Analysis of morpho-physiological response in *Brassica juncea* opposing cadmium spiked soil

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Dedication

I dedicated to my exceptional parents and adored siblings, whose tremendous support and cooperation led me to this wonderful accomplishment

Acknowledgment

All praises to **Almighty ALLAH** ﷻ, and My Beloved Prophet **Hazrat MUHAMMAD e MUSTAFA** ﷺ. I could complete my research work only because of Allah's help and blessing. He always granted me undoubtedly much more than I deserved.

"My Success is Only by Allah"

Durood-o-Salaam to our beloved Prophet **Hazrat MUHAMMAD e MUSTAFA** ﷺ. The last prophet of Allah and the prophet who had brought us from the darkness to the lightness.

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Abstract

Brassica Juncea, a “mustard plant,” is the second largest edible oil crop production source. This plant has a unique ability to store metal ions, thus recognized as a hyper-accumulator. Metal ions include cadmium which damages the plant at a morphological, physiological, and biochemical level. The previous study suggested that the different organic amendments inhibit the translocation of cadmium ions in plants. Thus, this research has been designed and performed with varying combinations of biological aspects, including - biochar, PGPR bacteria, and co-planting. The primary purpose is to identify the best combination for preventing cadmium ions translocation in the mustard plant. Eight treatments were made with different varieties, including one control and seven experimental groups. Phenotypical analysis revealed that cadmium reduce plant growth while the different combination of biological compositions helps the plant growth and yield quality. Moreover, the biochemical analysis identified that mustard plants with cadmium have higher antioxidant enzymes than other treatments. Furthermore, it has proven that cadmium negatively impacts the mustard plant; morphological, physiological, and biochemical aspects in term of phytoremediation, correspondence, with the help of different compound mixtures, its toxicity can reduce to a certain level. Research concludes that a combination of biochar, PGPR bacteria, and inter-cropping (T8) give competitively equivalent result as negative control (T1). This prove that if such combination can enhance growth parameters of hyperaccumulator plants than in future its use for non-hyperaccumulator plants more specifically crops that show highly effective under cadmium stress.

Chapter 1: Introduction

1. Brassica Juncea

Brassica Juncea belongs to the "Brassicaceae or Cruciferae" family with the common name "Brown mustard, Indian mustard, or Chinese mustard," used for oil production and vegetable source. Its production occurs annually as *B. Juncea* is a Rabi crop cultivated from October to December and harvested in April. The preference of soil for *B. Juncea* is sandy, loamy, or clay with a pH; of mildly acid, neutral, or mildly basic (Wechter, Farnham, Smith, & Keinath, 2007).

The growing availability of plants takes place in Africa, Asia, and northern America regions. The Brassica family plants are the second-largest oilseed crop worldwide (FAO, 2017). Brassica genus consists of thirty-seven species. Four are widely cultivated as oilseeds *(B. juncea, B.; Rapa, B. napus, and B. carinate)* (Raymer, 2002). *B. juncea* is derived from two diploid species *B. Rapa (*AA) and *B. nigra* (BB), with the allopolyploid chromosomal arrangement (AABB) (S. Chen et al., 2013). Cultivation of *B. juncea* began about 6000-7000 years ago in China and has thrived in India since 2300 BC (Prakash, 1980).

The average height of the plant is about 1-2 feet long with obviated, ovate, and petioled in shape leaves; meanwhile, its flowers have four sepals and four yellow petals with two longer and two shorter stamens. The overall life span of the plants has considered from 40-65 days. *B. Juncea* seeds are round with different shades of brown color (Wechter et al., 2007).

The significant life cycle stages have characterized brassica growth: a pre-emerging stage, seedling, rosette, budding, flowering, and ripening. The pre-emerging stage takes 4-10 days; in this stage, the plant is susceptible to soil-borne, biotic, and abiotic stress. Therefore, seed treatment is necessary at this stage. The young plant emerged from the soil with cotyledons and active

hypocotyl in the seedling stage. The third stage is the rosette stage, categorized by developed new leaves. Mustard plants that grow in spring remain in this stage for several weeks. While Mustard that is planted in winter also stays at this stage for several weeks. When the rosette stage ends, it has a maximum number of leaves area index. The fourth stage is the budding stage; in this stage, the upgrading temperature initiates bolting and budding in the plant. The plant's leaf area and 30-60% dry matter reach their maximum stage. The foliage accumulation is required to provide sugar during flowering and pod formation. The fifth stage is the flowering stage continues from 14-21 days after the plantations. It has been observed that 3-5 flowers open daily while 40-50% of flowers develop into proper pods. The sixth and last stage is the ripening stage, when petals of the last flowers fall from it on the main stem and pods fill to complete 35-45 days after the flower initiates. Pods contain seeds with 40% of moisture in them. At this stage, the crop has considered ripe and ready for swathing when pods turn their colors (Vélez, 2017)

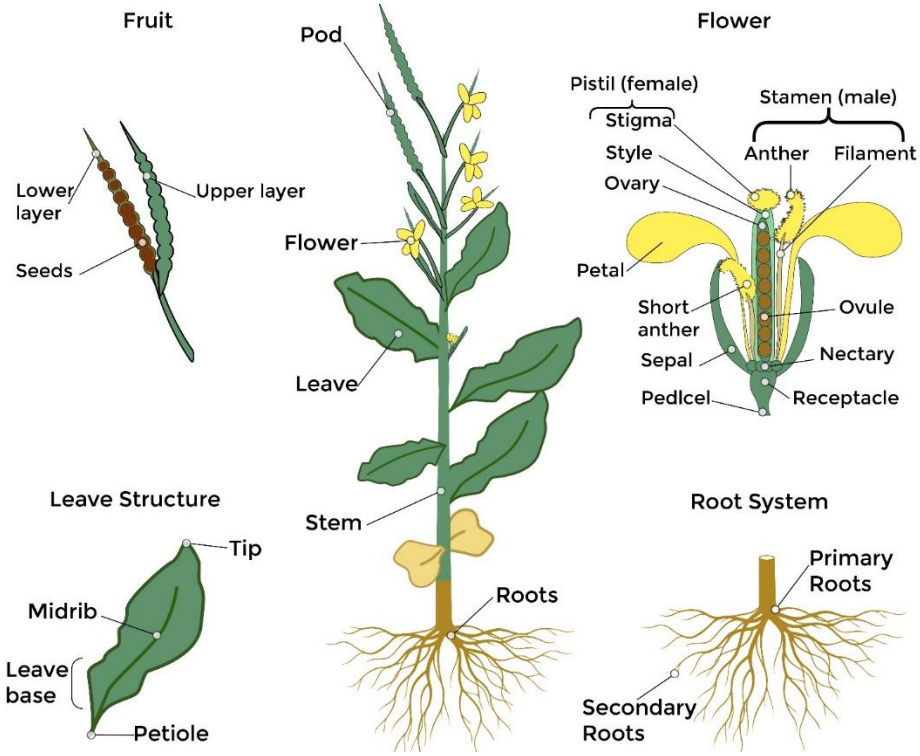

Figure 1 Mustard plant morphology explain different part of plants.

1.1. Taxonomy and Morphological description

1.1.1. Taxonomy

Indian mustard is one of the important oilseed self-pollinated crops. The word “Brassica juncea” has been driven from two Latin words, “Brassica,” which means cabbage, and “juncea,” which means Mustard. The Brassicaceae family has vast economic importance in different fields. It contains thirty-seven species with complex taxonomy. In 1999 Gomez-Campo presented a comprehensive classification of the *Brassica* family. In this classification, he divides the *Brassica* into sub-genera, i.e., *Brassicaria* and *Brassica*. Later, the same author subdivided the two genera into Brassica and *Guenthera* with subgenus *Brassicaria* due to distinct morphological characteristics. The sub-genera distribution is based on a stylar portion of the pistils without seed primordia and other specified traits, i.e., stem structure, leave the area, and leave shape. He further suggests that the species belong to subgenus *Brassicaria* under the generic denomination of *Guenthera Andr.* Later, molecular discoveries confirmed that *Guenthera* should be separated from the Brassica family. Whereas the problem considered was the classification into sections within Brassica itself. The Brassica genus includes six interrelated species considered of worldwide economic importance. In 1935 study concluded that the cytology of the genus established the relationship among the six genus genomes. B. Juncea has an allopolyploid chromosomal arrangement derived from two diploid species B. Rapa (AA) and B. nigra (BB) as (AABB 2n = 4 × = 36) (Gómez-Campo, 1999).

