when feeding ruminants, but it is required to mix sunflower seed meal with an alternative protein source that contains more lysine when feeding pigs or poultry (2022).

1.1.5 Sunflower Seed Crushing Process

The principal factor in choosing the crushing method to be applied to obtain the oil is the oil content. If the hulls are removed, sunflower seeds can have an oil content of up to 50%, which is higher than the average level of over 40%. Soybeans, in comparison, only have 18% oil. The two main methods of crushing used to process oilseeds are solvent extraction and mechanical crushing. A continuous feed expeller is used in the mechanical procedure to squeeze the oil out of the seeds using force. Compared to solvent extraction, the expeller technique uses more energy, costs more to maintain, and leaves 3–10% more oil in the cake. A chemical is used in the solvent process to separate the oil from the seed. As less oil is left in the leftover cake after solvent extraction, it is practically more effective. Nevertheless, solvent extraction becomes economically inefficient with oilseeds containing more than roughly 25% oil since more solvent is needed to obtain the oil. There are several reasons why a combination of mechanical and solvent processing has become the industry standard for sunflower seed processing, including its efficiency, cost-effectiveness, and ability to extract a high percentage of oil from the seeds. (Etienne Le Clef, 2015).

1.1.6 Oil Preparation: Refining, Bleaching, and Deodorizing

Like other vegetable oils, the sunflower seed oil is refined before it is used in culinary products. Free fatty acids and other impurities are taken out of the oil by refining, the color is lightened by bleaching, and the smell of the oil is taken out by deodorizing. Gums can cause larger losses during the refining process and can also settle in oil that has been stored if they are left in the oil. Degumming is performed by hydration, a procedure in which oil and water are combined to separate gums and other impurities. The main aim of degumming is to remove phospholipids from sunflower oil (Segers JC, 1990) as they can cause off-flavors and a dark color in the oil. Various methods such as settling, centrifuging, or other common techniques are used to remove these impurities. Although there is typically an ample supply of soy lecithin in the market, the gums removed during degumming can be further processed

and marketed as lecithin. Degumming is often carried out at the crushing mill rather than the refinery, although it is easier to perform degumming with fresh oils.

During the refining process, nearly all free fatty acids are converted into oil-insoluble soaps using alkali solutions such as sodium hydroxide. The soap stock can then be removed and sold, although there is currently less demand for soap supplies than there was three decades ago due to the increased use of detergents. The soap stock can be used as an alternative source of industrial fatty acids. Bleaching sunflower oil involves removing certain components that cause pigmentation in the oil. Natural bleaching earth or synthetic materials such as bentonite or montmorillonite are used as adsorbent materials. Some degumming is done during the bleaching process, which involves heating the oil and substance to between 220 and 230 °F, after which the color pigment-containing bleaching material is filtered off. (DA., 2005).

To prepare sunflower seed oil for its intended use, additional processing steps may be necessary. This can include hydrogenation, dewaxing, or deodorization. Dewaxed oil is commonly sold as cooking oil or used in the production of salad dressing and mayonnaise. Hydrogenated oils, which are produced by hydrogenation, are frequently used in the production of margarine, confectionery fats, and shortening.

Initial dewaxing procedures may not remove all the wax from sunflower seed oil, so a second dewaxing step may be necessary. In this process, the oil is chilled in holding tanks, which causes the waxes to accumulate. The oil is then mixed with diatomaceous earth, and the waxes adhere to the filters as the oil passes through.

Deodorization is the final and most important step in the processing of edible oils. Its purpose is to remove contaminants that can affect the oil's taste. Deodorization removes off-flavors that were created during earlier stages of refining, including oxidation products such as hydroperoxides, aldehydes, ketones, and epoxides, as well as volatile components such as FFA or impurities. The oil is purified using steam and pressure to eliminate impurities such as free fatty acids, alcohols, hydrocarbons, and breakdown products of peroxides and pigments. This results in a bland-tasting, light-yellow oil suitable for cooking or use as a salad dressing. Alternatively, the oil can be further processed into a hydrogenated or partially hydrogenated product that can be used to make margarine or shortening.

Hydrogenation is a chemical process in which hydrogen gas and oil react in the presence of a catalyst, typically nickel. This process improves the stability of the product and raises its melting point. Hydrogenation also slows down oxidation, which can cause taste loss. During hydrogenation, unsaturated bonds in the fat molecule are converted to saturated ones. The oil that has been lightly hydrogenated may still be liquid at room temperature, but additional hydrogenation turns the oil solid. (Aguirre MR, 2014)

1.1.6.1 Vegetable Oil Market

Margarine, baking and frying fats, salad and cooking oils, and other culinary items like baking and frying fats are the main uses for sunflower seed oil. Oil is a minor component of industrial goods like plastics, paint, soap, and animal feed. Traditionally, the major portion of domestic sunflower seed oil demand has been for salad and cooking oil. The usage of baking and frying fats, salad and cooking oils, and other fats increased significantly. This increase is frequently correlated with rising consumer affluence, rising prepared food consumption, and rising restaurant service demand. Despite rising wages, per capita consumption of fats and oils has stabilized since 1987, in part because of shifting consumption patterns that have emphasized lower-fat domestic markets, sunflower seed oil competes with oils like soybean, cottonseed, and, more recently, canola oil. The usage of baking and frying fats, salad and cooking oils, and other fats increased significantly. This increase is frequently correlated with rising consumer affluence, rising prepared food consumption, and rising restaurant service demand. The market for consumer-visible oils, like bottled cooking oils and margarine, and the production of goods that require fixed ingredient mixtures for flavor or stability is where premium oils are most in demand. **Figure 2** explains the marketing flow of sunflower seeds.

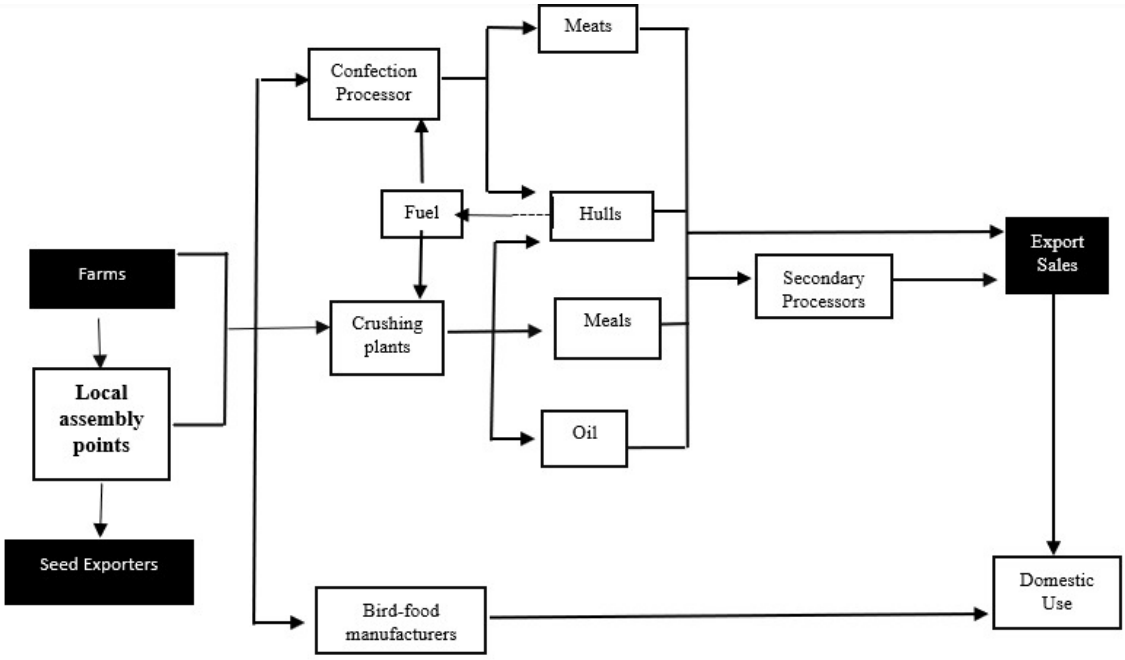

Figure 2: Marketing flow of Sunflower seed.

1.2 Bacillus *subtilis*

Bacillus *subtilis* is an aerobic, gram-positive common PGPR commonly utilized to produce heterologous proteins(Yuan Su, 03 September 2020). It contains cells that are rod-shaped and average 2 to 6m long and 1m in diameter. Between 30 and 35°C is the optimum temperature for growth, which shortens doubling periods to 20 min. (Jeffery Errington, 2020). Under specific growth circumstances, the cells can form long chains connected by septal wall material that hasn't been cleaved. When resources are scarce, the cells may undergo a difficult 2-cell differentiation process that results in an endospore. The mother cell that is enclosing this endospore is subsequently destroyed, causing it to be removed. The single-celled membrane facilitates the secretion of proteins and makes the downstream processing simpler.

Bacillus *subtilis* possesses excellent physiological characteristics; it grows fast, has a fermentation cycle of 48 hours, and can be grown on cheap substrates(Chen J, 2016). It can change its growing conditions according to the changing environment using various enzymes to degrade several different substrates. This bacterial specie is quite beneficial to industries and for medicinal purposes, as it has an excellent ability to secrete enzymes and is considered safe(Lidia Westers, 2004).

1.2.1 Taxonomy

The groundbreaking mutagenesis research over 60 years ago marked the beginning of the establishment of B. *subtilis* as a bacterial model system(P R BURKHOLDER, 1947). It was later discovered that strain 168, a tryptophan auxotrophic mutant obtained in those trials, was naturally capable of genetic modification(Spizizen, 1958). A scientific community devoted to examining the genetics and growth of this Gram-positive spore-former and to using its biotechnological prospects soon emerged because of this finding. The taxonomy of Bacillus *subtilis* is explained in **Figure 3**.

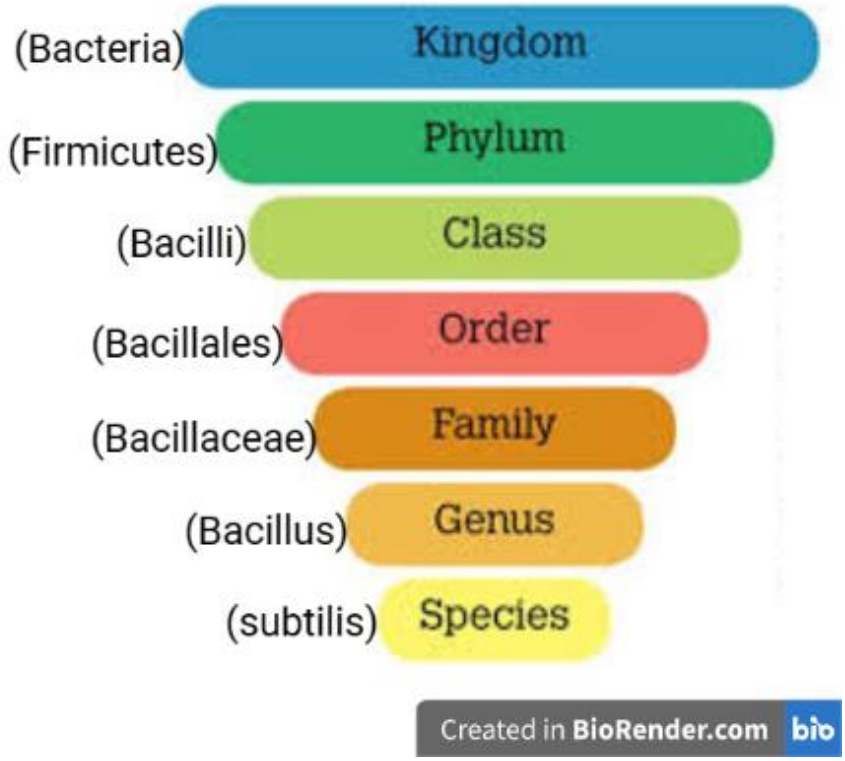

Figure 3: Taxonomy of Bacillus subtilis.

1.2.2 Habitat

Both terrestrial and marine settings are home to Bacillus *subtilis*. Therefore, this bacterial specie is present everywhere in the ecosystem and can adapt easily to different environmental conditions. As a coping mechanism against stress conditions and nutrient unavailability, Bacillus *subtilis* produces highly resistant dormant endospores which are airborne and can be dispersed easily with the wind to travel large distances, so they can land on different

surfaces(Ashlee M. Earl, 2010; Jaenicke, 2005). Therefore, it cannot be concluded whether the stain was growing on that surface or not.

An experiment conducted to identify the vegetative and spore form of Bacillus *subtilis* in different soils by using fluorescent antibodies revealed that this bacterial strain remained in its vegetative form in the presence of organic matter, and its vegetative cells continue to proliferate until the nutrients are depleted from the soil(J. R. NORRIS, 1961). When the soil is deprived of the nutrients required for its growth, B.*subtilis* transforms its vegetative cells into spores.

1.2.3 Role in Plant Growth

The most prevalent bacterium in plant rhizospheres is Bacillus *subtilis*. Because it can grow near the roots of many other plants, it aids in the growth of those plants. (F.M. Cazorla, 2007). This bacterial specie promotes plant growth in either of three ways: it competes for nutrients with other bacterial species that are harmful to plant growth, the plant defense mechanism against harmful pathogens is activated by these bacteria, or this strain of bacteria makes certain nutrients readily available to plants (Krzysztofa Nagórska, 2007).

Bacillus *subtilis* is commonly found in feces, as it can be present in plants and consumed by animals. While there is anecdotal evidence to suggest that B. *subtilis* may play a role in the gastrointestinal tract, its passage through animal GI tracts may have consequences.(Nguyen K M Tam, 2006; Teresa M Barbosa 1, 2005). B. *subtilis* has been marketed as a probiotic due to its ability to help maintain or restore "healthy" bacterial populations. (Huynh A Hong, 2005). Some commercially available fermented foods, such as natto, also contain B. *subtilis*. However, it is unclear how B. *subtilis* imparts its probiotic properties given its role in promoting plant growth (Y Inatsu, 2006).

Previously, B. *subtilis* was thought to be an obligate aerobic bacterium that passed through the GI tract as a spore, with any benefits derived from spore-specific intrinsic characteristics. However, recent research suggests that B. *subtilis* can complete its entire life cycle inside the GI tract, transitioning from spore to vegetative cell and then sporulating once more. (Gabriella Casula, 2002; T T Hoa, 2001).

Analysis of the B. *subtilis* 168 genome sequences indicates that no genes encoding recognized virulence factors were found, supporting the idea that this bacterium is not a pathogen (F

Kunst, 1997). Interestingly, the genome encodes a lot of pathways for using chemicals obtained from plants, which supports the idea that this specie is closely linked to plants. B. *subtilis* was shown to include genes for a potential respiratory nitrate reductase, which disproved the theory that it was an obligate aerobe. As a result, it was hypothesized that B. *subtilis* may develop anaerobically by utilizing nitrate as an electron acceptor instead of oxygen. Since then, experimental evidence has shown that B. *subtilis* can grow anaerobically when nitrate is present. The finding that B. *subtilis* can develop anaerobically lends additional credence to the hypothesis that vegetative life is possible in the GI tract of animals, which is primarily anaerobic(Martha J Folmsbee, 2004).

A significant amount of B. *subtilis*' genome (around 4%), as shown by the genome sequence, is focused on producing secondary metabolites(Stein, 2005). B. *subtilis* is probably able to compete in the natural environment because of several of these substances because they are effective fungal and bacterial inhibitors, encourage plant development, and act as probiotics.

1.2.4 Applications

The CRISPR-cas9/cpf1 technique has been developed to genetically modify B. *subtilis* for various commercial applications, including the production of xylanase, cellulase, lipase, lichenase, and α-amylase, using the organism's protein secretion system, ribosomal binding sites, and artificial promoter (Da-Eun Jeong, 2018). B. *subtilis* has multiple benefits as a multipurpose probiotic, including preventing the growth of pathogenic bacteria, improving nutrient absorption, and serving as an excellent bioreactor host (Olmos J, 2020). It can also produce various compounds, such as riboflavin, menaquinone-7, inositol, and N-acetylglucosamine (Gu Y, 2019; Gu Y, 2018). In agriculture, inoculating the correct amount of B. *subtilis* can enhance the humus and carbon content of compost, recovering soil quality and increasing crop development. Moreover, B. *subtilis* can produce complex biofilms that can generate useful biomaterials, including surface growth factors, antibiotics, lysozyme, and antimicrobial peptides for use in medical products. These biomaterials can be produced using living biological material (Yuan Su, 03 September 2020). Furthermore, these biofilms can be utilized as a biomaterial in 3D printing (Duan M, 2020). **Figure 4** shows the summary of industrial applications of B.*subtilis*.

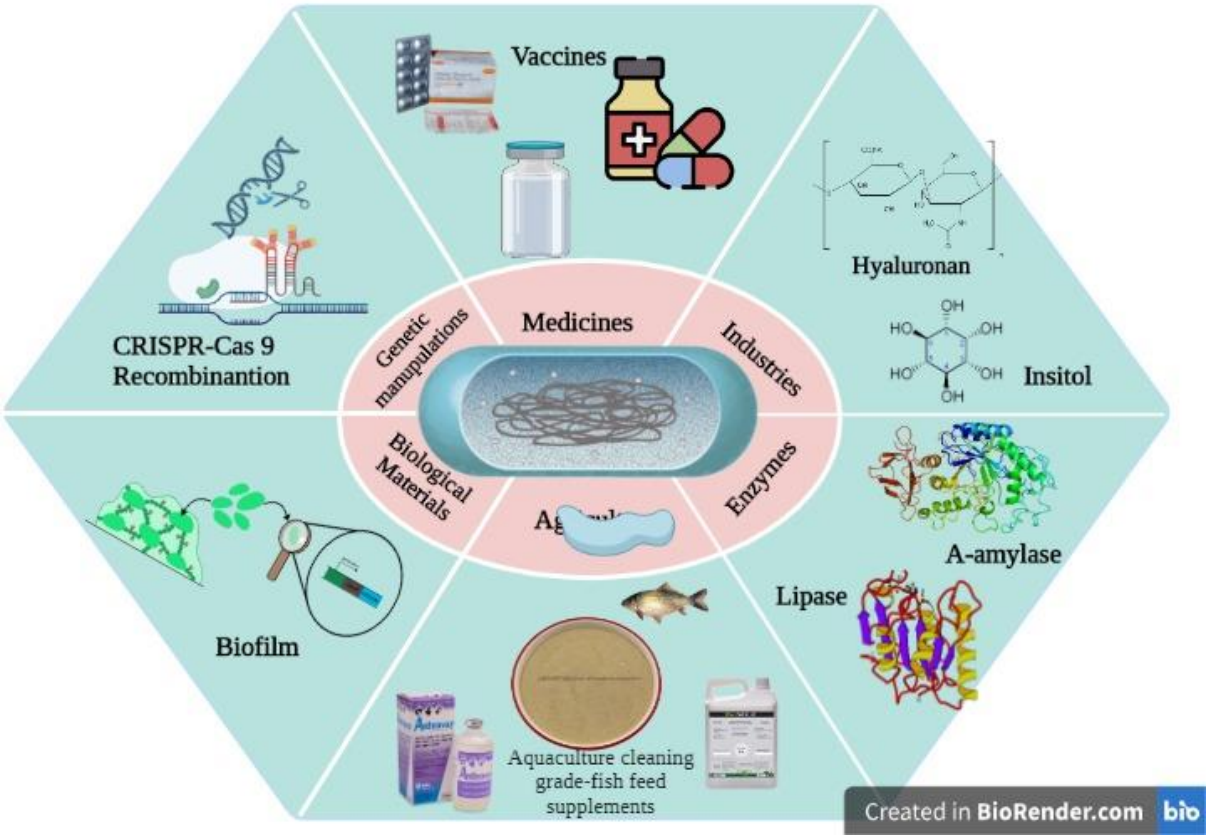

Figure 4: Industrial applications of Bacillus subtilis.

1.3 Effect of Cadmium Stress on Helianthus annus

At the beginning of the 20th century, the industrial revolution and rapid population growth caused an increase in heavy metal pollution, leading to significant environmental and health issues worldwide. Agricultural areas around the world have been contaminated due to the use of agricultural additives such as pesticides, mineral fertilizers, and urban composts, as well as waste incinerator emissions, car exhausts, mining leftovers, and smelting industries (Ahmed A. Abdelhafez, 2014). Heavy metals, which are not biodegradable, act as mutagenic and carcinogenic agents and pose a significant hazard to the ecosystem and all living things (Wei Wu, 2018). Furthermore, the high levels of heavy metals in soil can have negative impacts on plant growth and agricultural production.

Cadmium (Cd) contamination in soil is a growing problem caused by industrial contamination, the use of commercial fertilizers, or pollution from bedrock. Cadmium can affect various plant processes such as photosynthesis, nitrogen and carbohydrate metabolism, and the development of chlorophyll and the light-harvesting chlorophyll a/chlorophyll b protein complex II. It has also been shown to impact gene expression, transpiration rates, protein patterns, plant cell respiration, and enzyme activity (Helena Azevedo, 2005). The amount of cadmium (Cd) in the soil varies between 0.1 and 1.0 mg kg^{-1} (Mutlu, 2012). High levels of cadmium in the soil can be found in fruits and vegetative parts of plants, even after several decades.

1.3.1 Morphological Changes

Since cadmium toxicity causes the stomata to close and the plant tissues to take in less CO_2 than they would otherwise, the availability of cadmium in the soil causes a decline in plant development. This drop is a result of the plant's reduced ability to perform photosynthesis and transpiration(Parameswaran Aravind, 2005). Cadmium can alter the root's morphology, which can lead to an ion imbalance that interferes with the delivery of various nutrients to the plant, including magnesium(M. V. Pérez Chaca, 2014). Cadmium poisoning also slows down the formation of the root system, which may lead to a disturbance in the activity of certain enzymes and have an adverse influence on how cells divide. There was a reported decline in the amount of chlorophyll and carotene, as well as the development of the entire plant,

including its roots, and its leaves. It also had an impact on the structure of the leaves and roots(Almuwayhi, 2021).

Cadmium ions disrupt chromosomes to suppress mitotic activity in meristematic tissues, limit root extension, and depolymerize the cell cytoskeleton(C.S. Seth, 2008). They also increase parenchyma cells and, as a result, cortical tissue development. This improves root diameter and makes the plant more resistant to ionic solutes(Ivana Maksimovic, 2007). Under cadmium stress, the root's ability to take up nutrients and water is dependent on the length of the root; therefore, under stress, the root's ability to take up nutrients and water is decreased due to a decrease in root length, surface area, and the number of tips, as well as an increase in root diameter(Andrea Staňová, 2012).

Cadmium concentrations in leaves typically range from 0.05 to 0.2 mg kg^{-1}; however, levels greater than 5 to 10 mg kg^{-1} are harmful to plants. According to studies, young leaves contain more Cadmium than older ones(F Pietrini, 2010). In stressful conditions, the shoots and leaves exhibit necrosis, chlorosis, desiccation, and stunting. According to research, when plants are under cadmium stress, there is an impact on seed production and germination. Under the influence of Cadmium, seed imbibition, and water-repressing content also affect germination and growth(Veria Alvarado, 2005).

1.3.2 Physiological Changes

Chlorosis and stunted growth in plants are two visible indicators of cadmium toxicity. Increased toxicity prevents plant development and causes necrosis. Since cadmium poisoning prevents plants from fixing carbon, it also lowers their chlorophyll levels and photosynthetic activity(P. Das, 1997). Exposure to cadmium in the soil promotes osmotic stress in plants, which damages their physiological health by lowering leaf RWC, stomatal conductance, and transpiration. Additionally, cadmium prevents plants from absorbing Fe and Zn, which results in leaf chlorosis. Cadmium frequently impairs the transport and absorption of Ca, P, Mg, K, and Mn.(Fasih Ullah Haider, 2021; Hao Zhang, 2019).

The cytoplasmic membranes can be damaged by Cadmium toxicity, which lowers the potassium content of the plant's leaves(Balaji B. Maruthi Sridhar, 2007). Moreover, it degrades lipids, which disturbs the amounts of several elements inside the plant. Chlorophyll levels in plants drop when Cadmium is present, which is caused by modifications to the

chloroplasts that impede the production of chlorophyll (Parameswaran Aravind, 2005; Zhao Zhong-qiu, 2005). Protein synthesis is inhibited by Cadmium poisoning, which may eventually prevent plant tissues from producing proteins. The toxicity of Cadmium affects the process of shutting the stomata in the leaves, which reduces photosynthesis and thus lowers the number of carbohydrates in plant leaves(Balaji B. Maruthi Sridhar, 2007; Parameswaran Aravind, 2005).

High quantities of cadmium can build up in the vegetative system, causing a disturbance in the activity of enzymes and inhibiting cellular division. Moreover, it binds to enzymes, which changes how carbohydrates are metabolized and lower the amount of glucose in leaves(Claudia Cosio, 2004; Muhammad Arshadullah, 2007).

1.3.3 Biochemical Changes

Reactive oxygen species (ROS) are produced excessively because of cadmium toxicity, which damages plant membranes and kills cell organelles and biomolecules. MDA concentration is a common indicator of oxidative damage in plant cells. The primary sign for the transmission of lipid peroxidation is thought to be the lipids in cell membranes. In plant tissues, this process destroys the cell membrane as well as induced free radicals(I. H. Shamsi, 2008; Ian M Møller, 2007). By the activation of the oxidase enzyme, disturbance of the electron transport chain, and interaction with the anti-oxidative defense mechanism, Cadmium ions cause oxidative stress in crops(Andres Schützendübel, 2002).

The excessive buildup of Cadmium initiates the production of ROS, which leads to membrane lipid peroxidation, enzyme inhabitation, structural alterations in metabolites, and disturbance of metabolic pathways. ROS also alters DNA and RNA structure, which causes cell death(Priscila L Gratão, 2005). Moreover, cadmium stress alters the amounts of sucrose and hexose (a plant sugar), interfering with cellular processes. Sugar content increases in plants are linked to cell swelling and disturbance(Masa-aki Ohto, 2005).

1.4 Bioremediation

Heavy metal contamination is a serious threat to the ecosystem and public health due to its toxic and non-biodegradable nature. Cadmium (Cd), a class-I carcinogen and powerful nephrotoxin is among the most harmful heavy metals. In situ, the immobilization of Cd in the soil is a cost-effective and feasible approach for remediation, as agricultural output can continue during the process. The addition of microorganisms to immobilize heavy metals and promote plant growth is an environmentally friendly alternative to chemical sorbents, as it reduces secondary pollution and maintains soil quality. Therefore, using living microorganisms for bioremediation in large Cd-contaminated areas is a viable solute ion. (Hang Ma, 2020). Bacillus *subtilis* has been studied for its bioremediation potential.

1.4.1 Mechanism of Bioremediation

For heavy metals elimination from the environment, Bacillus species employ a variety of methods including bioprecipitation, bioaccumulation, and biosorption.

1.4.1.1 Biosorption

The method of biosorption for heavy metal removal is a physicochemical process based on cell membranes and does not require metabolism. This process utilizes negatively charged substances found in cell membranes to attract heavy metal ions. Typically, non-living biomass is used for biosorption, as it accelerates the process more effectively. The effectiveness of this approach is influenced by various factors, such as surface characteristics, pH, temperature, and electrostatic interactions (Tiquia-Arashiro, 2018; Zabochnicka-Świątek M., 2014). To optimize the biosorption process, it is crucial to understand the mechanisms involved in the sorption process. Three processes, including ion exchange, complexation, and physical adsorption induced by intermolecular interaction, are responsible for heavy metal ions' attachment to functional groups in cell membranes, mainly through Van der Waals forces (Babák L., 2012). Different mechanisms of bacterial biosorption have been identified in **Figure 5**.

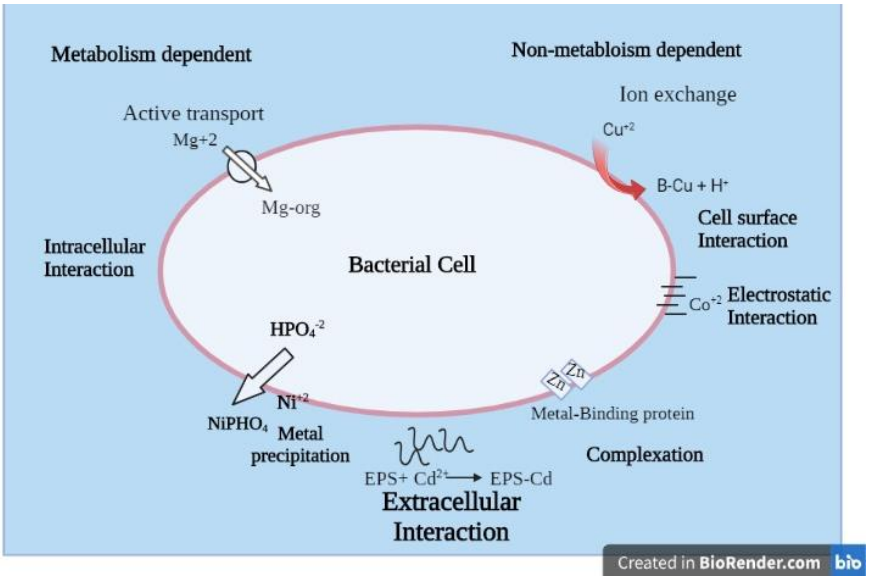

Figure 5: Different mechanisms of Bacterial biosorption.

1.4.1.2 Bioaccumulation

The cellular energy-dependent process of bioaccumulation is carried out by active metabolic bacteria. Factors such as microbial internal structure, genetic and physiological capacity, and environmental conditions impact the efficiency of heavy metal absorption through bioaccumulation (Issazadeh K., 2013; Vijayaraghavan K., 2008). The charge of the cell surface and temperature also affect the bioaccumulation process (Srinath T., 2002). The use of metallothionein for heavy metal binding is the most well-known bioaccumulation process, and the bmtA gene can encode this protein (D.H, 1986). Metallothionein is a cysteine-rich protein that aids in the bioaccumulation of heavy metals inside cells, and bacterial cells often produce it in response to increased metal exposure (Blindauer C.A., 2002; Liu T., 2003). The mechanism of bioaccumulation is explained in **Figure 6**.

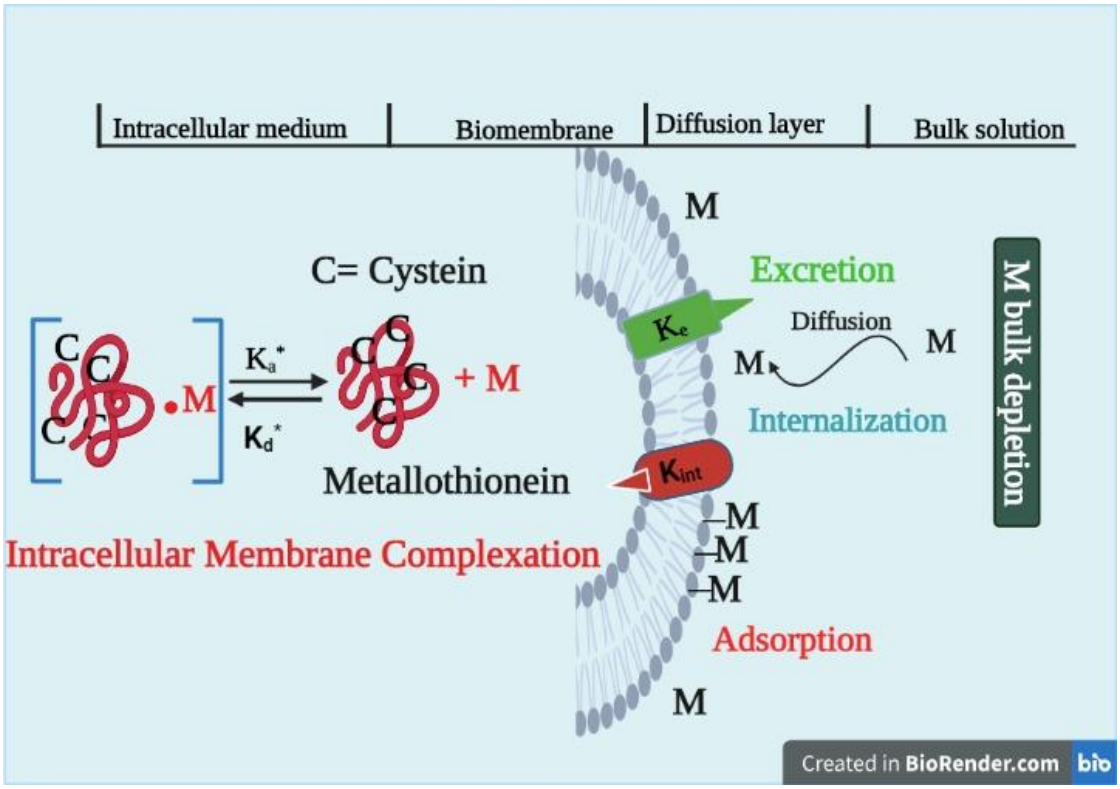

Figure 6: Bioaccumulation of metals.

1.4.1.3 Bioprecipitation

Another bioremediation technique that has been discovered in bacteria is bioprecipitation. To decrease their bioavailability and toxicity, this method includes changing the amount of free metals to insoluble complexes(Monika Wróbel, 2023). By accelerating oxidative and reductive reactions, microorganisms can speed up the precipitation of pollutants including Pb, Cd, Cr, Fe, and U. It has also been shown that some microorganisms may liberate phosphates and promote the deposition of metal phosphates, while other bacteria can precipitate alkanes to create hydroxides or carbonates(Kaksonen A.H., 2007). **Figure 7** shows the bioprecipitation of heavy metals.

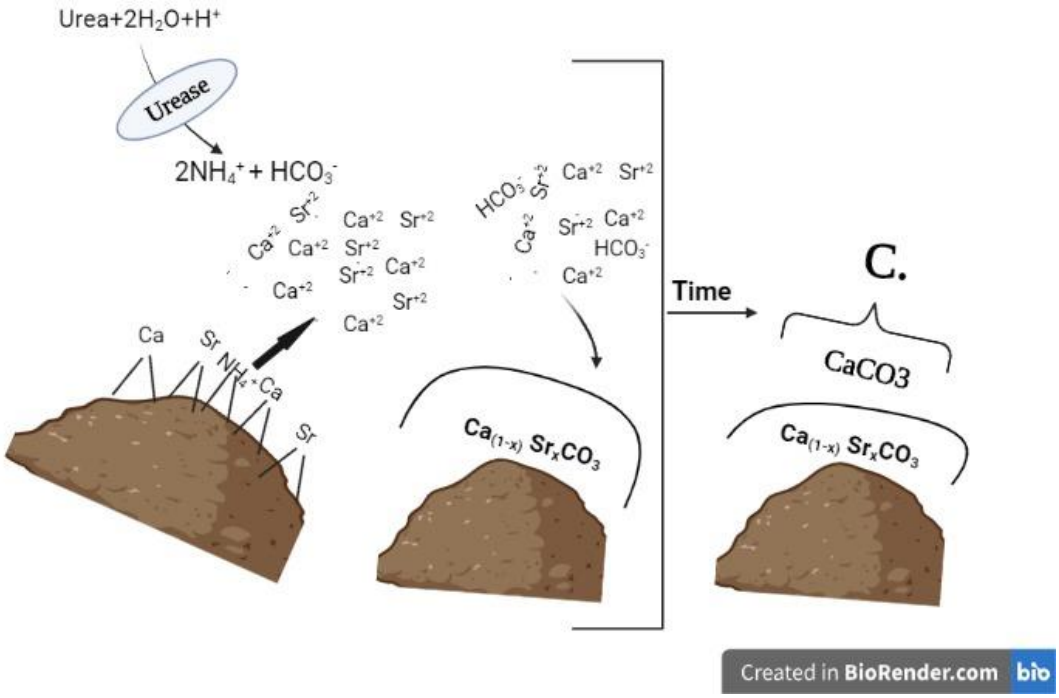

Figure 7: Bioprecipitation of heavy metals.

1.5 Phytoremediation

Heavy metals are toxic for plants and animals, and their presence in the soil is extensively growing daily, turning it into a significant concern for the survival of living organisms. The cadmium (Cd) toxicity cannot be neglected due to its vast effect on every aspect of a living organism. The primary sources of Cd in environments are sewage, phosphate fertilizers, mining, industrialization, and neutral disasters (Rizwan, 2016). The exclusion of HMs from the soil needs a comprehensive and cheap mechanism for cleanup. For this purpose, phytoremediation is widely used as an in-situ process. Many advantages and disadvantages of phytoremediation have been reported in the literature, likewise in terms of benefits: it's cost-effective and easy to operate contaminants whereas, disadvantages: its selective metals uptakes plants, slow growth, and long cleanup process.

It's hard to simultaneously understand any plant's phytoremediation potential and Cd tolerance. For this purpose, the helianthus crop has been studied and used widely because of its high biomass production, short life span, and ability to store HMs. Moreover, Helianthus crops also have substantial economic importance in oil production. Thus, plants that grow for phytoremediation can’t be used for food purposes (Asad, 2018).

The cadmium accumulation occurs in a different part of Helianthus species without showing a huge impartation on its growth and development; thus, such plants are further used for biofuel after total gain.

Although Helianthus species have enormous tolerance against Cd and accumulate. The Cd accumulation occurs in above-ground parts: specifically in shoots, leaves, and seeds. Cd accumulation adversely affects the plant through stunted growth, low biomass, decreased chlorophyll quality, and low yield. Different exogenous applications in the soil have been introduced to boost plant growth. Some organic and inorganic approaches have been submitted for Brassica Cd uptake enhancement and extensive tolerance under Cd stress. The organic methods include soil microbes, co-cropping, and biochar, while inorganic approaches include fertilizer management, EDTA, chemical treatments, and physical treatments.(Angelova, 2008)

The uptake of Cd via different Helianthus species varies from one another. Helianthus species cultivators select Cd-tolerant plants for phytoextraction. Some species have higher transcription factors that enhance phytoextraction than others.

1.5.1 Mechanism of Phytoremediation

The Cadmium uptake and accumulation in different plants conquered the great attention of scientists due to its toxic effect on plants, animals, and humans. The proliferation of Cd in plants depends on various factors involved, including (soil pH, soil type, Cd level in soil, Cd rhizosphere speciation, organic matter contents, and harmful or beneficial microbes).

The uptake concentration of Cd in the plant's upper part depends upon the soil's pH concentration. Different studies show that the uptake of Cd also depends upon the type of experiments (hydroponic vs. soil), rhizosphere volume, Cd contents, exposure duration, and soil type. Furthermore, uptake regulation also depends upon root morphological characteristics such as (root length, surface area, root hair, and root volume) (Armas, 2015). There are different phytoremediation mechanisms shown in **Figure 8**.

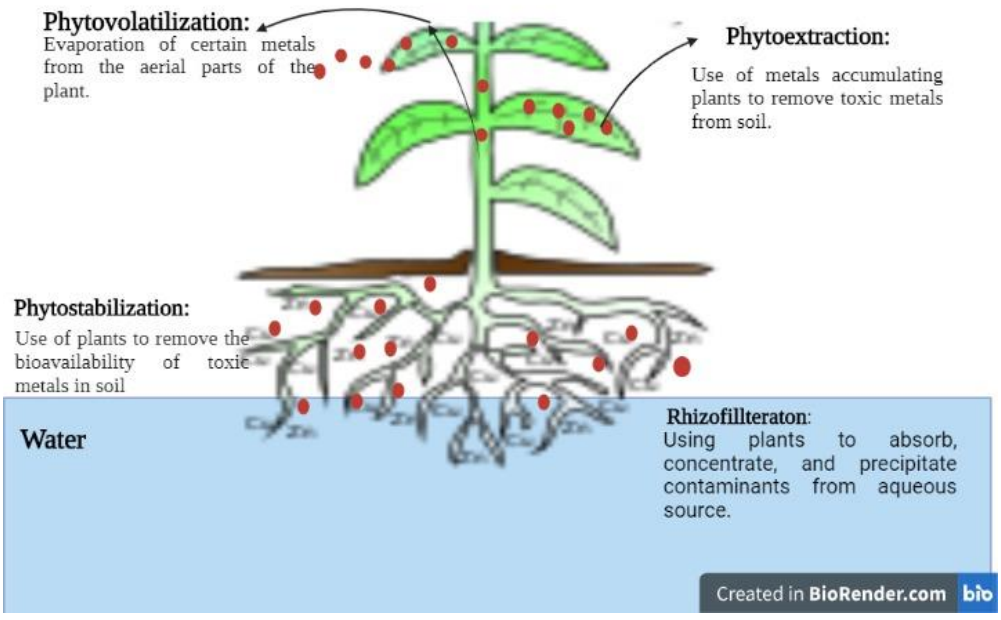

Figure 8: Different phytoremediation mechanisms.

1.6 Objectives

1. Identification of the effects of cadmium in sunflowers as a phytoremediator under the presence of bacteria showing resistant properties against cadmium.
2. Investigation of the antioxidant properties of sunflowers further down cadmium stress in different concentrations.
3. Investigation of the root and shoot structures and their interaction with bacteria under the presence of cadmium stress.