Table 1 Taxonomy of Brassica Juncea

KINGDOM	**PLANTAE – (VEGETAL)**
SUBKINGDOM	Viridiplantae – (green plants)
INFRAKINGDOM	Streptophyta– (land plants)
SUPERDIVISION	Embryophyta
DIVISION	Tracheophyta– (vascular plants, tracheophytes)
SUBDIVISION	Spermatophytina– (spermatophytes, seed plants)
CLASS	Magnoliopsida
SUPERORDER	Rosanne
ORDER	Brassicales
FAMILY	Brassicaceae– (mustards, crucifers)
GENUS	Brassica L.– (mustard)
SPECIES	Brassica juncea (L.) Czern. – (Chinese or Indian mustard)

1.1.2. Morphology

The Brassicaceae family consists of herbaceous plants based on annual, biennial, or perennial life cycles. The leaves of plants have alternate organizations with a basal rosette or terminal rosette. The arrangement of flowers is uniform with four saccate sepals and clawed petals, and the petals can be asymmetric with cross-like structures. Tetradynamous flowers have six stamens, four are longer and arranged in a cross-format, and two are shorter. The pistil consists of two fused carpels with a small style and a bilobate stigma. Under the developmental stage, the false septum known as replum converts the superior ovary with unilocular properties to bilocular.

The pollination process is expedited by entomogamy and nectar glands at the base of longer stamens stored on sepals. The fruit of the plant is present in a capsule named siliqua. The siliqua has two valves that are further modified as a part of carpels; the seeds are attached with dehisce

and replum from the bottom to upward. The siliqua part is sometimes separate and creates a loment between seeds. It expel the seed or evolved in a shot of samara (isatis) (Gómez-Campo, 1980).

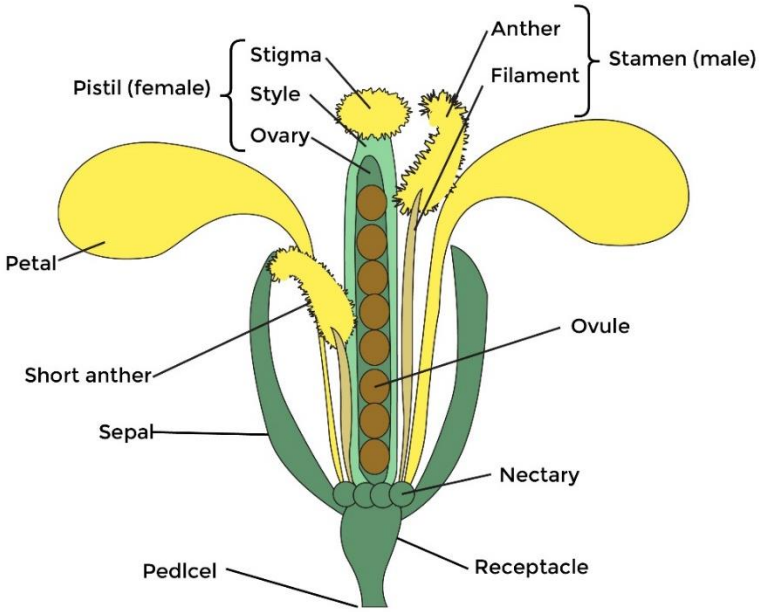

Figure 2 Mustard plant flower morphology show different interal part of flowers

1.2.Nutritional value of Brassica Juncea

The brassica family is known for oil production and contains high minerals and vitamins in its leaves and seeds. The leaves of the plants consume food, while the seed of the plants been used as edible oil for 1000 years. The leaves and seeds of the plants are the primary source of carbohydrates, proteins, dietary fibers, minerals (Na, Ca, Mg, Zn, Fe, P, and K), and vitamins (A, B1, B2, B3, B6, B9, C, E, and K). This plant has phytochemicals (Brassicasterol, sitosterol, campesterol), glycosides, and flavonoids in the form of phenolic compounds (Jahangir, Kim, Choi, & Verpoorte, 2009). Table 2 shows the different concentrations of minerals, vitamins, and other compounds present in B. juncea according to the report of the USA Agriculture Department 2019 (Agriculture, 2019).

The brassica family was enriched with vitamins and minerals than other vegetables and crops. In comparing vitamin contents with other vegetable crops, brassica shows a high vitamin C, vitamin B-6, vitamin A, vitamin K, β-carotene, lutein, and carotenoid concentration than other foods. *B. juncea* has a high level of folate (B-9), which prevent cancer, neural tube defects, and vascular diseases (Jahangir et al., 2009). Additionally, these plants have massive content of minerals in them. According to (Lucarini, Canali, Cappelloni, Di Lullo, & Lombardi-Boccia, 1999), calcium in brassica is higher than in other vegetables. It shows exceptional bioavailability since the plant has a low level of oxalic and phytic acid, which makes the brassica a reliable source of calcium. Meanwhile, a high level of potassium and calcium is an essential mineral that plays a vital role in different metabolic pathways (protein synthesis, phytochemical synthesis, carbohydrate metabolism, and flavonoid synthesis) (Cartea, Lema, Francisco, & Velasco, 2011).

1.2.1 Glucosinolates

Glucosinolates are the class of phytochemicals present in the Brassicaceae family. The breakdown of glucosinolates products contains prevention from cancer, cardio-protection activity, and anti-bacterial and anti-inflammatory properties (Smith, Lund, Clarke, Bennett, & Johnson, 2005; Traka & Mithen, 2009). These compounds are hydrolyzed by myrosinase (enzyme) into allyl-isothiocyanate during the oil formation process. The allyl-isothiocyanate combination develops a pungent taste in brassica vegetables as they are responsible for their flavors (Fenwick, Griffiths, & Heaney, 1983). Glucosinolates are the essential secondary metabolites in the Brassicaceae family. These molecules comprise β-thiol-glucoside N-hydroxy sulfate along with side chains of the β-D-glucopyranose moiety. These chemicals are further classified into three different classes as follows; aliphatic, indolic, and aromatic, according to amino acids and other precursors (Giamoustaris & Mithen, 1996)

Glucosinolate degradation was recognized long ago and benefits humans for nutrition and plant defense. Brassica foods, if considered functional, will provide an adequate number of bioactive components to improve health (Ciska, Martyniak-Przybyszewska, & Kozlowska, 2000).

1.2.2 Antioxidant properties

In addition, glucosinolate Brassica vegetables improve health associated with antioxidant properties known as “phenolic compounds.” Phenolic compounds refer to many mixtures widely dispersed in the plant kingdom. These compounds depict antioxidant activity by the inhibition mechanism of carcinogenic compounds via inducing the process of detoxifying Reactive oxygen species (ROS). The following characteristics classify phenolic compounds in B. Juncea:- the number of carbons, atoms arrangement, molecular weight, and aromatic and aliphatic properties (Jahangir et al., 2009; Kapusta-Duch, Kopec, Piatkowska, Borczak, & Leszczynska, 2012; Morales-López et al., 2017). The two major groups of phenolic compounds are flavonoids and

non-flavonoids. These molecules are found as a conjugate with an organic acid or sugar molecules. (Crozier, Clifford, & Ashihara, 2008)The antioxidant activity of phenolic compounds, specifical flavonoids that possess biological activities against tumors, is one of the crucial contests (Podsędek, 2007).