Chapter 2: Literature Review

2.1 Cadmium Contamination of Agricultural Land

Cadmium contamination in agricultural soils can be caused by some factors, including fertilizers, industrial emissions, sewage sludge, irrigational water, air contamination, and contaminated animal dung (Sardar, Heavy metals contamination and what are the impacts on living organisms.', 2013). When used in excess, some fertilizers contain significant quantities of cadmium, which can contaminate the soil. Mining, smelting, and manufacturing can all cause cadmium to be released into the air and water, contaminating the soil (Kumari, 2021). If sewage sludge, which is commonly used as a fertilizer, has been contaminated by industrial or municipal sources, it might contain significant quantities of cadmium. Cadmium can be found in irrigation water, especially if it has been contaminated by industrial operations (Sardar, Heavy metals contamination and what are the impacts on living organisms., 2013). Cadmium can be deposited in agricultural soils from the air, particularly near industrial sites. Cattle raised in cadmium-affected locations may excrete contaminated manure, which can contaminate agricultural soils. It is crucial to highlight that high levels of cadmium in agricultural soils can be detrimental to human health and the environment, thus actions should be made to prevent and minimize pollution (Alengebawy, 2021).

2.2 Phytoremediation and Bioremediation

Phytoremediation and bioremediation are potential strategies for reducing the environmental impact of contaminants. Plants are used in phytoremediation to remove contaminants from the soil, whereas microorganisms are used in bioremediation to break down pollutants (Islam, 2022). In recent years, experts have merged these two strategies to improve the results of contaminated soil remediation. Sunflowers (Helianthus annuus) and Bacillus *subtilis*, respectively, have been widely explored in phytoremediation and bioremediation (Castiglione, 2019). The purpose of this literature review is to investigate the utilization of sunflowers and B. *subtilis* in polluted soil phytoremediation and bioremediation.

2.3 Sunflower in Phytoremediation

Because of their propensity to absorb heavy metals from the soil, sunflowers have been discovered to be efficient in phytoremediation. Sunflower roots are particularly useful in this sense since they can reach deep into the soil and absorb contaminants such as lead, cadmium, zinc, and copper. (Jadia C. D., 2008)evaluated the utilization of sunflowers in the phytoremediation of cadmium, zinc, copper, and nickel-contaminated soils. The study discovered that sunflowers were successful at removing heavy metals from the soil, with the roots absorbing most of the pollutants. (Lyubun, 2002)evaluated the utilization of sunflowers in the phytoremediation of arsenic-contaminated soil. Sunflowers were discovered to be effective at accumulating arsenic in their roots and shoots, and they might potentially be utilized to remediate arsenic-contaminated soil. Sunflowers have been shown to increase soil microbial activity in addition to their potential to absorb heavy metals. The influence of sunflowers on microbiological activity in petroleum hydrocarbon-contaminated soil was studied by (Kaimi, 2007). The results of the study concluded that sunflowers increased microbial activity in the soil, resulting in more petroleum hydrocarbon breakdown.

2.4 Bacillus *subtilis* in Bioremediation

Bacillus *subtilis* is a Gram-positive, spore-forming bacterium that has received a great deal of attention in bioremediation due to its ability to digest a wide range of contaminants. B. *subtilis* generates enzymes capable of degrading pollutants such as polycyclic aromatic hydrocarbons (PAHs), benzene, toluene, and xylene (Mohsin, 2021). evaluated the utilization of B. *subtilis* in the bioremediation of PAH-contaminated soil. The study discovered that B. *subtilis* degraded the PAHs effectively, with degradation rates of up to 70% seen in some situations. explored the utilization of B. *subtilis* in the bioremediation of soil contaminated with benzene, toluene, and xylene in another study. The study discovered that B. *subtilis* degraded these contaminants well, with degradation rates of up to 90% seen in some cases. *Bacillus subtilis* has been extensively researched for its capacity to digest a wide range of pollutants, including heavy metals. B. *subtilis* was found to successfully break down cadmium in polluted soil in a study (Mo, 2021). The researchers discovered that B. *subtilis* stimulated sunflower development and increased their propensity to collect cadmium.

2.5 Combination of Phytoremediation and Bioremediation

(Cheng, 2022)investigated the use of sunflowers and B. *subtilis* in the combined phytoremediation and bioremediation. The combination of phytoremediation and bioremediation is successful in soil remediation. Plants are utilized in this strategy to ingest contaminants from the soil, which are then destroyed by microbes. In this approach, sunflowers, and B. *subtilis* have been widely explored. Phytoremediation and bioremediation are potential options for contaminated soil cleanup (Ward, 2004). Sunflower (Helianthus annuus) has been discovered to be an excellent heavy metal accumulator, whereas Bacillus *subtilis* is a soil bacterium with the ability to digest a variety of pollutants (Jadia F. , 2008). Much research has been conducted to look at the potential of sunflowers for the phytoremediation of cadmium-contaminated soil. Sunflowers, for example, were found to be effective at removing cadmium from the soil in a study by.

A combination of sunflower and B. *subtilis* to remediate cadmium-contaminated soil was used. The study found that combining sunflower and B. *subtilis* was more successful than either sunflower or *B. subtilis* alone in eliminating cadmium from the soil (Xie, 2022). The researchers also noticed that the combination of sunflower and B. *subtilis* resulted in greater biomass and chlorophyll content in sunflowers. Overall, the studies reviewed here show that sunflowers and B. *subtilis* have the potential for phytoremediation and bioremediation of cadmium-contaminated soil. Sunflower and B. *subtilis* together may provide a more successful remediation solution than either approach alone. Further research is needed, however, to optimize the conditions for using sunflower and B. *subtilis* in phytoremediation and bioremediation of contaminated soil.

Bacillus *subtilis* is a well-known plant growth-promoting bacterium that may colonize plant roots and promote plant development through the production of plant growth hormones, siderophores, and enzymes that improve nutrient uptake (Bhattacharyya, 2012). B. *subtilis* can also boost plant resilience to biotic and abiotic stressors such as drought, salt, and heavy metal stress. The sunflower (Helianthus annuus) is a well-known hyperaccumulator plant, capable of tolerating and accumulating high quantities of heavy metals such as cadmium in its tissues (Prasad, 1999). Sunflower has a deep root system that may take heavy metals from the soil and transport them to the plant's aerial portions. As a result, sunflowers combined

with B. *subtilis* may be an effective tool for the phytoremediation of cadmium-contaminated soil.

The efficacy of heavy metal phytoremediation is determined by plant species, heavy metal type, and concentration, and the presence of PGPMs in the soil (Sharma, 2023). Sunflower is a well-known hyperaccumulator plant, meaning that it can withstand and collect high levels of heavy metals like cadmium in its tissues. Cadmium hyperaccumulation in sunflowers is mostly owing to the presence of photoheating (PCs) in their tissues (Alsafran, 2022). PCs are tiny peptides that can bind to and detoxify heavy metals. Sunflower has a deep root system that may take heavy metals from the soil and transport them to the plant's aerial portions (McIntyre, 2003). for example, studied the phytoremediation capability of sunflowers for cadmium-contaminated soil. The results showed that sunflowers can accumulate up to 228 mg Cd kg^{-1} of cadmium in their shoots (de Andrade, 2008). The study also discovered that after the growth of sunflowers, the concentration of cadmium in the soil reduced dramatically.

2.6 Research questions and hypothesis

- What is the effectiveness of plant-microbe-based phytoremediation and bioremediation for cadmium-contaminated soils?
- How is the phytoremediation role of the sunflower affected by Bacillus *subtilis*?
- What is the individual role of sunflowers in the phytoremediation of cadmium-contaminated soil?

2.6.1 Hypothesis

Phytoremediation and bioremediation based on plant-microbe interactions can successfully remove cadmium from contaminated soils by enhancing cadmium uptake, sequestration, and breakdown in the soil. Furthermore, by fostering soil ecological restoration, the utilization of native plant-microbe combinations can improve the sustainability and long-term effectiveness of remediation activities.

2.7 Search strategy

A potential search strategy for finding information on plant-microbe-based phytoremediation and bioremediation of cadmium-contaminated soil using sunflower and Bacillus *subtilis* was followed. Relevant keywords were used for research such as Phyto-remediation, Bioremediation, Cadmium Contaminated soil, Sunflower, Bacillus *subtilis*, Plant-microbe interactions, Heavy metal remediation, and Soil pollution.

Different search engines such as PubMed, Web of Science, Scopus, and Google Scholar were used for finding relevant data for research. Boolean operators (AND, OR) were used to refine the research. The search was narrowed by using different filters such as publication year and language etc. Previous research was evaluated based on titles, abstracts, and keywords. To extract the relevant information, selected articles were fully understood and analyzed. The reference lists of selected articles were used to find additional relevant studies. To cover the topic comprehensively, different combinations of keywords and phrases are used repetitively in different ways.

Chapter 3: Methodology

3.1 Wet Lab Analysis

3.1.1 Soil and Seeds Collection

For growing the plants, the sandy loom soil was collected from the peanut fields of the National Agriculture Research Centre (NARC), Islamabad, Pakistan. Fresh hybrid Helianthus annus (PARSON-3) seeds were gotten from the Oil and Seed Research Department of NARC for research purposes.

3.1.2 Autoclaving the soil

The soil obtained from NARC was sieved to remove unwanted material. It was then autoclaved at 121°C and 15 psi to kill the microorganisms (K. Oates, 1983).

3.1.3 Fungicide Preparation

To prevent the growth of fungus on seeds, a broad-spectrum fungicide was obtained from NARC. The fungicide solution was made by adding 2g of fungicide in 500 ml of distilled water.

3.1.4 $CdCl_2$ Solutions Preparation

Four different cadmium chloride solutions were prepared from the stock solution of 1000mM. These solutions were 25mM, 50mM, 75mM, and 100mM. They were prepared by diluting the stock solution with distilled water.

3.1.5 Seeds Germination

In a safety cabinet, the seeds were treated with 70% ethanol for 5 min to remove any fungus grown on their surfaces. This was followed by rinsing the seeds with distilled water and placing them on the filter paper for the ethanol to evaporate. When ethanol was wholly dried, the seeds were soaked in the fungicide solution for 2 min and washed with distilled water. The seeds were then spread on UV-sterilized germination paper placed inside the germination box. The germination box was covered in aluminum foil to prevent the interaction of seeds with light, and placed inside the incubator, which was set at 27°C, for 72 hours. After 3 days,

the seeds were germinated and ready to be transferred to the soil. The seeds which had the same germination rates were transferred to the soil.

3.1.6 Soil Preparation

Nine different treatments were prepared in the soil for plant growth, and three replicates of each treatment were prepared. Each pot contained 4 kg of autoclaved soil. The first treatment was the control group in which only the sunflower seeds were planted. $CdCl_2$ was not given to this group. All the other eight treatments were given $CdCl_2$ stress of different concentrations, with or without an organic variable: a bacterial strain of Bacillus *subtilis* BS-10, a common PGPR, depending on the required treatment. 40 ml of CdCl2 solution of the required concentration was added to each of the eight treatments, and 20 ml of bacterial culture solution was added to the required treatments. The soil was mixed thoroughly with the solutions and kept in the glasshouse of ASAB, NUST, Islamabad, Pakistan, to provide controlled conditions for optimum plant growth. After 5 days of adding the cadmium stress and bacterial solution to the soil, 4 seedlings were transferred to each of the pots, and the temperature was set at 25-28°C in the glasshouse. 30ml of distilled water was provided to the plants twice daily for a period of 45 days for plant growth. The experimental design is shown in **Table 3**.

Table 3: Treatments of the experiment

Treatment Code	Treatment
T1 (Control)	Sunflower + No Cadmium Stress
T2	Sunflower + 25mM CdCl2
T3	Sunflower + 50mM CdCl2
T4	Sunflower + 75mM CdCl2
T5	Sunflower + 100mM CdCl2
T6	Sunflower + 25mM CdCl2 + Bacterial solution
T7	Sunflower + 50mM CdCl2 + Bacterial solution
T8	Sunflower + 75mM CdCl2 + Bacterial solution
T9	Sunflower + 100mM CdCl2 + Bacterial solution

3.1.7 Soil Analysis

Soil analysis was performed before and after the experiment to identify its pH, electrical conductivity, and soil texture. The amount of nitrates, phosphates, and potassium was identified by the AB-DTPA soil test (Soltanpour, 1991). These tests were performed at NARC.

3.2 Morphological Analysis

3.2.1 Number of leaves and plant length

The number of leaves was counted after 40 days of sowing. Shoot length was also measured after 40 days of sowing using a measuring tape. After harvesting, root length was measured, followed by calculating the sum of the length of both roots and shoots which gives the total plant length.

3.2.2 Fresh and dry plant weight

After 40 days, plants were harvested, and the fresh weight of the roots, shoots, and leaves of the plants was determined using a weighing scale instantly after washing them with deionized water. The total fresh weight of the plant was then analyzed by calculating the sum of the fresh weights of roots, shoots, and leaves. The roots and shoots were left in an oven at 70°C for 2 days and weighed using the weighing scale to measure their dry weights (Wennan Su, 2019).

3.2.3 Leaf Area and Stem Girth

ImageJ software was used to calculate the area of the leaf by placing the leaf on graph paper and scanning it (Florin Sala, 2021). Shoot girth was calculated by using a vernier caliper.

3.3 Physiological Analysis

3.3.1 Soil Plant Analyzer Development

A chlorophyll meter SPAD was used to calculate the SPAD values of the leaves, which calculated the index of chlorophyll a and b. These values were taken on Day 40.

3.3.2 Relative Water Content of Leaves

To calculate the relative water content, the following method is used (Dorota Soltys Kalina, 2016). Fresh leaf was taken and weighed. It was then placed in distilled water for 2 days and weighed again. This weight was the turgor weight. Finally, the dry weight was calculated after air-drying the leaf for 3 days.

W_f = Fresh weight of leaf.

W_t = Turgor weight of leaf.

W_d = Dry weight of leaf.

$$Relative\ Water\ Content = \frac{W_f - W_d}{W_t - W_d} \times 100$$

3.3.3 Microscopy

Microscopy was performed to analyze the structural differences of roots and shoots in plants with different amounts of $CdCl_2$ and with or without the treatment of bacteria. Small sections of roots and shoots were cut with microtome and slides were prepared by staining them with hematoxylin and eosin. The slides were then visualized under the microscope at 10X magnification. (Andrew H Fischer, 2008).

3.4 Biochemical Assays

3.4.1 Superoxide Dismutase (SOD)

For SOD quantification, the following procedure was used (Kono, 1978). A 0.5g fresh plant leaf sample was homogenized in 3 ml of PBS. Another 5 ml of PBS buffer was added after transferring the homogenized mixture to a 10 ml tube. It was then centrifuged at 13000 rpm for 20 min at 4 °C. For SOD analysis, the plant extract supernatant was collected, and it was placed in another tube. It was then kept there for 24 hours at 4°C. 1 ml PBS buffer, 33 µl EDTA, and 66 µl of methionine, NBT, and riboflavin to 25 µl of the enzyme, the extract was added. The supernatant's absorbance at 560 nm was taken.

A_e = the optical density reading on a spectrophotometer

A_{ck} = the control tube's optical density value under light situations (at 4000 lux for 20 min).

V = the quantity of the buffer solution used to remove the enzyme.

W = The sample's fresh weight.

V_t = Quantity of enzyme extract required in reaction solution to analyze SOD.

$$\text{SOD activity} = \frac{O.D_{control} - O.D \times V}{0.5 \times \text{O.D}_{control} \times \text{W} \times \text{V}_t}$$

3.4.2 Peroxidase (POD)

For POD quantification, the following procedure was used (U., 1974). A 0.5g fresh plant leaf sample was homogenized in 3 ml of PBS. Another 5 ml of PBS buffer was added after transferring the mixture to a 10 ml tube. It was centrifuged at 13000 rpm for 20 min at 4 °C. For POD analysis, the plant extract supernatant was collected and placed in another tube. It was kept there for 24 hours at 4°C. Next, 2.7 ml PBS buffer, 100 µl Guaiacol, and 100 µl 30% hydrogen peroxides (H_2O_2) were added to 100 µl of enzyme extract. The supernatant's absorbance at 270 nm was taken.

W = Fresh weight of the sample.

V = Total amount of the buffer solution used to remove the enzyme.

a = The quantity of enzyme extract used in the reaction solution to test.

E = constant activity i.e., 26.6 mM/cm at 270 nm, Whereas the E value is 26.6 mM/cm.

$$POD\ activity\ = \frac{\mathrm{O.D}_{value}\ \times \frac{V}{V_t}}{\mathrm{E}\ \times\ W}$$

3.4.3 Catalase (CAT)

For CAT quantification, the following procedure was used (Aebi, 1984). A 0.5g fresh plant leaf sample was homogenized in 3 ml of PBS. Another 5 ml of PBS buffer was added after transferring the mixture to a 10 ml tube. It was centrifuged at 13000 rpm for 20 min at 4 °C. The plant extract supernatant was collected and placed in a different tube. It was kept at 4°C for 24 hours for CAT analysis. Next, 2.8 ml PBS buffer and 100 µl of 30% hydrogen peroxide (H_2O_2) were added to 100 µl of enzyme extract. The supernatant's absorbance was considered at 240 nm. The E value, however, is 39.4 mM/cm.

A = Optical density activity.

W = The sample's fresh weight.

V = Amount of buffer solution used in obtaining the enzyme.

a = Quantity of enzyme extract used in reaction solution to test.

E = activity constant i-e.,39.4mM/cm.

$$\text{CAT activity} = \frac{\text{O. D}_{value} \times \frac{\text{V}}{V_t}}{E \times \text{W}}$$

Chapter 4: Results

4.1 Soil Analysis

Soil analysis revealed that the soil used for the experiment has a silt loam texture. The pH of the soil initially used was 8.21, with 0.486 dS/m and 2.39 mg/kg nitrate, 7.88 mg/kg phosphorus, and 84 mg/kg potassium. AB-DTPA soil analysis suggested that the soil had low nitrate, adequate phosphorus, and a marginal amount of potassium. **Table 4** shows the pH, EC value, and the amounts of nitrate, phosphate, and potassium in the soils of each treatment after the experiment.

Table 4: Results of soil analysis after the experiment.

Treatments	pH	EC (dS/m)	Nitrate (mg/kg)	Phosphorus (mg/kg)	Potassium (mg/kg)
T1	8.24	0.483	2.32	7.81	82
T2	8.27	0.478	2.27	6.63	76
T3	8.30	0.367	2.47	8.10	76
T4	8.25	0.385	2.32	6.98	90
T5	8.29	0.471	2.36	7.71	94
T6	8.31	0.398	2.41	7.11	70
T7	8.23	0.480	4.09	8.57	62
T8	8.26	0.467	2.43	7.88	58
T9	8.02	0.459	4.27	9.48	70

4.2 Morphological Results

4.2.1 Number of Leaves

The number of leaves is calculated after 40 days for each treatment (T1 = Sunflower, T2 to T5= Sunflower + $CdCl_2$, T6 to T9 = Sunflower + $CdCl_2$ + Bacteria). R-Studio is used to calculate their mean values, standard deviation, 1st and 3rd quartile, and p-value.

Statistical analyses like the 2-way ANOVA and Tukey HSD Test are applied, which identifies the significant difference between different treatments as 0.001. X-axis represents the treatments from T2 to T9, and Y-axis represents the number of leaves. The graph compares the effect of Cd on sunflower when bacteria is absent with the effect of Cd on the plants when Bacillus *subtilis* is present. The error bar shows the maximum and minimum sum of the means and standard deviations of the treatments, as well as the difference between them. The differences in the results of the treatments are represented by lettering; the results with the same letters explain that there is no obvious difference between the treatments, while the results with different letters suggest an obvious difference.

The results in **Figure 9** show that the number of leaves decreases in plants with increasing $CdCl_2$ concentrations. However, with the presence of bacteria, there is an obvious increase in the number of leaves with increasing $CdCl_2$ concentrations. Since there is little effect of $CdCl_2$ at low concentrations, there is little to no effect of bacteria at these low concentrations, but at higher concentrations, $CdCl_2$ decreases the number of leaves of plants significantly. Therefore, the effect of bacteria is also prominent at higher concentrations. Bacillus *subtilis* increases the number of leaves in plants with higher concentrations of $CdCl_2$.

The mean value of the control, known as T1 (Sunflower only) is 12. When compared to the control (T1), there is a 10.33%, 25.75%, 72.25%, and 81.33% decrease in the number of leaves when 25mM, 50mM, 75mM, and 100mM $CdCl_2$ are added to the soil respectively. However, when the bacteria are added, the number of leaves decreases by 41.667%, 14.75, 37.5%, and 19.25%, compared to the control (T1), even when 25mM, 50mM, 75mM, and 100mM $CdCl_2$ are added to the soil respectively.

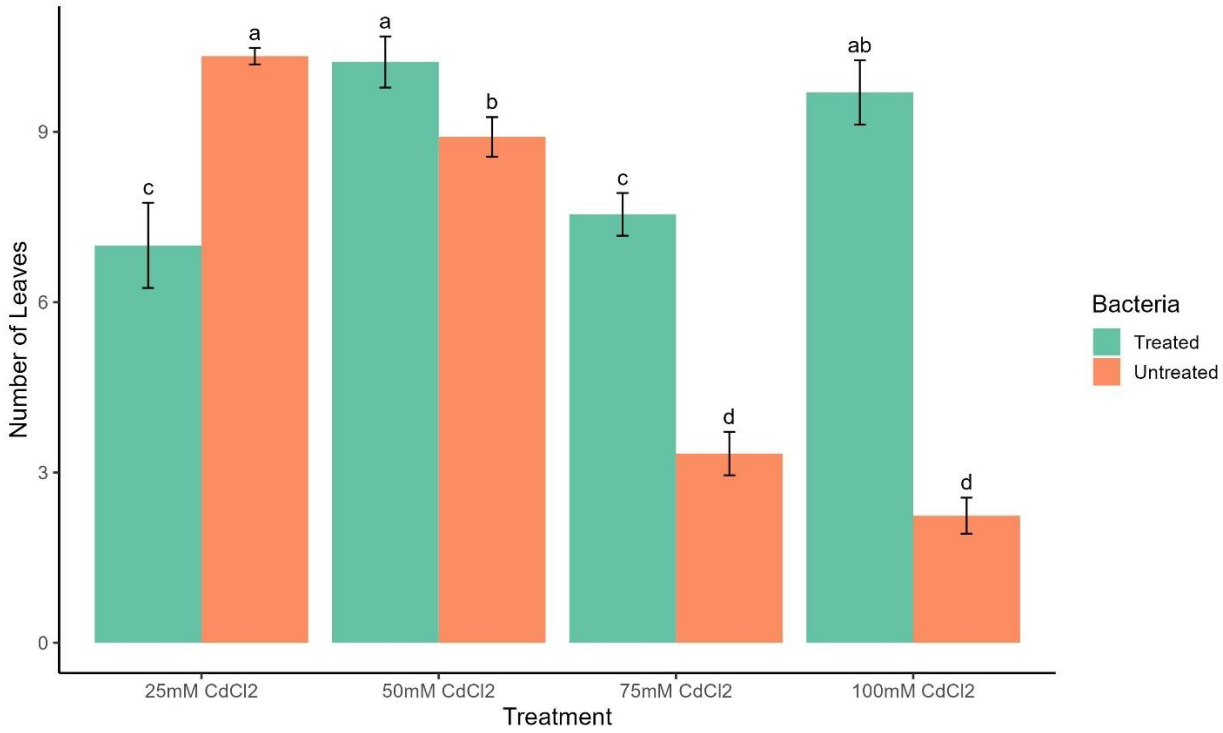

Figure 9: The number of leaves of sunflower plants given different concentrations of Cd stress. The analysis was conducted on day 40 of the experiment. The treatments included positive control groups and experimental groups with varying concentrations of $CdCl_2$ (25mM, 50mM, 75mM, and 100mM). The aim was to assess the relationship between plant growth and applied cadmium stress. Statistical significance was determined using two-way ANOVA, and Tukey's HSD test was applied for normal distribution data, with a significance level set at $p < 0.001$. Lower-case letters were used to indicate significant differences between the treatments.

4.2.2 Shoot Length

The shoot length is calculated after 40 days for each treatment (T1 = Sunflower, T2 to T5= Sunflower + $CdCl_2$, T6 to T9 = Sunflower + $CdCl_2$ + Bacteria). R-Studio is used to calculate their mean values, standard deviation, 1st and 3rd quartile, and p-value.

Statistical analyses like the 2-way ANOVA and Tukey HSD Test are applied, which identifies the significant difference between different treatments as 0.001. X-axis represents the treatments from T2 to T9, and Y-axis represents the shoot length. The graph compares the effect of Cd on sunflower when bacteria is absent with the effect of Cd on the plants when Bacillus *subtilis* is present. The error bar shows the maximum and minimum sum of the means and standard deviations of the treatments, as well as the difference between them. The differences in the results of the treatments are represented by lettering; the results with the same letters explain that there is no obvious difference between the treatments, while the results with different letters suggest an obvious difference.

The results in **Figure 10** show that the shoot length decreases in plants with increasing $CdCl_2$ concentrations. However, with the presence of bacteria, there is an obvious increase in the shoot length with increasing $CdCl_2$ concentrations. Since there is little effect of $CdCl_2$ at low concentrations, there is little to no effect of bacteria at these low concentrations, but at higher concentrations, $CdCl_2$ decreases the shoot length of plants significantly. Therefore, the effect of bacteria is also prominent at higher concentrations. Bacillus *subtilis* increases the shoot length in plants with higher concentrations of $CdCl_2$.

The mean value of the control, known as T1 (Sunflower only) is 45.28. When compared with the control (T1), there is a decrease of 7.33%, 8.44%, 61.62%, and 91.23% in the shoot length when 25mM, 50mM, 75mM, and 100mM CdCl2 is added to the soil respectively. With bacteria present, when the shoot length of control (T1) is compared to the shoot length of plants that are without bacteria treatments, there is a decrease of 5.63%, 7.93%, 32.16%, and 9.67% when 25mM, 50mM, 75mM, and 100mM CdCl2 are added to the soil respectively.

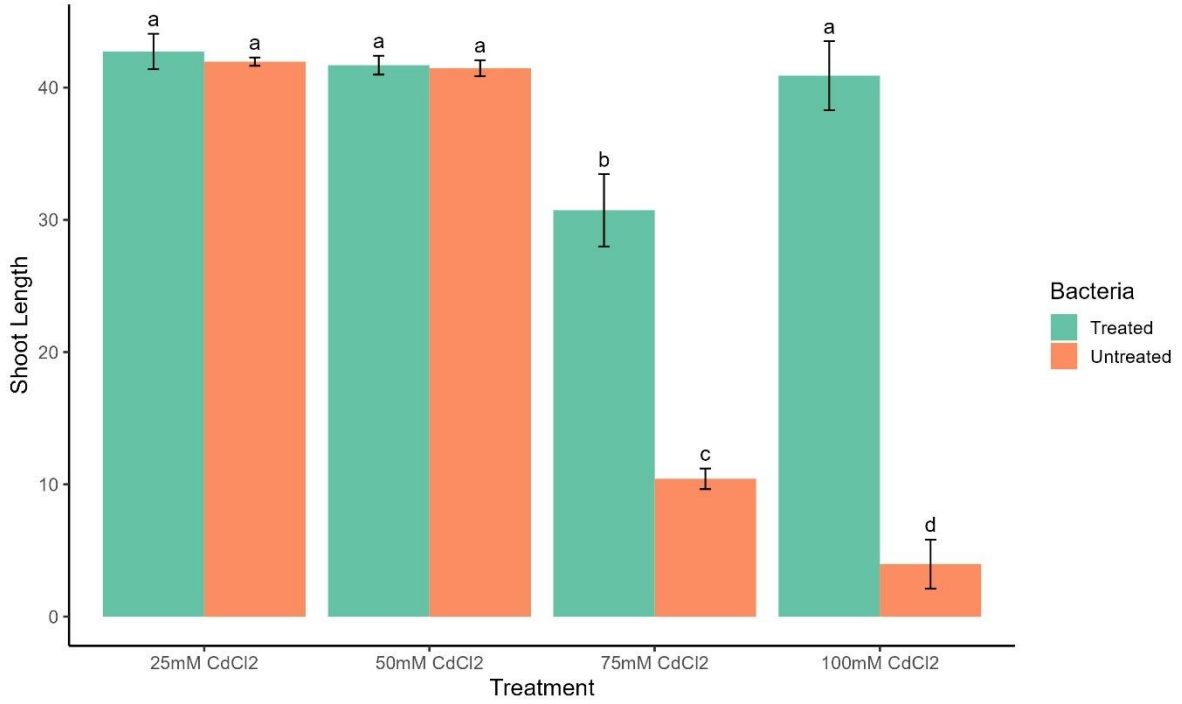

Figure 10: The shoot length of sunflower plants given different concentrations of Cd stress. The analysis was conducted on day 40 of the experiment. The treatments included positive control groups and experimental groups with varying concentrations of $CdCl_2$ (25mM, 50mM, 75mM, and 100mM). The aim was to assess the relationship between plant growth and applied cadmium stress. Statistical significance was determined using two-way ANOVA, and Tukey's HSD test was applied for normal distribution data, with a significance level set at $p < 0.001$. Lower-case letters were used to indicate significant differences between the treatments.

4.2.3 Root Length

The root length is calculated after 40 days for each treatment (T1 = Sunflower, T2 to T5= Sunflower + $CdCl_2$, T6 to T9 = Sunflower + $CdCl_2$ + Bacteria). R-Studio is used to calculate their mean values, standard deviation, 1st and 3rd quartile, and p-value.

Statistical analyses like the 2-way ANOVA and Tukey HSD Test are applied, which identifies the significant difference between different treatments as 0.001. X-axis represents the treatments from T2 to T9, and Y-axis represents the root length. The graph compares the effect of Cd on sunflower when bacteria is absent with the effect of Cd on the plants when Bacillus *subtilis* is present. The error bar shows the maximum and minimum sum of the means and standard deviations of the treatments, as well as the difference between them. The differences in the results of the treatments are represented by lettering; the results with the same letters explain that there is no obvious difference between the treatments, while the results with different letters suggest an obvious difference.

The results in **Figure 11** show that the root length decreases in plants with increasing $CdCl_2$ concentrations. However, with the presence of bacteria, there is an obvious increase in the number of leaves with increasing $CdCl_2$ concentrations. Since there is little effect of $CdCl_2$ at low concentrations, there is little to no effect of bacteria at these low concentrations, but at higher concentrations, $CdCl_2$ decreases the number of leaves of plants significantly. Therefore, the effect of bacteria is also prominent at higher concentrations. Bacillus *subtilis* increases the root length in plants with higher concentrations of $CdCl_2$.

The mean value of the control, known as T1 (Sunflower only) is 16.35. When compared to the control (T1), there is a decrease of 1.79%, 14.39%, 65.85%, and 72.72% when 25mM, 50mM, 75mM, and 100mM CdCl2 is added to the soil respectively. However, when the bacteria are added, the root length decreases by 14.68%, 6.61%, 33.76%, and 10.83% compared to the control (T1), even when 25mM, 50mM, 75mM, and 100mM CdCl2 are added to the soil respectively.

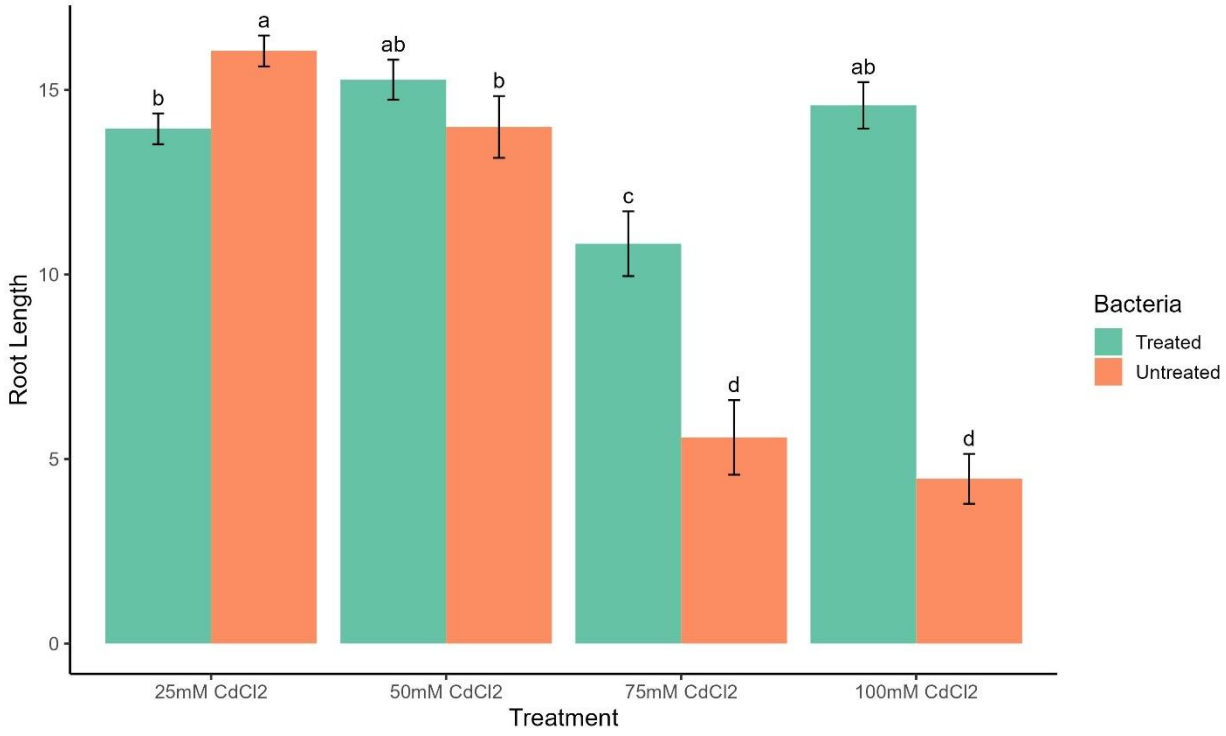

Figure 11: The root length of sunflower plants given different concentrations of Cd stress. The analysis was conducted on day 40 of the experiment. The treatments included positive control groups and experimental groups with varying concentrations of $CdCl_2$ (25mM, 50mM, 75mM, and 100mM). The aim was to assess the relationship between plant growth and applied cadmium stress. Statistical significance was determined using two-way ANOVA, and Tukey's HSD test was applied for normal distribution data, with a significance level set at $p < 0.001$. Lower-case letters were used to indicate significant differences between the treatments.

4.2.4 Total Plant Length

The total plant length is calculated after 40 days for each treatment (T1 = Sunflower, T2 to T5= Sunflower + $CdCl_2$, T6 to T9 = Sunflower + $CdCl_2$ + Bacteria). R-Studio is used to calculate their mean values, standard deviation, 1st and 3rd quartile, and p-value.

Statistical analyses like the 2-way ANOVA and Tukey HSD Test are applied, which identifies the significant difference between different treatments as 0.001. X-axis represents the treatments from T2 to T9, and Y-axis represents the total length of the plant. The graph compares the effect of Cd on sunflower when bacteria is absent with the effect of Cd on the plants when Bacillus *subtilis* is present. The error bar shows the maximum and minimum sum of the means and standard deviations of the treatments, as well as the difference between them. The differences in the results of the treatments are represented by lettering; the results with the same letters explain that there is no obvious difference between the treatments, while the results with different letters suggest an obvious difference.

The results in **Figure 12** show that the total plant length decreases in plants with increasing $CdCl_2$ concentrations. However, with the presence of bacteria, there is an obvious increase in the number of leaves with increasing $CdCl_2$ concentrations. Since there is little effect of $CdCl_2$ at low concentrations, there is little to no effect of bacteria at these low concentrations, but at higher concentrations, $CdCl_2$ decreases the number of leaves of plants significantly. Therefore, the effect of bacteria is also prominent at higher concentrations. Bacillus *subtilis* increases the total length in plants with higher concentrations of CdCl2.

The mean value of the control, known as T1 (Sunflower only) is 59.63. When compared to the control (T1), it decreases by 0.008%, 22%, 74.19%, and 86.4% when 25mM, 50mM, 75mM, and 100mM CdCl2 are added to the soil respectively. However, when the bacteria are added, the total plant length decrease is low as compared to the control (T1), even when 25mM, 50mM, 75mM, and 100mM CdCl2 are added to the soil, the decrease in value is 16.65%, 5.3%, 30.3%, and 11.4% respectively in contrast to the treatments without bacteria.

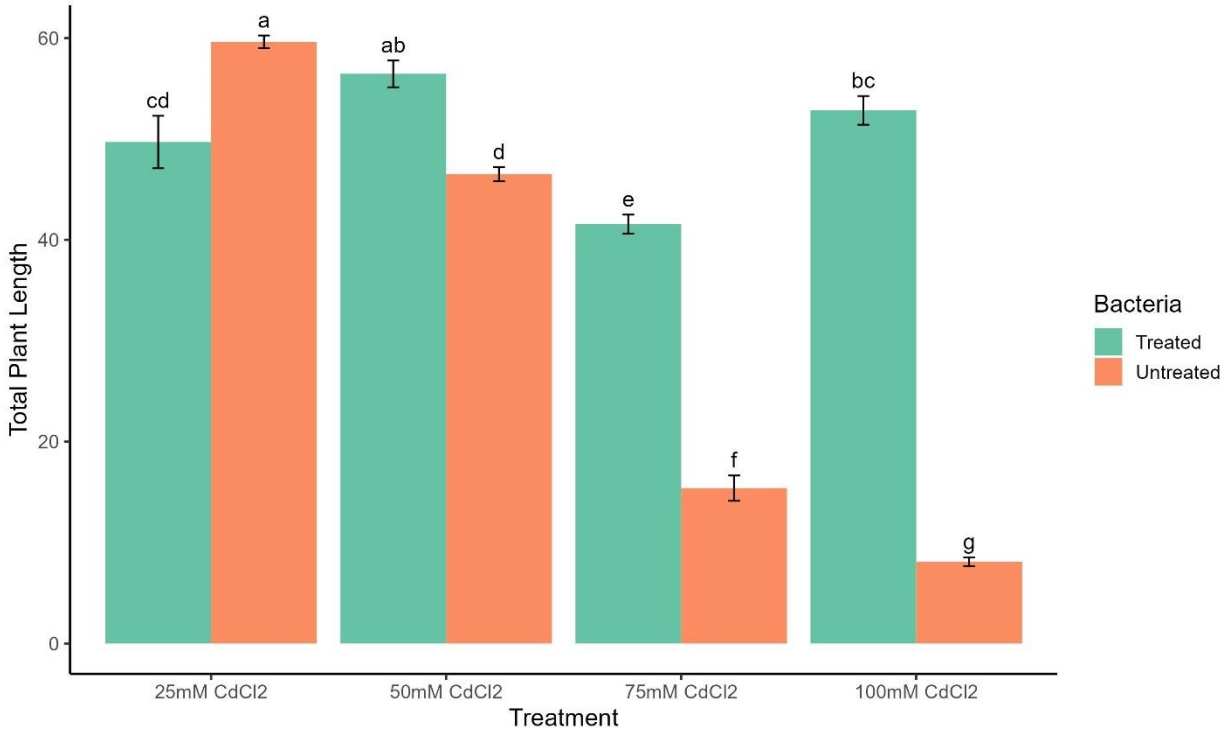

Figure 12: The total plant length of sunflower plants given different concentrations of Cd stress. The analysis was conducted on day 40 of the experiment. The treatments included positive control groups and experimental groups with varying concentrations of $CdCl_2$ (25mM, 50mM, 75mM, and 100mM). The aim was to assess the relationship between plant growth and applied cadmium stress. Statistical significance was determined using two-way ANOVA, and Tukey's HSD test was applied for normal distribution data, with a significance level set at $p < 0.001$. Lower-case letters were used to indicate significant differences between the treatments.

4.2.5 Fresh Shoot Weight

The fresh weight of shoots is calculated after 40 days for each treatment (T1 = Sunflower, T2 to T5= Sunflower + $CdCl_2$, T6 to T9 = Sunflower + $CdCl_2$ + Bacteria). R-Studio is used to calculate their mean values, standard deviation, 1st and 3rd quartile, and p-value.

Statistical analyses like the 2-way ANOVA and Tukey HSD Test are applied, which identifies the significant difference between different treatments as 0.001. X-axis represents the treatments from T2 to T9, and Y-axis represents the fresh shoot weight. The graph compares the effect of Cd on sunflower when bacteria is absent with the effect of Cd on the plants when Bacillus *subtilis* is present. The error bar shows the maximum and minimum sum of the means and standard deviations of the treatments, as well as the difference between them. The differences in the results of the treatments are represented by lettering; the results with the same letters explain that there is no obvious difference between the treatments, while the results with different letters suggest an obvious difference.

The results in **Figure 13** show that the shoot fresh weight decreases in plants with increasing $CdCl_2$ concentrations. However, with the presence of bacteria, there is an obvious increase in the number of leaves with increasing $CdCl_2$ concentrations. Since there is little effect of $CdCl_2$ at low concentrations, there is little to no effect of bacteria at these low concentrations, but at higher concentrations, $CdCl_2$ decreases the number of leaves of plants significantly. Therefore, the effect of bacteria is also prominent at higher concentrations. Bacillus *subtilis* increases the shoot fresh weight in plants with higher concentrations of $CdCl_2$.

The mean value of the control, known as T1 (Sunflower only) is 3.3. When compared to the control (T1), there is a gradual decrease initially at 25mM and 50mM concentrations of 5.53% and 8.18% respectively, but when 75mM and 100mM $CdCl_2$ is added to the soil, shoot fresh weight declines rapidly by 81.4% and 85.85% respectively. However, the shoot fresh weight decreases initially by 22.12% then increases by 10%, 4.55%, and 6.89% compared to the control (T1), when 25mM, 50mM, 75mM, and 100mM CdCl2 are added to the soil respectively along with the bacteria.