The oil of *B. Juncea* also contains a wide range of fatty acids, carbohydrates, proteins, and vitamins. Its seeds are the major source of oil. The average oil production is 35-40% per year with a varied 28-45% range. In the oil following major fatty acids compound are present; - linoleic acid 12-21%, oleic acid 8-33%, stearic acid 0.8-1.5%, palmitic acid 2-4%, arachidic acid 0.5-1.2%, nervonic acid 0.5-2%, lignoceric acid 0-1% and behenic acid 0-1%. The oil obtained from B. napus, and B. Rapa is like *B. juncea.* About 37-40% of the crude protein present in seed cakes produce through oil extraction. (Das, Bhattacherjee, & Ghosh, 2009; Service, 2001; Yokozawa, Kim, Cho, Choi, & Chung, 2002).

Table 2 Nutritional Value of Brassica Juncea

NUTRITIONAL VALUE	PER 100 GRAMS	UNIT	
WATER	91.8	g	
ENERGY	110 (26 kcal)	kJ	
CARBOHYDRATES	4.51	g	
SUGARS	1.41	g	
DIETARY FIBRE	2	g	
FAT	0.47	g	
PROTEIN	2.56	g	
TOTAL LIPID	0.47	g	
FIBER	2	g	
VITAMINS	Quantity		%DV
VITAMIN A EQUIV,	618	µg	77%
BETA-CAROTENE	7400	µg	69%
LUTEN ZEAXANTHIN/	10400	µg	
THIAMIN (BL)	0.041	mg	4%
RIBOFLAVIN (B2)	0.063	mg	5%
NIACIN (B3)	0.433	mg	3%
PANTOTHENIC ACID (35)	0.12	mg	2%
VITAMIN B6	0.098	mg	1%
FOLATE (B9)	9	mg	8%
VITAMIN C	25.3	mg	30%
VITAMIN E	1.78	mg	12%
VITAMIN K	592.7	mg	564%
MINERALS	Quantity		%DV
CALCIUM	118	mg	12%
IRON	0.87	mg	7%
MAGNESIUM	13	mg	4%
PHOSPHORUS	42	mg	6%
POTASSIUM	162	mg	3%
SODIUM	9	mg	1%
ZINC	0.22	mg	2%
COPPER	0.146	mg	0.5%

1.3.Economic importance

Around 28 million hectares of land are cultivated with *B. Juncea*, with an annual production of 58 Mt. It is considered the third-largest oil production plant after palm oil and soybeans (FAO, 2017). In 2021, Canada, the Europe Union, China, and India produced 19.49, 16.26, 14.06, and 8.5 Mt rapeseeds, respectively (Service, 2001). The leaves, roots, young flowers, and stems are used as vegetable food. In contrast, the seeds are used for edible oil in different regions of the world, specifically Pakistan, Bangladesh, China, India, France, Germany, Korea, Japan, and America. These plants are consumed in different culinary items due to their high nutritional value (Grubben & Denton, 2004).

Young plants have been eaten as raw salads or cooked vegetables, whereas older leaves can be eaten as boiled, canned, pickled, or salads. A different protein extract from its leaves is utilized for stuffing pies with banana pulp. Young flowers and stems are sweet and used to eat in raw or cooked form. Moreover, In Nepal and China, the thick stem of B. Juncea uses to make pickles. The seeds of plants convert to powdered form and are used to add food for spicy flavors produced by ally isothiocyanate.

Brassica family is not immensely popular in the therapeutic field; moreover, still used in different remedies against colds, arthritis, rheumatism, skeletal pains, etc. The mustard plant has antibiotic, anti-inflammation, diuretic, digestant, rubefacient, antioxidant and anodyne properties that benefit the medicinal field. Traditional restorative practices of Ayurveda in 3000 BC also explains various medicines and food preparation from the different part of this plant. While in the traditional medicine of China and India, mustard, its seeds, are used to cure exogenous and endogenous maladies. Recently, this plant has been utilized in modern medicine practices in Europe and North America. The healing attributes of *B. juncea* that link

with their various phytochemical presence lead to therapeutic fields (Malan, Walia, Saini, & Gupta, 2011).

A study conducted (Fadhil, Saleh, & Altamer, 2020) shows the conversion of mustard seed oil to biofuel with KOH- catalyzed transesterification through methanol as a co-solvent and methyl esters as a purifying agent. They perform the ASTM D6751 test confirming mustard oil conversion to biofuel.

1.4. Stress Inductions in *Brassica Juncea*

Plants are sessile and face several biotic and abiotic stresses in their life period. Biotic factors include attack and diseases imprinting from different living organisms, e.g. (animals, insects, bacteria, fungi, and viruses) which attacked them for their shelter and nutrition uptake. Meanwhile, abiotic factors include metal toxicity, temperature stress, and water shortage, negatively impacting growth, yield, and food quality (A. Sharma et al., 2020).

Eventually, plants encounter these factors in regular interaction. Therefore, they possess an internal defense mechanism; with these strategies, plants protect themselves from different stresses (Krasensky & Jonak, 2012). Several studies have identified that altered B. juncea growth mechanism includes heavy metal stress, drought stress, salinity stress, and temperature stress (Kapoor et al., 2019; Ram et al., 2016; Srivastava, Srivastava, Lokhande, D'Souza, & Suprasanna, 2015; Toosi, Bakar, & Azizi, 2014). The abiotic factors, specifically heavy metals, and stress integer, alter physiological, morpho-anatomical, and biochemical aspects of *B. Juncea.* To survive against abiotic factors, plants accumulate an antioxidant defense system (Jan et al., 2017).

The quantity of heavy metals in the soil is intensified over time, and it has become a significant issue for our ecosystem as these pollutants affect agronomic fields. Heavy metals (HMs) are a

substantial concern for plant scientists due to their effect on crops (Ashfaque, Inam, Iqbal, & Sahay, 2017). HMs accumulation in crops disturbs various physiological aspects of the plant body, food quality, the food chain, and living organisms that consume this food (Mudgal, Madaan, Mudgal, Singh, & Mishra, 2010). The major HMs that disrupt the plants are (Cadmium (Cd), Aluminum (Al), Mercury (Hg), Methylmercury (Methyl-As), Lead (Pb), Arsenic (As), and Nickel (Ni)) significantly proved their toxic effect in crops. HMs toxicity express toxicity on the genotypic, phenotypic, morphologic, and metabolomic levels. Our primary concern is cadmium metal, as its extensively present in our soil. Different studies reveal that Cd metals reduce growth (length of root or shoot, dry plant mass, photosynthesis, yield, and leaf area). The growth inhibition in *B. Juncea* plants was attributed to Cd invasion (Gill, Khan, Anjum, & Tuteja, 2011; Sarvajeet Singh Gill, Nafees A Khan, & Narendra Tuteja, 2011).

1.5. Cadmium Stress effect on *Brassica Juncea*

Cadmium (Cd) is a heavy metal (HMs) present in Cd^{+2} with 0.1-1.0 mg kg^{-1} in soil. The dispersion of Cd in soil persists for several decades (Mutlu, Lee, Park, Yu, & Lee, 2012). Cd is found in fruits and vegetative parts of plants if its concentration is high in the soil. Cd influences enzymatic activities and the nutritional quality of *B. Juncea* (Irfan, Ahmad, & Hayat, 2014). Some plant has special Cd-binding enzymes that contain protein bound with cadmium and play a vital role in growth stimulation and photosynthesis against the presence of Cd as tolerant. Such plants are also known as hyperaccumulators. The *B.Juncea* also has the properties of hyperaccumulation, and a study shows that B. Juncea stored up to 100 mg Cd kg-1 in dry biomass (McGrath & Zhao, 2003). It is also observed that in hyperaccumulator plants, the concentration of chlorophyll increases and decreases in typical plants (Zhou & Qiu, 2005).