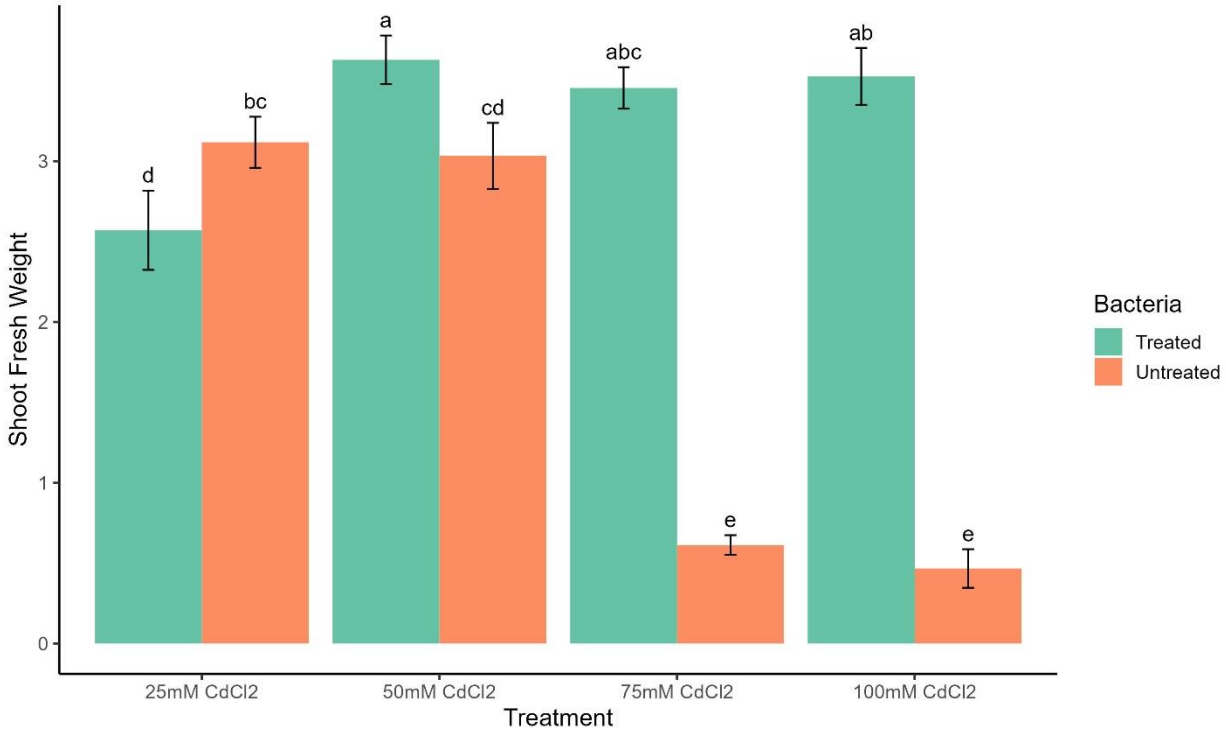

Figure 13: The fresh weight of shoots of sunflower plants given different concentrations of Cd stress. The analysis was conducted on day 40 of the experiment. The treatments included positive control groups and experimental groups with varying concentrations of $CdCl_2$ (25mM, 50mM, 75mM, and 100mM). The aim was to assess the relationship between plant growth and applied cadmium stress. Statistical significance was determined using two-way ANOVA, and Tukey's HSD test was applied for normal distribution data, with a significance level set at $p < 0.001$. Lower-case letters were used to indicate significant differences between the treatments.

4.2.6 Fresh Root Weight

Root fresh weight was calculated after 40 days for each treatment (T1 = Sunflower, T2 to T5= Sunflower + $CdCl_2$, T6 to T9 = Sunflower + $CdCl_2$ + Bacteria) by using a weighing balance. R-Studio is used to calculate their mean values, standard deviation, 1st and 3rd quartile, and p-value.

Statistical analyses like the 2-way ANOVA and Tukey HSD Test are applied, which identifies the significant difference between different treatments as 0.001. X-axis represents the treatments from T2 to T9, and Y-axis represents the root fresh weight. The graph compares the effect of Cd on sunflower when bacteria is absent with the effect of Cd on the plants when Bacillus *subtilis* is present. The error bar shows the maximum and minimum sum of the means and standard deviations of the treatments, as well as the difference between them. The differences in the results of the treatments are represented by lettering; the result with the same letters explains that there is no obvious difference between the treatments, while the results with different letters suggest an obvious difference.

The results in **Figure 14** show that the root fresh weight decreases in plants with increasing $CdCl_2$ concentrations. But bacteria showed good resistance even at lower cadmium concentrations. At 50mM $CdCl_2$ at both, the resistance and impact of cadmium were the same. However, with the presence of bacteria, there is an obvious increase in root fresh weight despite increasing $CdCl_2$ concentrations. At higher Cd concentrations, the bacterial role was obvious and helped to increase root fresh weight despite higher concentrations of cadmium. Therefore, the effect of bacteria is more prominent at higher concentrations. Bacillus *subtilis* increases the root fresh weight in the plants both at higher and lower concentrations of $CdCl_2$.

The mean value of the control, known as T1 (Sunflower only) is 1.25. When compared to the control (T1), there is an increase of 46.4% and 67.74% and a decrease of 43.2%, and 62.86% when 25mM, 50mM $CdCl_2$, 75mM, and 100mM $CdCl_2$ are added to the soil respectively. However, when the bacteria are added, the root fresh weight increases by 91.3% and 69.53%, and then decreases by 10.4%, and 31.85% when 25mM, 50mM, 75mM, and 100mM CdCl2 are added to the soil respectively.

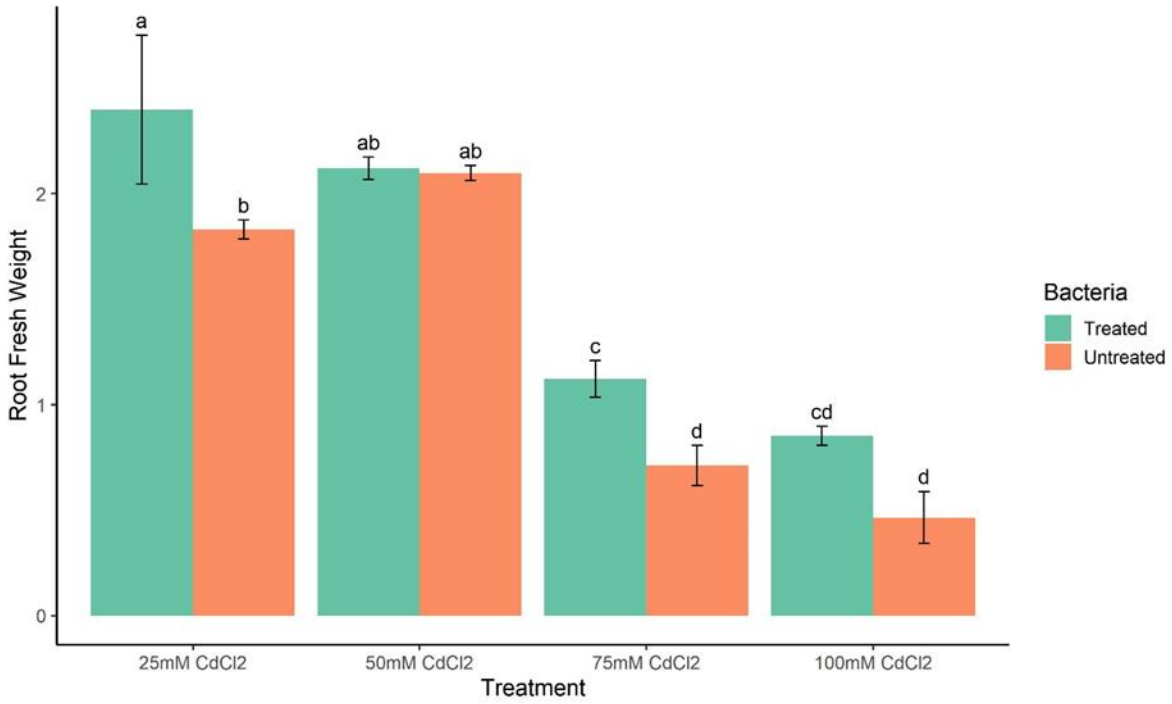

Figure 14: The fresh weight of roots of sunflower plants given different concentrations of Cd stress. The analysis was conducted on day 40 of the experiment. The treatments included positive control groups and experimental groups with varying concentrations of $CdCl_2$ (25mM, 50mM, 75mM, and 100mM). The aim was to assess the relationship between plant growth and applied cadmium stress. Statistical significance was determined using two-way ANOVA, and Tukey's HSD test was applied for normal distribution data, with a significance level set at $p < 0.001$. Lower-case letters were used to indicate significant differences between the treatments.

4.2.7 Fresh Weight of Leaves

Leaves fresh weight was calculated after 40 days for each treatment (T1 = Sunflower, T2 to T5= Sunflower + $CdCl_2$, T6 to T9 = Sunflower + $CdCl_2$ + Bacteria) by using a weighing balance. R-Studio is used to calculate their mean values, standard deviation, 1st and 3rd quartile, and p-value.

Statistical analyses like the 2-way ANOVA and Tukey HSD Test are applied, which identifies the significant difference between different treatments as 0.001. X-axis represents the treatments from T2 to T9, and Y-axis represents the leaf's fresh weight. The graph compares the effect of Cd on sunflower when bacteria is absent with the effect of Cd on the plants when Bacillus *subtilis* is present. The error bar shows the maximum and minimum sum of the means and standard deviations of the treatments, as well as the difference between them. The differences in the results of the treatments are represented by lettering; the result with the same letters explains that there is no obvious difference between the treatments, while the results with different letters suggest an obvious difference.

The results in **Figure 15** show that the fresh weight of leaves decreases in plants with increasing $CdCl_2$ concentrations. At 25mM $CdCl_2$, the fresh weight of leaves is very low due to less activity of bacteria. Afterward, with increasing cadmium concentration, the bacterial activity also increased and an obvious increase in leaves fresh weight despite increasing $CdCl_2$ concentrations can be observed. At higher concentrations of cadmium, $CdCl_2$ decreases the fresh weight of leaves of plants significantly which is opposed by bacteria, and no obvious decrease in fresh weight of leaves can be observed. Therefore, the effect of bacteria is also prominent at higher concentrations. Bacillus *subtilis* increases the leaves' fresh weight in the plants even at higher concentrations of $CdCl_2$.

The mean value of the control, known as T1 (Sunflower only) is 0.76. When compared to the control (T1), there is an increase of 18.85% and 26.8% and a decrease of 63.95%, and 76.97% in leaves fresh weight when 25mM, 50mM, 75mM, and 100mM $CdCl_2$ is added to the soil respectively. However, with bacteria present, compared to the control (T1), a decrease of 15.79% and an increase of 44.47%, 9.7%, and 22.76% when 25mM, 50mM,75mM, and 100Mm CdCl2 are also added to the soil respectively.

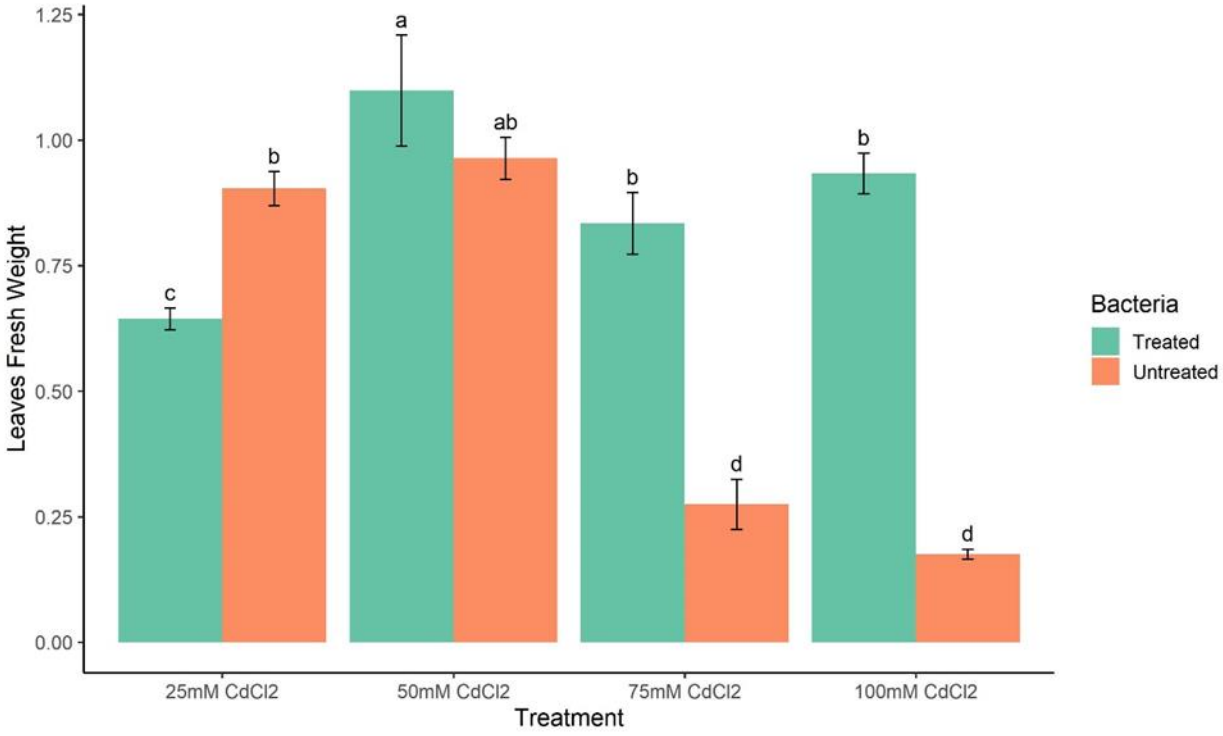

Figure 15: The fresh weight of leaves of sunflower plants given different concentrations of Cd stress. The analysis was conducted on day 40 of the experiment. The treatments included positive control groups and experimental groups with varying concentrations of $CdCl_2$ (25mM, 50mM, 75mM, and 100mM). The aim was to assess the relationship between plant growth and applied cadmium stress. Statistical significance was determined using two-way ANOVA, and Tukey's HSD test was applied for normal distribution data, with a significance level set at $p < 0.001$. Lower-case letters were used to indicate significant differences between the treatments.

4.2.8 Total Plant Fresh Weight

Total plant fresh weight was calculated after 40 days for each treatment (T1 = Sunflower, T2 to T5= Sunflower + $CdCl_2$, T6 to T9 = Sunflower + $CdCl_2$ + Bacteria) by using weighing balance. R-Studio is used to calculate their mean values, standard deviation, 1st and 3rd quartile, and p-value.

Statistical analyses like the 2-way ANOVA and Tukey HSD Test are applied, which identifies the significant difference between different treatments as 0.001. X-axis represents the treatments from T2 to T9, and Y-axis represents the stem girth. The graph compares the effect of Cd on sunflower when bacteria is absent with the effect of Cd on the plants when Bacillus *subtilis* is present. The error bar shows the maximum and minimum sum of the means and standard deviations of the treatments, as well as the difference between them. The differences in the results of the treatments are represented by lettering; the result with the same letters explains that there is no obvious difference between the treatments, while the results with different letters suggest an obvious difference.

The results in **Figure 16** show that the total plant weight decreases in plants with increasing $CdCl_2$ concentrations. At lower concentrations of cadmium, the effect of bacteria is low, so the plant's total weight has reduced. However, with the presence of bacteria, there is an obvious increase in total plant weight despite increasing $CdCl_2$ concentrations. Therefore, the effect of bacteria is more obvious at higher cadmium concentrations. Bacillus *subtilis* increases the total plant weight in the plants even at higher concentrations of $CdCl_2$.

The mean value of the control, known as T1 (Sunflower only) is 5.23. When compared to the control (T1), there is an increase of 10.61% and 16.54% and a decrease of 73.42% and 82.62% when 25mM, 50mM, 75mM, and 100mM $CdCl_2$ were added to the soil respectively. However, when the bacteria are added, the total plant weight decreases by 0.51%, increases by 29.1%, decreases by 1.87%, and again increases by 2.49% even when 25mM, 50mM, 75mM, and 100mM $CdCl_2$ are added to the soil respectively.

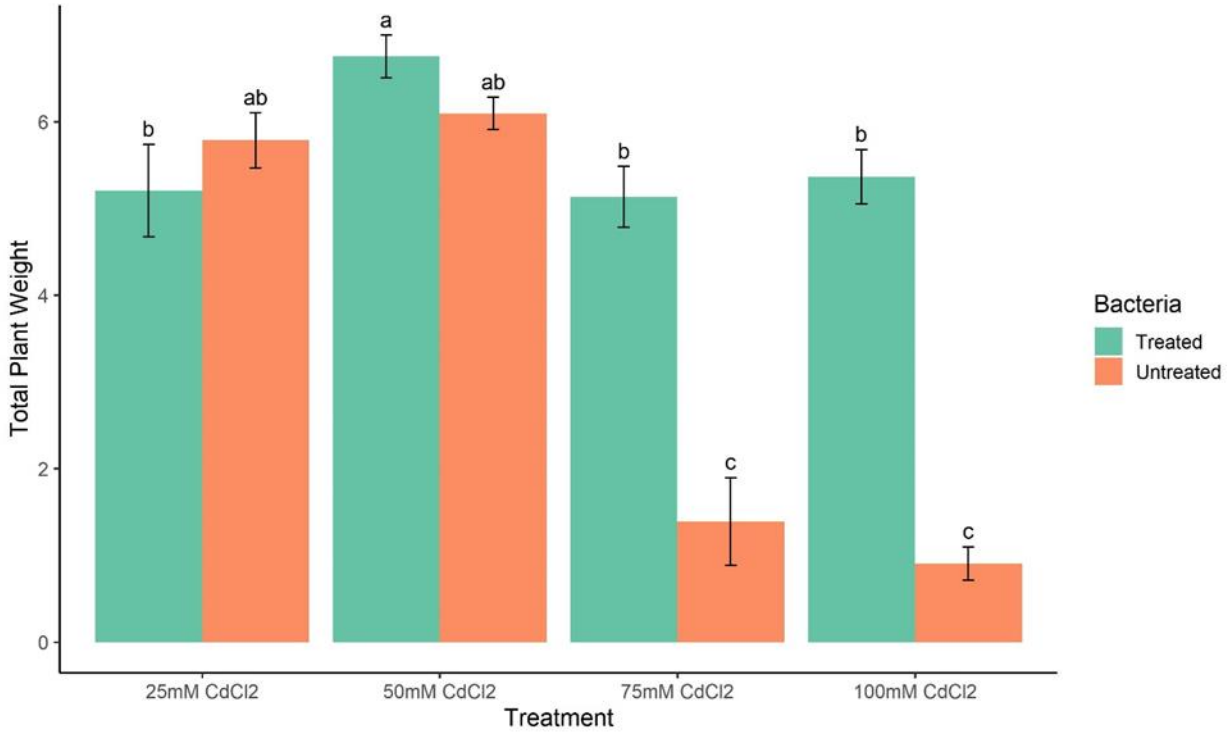

Figure 16: The total plant weight of sunflower plants given different concentrations of Cd stress. The analysis was conducted on day 40 of the experiment. The treatments included positive control groups and experimental groups with varying concentrations of $CdCl_2$ (25mM, 50mM, 75mM, and 100mM). The aim was to assess the relationship between plant growth and applied cadmium stress. Statistical significance was determined using two-way ANOVA, and Tukey's HSD test was applied for normal distribution data, with a significance level set at $p < 0.001$. Lower-case letters were used to indicate significant differences between the treatments.

4.2.9 Dry Root Weight

Root dry weight was calculated after 40 days for each treatment (T1 = Sunflower, T2 to T5= Sunflower + $CdCl_2$, T6 to T9 = Sunflower + $CdCl_2$ + Bacteria) by using a weighing balance. R-Studio is used to calculate their mean values, standard deviation, 1st and 3rd quartile, and p-value.

Statistical analyses like the 2-way ANOVA and Tukey HSD Test are applied, which identifies the significant difference between different treatments as 0.001. X-axis represents the treatments from T2 to T9, and Y-axis represents the root dry weight. The graph compares the effect of Cd on sunflower when bacteria is absent with the effect of Cd on the plants when Bacillus *subtilis* is present. The error bar shows the maximum and minimum sum of the means and standard deviations of the treatments, as well as the difference between them. The differences in the results of the treatments are represented by lettering; the result with the same letters explains that there is no obvious difference between the treatments, while the results with different letters suggest an obvious difference.

The results in **Figure 17** show that the root dry weight decreases in plants with increasing $CdCl_2$ concentrations. However, with the presence of bacteria, there is an obvious increase in shoot dry weight despite increasing $CdCl_2$ concentrations. Since there is little effect of $CdCl_2$ at low concentrations, there is little to no effect of bacteria at these low concentrations, but at higher concentrations, $CdCl_2$ decreases the root dry weight of plants significantly. Therefore, the effect of bacteria is also prominent at higher concentrations. Bacillus *subtilis* increases the root dry weight in the plants even at higher concentrations of $CdCl_2$.