1.5.1. Morphological Change

At the morphological level, many changes are observed under the cadmium stress in different plants. Cadmium toxicity decreases dry mass, root length, and yield quantity and enhances root diameter. Under Cd stress, the roots of *B. Juncea* become thicker and shorter with the inhibition of root elongation (Gratão, Polle, Lea, & Azevedo, 2005; Lux, Martinka, Vaculík, & White, 2011). Cadmium ions inhibit root elongation and depolymerize the cell cytoskeleton; it distorts chromosomes to repress mitotic activity in meristematic tissues (Seth, Misra, Chauhan, & Singh, 2008). Cd stress enhances parenchyma cells, and cortical tissue growth as a result of this root diameter also increases, leading the plant toward resistance against ionic solutes (Maksimović, Kastori, Krstić, & Luković, 2007). The roots absorption potentiality depends on root length under Cd stress; however, root length, surface area, and the number of tips decrease, and root diameter increases in B. Juncea under stress, reducing the capacity of root to acquire nutrients and water. Some other studies show that Cd also influences root system architectures in several species. Therefore, root morphological parameters have been used to assess Cd toxicity (Staňová et al., 2012; Wei, Li, Zhan, Wang, & Zhu, 2012).

The normal 0.05- 0.2 mg kg-1 Concentration of cadmium present in leaves while >5-10 mg kg-1 are toxic for plants. Studies show that young leaves have high Cd concentration than old leaves. Shoot and leaves show necrosis, chlorosis, desiccation and stunting under stress conditions (Pietrini et al., 2010). Some studies show that Cd also affects seed yield and germination when plants are exposed to stress. The seed imbibition and water repressing content also impact germination and growth under the presence of Cd (Alvarado & Bradford, 2005).

1.5.2. Physiological Change

The toxic cadmium also interacts with the minerals present in plants as nutrients, including iron (Fe), Potassium (K), Copper (Cu), Silicon (Si), magnesium (Mg) and Calcium (Ca) (Nedjimi & Daoud, 2009) and cations Zn+2, Fe+2, Mn+2, Mg+2 and Si+2. A study conducted by (Feng et al (2013) reported that adding calcium, magnesium, silicon, and phosphate fertilizer significantly reduces Cd toxicity in soil (Feng et al., 2013). The Cd in plants influences the nutrients transported in leaves and roots by inhibiting nutrition transport. This load other metals in the aerial part of plants with phytochelatin production (Sandalio, Dalurzo, Gomez, Romero-Puertas, & Del Rio, 2001)

Nelson, in 1986 stated in his study that Ca and Cd vie identical Calcium channels in plants. Cd pervades plasma membranes through Ca channels in guard cells and root cells (Perfus-Barbeoch, Leonhardt, Vavasseur, & Forestier, 2002; White, 2000). In guard cells, Cd interacts with Ca metabolites, resulting in a disturbance in the signaling pathway thus leading toward stomatal closure, decreased transpiration rate, inhibiting cell growth, and translocation (Perfus-Barbeoch et al., 2002; Sipos et al., 2013).

The accumulation of Nitrogen was severely affected in the presence of cadmium stress at a metabolic level. The presence of the Cd ion inhibits the uptake of nitrogen, which assimilates the enzyme activity and nitrate pathway (Chang et al., 2013; Sánchez-Pardo, Carpena, & Zornoza, 2013). Chang et al stated that different enzymes involved in ammonium absorption declines extensively in cadmium stress. it shows the negative influence of the downregulation of nitrogen in plants. Cd stress also influences sugar metabolic and Carbon metabolic pathways.

Cadmium in soil subdues water uptake by decreasing transpiration rate, stomatal conductance, and leaf water content. These circumstances damaged the physiological aspects of the cell by reducing

intracellular space, chloroplasts quantity, and enlargement of the cell wall. Cd stress also affects Cell membrane permeability, called the reduction of water content in cells (Fernández et al., 2013).

Under cadmium stress, photochemical efficiency, photosynthetic activity, and chlorophyll content are considered sensitive indicators toward heavy metals stress. Different studies show that Cd effectively inhibits photosynthesis in plants. Cd suppresses the bending state of a protein, thereby damaging the photosynthetic pathways and enzymes involved in it. Cd binds competitively at all the essential Ca-binding enzymatic sites of Photosystem-I (PS-I) and Photosystem-II (PS-II), more specifically water-splitting enzymes, and inhibits the production of hydrogen ion (H+), electron (e+) and oxygen (O2) that are necessary components of the photosynthesis process (F. Chen, Wang, Zhang, & Wu, 2008; Küpper, Lombi, Zhao, & McGrath, 2000)

1.5.3. Biochemical Change

Many researchers have found the Cd toxic effects on a plant biochemical level. The common aspect analyzed after Cd exposure is an upsurge of reactive oxygen species (ROS) in plant cells (Semane et al., 2007; S. S. Sharma & Dietz, 2009). The mutual observation of oxidative damage in plant cells is MDA content. The cell membrane lipids are considered the main indicator for the transmission of lipid peroxidation. The abolishment of the cell membrane and incanted free radicals occur through this process in plant tissues (Moller, Jensen, & Hansson, 2007; Shamsi, Wei, Zhang, Jilani, & Hassan, 2008). The Cd ions trigger oxidative stress mechanism in crops through the activation of oxidase enzyme, interruption of electron transport chain, and interaction with anti-oxidative defense mechanism (Schutzendubel & Polle, 2002).

The extensive accumulation of Cd triggers ROS progression that initiate membrane lipid peroxidation, inhabitation of the enzyme, structural changes in metabolites, disturbance in metabolic pathways. ROS also damage the structure of DNA and RNA resulting into cell death

(Gratão et al., 2005). Cd stress also causes inflection in sucrose and hexose (plant sugar) levels, disturbing cellular activities. A higher quantity of sugar in plants is associated with cell expansion and disruption (Ohto, Fischer, Goldberg, Nakamura, & Harada, 2005).

The plant has a cleanup system in them as a defense mechanism that leads to normal cellular function for the avoidance of ROS species damage. For a balance function, the activity of antioxidative enzymes and ROS production should work in an equilibrium mechanism to avoid damage. *B. Juncea* has developed a compound process for an enzymatic and non-enzymatic antioxidant system to reduce oxidative stress. The enzyme that works against ROS includes:- superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) (Apel & Hirt, 2004; Horváth, Pál, Szalai, Páldi, & Janda, 2007). The photosynthetic membrane functions are maintained under ROS stress with the non-enzymatic antioxidant. The enzymatic process activates the non-enzymatic antioxidant for ROS scavenging (Mittler, 2002).

1.6. 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 et al., 2016; Shahid, Dumat, Khalid, Niazi, & Antunes, 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 (S. S. Gill, N. A. Khan, & N. Tuteja, 2011; Ma, Cao, Tan, Si, & Wu, 2017).

It is hard to simultaneously understand any plant's phytoremediation potential and Cd tolerance. For this purpose, the brassica crop has been studied and used widely because of its high biomass production, short life span, and ability to store HMs. Moreover, Brassica crops also have a substantial economic importance in oil production. Thus, plants that grow for phytoremediation can't be used for food purposes (Cojocaru, Gusiatin, & Cretescu, 2016).

The cadmium accumulation occurs in a different part of Brassica species without showing huge impartation on its growth and development; thus, such plants are further used for biofuel after total gain (Romih, Grabner, Lakota, & Ribarič-Lasnik, 2012). Although Brassica 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 application in the soil has 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 (Sharmila, Kumari, Singh, Prasad, & Pardha-Saradhi, 2017; Zhichao Wu, Zhao, Sun, Tan, Tang, Nie, Qu, et al., 2015). However, inorganic treatments have some disadvantages for the soil and plant's growth; therefore, plant scientists prefer organic amendment (Marchand et al., 2016; Tang et al., 2016; Yao et al., 2017).

The uptake of Cd via different Brassica species varies from one another. Brassica species cultivators select Cd tolerant plants for phytoextraction. Some species have higher transcription

factors that enhance phytoextraction than others. It has been observed that black seeded *Brassica Rapa* has a high growth rate and biomass with the concern as a yellow seeded variety under 100 μM Cd stress. B. Juncea produces more biomass than other Brassica species (Xuan et al., 2015; Yu et al., 2014).

1.6.1. Mechanism of Phytoremediation

The Cadmium uptake and accumulation in different plants conquered 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) (Rizwan et al., 2016; Ru, Xing, & Su, 2006).