The mean value of the control, known as T1 (Sunflower only) is 0.095. When compared to the control (T1), there is an increase of 60.53%, 64.92%, and a decrease of 52.53%, and 64.39% when 25mM, 50mM, 75mM, and 100mM CdCl2 is added to the soil respectively. However, when the bacteria are added, the root dry weight first increases by 49.82%, 28.81%, and then decreases by 27.19% and 31.3% compared to the control (T1), even when 25mM, 50mM, 75m,M and 100mM CdCl2 are added to the soil respectively.

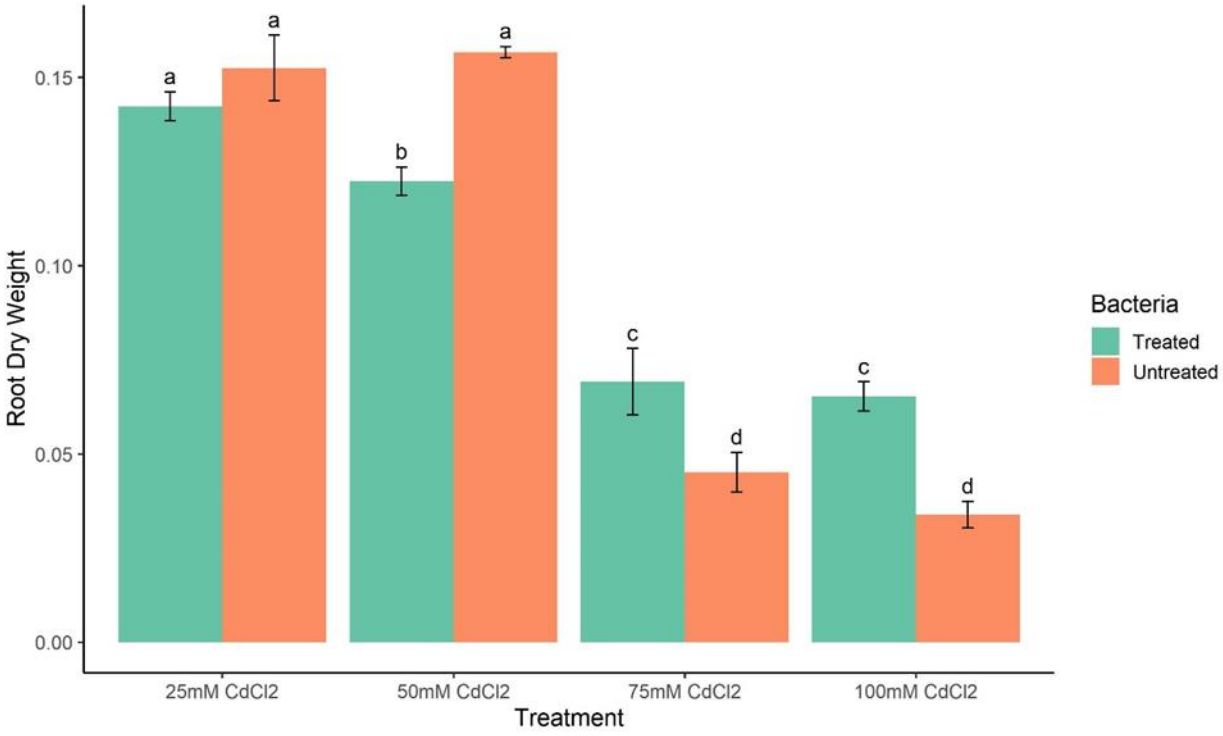

Figure 17: The dry weight of roots of sunflower plants given different concentrations of Cd stress. The analysis was conducted on day 40 of the experiment. The treatments included positive control groups and experimental groups with varying concentrations of CdCl2 (25mM, 50mM, 75mM, and 100mM). The aim was to assess the relationship between plant growth and applied cadmium stress. Statistical significance was determined using two-way ANOVA, and Tukey's HSD test was applied for normal distribution data, with a significance level set at $p < 0.001$. Lower-case letters were used to indicate significant differences between the treatments.

4.2.10 Dry Shoot Weight

Shoot dry weight was calculated after 40 days for each treatment (T1 = Sunflower, T2 to T5= Sunflower + $CdCl_2$, T6 to T9 = Sunflower + $CdCl_2$ + Bacteria) by using a weighing balance. R-Studio is used to calculate their mean values, standard deviation, 1st and 3rd quartile, and p-value.

Statistical analyses like the 2-way ANOVA and Tukey HSD Test are applied, which identifies the significant difference between different treatments as 0.001. X-axis represents the treatments from T2 to T9, and Y-axis represents the shoot dry weight. The graph compares the effect of Cd on sunflower when bacteria is absent with the effect of Cd on the plants when Bacillus *subtilis* is present. The error bar shows the maximum and minimum sum of the means and standard deviations of the treatments, as well as the difference between them. The differences in the results of the treatments are represented by lettering; the result with the same letters explains that there is no obvious difference between the treatments, while the results with different letters suggest an obvious difference.

The results in **Figure 18** show that the shoot dry weight decreases in plants with increasing $CdCl_2$ concentrations. This effect is mitigated by bacteria at higher concentrations. The presence of bacteria has resulted in an obvious increase in shoot dry weight despite increasing $CdCl_2$ concentrations. Since there is little effect of $CdCl_2$ at low concentrations, there is little to no effect of bacteria at these low concentrations, Therefore, a more prevalent effect of cadmium can be observed in physiological nature. But at higher concentrations, $CdCl_2$ decreases the shoot dry weight of plants significantly. Therefore, the effect of bacteria is also prominent at higher concentrations. Bacillus *subtilis* increases the shoot dry weight in the plants even at higher concentrations of $CdCl_2$.

The mean value of the control, known as T1 (Sunflower only) is 0.354. When compared to the control (T1), there is a decrease of 3.96%, an increase of 6.78%, and again a decrease of 80.96%, and 88.74%, when 25mM, 50mM, 75mM, and 100mM $CdCl_2$ is added to the soil respectively. However, when the bacteria are added, the root length decreases by 20.904%, 4.72%, 39.55%, and 8.19%. compared to the control (T1), even when 25mM, 50mM, 75mM and 100mM $CdCl_2$ are added to the soil respectively.

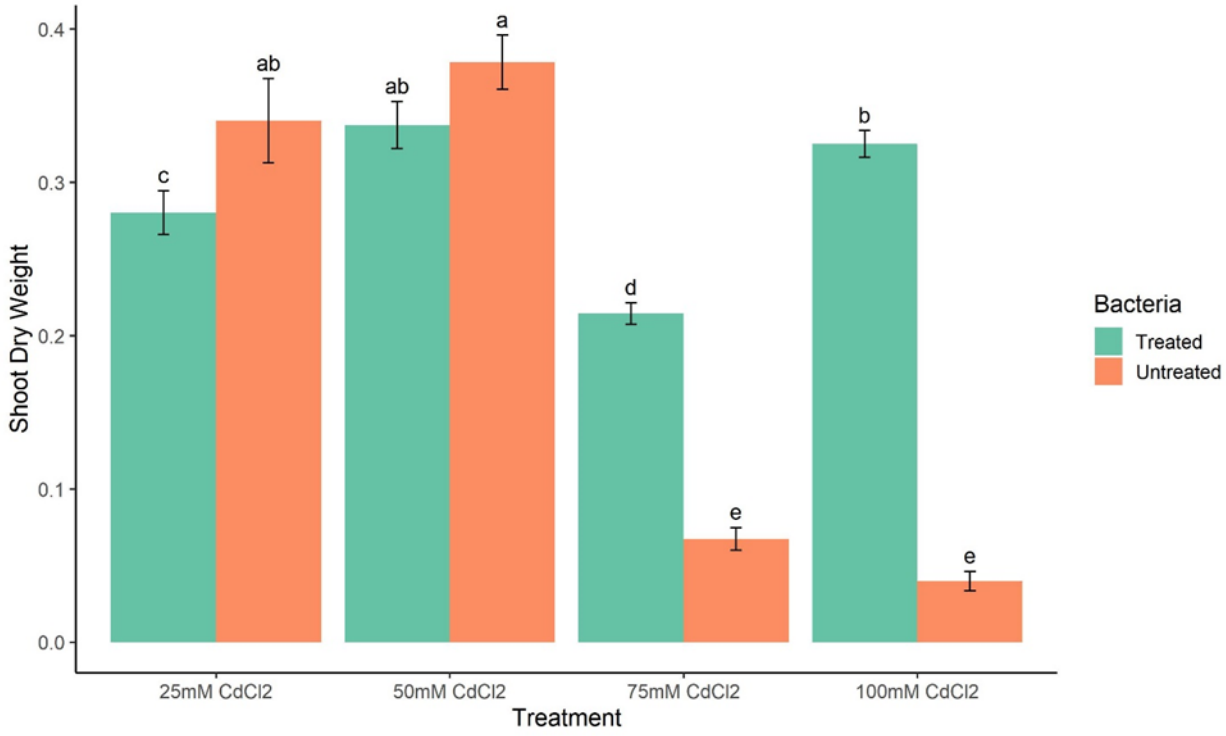

Figure 18: The dry weight of shoots of sunflower plants given different concentrations of Cd stress. The analysis was conducted on day 40 of the experiment. The treatments included positive control groups and experimental groups with varying concentrations of $CdCl_2$ (25mM, 50mM, 75mM, and 100mM). The aim was to assess the relationship between plant growth and applied cadmium stress. Statistical significance was determined using two-way ANOVA, and Tukey's HSD test was applied for normal distribution data, with a significance level set at $p < 0.001$. Lower-case letters were used to indicate significant differences between the treatments.

4.2.11 Stem Girth

The stem girth is calculated after 40 days for each treatment (T1 = Sunflower, T2 to T5= Sunflower + $CdCl_2$, T6 to T9 = Sunflower + $CdCl_2$ + Bacteria). R-Studio is used to calculate their mean values, standard deviation, 1st and 3rd quartile, and p-value.

Statistical analyses like the 2-way ANOVA and Tukey HSD Test are applied, which identifies the significant difference between different treatments as 0.001. X-axis represents the treatments from T2 to T9, and Y-axis represents the stem girth. The graph compares the effect of Cd on sunflower when bacteria is absent with the effect of Cd on the plants when Bacillus *subtilis* is present. The error bar shows the maximum and minimum sum of the means and standard deviations of the treatments, as well as the difference between them. The differences in the results of the treatments are represented by lettering; the results with the same letters explain that there is no obvious difference between the treatments, while the results with different letters suggest an obvious difference.

The results in **Figure 19** show that the stem girth decreases in plants with increasing $CdCl_2$ concentrations. However, with the presence of bacteria, there is an obvious increase in the stem girth with increasing $CdCl_2$ concentrations. Since there is little effect of $CdCl_2$ at low concentrations, there is little to no effect of bacteria at these low concentrations, but at higher concentrations, $CdCl_2$ decreases the stem girth of leaves of plants significantly. Therefore, the effect of bacteria is also prominent at higher concentrations. It is evident from the graph that the bacteria work well for 100mM $CdCl_2$ concentration, as the most significant increase in stem girth was shown at this concentration. Bacillus *subtilis* increases the stem girth of leaves in plants with higher concentrations of $CdCl_2$.

The mean value of the control, known as T1 (Sunflower only) is 0.27. When compared to the control (T1), there is a 17.4%, 16.67%, 52.59%, and 84.82% decrease in stem girth when 25mM, 50mM, 75mM, and 100mM $CdCl_2$ are added to the soil respectively. However, with bacteria present, compared to the control (T1), a decrease of 37%, 18.5%, and 22.2%, and an increase of 66.67% in stem girth is observed when 25mM, 50mM, 75mM, and 100mM $CdCl_2$ are also added to the soil respectively.

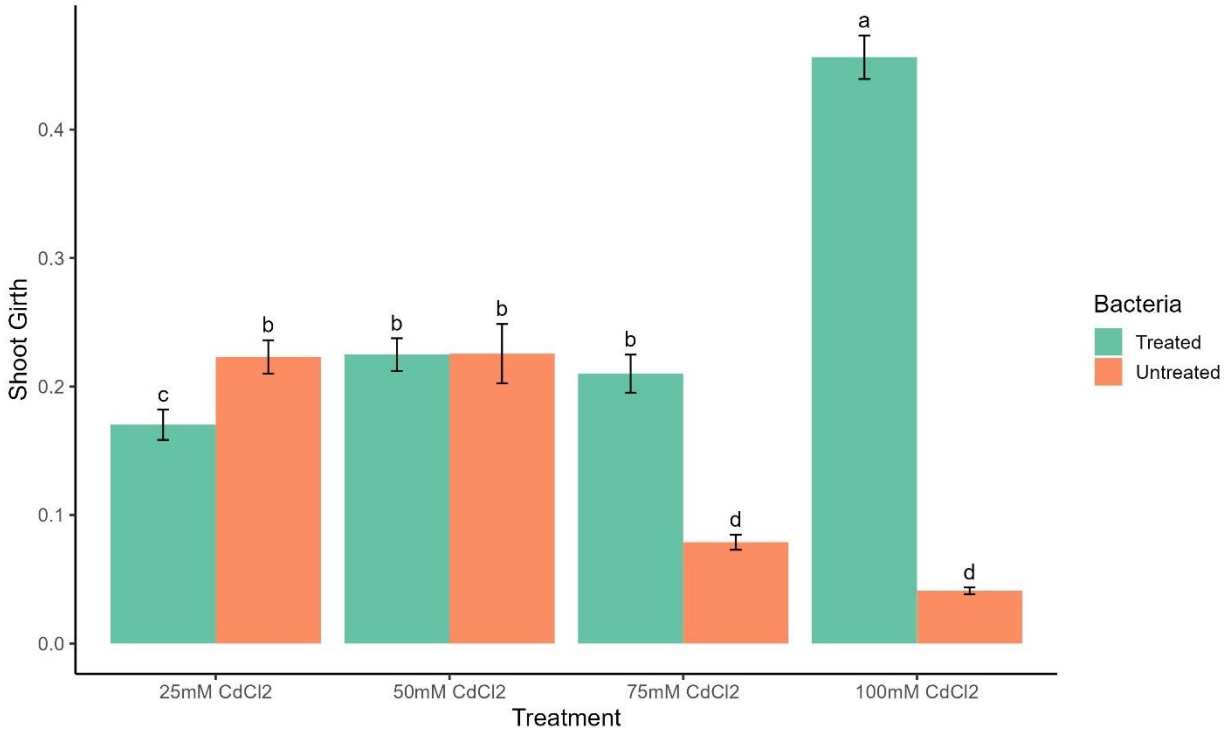

Figure 19: The shoot girth of sunflower plants given different concentrations of Cd stress. The analysis was conducted on day 40 of the experiment. The treatments included positive control groups and experimental groups with varying concentrations of $CdCl_2$ (25mM, 50mM, 75mM, and 100mM). The aim was to assess the relationship between plant growth and applied cadmium stress. Statistical significance was determined using two-way ANOVA, and Tukey's HSD test was applied for normal distribution data, with a significance level set at $p < 0.001$. Lower-case letters were used to indicate significant differences between the treatments.

4.2.12 Leaf Area

The leaf area is calculated after 40 days for each treatment (T1 = Sunflower, T2 to T5= Sunflower + $CdCl_2$, T6 to T9 = Sunflower + $CdCl_2$ + Bacteria). R-Studio is used to calculate their mean values, standard deviation, 1st and 3rd quartile, and p-value.

Statistical analyses like the 2-way ANOVA and Tukey HSD Test are applied, which identifies the significant difference between different treatments as 0.001. X-axis represents the treatments from T2 to T9, and Y-axis represents the leaf area. The graph compares the effect of Cd on sunflower when bacteria is absent with the effect of Cd on the plants when Bacillus *subtilis* is present. The error bar shows the maximum and minimum sum of the means and standard deviations of the treatments, as well as the difference between them. The differences in the results of the treatments are represented by lettering; the results with the same letters explain that there is no obvious difference between the treatments, while the results with different letters suggest an obvious difference.

The results in **Figure 20** show that the leaf area decreases in plants with increasing $CdCl_2$ concentrations. However, with the presence of bacteria, there is an obvious increase in the leaf area with increasing $CdCl_2$ concentrations. Since there is little effect of $CdCl_2$ at low concentrations, there is little to no effect of bacteria at these low concentrations, but at higher concentrations, $CdCl_2$ decreases the leaf area of plants significantly. Therefore, the effect of bacteria is also prominent at higher concentrations. Bacillus *subtilis* increases the leaf area in plants with higher concentrations of $CdCl_2$.

The mean value of the control, known as T1 (Sunflower only) is 6.5. When compared with the control (T1), there is an increase of 34.05% and 46.08% and a 51.54% and 72.22% decrease in the leaf area when 25mM, 50mM, 75mM, and 100mM $CdCl_2$ are added to the soil respectively. With bacteria present, when the leaf area of control (T1) is compared to the leaf area of other treatments, there is a 5.54% and 34.62% increase and then an increase of 26.6%, and 10.77% increase when 25mM, 50mM, 75mM, and 100mM $CdCl_2$ are added to the soil respectively.

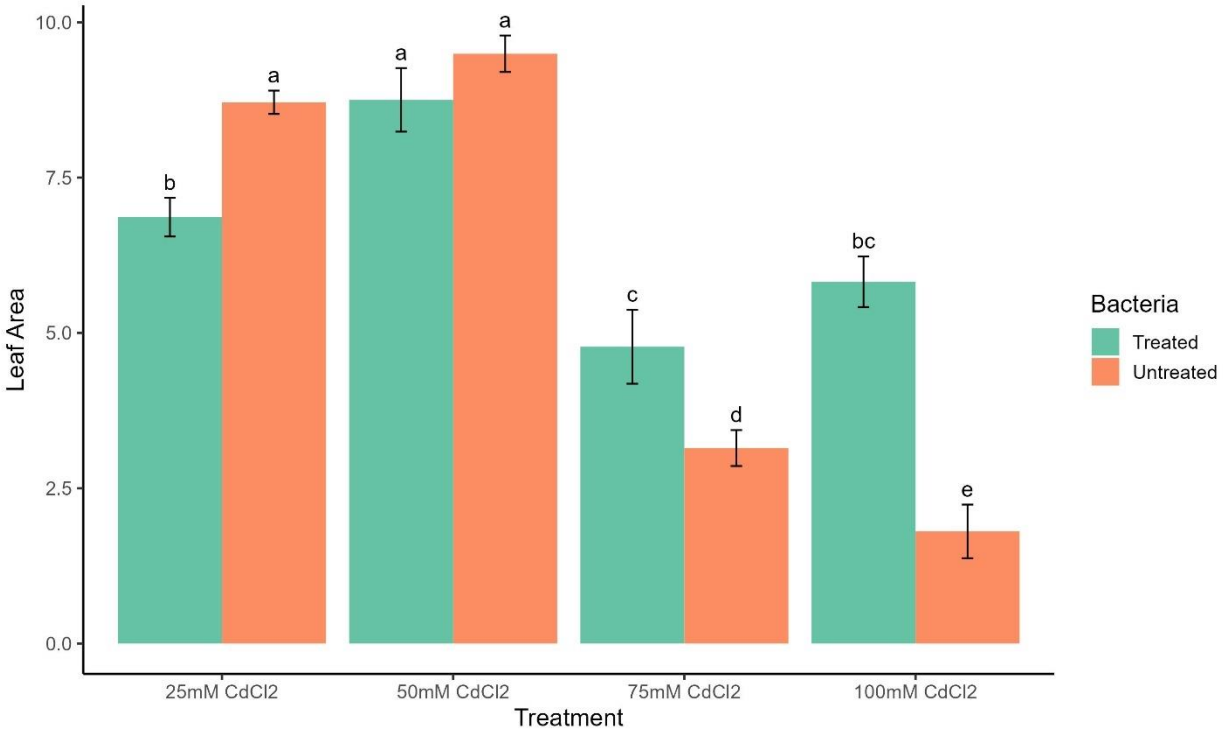

Figure 20: The leaf area of sunflower plants given different concentrations of Cd stress. The analysis was conducted on day 40 of the experiment. The treatments included positive control groups and experimental groups with varying concentrations of $CdCl_2$ (25mM, 50mM, 75mM, and 100mM). The aim was to assess the relationship between plant growth and applied cadmium stress. Statistical significance was determined using two-way ANOVA, and Tukey's HSD test was applied for normal distribution data, with a significance level set at $p < 0.001$. Lower-case letters were used to indicate significant differences between the treatments.

4.3 Physiological Analysis

4.3.1 SPAD Value

The SPAD values of leaves are calculated after 40 days for each treatment (T1 = Sunflower, T2 to T5= Sunflower + $CdCl_2$, T6 to T9 = Sunflower + $CdCl_2$ + Bacteria). R-Studio is used to calculate their mean values, standard deviation, 1st and 3rd quartile, and p-value.