The uptake concentration of Cd in the plant's upper part depends upon the soil's pH concentration. In previous studies, it has been concluded that *B. juncea* uptake Cd three times more at 5.5 pH compared with the soil of 6.5 and 7.5, whereas the Nitrogen deficiency is higher in pH 5.5 than the comparisons of other (Zaurov, Perdomo, & Raskin, 1999). The Cd uptake also depends on Cd speciation in rhizospheric soil. Cd binds with carbonate produced by Cd accumulator Brassica in the rhizosphere was higher than non-accumulator Brassica. Accumulator species stored even insoluble Cd in them compared with non-accumulator species (Dechun, Jianping, Weiping, & Woonchung, 2009; Ru et al., 2006; Yang et al., 2016). 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 et al., 2015; Xia, Deng, Zhang, Liu, & Shi, 2016).

The Cd uptake from roots mainly translocate to shoots via xylem tissue by binding with organic acids, depending upon Brassica species (Zhichao Wu, Zhao, Sun, Tan, Tang, Nie, & Hu, 2015). The long-distance transport from roots to shoots and leaves held via phytochelatins (PCs) in *B. Juncea* under Cd stress, the PCs-Cd complex increase with the increasing Cd quantity. It has also been reported that Cd translocation in Brassica species is relatively easy to compare with other metals such as lead (Pb), Mercury (Hg) or zinc (Zn) (Angelova, Ivanova, Todorov, & Ivanov, 2008).

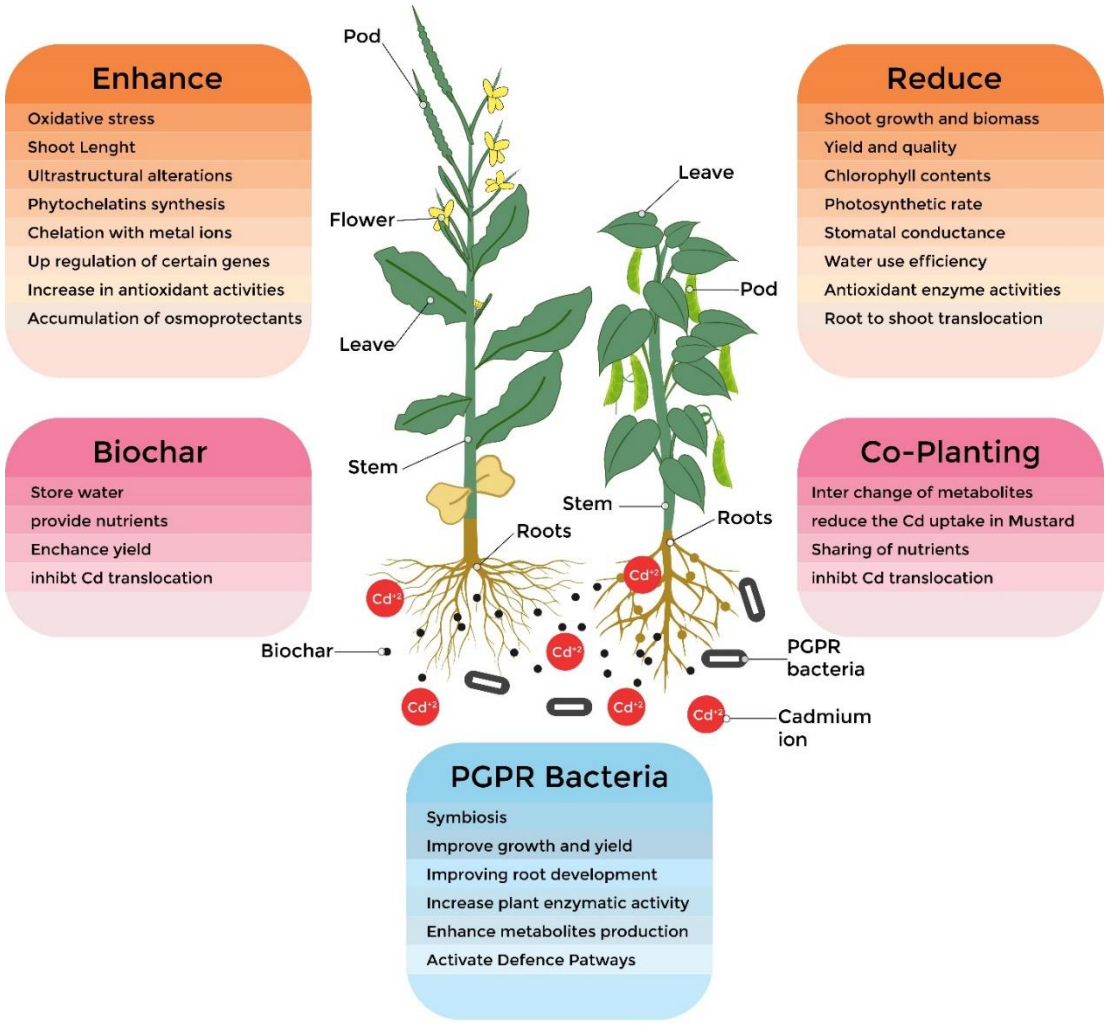

Figure 3 different effects of cadmium on plants and benefits impact of biochar,co-planting and PGPR.

Aim and Objective

The study aims to evaluate the effect of organic amendment and co-planting on the growth and development of *Brassica juncea* with the given objective.

- To Identify the morpho-physiological responses in brassica juncea against Cd stress
- To evaluate antioxidant activities in brassica juncea towards the response of Cd stress with bacterial strain, biochar, and legumes co-planting.

Chapter 2: Review of Literature

2.1.Persistence of Heavy Metal

(Pallavi Sharma & Dubey, 2006) review that the presence of toxic heavy metals gradually increases in soil over time. HMs in high concentrations show potential harmful effects on plant growth and metabolism. Different methods have been introduced for the accretion of these metals from the soil, but most of them are expensive, hazardous to the environment or slow. In the interim, chemical, physical, and biological procedures have been used for soil remediation. Chemical remediation includes different chemicals. Fortunately, a single chemical can't use against all HMs (Chaney & Oliver, 1996).

Additionally, chemical remediation is complicated and hazardous for plants and microbes that reside in the soil. Physical methods consume ample time and machines for this purpose as it has become economical for remediation. The scientist introduced a new method known as "bioremediation." Bioremediation restores contaminated sites and cleans the environment (A. J. Baker, McGrath, Reeves, & Smith, 2020).

(Viehweger, 2014) perform a study that the bioremediation rate is directly proportional to plant growth and the total amount of remediation correlates with plant biomass. *Brassica Juncea's* up-graded translocation of Cd from root to shoot significantly reduces metal presence in soil. When plants accumulate a high amount of Cd, their growth and metabolic process is induced negatively. Hyperaccumulation plants have some singular metabolites produced thru genes that have activated in the presence of Cd ions. These metabolites are known as Chelators. They contribute to metal detoxification on the cytosolic level.

2.2.Synthesis of Phyto-chelators

(A. Baker, McGrath, Sidoli, & Reeves, 1994) confirm that the HMs accumulation typically happened in shoot more than root. (Ucer, Uyanik, & Kutbay, 2013). Their study plant shows the capacity to accumulate 100 mg kg-1 of Cd. Whereas, (Thijs, Langill, & Vangronsveld, 2017) stated that the high accumulation capacity of plants is enhanced by specific proteins and metabolites that work as chelators, correspondingly with the analysis of the overexpression transport system. There are approx. 720 plant species registered globally as hyperaccumulator plants and accumulate different heavy metals at a time. The following study suggests seven plants for cadmium accumulation (Reeves et al., 2018). (Hörger, Fones, & Preston, 2013) stated that the accumulation of metal ions induces defence against pathogens and herbivores. The transformation of Cd from roots to shoots, fruits, seeds and leaves via the phloem transport channel (Turgeon & Wolf, 2009).

2.3. Phyto-chelators

Metal ions bound to ligands and proteins have a low molecular mass in plants, while a minute quantity of metal ions is present as free. Hyperaccumulator plants produce numerous metal-binding ligands, including thiols group compounds likewise: - nicotianamine (NA), glutathione, metalothioneins (MTs) and Phyto-chelators (PCs). A study by (Krämer, Cotter-Howells, Charnock, Baker, & Smith, 1996) shows that Histidine (His) is an amino acid that comprises hyperaccumulation by acting as nitrogen donor ligands in the roots. The central complex has been found with Cadmium (Cd), Nickel (Ni) and Zinc (Zn).

Another study concludes that (Stephan & Scholz, 1993) Nicotianamine (NA) is a metal chelator found in every plant. The enzyme NA synthase (NAS) controls the trimerization of S-adenosylmethionine (SAM) (NAS). Nicotianamine is involved in micronutrient transportation in plants used in iron metabolism. Other reports show that NA bind with Cu and Cd in different

plants. The enhancement of Cd accumulation depends upon the NAS expression in the plant. Cadmium stress causes a reduction in plant growth, photosynthesis, food, oil quality, morphology, and physiology.

The other most crucial metal detoxifying ligands are metallothionins (MTs), present in all plants and animals. Their principal function is to maintain the homeostasis of metal ions in hyperaccumulator and non-hyperaccumulator plants under normal physiological conditions. MTs family has three most important types that express under the presence of different HMs (MT-I, MT-II, MT-III). MT-III has been observed as an activator under the presence of Cd ion stress (Jack et al., 2007)

2.4. Reactive Oxygen Species and metal Detoxification

In several studies, glutathione (GSH) is vital for maintaining cellular ROS homeostasis and has been implicated in plant metal detoxification. Previous studies show that thiol ligands play a minor role in hyperaccumulation. (Freeman & Salt, 2007) His studies showed increased activity of the assimilatory sulfur pathway, mitochondrial serine acetyltransferase (SATm), and increased excessive production of GSH. TgSATm-expressing Arabidopsis also showed it improved metal resistance (van de Mortel et al., 2008). They found that Cd exposure increased sulfate synthesis and GSH metabolism in T. caerulescens (also known as N. caerulescens) and increased foliar and root GSH production in metal hyperaccumulators.

2.5. Assistances of biochar

In soil, biochar provides nutrients (carbon, nitrogen, calcium, and phosphorus). It also helps to store water due to its porous structure that keeps the plant hydrated for longer-term than expected. There are diverse types of biochar on physicochemical properties (pore structure, surface area, phosphorus quantity, and functional groups). Biochar production at a high pyrolysis temperature

requires a large surface area, huge porosity, normal pH, and low cation exchange capacity (CEC) compared with the biochar produced at carbonization degree. Biochar properties are also related to certain variables, including lignin, cellulose, and moisture (Trampczynska, Küpper, Meyer-Klaucke, Schmidt, & Clemens, 2010). Biochar is produced under the 350C- 650 C derived from different plants such as palm kernel shells, corn cobs, cocoa pod husks, rice husks and wheat husks used as nutrients booster for new plants.

The cadmium effect on plants and humans cannot be neglected to protect public health from contaminated food with Cd; different biological amendments are required. Food and Agriculture Organization and World Health Organization (FAO, 2017). Studies show that the new vegetable maximum limit of Cd permissible is 0.05-0.2 mg kg 1.z Previous studies confirm that acidic soil or pH from 3-5.5 overrides Cd in accumulation (Huang et al., 2017). Limiting acidic soil will reduce the sorption of Cd in plants (Zhipeng Wu et al., 2014; Zhichao Wu et al., 2016). Biochar improves soil qualities and limits acidic soil properties. It has also been shown that biochar lowers the mobility of toxic heavy metals in soil due to the activation of its functional groups, includes (carboxylic acid (–COOH), –C=O and inorganic ionic PO_4^-).

Until now, no research has been reported that explains the impacts of different biochar characteristics on Cd and Pb phytoavailability in toxic metal polluted soil types and their effect on metal uptake by vegetable crops. As a result, this study aimed to see how biochars made from three different feedstocks affected soil phytoavailable Cd concentrations and plant uptake in three different soils. We anticipated that biochar feedstocks might vary the physiochemical characteristics of biochar, resulting in various modes of action in both acidic and alkaline soils. Biochar might change soil pH, affecting soil Cd and Pb phytomobility and plant absorption (Houssou et al., 2022).

2.6. Assistance of PGPR

Whereas soil microbes are also negatively affected under Cd stress, many microbes develop resistance against Cd-stress in rhizosphere soil. Different Cd-resistant microbes have shown the potential to enhance Cd uptake in plants. There is another possibility that Cd-resistant microbes either improve or reduce Cd uptake in plants. Different organic acid has been reported in various plants that help in mineral solubilization includes: - (indole-3-acetic acid (IAA) and 1-aminocyclopropane-1-carboxylate (ACC). These organic acids help to uptake Cd in the upper part of the plant under the presence of microbes (Z. Deng & Cao, 2017; Zhipeng, Weidong, Shenglu, & Shaohua, 2016).

Microbes mainly reduce the soil pH and help slow the Cd uptake (Jing et al., 2014). Microbes also enhanced Cd tolerance in Brassica species by reducing ROS production in plants with mutual assistance (Panwar et al., 2011). Plant growth-promoting rhizobacteria (PGPR) are microbes frequently used in heavy metal cleanup (Pramanik, Mitra, Sarkar, & Maiti, 2018). Plants have a symbiotic connection with PGPRs, which boost plant growth and competitiveness in space and nutrients gain, enhancing plant resistance to external challenges. These microorganisms get their sustenance from root exudates, providing many benefits to the plants, including synthesizing growth hormones (Miransari & Smith, 2014).

2.7. Characteristic of Brassica as hyperaccumulator

Brassicales have been studied extensively in phytoremediation because they can efficiently transport heavy metals from roots to shoots and survive elevated soil metal content. They also have a fast-growing habit and produce a lot of biomass (Marchiol, Assolari, Sacco, & Zerbi, 2004). Empirical research suggests that several Brassicas have an increased amount of heavy metal accumulation, indicating that they have a high tolerance level to heavy metal stress. The only

concern associated with using brassica vegetables for phytoremediation is that brassica oil may be contaminated due to elevated levels of metal collected in the seed, resulting in the food chain and environmental toxicity (Park, Kim, & Kim, 2012).

However, experimental research show evidence (Park et al., 2012) that heavy metals are not absorbed into the oil during the extraction process; hence there is no risk of contamination in the food chain. Furthermore, there is a study on using PGPRs to improve heavy metal uptake by accumulator plants. This study aimed to see how Cd accumulated in B. oleraceae under controlled conditions. Furthermore, the plants were infected with PGPR strains and grown at different doses of Cd to see how microbial strains affected heavy metal uptake by plants. Experimental plants can survive Cd toxicity in a controlled environment (Asad, Rehman, Ahmad, & Umer, 2018).

2.8. Assistance through Intercropping

Intercropping or co-planting is an ancient agricultural strategy involving two or more crop species growing together and co-existing for a specific period (Brooker et al. 2015). Co-planting was coined to describe how plants improve each other phytoremediation potential via utilizing nutrients, water, soil space, and lights together (Wu et al. 2007; Sun et al. 2018; Zeng, Guo, Xiao, Peng, Feng, et al. 2019). Previous studies have primarily focused on the coplanting patterns of crops and hyperaccumulators in the soil to restrain the levels of heavy metal contamination, reducing heavy metal accumulation in crops. These studies conduct by (L. Deng et al., 2016) on Sedum plumbizincicola and maize (Zea mays), Thlaspi caerulescens and Ryegrass (Jiang et al., 2010), and Solanum nigrum and "welsh" onion (Allium cepa) (S. Wang, Wei, Ji, & Bai, 2015) show some benefits toward the environment aginst heavy metals.

The research also found that the hyperaccumulator plants diminish the HMs accumulation with co-planting, which aided in safe agricultural production and the remediation of contaminated soil in these experiments.