Statistical analyses like the 2-way ANOVA and Tukey HSD Test are applied, which identifies the significant difference between different treatments as 0.001. X-axis represents the treatments from T2 to T9, and Y-axis represents the SPAD values. The graph compares the effect of Cd on sunflower when bacteria is absent with the effect of Cd on the plants when Bacillus *subtilis* is present. The error bar shows the maximum and minimum sum of the means and standard deviations of the treatments, as well as the difference between them. The differences in the results of the treatments are represented by lettering; the results with the same letters explain that there is no obvious difference between the treatments, while the results with different letters suggest an obvious difference.

The results in **Figure 21** show that the SPAD values decrease in plants with increasing $CdCl_2$ concentrations. However, with the presence of bacteria, there is an obvious increase in the SPAD values with increasing $CdCl_2$ concentrations. Since there was little effect of $CdCl_2$ at low concentrations, there was little to no effect of bacteria at these low concentrations, but at higher concentrations, $CdCl_2$ decreases the SPAD values of leaves of plants significantly. Therefore, the effect of bacteria is also prominent at higher concentrations. Bacillus *subtilis* increases the SPAD values of leaves in plants with higher concentrations of $CdCl_2$.

The mean value of the control, known as T1 (Sunflower only) is 25.3. When compared to the control (T1), there is an 18.83%, 22.26%, 71.3%, and 77.81% decrease in SPAD values of leaves when 25mM, 50mM, 75mM, and 100mM $CdCl_2$ are added to the soil respectively. On the contrary, compared to the control, a 43.87%, 22.41%, 43.8%, and 20.15% decrease was observed when 25mM, 50mM, 75mM, and 100mM $CdCl_2$ are added to the soil respectively along with the bacteria.

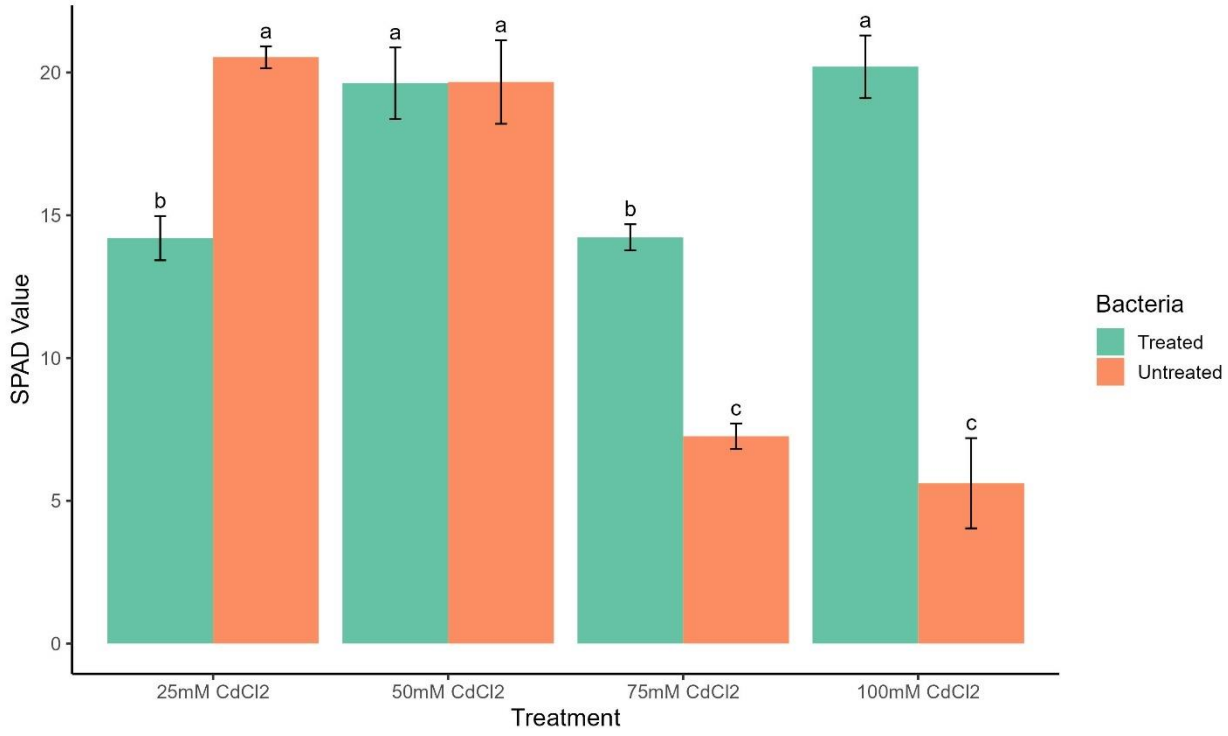

Figure 21: The SPAD values of leaves of sunflower plants given different concentrations of Cd stress. The analysis was conducted on day 40 of the experiment. The treatments included positive control groups and experimental groups with varying concentrations of $CdCl_2$ (25mM, 50mM, 75mM, and 100mM). The aim was to assess the relationship between plant growth and applied cadmium stress. Statistical significance was determined using two-way ANOVA, and Tukey's HSD test was applied for normal distribution data, with a significance level set at $p < 0.001$. Lower-case letters were used to indicate significant differences between the treatments..

4.3.2 Relative Water Content

The relative water content of leaves is calculated after 40 days for each treatment (T1 = Sunflower, T2 to T5= Sunflower + $CdCl_2$, T6 to T9 = Sunflower + $CdCl_2$ + Bacteria). R-Studio is used to calculate their mean values, standard deviation, 1st and 3rd quartile, and p-value.

Statistical analyses like the 2-way ANOVA and Tukey HSD Test are applied, which identifies the significant difference between different treatments as 0.001. X-axis represents the treatments from T2 to T9, and Y-axis represents the relative water content of leaves. The graph compares the effect of Cd on sunflower when bacteria is absent with the effect of Cd on the plants when Bacillus *subtilis* is present. The error bar shows the maximum and minimum sum of the means and standard deviations of the treatments, as well as the difference between them. The differences in the results of the treatments are represented by lettering; the results with the same letters explain that there is no obvious difference between the treatments, while the results with different letters suggest an obvious difference.

The results in **Figure 22** show that the RWC of leaves decreases in plants with increasing $CdCl_2$ concentrations. The effect of bacteria on the relative water content of the leaves is not very prominent.

The mean value of the control, known as T1 (Sunflower only) is 52.5. When compared to the control (T1), the RWC of leaves first increases by 11.5% and then decreases by 7.2%, 5.4%, and 32.98% when 25mM, 50mM, 75mM, and 100mM $CdCl_2$ are added to the soil respectively. This shows that an obvious decrease in the RWC of leaves was observed in 100mM $CdCl_2$ concentration only. When bacteria are added, compared to the control (T1), there is a 4.32% increase, and then a 2.8%, 16.99%, and 29.58% decrease in RWC even when 25mM, 50mM, 75mM, and 100mM $CdCl_2$ is added to the soil respectively.

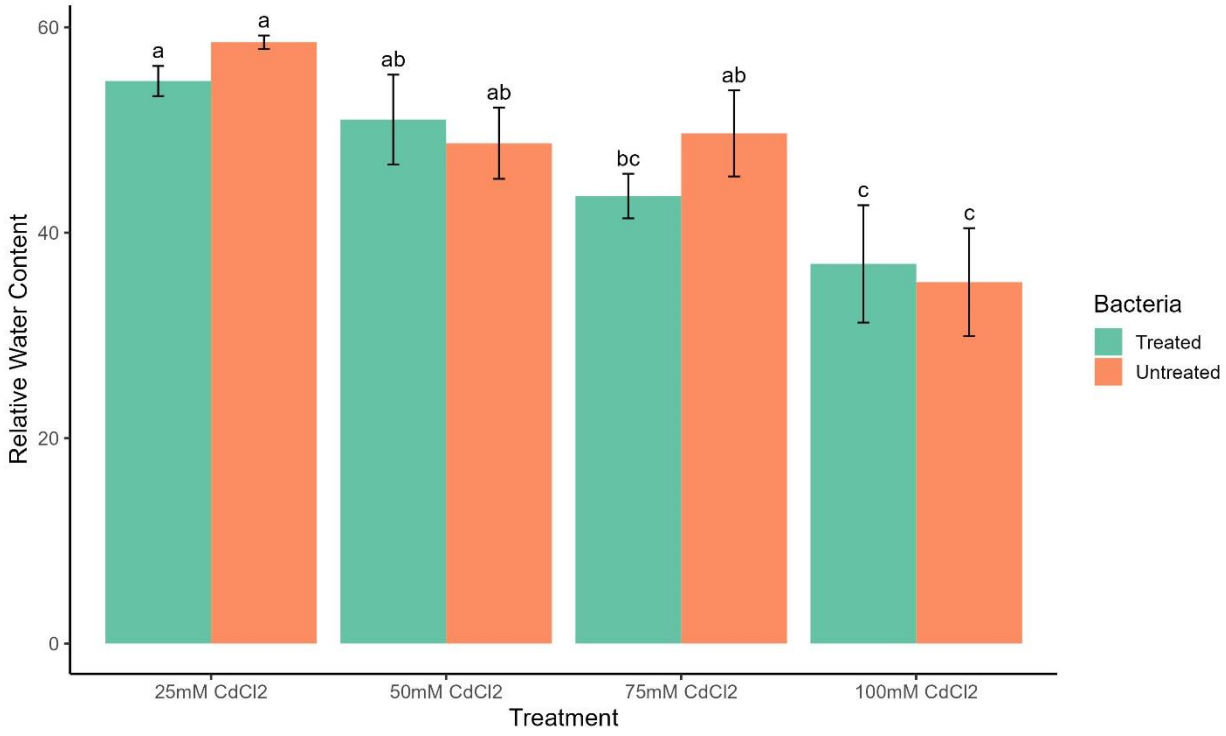

Figure 22: The RWC of leaves of sunflower plants given different concentrations of Cd stress. The analysis was conducted on day 40 of the experiment. The treatments included positive control groups and experimental groups with varying concentrations of $CdCl_2$ (25mM, 50mM, 75mM, and 100mM). The aim was to assess the relationship between plant growth and applied cadmium stress. Statistical significance was determined using two-way ANOVA, and Tukey's HSD test was applied for normal distribution data, with a significance level set at $p < 0.001$. Lower-case letters were used to indicate significant differences between the treatments.

4.3.3 Microscopy

Microscopy of roots and shoots was performed by visualizing them at 10X magnification. The results of compound microscopy showed that cadmium toxicity caused structural distortion in both stem cells and root cells. The damage caused to cells at higher concentrations of cadmium shows cadmium toxicity. The results of microscopy have clearly shown that with increasing cadmium concentration, the cell structures are distorted.

Figure 23 shows the root and shoot structure of negative control. A shows the structure of the shoot while B shows the structure of the roots. According to these images, the root and shoot structures are intact and remain undistorted.

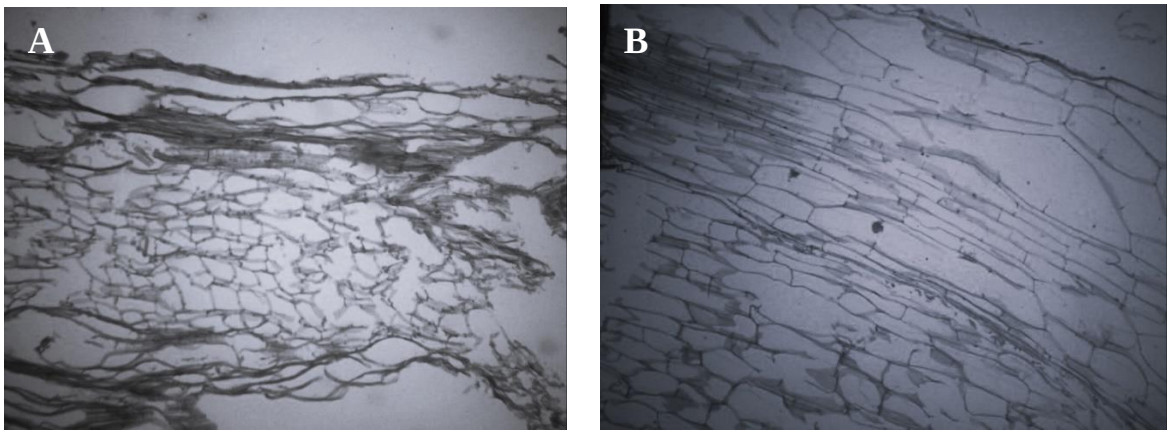

Figure 23: Microscopic view of a cross-section at 10X magnification obtained from a plant sample of control. (A) Shoot cross-section (B) Root cross-section. The figure provides an overview of the internal organization and tissue composition of shoots and roots.

Figure 24 shows the structure of roots and shoots at 50mM $CdCl_2$ concentration. A shows the structure of shoots while B shows the structure of roots. The cells in shoots and roots are damaged which explains the cadmium toxicity and its effect on the structures of roots and shoots.

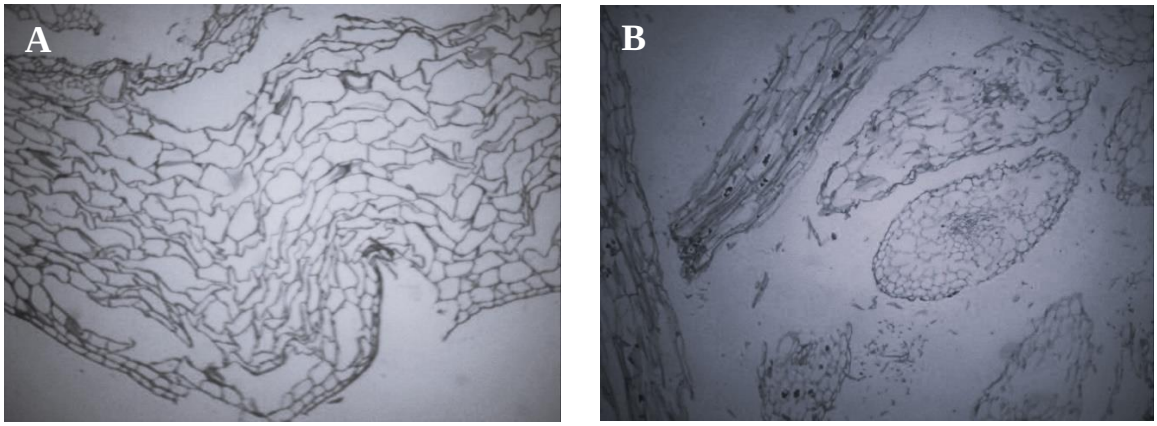

Figure 24: Microscopic view of a cross-section at 10X magnification obtained from a plant sample of positive control (50mM $CdCl_2$ in the absence of bacteria). (A) Shoot cross-section (B) Root cross-section. The figure provides an overview of the internal organization and tissue composition of shoots and roots.

Figure 25 explains the root and shoot structure at 100mM $CdCl_2$ in the absence of bacteria. A shows the structure of the shoot while B is the structure of the root. These structures compared to the ones in Figure 24 are more damaged, explaining the high stress conditions in plants.

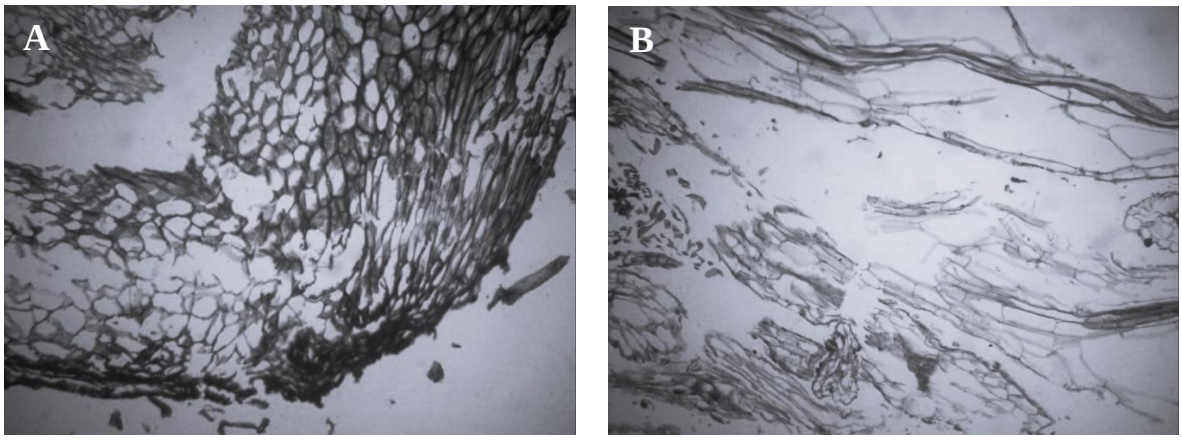

Figure 25: Microscopic view of a cross-section at 10X magnification obtained from a plant sample of positive control (100mM $CdCl_2$ in the absence of bacteria). (A) Shoot cross-section (B) Root cross-section. The figure provides an overview of the internal organization and tissue composition of shoots and roots.

Figure 26 shows the root and shoot structures of plants growing in 50mM $CdCl_2$ concentration after the addition of bacterial treatments. A shows the structure of shoots while B shows the structure of roots. The presence of bacteria minimized the damage to plant structures. Compared to Figure 24, structures in Figure 26 show improvement in the cell structures despite $CdCl_2$ being present in them.

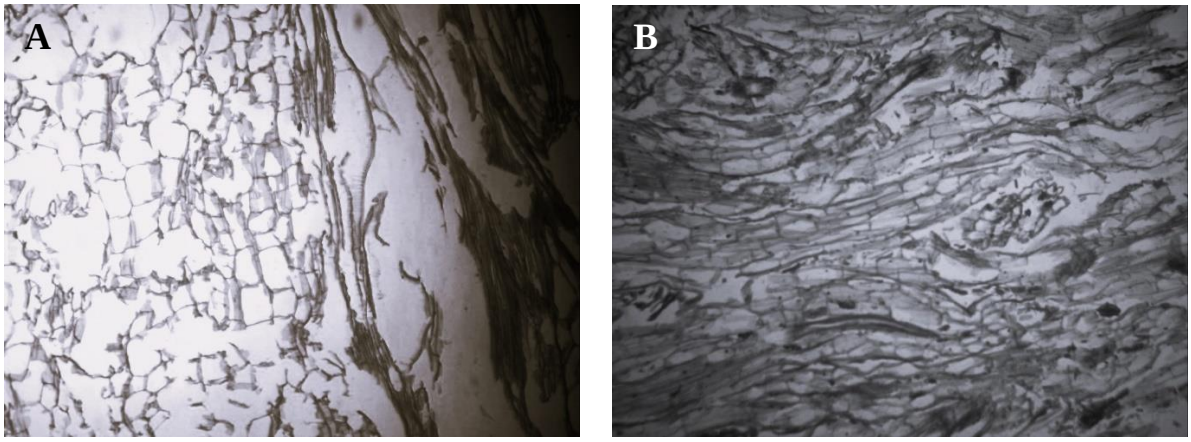

Figure 26: Microscopic view of a cross-section at 10X magnification obtained from a plant sample of the experimental group (50mM $CdCl_2$ in the presence of bacteria). (A) Shoot cross-section (B) Root cross-section. The figure provides an overview of the internal organization and tissue composition of shoots and roots.

Figure 27 represents the structures of roots and shoots in plants growing in soil with 100mM $CdCl_2$ concentration in the presence of bacterial treatment. A represents the structure of shoots while B shows the structure of roots. This figure identifies the potential role of bacteria in mitigating the harmful effects of heavy metal toxicity on plant growth and development. The damage to root and shoot structures is minimized when compared to the treatment with the same $CdCl_2$ concentration in the absence of bacteria.

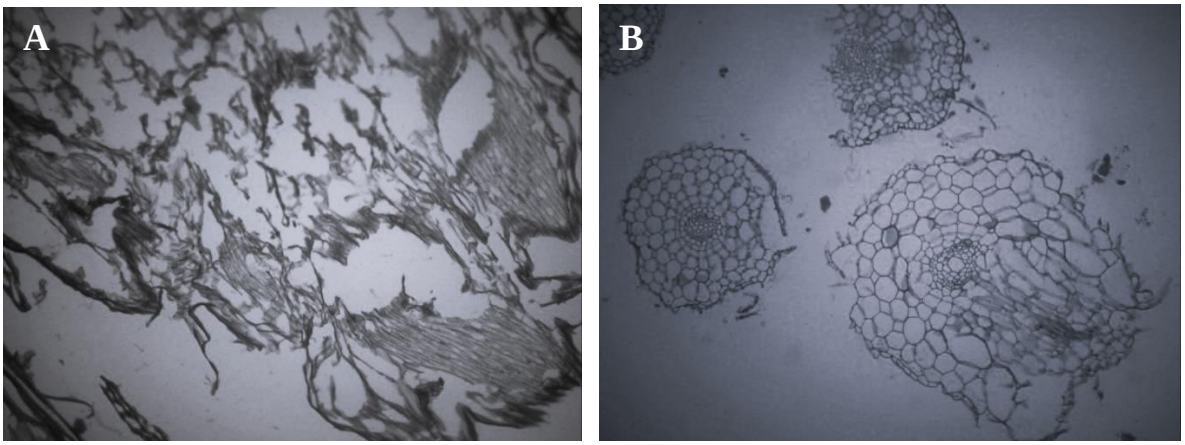

Figure 27: Microscopic view of a cross-section at 10X magnification obtained from a plant sample of the experimental group (100mM $CdCl_2$ in the presence of bacteria). (A) Shoot cross-section (B) Root cross-section. The figure provides an overview of the internal organization and tissue composition of shoots and roots.