Pteris vittata co-planted with the metal (loid)-tolerant species Morus alba and Broussonetia papyrifera (Zeng et al., 2019) can optimize planting structure, remediate contaminated soil at various depths, and improve phytoremediation efficiency. The significant effects of co-planting are also proposed to be facilitation and competition, which are always present simultaneously (Kutrowska et al., 2017). The kind of metal, its quantity and the interaction with plant species among co-existing plants all influence plant growth and metal accumulation in diverse co-planting patterns (Ling, Shen, Gao, Gu, & Yang, 2007).

In comparison to shrubs, arbour trees provide more ecological and economic benefits. There is currently limited information on the phytoremediation of co-planting in arbour trees, its hyperaccumulators properties towards metal-polluted soil. Solanum nigrum, a perennial weed that can survive high levels of Cu, Pb, Ni, and Zn, is classified as the Cd-hyperaccumulator (Rizwan et al., 2016). Tall and straight arbor trees with magnificent crown shape and leaf color in autumn, Quercus nuttallii and Quercus pagoda have been widely imported and used in greening in China's subtropical zones in recent decades and are also highly resistant to heavy metals-induced abiotic stressors (Suresh Kumar, Dahms, Won, Lee, & Shin, 2015). The phytoremediation capacity of heavy metals utilizing Q. nuttallii and Q. pagoda co-planting with Q. nuttallii and Q. pagoda has yet to be determined.

Chapter 3: Material and methods

3.1 Wet lab analysis

3.1.1. Seed and soil collection

Fresh seeds of *B. Juncea* were collected for experimental purposes from the oil and seed research department at the National Agriculture Research Center (NARC) Islamabad, Pakistan. The sandy, loamy soil was also collected from NARC peanut fields for plant growth.

3.1.2. Seed germination

Seeds' surfaces were sterilized with 70% ethanol for 1 min, followed by washing through distilled water. Seeds were then spread on filter paper inside the safety cabinet to evaporate the maximum amount of ethanol. After dried from ethanol, the seeds were aligned on UV-sterilized germination paper in a germination box and wrapped with aluminum foil, so the interaction with light was limited and shifted to a dark place at 25℃-28℃ for 48 hours to break seed dormancy after three days *B. Juncea* seeds appropriately germinated and were ready for soil transformation. Seeds with equal germination rates were transferred in pots.

3.1.3. Soil analysis

Soil analysis was conducted at National Agriculture Research Center (NARC) Islamabad. The texture of the soil is sandy loam with 7.78 pH, containing a concentration of organic matter of 0.49%. The saturation percentage (SP) is 32, respectably (Estefan, 2013)

3.1.4. Soil preparation

Eight different treatment was prepared in the soil using for plant growth. The first treatment was taken as a control group as it does not contain cadmium stress. At the same time, the other seven

treatments have cadmium stress with different organic variables, including bacterial strain, also known as plant growth-promoting rhizobacteria *(Rhizobium legumin Sarum,)* Biochar *(Wheat Husk)* and co-planting *(Vigna radiata)* illustrated in the given table 3. Variables provide as follows concentrations: - biochar as 1.2%, rhizosphere as 0.1%, along with one legume plant *(Vigna radiata)* as a co-plant variable. There were two batches. The first batch contained seven replicates, and the second had ten replicates with soil per pot of 1kg. Ten millimole *Cadmium chloride* ($CdCl_2$) solution was prepared, and 10ml solutions per pot were added to the required treatments. After adding cadmium stress solution, the soil and solution are mixed by providing water daily for ten days at 25℃. Seedlings were transferred in the soil for ten days with growth conditions kept as 25℃-28℃ with light.

Table 3 Treatments Table

CODES	**TREATMENT**
T1	Sole Brassica+ no cadmium
T2	Sole Brassica + cadmium
T3	Brassica+ cadmium + Rhizosphere Bacteria
T4	Brassica+ cadmium + biochar
T5	Brassica + cadmium + biochar + Rhizosphere Bacteria
T6	Cadmium + co-planted with mung bean
T7	Brassica+ cadmium+ Rhizosphere Bacteria+ co-planted
T8	Brassica+ cadmium + Rhizosphere Bacteria + Biochar+ co-planted

3.1.5. Water holding capacity

For the water holding capacity of the soil, we take six pots containing 1kg of soil, three with simple soil (control group), and 3 with a mixture of biochar and bacterial strain (experimental group). There are several holes at the bottom of each pot. The tissue paper was put inside them. The three control pots were placed in one water tub, and the three experimental pots were placed in the other water tub. Water was added to all six pots till it is started dropping from the bottom. After 4 hours, all pots are weight again with electrical balance measured as W_1. Soil was dried for 2 hours at 100 C in an oven, and calculated its weight measured as (W_a). The pot was air-dried at room temperature, and its weight was calculated as (W_b). For the W_2 value, W_2=, W_a + W_b, and water holding capacity is measured by a formula (D. Y. Wang, Yan, Song, & Wang, 2014).

$$100\%\, WHC = \frac{W_1 + W_2}{W_2} * 100$$

Table 4 Water Holding Capacity

		W_1	**W_A**	**W_B**	**W_2= W_A+ W_B**	**WHC= {(W_1- W_2)/ W_2} *100**	**100% WHC IN 1000KG SOIL**	**70% OF WHC**	**AVG WATER CONTENT**
CONTROL GROUP	R1	1250	1000	45g	1045	19.61	250ml	171ml	210.5
	R2	1261	1007	47g	1054	19.63	250ml	173ml	211.5
	R3	1252	1002	47g	1049	19.35	250ml	170ml	210
EXPERIMENT GROUP	R1	1250	960	47g	1007	24.13	250ml	183ml	216.5
	R2	1254	983	46g	1029	21.86	250ml	188ml	219
	R3	1250	990	48g	1038	20.42	250ml	186ml	218

3.1.6. Morphological analysis

The number of leaves was counted on days 30th and 65th after sowing. Shoot height and weight were measured with a measuring scale on the 30th from rhizome boxes and the 65th day from pots. The total root length and weight were measured on the 30th day from rhizome boxes and the 65th day from pots (harvesting day). The number of pods was counted on the 65th day. The fresh root and shoot were washed with deionized water, calculated fresh weight, then air-dried for moisture evaporation at 70℃ for 2 hours in an air-dried machine and collected dry weight of samples (Aina, Amoo, Mugivhisa, & Olowoyo, 2019)

3.1.6.1. Scanning Election Microscopy

Scanning electron microscopy was performed for dry leaf samples under the TESCAN MIRA – SEM microscope.

3.1.6.2. Leave Area

The leaf area was measured with the software Image J (Rasband, 2011). The leaves were drawn on graph sheets, scanned with a scanner, and identified different parameters through software. Includes leave density, area, mean, and median.

3.1.7. Physiological analysis

3.1.7.1. Chlorophyll Pigment

The Chlorophyll content was measured by the following method (Arnon, 1949). Take a 0.5g fresh plant sample and homogenize it with 2ml of 80% acetone. Centrifuge it at 13000 rpm for 20mins at 4 degrees ℃. Collect the supernatant of plant extract for chlorophyll analysis. The absorbance of the supernatant was collected at 645 and 663 nm for chlorophyll a and chlorophyll b, respectively.

$$Total\ Chlorophyll = (Absorbance_{645} \times 20.2) + (Absorbance_{663} \times 8.3) \times ({}^{V}/_{1000} \times W)$$

$$Chlorophyll\ A\ content = \{Absorbance_{663} \times 0.058\} - \{Absorbance_{645} \times 0.032\}$$

$$Chlorophyll\ B\ content = \{Absorbance_{645} \times 0.096\} - \{Absorbance_{663} \times 0.01872\}$$

3.1.8. Biochemical assay

3.1.8.1. Superoxide dismutase (SOD)

The Antioxidant assay was measured following the method (Kono, 1978). Take a 0.5g fresh plant sample and homogenize it using 3ml of phosphate-buffered saline (PBS). Transfer it to a 10ml tube and further add 5ml PBS buffer. Centrifuge it at 13000 rpm for 20mins at 4 degrees ℃. Collect the supernatant of plant extract in another tube and store it for 24 hours at 4℃ for SOD analysis. Then take 25µl enzyme extract and add 1ml PBS buffer, 33µl EDTA, and 66µl (methionine, NBT, and Riboflavin). Take the absorbance value of the supernatant at 560 nm.