4.4 Biochemical Analysis

4.4.1 Superoxide Dismutase (SOD)

The activity of superoxide dismutase in fresh leaves is calculated after 40 days for each treatment (T1 = Sunflower, T2 to T5= Sunflower + $CdCl_2$, T6 to T9 = Sunflower + $CdCl_2$ + Bacteria). R-Studio is used to calculate their mean values, standard deviation, 1st and 3rd quartile, and p-value.

Statistical analyses like the 2-way ANOVA and Tukey HSD Test are applied, which identifies the significant difference between different treatments as 0.001. X-axis represents the treatments from T2 to T9, and Y-axis represents the activity of SOD. The graph compares the effect of Cd on sunflower when bacteria is absent with the effect of Cd on the plants when Bacillus *subtilis* is present. The error bar shows the maximum and minimum sum of the means and standard deviations of the treatments, as well as the difference between them. The differences in the results of the treatments are represented by lettering; the results with the same letters explain that there is no obvious difference between the treatments, while the results with different letters suggest an obvious difference.

The graph in **Figure 28** shows that initially, the activity of SOD in fresh leaves was higher, but when the concentration of $CdCl_2$ increases, the activity of SOD decreases. The difference in SOD activity at different concentrations of $CdCl_2$ is not prominent. The addition of bacteria to cadmium-contaminated soil increases the activity of SOD in leaves with increasing $CdCl_2$ concentrations. The activity of SOD at higher concentrations of $CdCl_2$, with or without bacteria remains almost the same. However, there is a significant difference in the activity of SOD at low concentrations of $CdCl_2$, with or without bacteria.

The mean SOD activity of control (T1) is 0.564 U/ml. When compared to the control (T1), the activity of SOD increases by 25.8%, 4.08%, 13.48%, and 15.78% when 25mM, 50mM, 75mM, and 100mM $CdCl_2$ are added to the soil respectively. When bacteria are added, compared to the control (T1), there is a 4.08%, 18.44%, 18.44%, and 18.26% increase in SOD activity even when 25mM, 50mM, 75mM, and 100mM $CdCl_2$ is added to the soil respectively. The increase in SOD activity when compared to the control (T1) explains the stress situation in which the plants are growing.

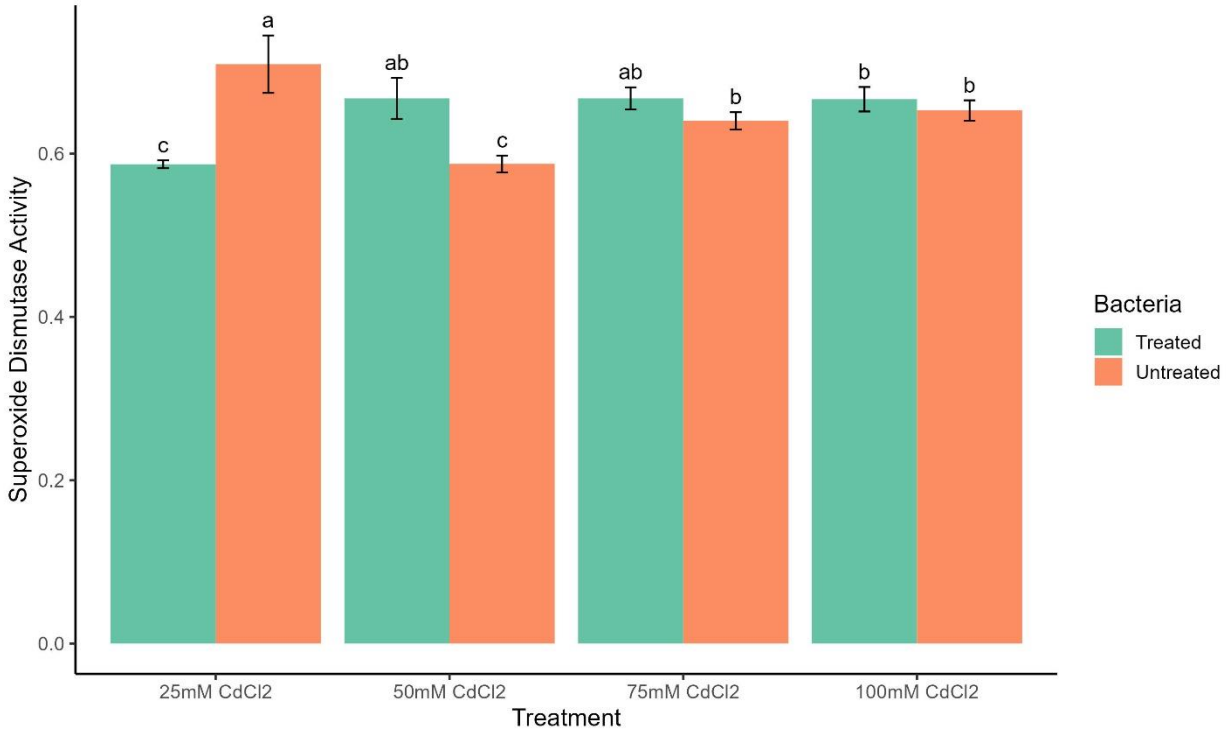

Figure 28: The SOD activity in sunflower plants given different concentrations of Cd stress. The plants were analyzed on day 40. The treatments are as follows: Positive control; 25mM $CdCl_2$, 50mM $CdCl_2$, 75mM $CdCl_2$, 100mM $CdCl_2$, and Experimental groups; 25mM $CdCl_2$, 50mM $CdCl_2$, 75mM $CdCl_2$ and 100mM $CdCl_2$. Data presents the interaction between plant growth and the applied cadmium stress. Significance was calculated using two-way ANOVA under Tukey's HSD test for normal distribution data (honestly significant difference, $p < 0.001$). The lower-case letters show the significant difference between treatments.

4.4.2 Peroxidase (POD)

The activity of peroxidase in fresh leaves is calculated after 40 days for each treatment (T1 = Sunflower, T2 to T5= Sunflower + $CdCl_2$, T6 to T9 = Sunflower + $CdCl_2$ + Bacteria). R-Studio is used to calculate their mean values, standard deviation, 1st and 3rd quartile, and p-value.

Statistical analyses like the 2-way ANOVA and Tukey HSD Test are applied, which identifies the significant difference between different treatments as 0.001. X-axis represents the treatments from T2 to T9, and Y-axis represents the activity of POD. The graph compares the effect of Cd on sunflower when bacteria is absent with the effect of Cd on the plants when Bacillus *subtilis* is present. The error bar shows the maximum and minimum sum of the means and standard deviations of the treatments, as well as the difference between them. The differences in the results of the treatments are represented by lettering; the results with the same letters explain that there is no obvious difference between the treatments, while the results with different letters suggest an obvious difference.

The graph in **Figure 29** shows that initially, the activity of POD in fresh leaves increases, but when the concentration of $CdCl_2$ increases, the activity of POD starts to decrease. The addition of bacteria to cadmium-contaminated soil significantly increases the activity of POD in leaves with increasing $CdCl_2$ concentrations. POD activity in leaves of plants with the presence of bacteria continues to decrease when the concentration of $CdCl_2$ increases.

The mean POD activity of control (T1) is 0.000805 U/ml. When compared to the control (T1), the activity of POD increases by 25.22%, 57.76%, 30.19%, and 18.63% when 25mM, 50mM, 75mM, and 100mM $CdCl_2$ are added to the soil respectively. When bacteria are added, compared to the control (T1), there is a 145.34%, 122.86%, 125.84%, and 30.81% increase in POD activity even when 25mM, 50mM, 75mM, and 100mM $CdCl_2$ is added to the soil respectively. The increase in POD activity when compared to the control (T1) explains the stress situation in which the plants are growing.

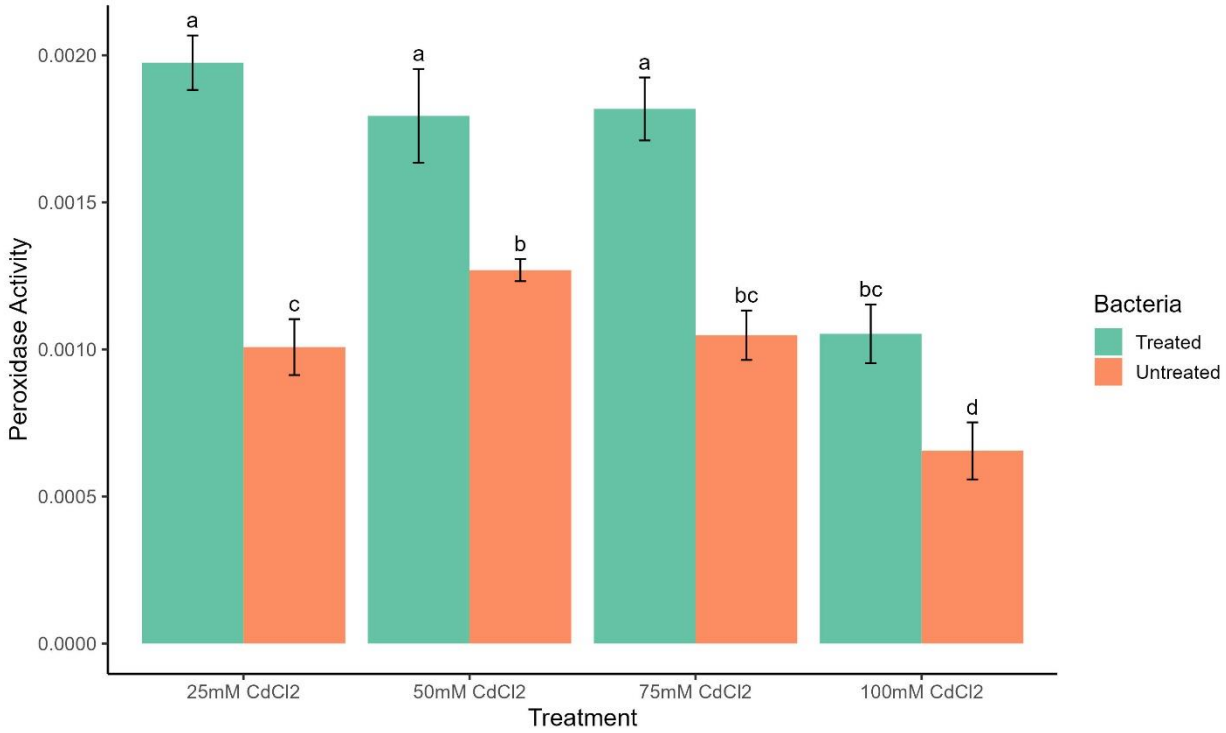

Figure 29: The POD activity in sunflower plants given different concentrations of Cd stress. The plants were analyzed on day 40. The treatments are as follows: Positive control; 25mM $CdCl_2$, 50mM $CdCl_2$, 75mM $CdCl_2$, 100mM $CdCl_2$, and Experimental groups; 25mM $CdCl_2$, 50mM $CdCl_2$, 75mM $CdCl_2$ and 100mM $CdCl_2$. Data presents the interaction between plant growth and the applied cadmium stress. Significance was calculated using two-way ANOVA under Tukey's HSD test for normal distribution data (honestly significant difference, $p < 0.001$). The lower-case letters show the significant difference between treatments.

4.4.3 Catalase (CAT)

The activity of catalase dismutase in fresh leaves is calculated after 40 days for each treatment (T1 = Sunflower, T2 to T5= Sunflower + $CdCl_2$, T6 to T9 = Sunflower + $CdCl_2$ + Bacteria). R-Studio is used to calculate their mean values, standard deviation, 1st and 3rd quartile, and p-value.

Statistical analyses like the 2-way ANOVA and Tukey HSD Test are applied, which identifies the significant difference between different treatments as 0.001. X-axis represents the treatments from T2 to T9, and Y-axis represents the activity of CAT. The graph compares the effect of Cd on sunflower when bacteria is absent with the effect of Cd on the plants when Bacillus *subtilis* is present. The error bar shows the maximum and minimum sum of the means and standard deviations of the treatments, as well as the difference between them. The differences in the results of the treatments are represented by lettering; the results with the same letters explain that there is no obvious difference between the treatments, while the results with different letters suggest an obvious difference.

The graph in **Figure 30** shows that initially, the activity of CAT in fresh leaves was less, but when the concentration of $CdCl_2$ increases, there is a prominent increase in the activity of CAT. The addition of bacteria to cadmium-contaminated soil increases the activity of CAT, especially at lower concentrations of $CdCl_2$. However, there is an obvious decrease in the activity of CAT in leaves with increasing $CdCl_2$ concentrations.

The mean CAT activity of control (T1) is 0.0000863 U/ml. When compared to the control (T1), the activity of CAT decreases by 64.71% and 82.35%, and an increase of 123.52% and 135.55% when 25mM, 50mM, 75mM, and 100mM $CdCl_2$ are added to the soil respectively. When bacteria are added, compared to the control (T1), there is a 141.16%, 83.99%, 20.58%, and 82.34% increase in CAT activity even when 25mM, 50mM, 75mM, and 100mM $CdCl_2$ is added to the soil respectively. The increase in CAT activity when compared to the control (T1) explains the stress situation in which the plants are growing.

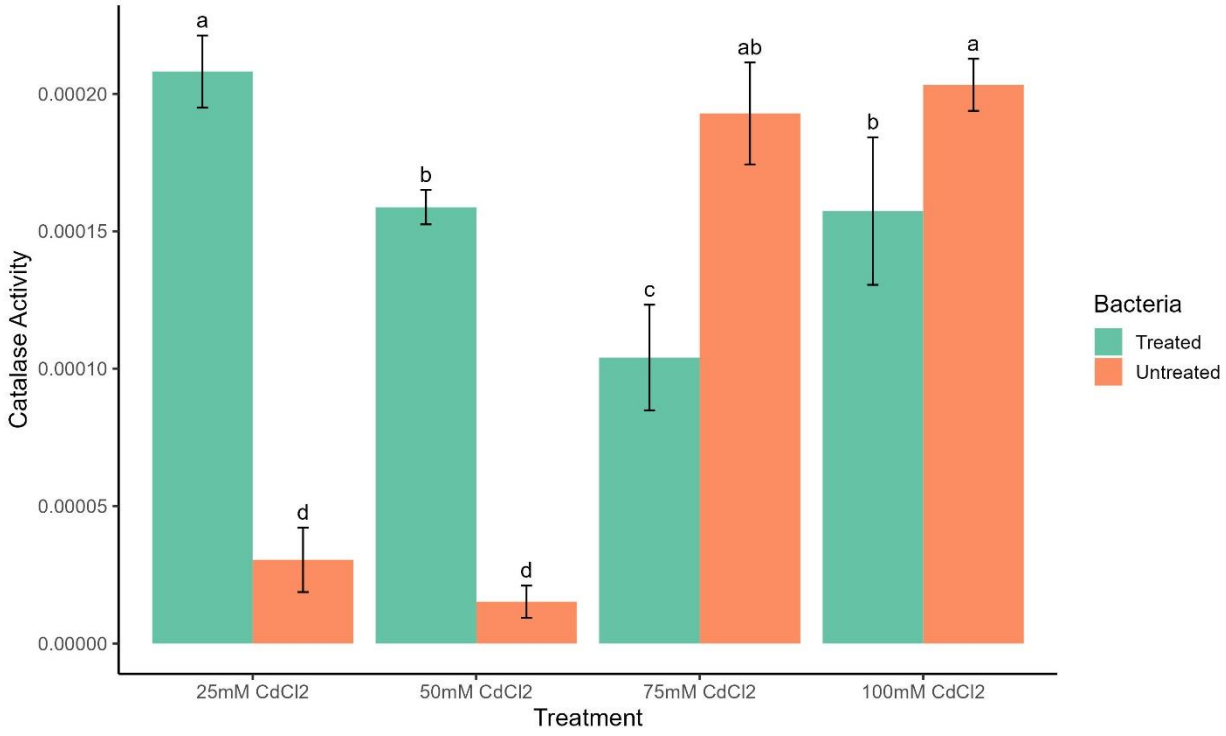

Figure 30: The CAT activity in sunflower plants given different concentrations of Cd stress. The plants were analyzed on day 40. The treatments are as follows: Positive control; 25mM $CdCl_2$, 50mM $CdCl_2$, 75mM $CdCl_2$, 100mM $CdCl_2$, and Experimental groups; 25mM $CdCl_2$, 50mM $CdCl_2$, 75mM $CdCl_2$ and 100mM $CdCl_2$. Data presents the interaction between plant growth and the applied cadmium stress. Significance was calculated using two-way ANOVA under Tukey's HSD test for normal distribution data (honestly significant difference, $p < 0.001$). The lower-case letters show the significant difference between treatments.

Chapter 5: Discussion

The sunflower is considered a hyperaccumulator plant, which means that it can build up high amounts of heavy metals in its tissues with no significant toxic effects. However, this can have a significant impact on the food chain. Cadmium is a heavy metal that is stored in the tissues of plants, animals, and humans, and can cause various health problems, including kidney damage, bone mineral density loss, and cancer. If sunflowers take cadmium from the soil, the metal can be transported to various parts of the plant. In this way, cadmium can continue up the food chain, resulting in higher concentrations of cadmium in the tissues of top predators. Therefore, it is important to monitor the cadmium uptake in sunflowers and take measures to reduce contamination in the soil to inhibit its accumulation in the food chain.

The need for effective and environmentally friendly strategies to address the detrimental effects of Cd pollution is crucial. Bioremediation and phytoremediation have emerged as promising approaches for remediating soils contaminated with Cd. In this study, we focused on evaluating the effectiveness of Bacillus subtilis-based bioremediation and sunflower-based phytoremediation for remediating Cd-contaminated soils.

The study demonstrated the effectiveness of sunflower-Bacillus *subtilis*-based phytoremediation and bioremediation for Cd-contaminated soils. Sunflower is commonly utilized for phytoremediation due to its capability to extract heavy metals from soil through uptake and translocation, as well as its fast growth rate and high biomass production. Bacillus subtilis, on the other hand, is employed for bioremediation due to its capacity to degrade or transform heavy metals in soil. The bacteria possess enzymes like proteases, phosphatases, and dehydrogenases that aid in the degradation of organic matter and mobilization of insoluble nutrients. They can also create chelating agents such as siderophores that decrease the availability of heavy metals in soil.

The study results demonstrated that increasing concentrations of $CdCl_2$ had harmful effects on the morphological and physiological characteristics of sunflower plants. This could be attributed to the elevated uptake and accumulation of Cd in plant tissues, leading to inhibition of growth and development. However, the introduction of Bacillus subtilis positively influenced the growth and physiological parameters of sunflower plants, indicating its ability to alleviate the negative impacts of Cd on plant growth. Bacillus subtilis effectively promoted

plant growth and reduced Cd toxicity, potentially by impeding the translocation of Cd in sunflower plants. The bacteria's production of the substances essential for plant growth and nutrient solubilization likely contributed to the observed improvements. Additionally, the manufacturing of chelating agents by *Bacillus subtilis* aided in the reduction of Cd.

When subjected to heavy metal stress, plants activate various defense responses. Antioxidant enzymes such as SOD, POD, and CAT, as well as protective proteins like heat shock proteins, play important roles in safeguarding plants from oxidative damage. The study revealed increased activity of antioxidant enzymes in response to different concentrations of $CdCl_2$. These enzymes facilitate the breakdown of reactive oxygen species (ROS) into less harmful compounds, thereby reducing oxidative stress and preserving cell integrity. Furthermore, Bacillus subtilis can activate plant defense mechanisms, safeguarding plants from other stress conditions while also improving their morphological and physiological parameters.

The presence of bacteria also appeared to mitigate structural damage caused by $CdCl_2$, suggesting their potential role in minimizing the detrimental effects of heavy metal toxicity on plant development. However, further investigations are necessary to fully understand the specific mechanisms involved in this protective effect.

Bacillus subtilis has the ability for bioremediation of Cd-contaminated soils. The bacteria can mitigate the negative effects of $CdCl_2$ on the sunflower plants, leading to improved growth and physiological parameters. This could be due to the ability of *Bacillus subtilis* to transform or degrade $CdCl_2$, lowering its toxicity to the plants. Additionally, the bacteria may have stimulated plant growth through nutrient cycling or other mechanisms. The use of sunflower for phytoremediation in the experiment also showed promise. Although the plants were negatively affected by increasing $CdCl_2$ concentrations, they were able to tolerate the presence of the heavy metal and exhibited some degree of $CdCl_2$ uptake.

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