Ae = OD value on the spectrophotometer

Ack = OD value for the control tube under light conditions (at 4000 lux for 20 minutes)

V= Total volume of the buffer solution used to extract the enzyme

W= Fresh weight of the sample

Vt = Amount of enzyme extract used in reaction solution to test SOD

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

3.1.8.2. Peroxidases (POD)

The Antioxidant assay for peroxidase (POD) was measured by following the method given by (Bergmeyer, 1974). Take a 0.5g fresh plant sample and homogenize it using 3ml of phosphate-buffered saline (PBS). Transfer it to a 10ml tube and further add 5ml PBS buffer. Centrifuge it at 13000 rpm for 20mins at 4 degrees ℃. Collect the supernatant of plant extract in another tube and store it for 24 hours at 4℃ for POD analysis. Then take 100µl enzyme extract and add 2.7ml PBS buffer, 100µl, Guaicol, and 100µl 30% hydrogen peroxides (H_2O_2). Take the absorbance value of the supernatant at 270 nm

W means Fresh weight of the sample

V means Total volume of the buffer solution used to extract the enzyme, a means

The amount of enzyme extract used in the reaction solution to test E means constant activity, i-e.,26.6mM/cm270 nm. Whereas the E value is 26.6 mM/cm

$$POD\ activity = \frac{O.D_{value} \times V/_{vt}}{E \times W}$$

3.1.8.3. Catalase (CAT)

The Antioxidant assay for Catalase (CAT) was measured by the following method (Aebi, 1984). Take a 0.5g fresh plant sample and homogenize it using 3ml of phosphate-buffered saline (PBS). Transfer it to a 10ml tube and further add 5ml PBS buffer. Centrifuge it at 13000 rpm for 20mins at 4 degrees ℃. Collect the supernatant of plant extract in another tube and store it for 24 hours at 4℃ for CAT analysis. Then take 100µl enzyme extract to add 2.8ml PBS buffer and 100µl 30% hydrogen peroxides (H2O2). Take the absorbance value of the supernatant at 240 nm. Whereas the E value is 39.4 mM/cm

A means Activity of OD value

W means Fresh weight of the sample

V means Total volume of the buffer solution used to extract the enzyme a means Amount of enzyme extract used in reaction solution to test E means activity constant i-e.,39.4mM/cm

$$CAT\ activity = \frac{O.D_{value} \times V/_{vt}}{E \times W}$$

3.1.8.4. Ascorbate peroxidase (APX)

Ascorbate peroxidase (APX) was measured by following the method (Habib, Chaudhary, & Zia, 2014). Take a 0.5g fresh plant sample and homogenize it using 3ml of phosphate-buffered saline (PBS). Transfer it to a 10ml tube and further add 5ml PBS buffer. Centrifuge it at 13000 rpm for 20mins at 4 degrees ℃. Collect the supernatant of plant extract in another tube and store it for 24 hours at 4℃ for POD analysis. Then take 100µl enzyme extract and add 2.7ml PBS buffer, 100µl 30% hydrogen peroxides (H2O2), and 100µl of Ascorbic acid (ASA). Take the absorbance value of the supernatant at 290 nm. Whereas the E value is 2.8 mM/cm

Activity A means OD value

W means Fresh weight of the sample

V means Total volume of the buffer solution used to extract the enzyme, a means

The amount of enzyme extract used in the reaction solution to test E means constant activity, i-e.,2.8mM/cm

$$APX\ activity = \frac{O.D_{value} \times V/_{vt}}{E \times W}$$

3.1.8.5. Melo-dialdehyde Content (MDA)

The Melo-dialdehyde content (MDA) was measured by the following method (T. Chen & Zhang, 2016). Take a 0.5g fresh plant sample and homogenize it using 3ml of phosphate-buffered saline (PBS). Transfer it to a 10ml tube and further add 5ml PBS buffer. Centrifuge it at 13000 rpm for 20mins at 4 degrees ℃. Collect the supernatant of plant extract in another tube and store it for 24 hours at 4℃ for MOA analysis. Add one hundred µl crude protein/enzyme extract from each sample into 1 ml 0.25% TBA solution in a 1.5 ml centrifuge tube. A total of 1 ml 0.25% TBA solution with one hundred µl 100 mM PBS (pH7.8) serves as a reference. Boil the reaction mixture in a boiling water bath for 15 min. The reaction mixture turns red after boiling. Cool down reaction mixture on ice for 5 min. Pipette 200 µl of the reaction mixture and measure the absorbance at 532 nm and 600 nm—the Value of E as 532 (mM-1 cm-1).

A532: the absorbance at 532 nm

A600: the absorbance at 600 nm

Vr: the volume of the reaction mixture

V: total volume of crude enzyme solution

Vt: volume of crude enzyme used in the testing tube

Cp: crude protein concentration (mg/ml)

E: the extinction coefficient of MDA-TBA at 532 (mM-1 cm-1)

$$MOA\ Activity = (A_{532} - A_{600}) \times V_r \times \frac{\frac{V}{V_t}}{E} \times 1000/C_p$$

Chapter 4: Result

4.1 Length

4.1.1 Root length

The root length of the mustard plant has measured with measuring scare on 30th and 65th day for each treatment (T1 =BJ, T2 = BJ+ Cd, T3 = BJ +Cd+ PGPR, T4 = BJ+ Cd+ Biochar, T5 = BJ +Cd+ PGPR+ Biochar, T6 = BJ+ Cd+ Co-plant, T7 = BJ+ Cd+ PGPR+ Co-plant, T8 = BJ+ Cd + PGPR+ Biochar+ Co-plant). Their mean values, standard deviation (SD), and p-value have been calculated with R studio.

The results show a massive reduction in treatment two concerning other treatments. It concludes that the presence of Cd reduces the root length compared with other treatments having different combinations of biochar, PGPR and co-planting. Root length results in *figure 4* show the variation among 30^{th} and 65^{th} days treatments. It shows that on the 30th-day timeframe, the root length was less affected than 65 days. The mustard plants show a massive reduction in root length on 65 days in Cd soil.

T1, T5, T7, and T8 show non-significance results with each other in *figure 4*, However T3, T6 are non-significance with each other but show significance results towards the treatments having a lettering represent “AB”. The lowest root length on the timeframe of 65-days has noticeable in T2 and T4. Moreover, on 30^{th} day timeframe T1, T5, T6, T7 and T8 show non-significant results towards each other but show meaningful results with T2 and T4. This confirm that the presence of different biological mixtures helps to induce resistive parameters against cadmium stress. However, T4 (contain biochar only) did not show as much significance results as provide in combination form.

4.1.2 Shoot Length

The shoot length of the mustard plant has measured with measuring scare on 30th and 65th day for each treatment (T1 =BJ, T2 = BJ+ Cd, T3 = BJ +Cd+ PGPR, T4 = BJ+ Cd+ Biochar, T5 = BJ +Cd+ PGPR+ Biochar, T6 = BJ+ Cd+ Co-plant, T7 = BJ+ Cd+ PGPR+ Co-plant, T8 = BJ+ Cd + PGPR+ Biochar+ Co-plant). Their mean values, standard deviation (SD), and p-value have been calculated with R studio.

Shoot length results explain in *figure 5* shows the variation among 30^{th} and 65^{th} days treatments. It shows that on the 30th-day timeframe, the shoot length has reduced up to 50% on T2 and T4 for T1, while other treatments that include the compositions of Biochar, co-planting, and PGPR show non-significant results with T1. 65^{th}-day timeframe, a 61% reduction was noticed in T2; however, a 30% reduction was noticed in T4 on shoot length compared to T1. On the other hand, T5, T6, T7, and T8 show non-significance results compare with T1 on 65 days. It illustrates that plants on 30 days were less affected compared to 65 days. The mustard plants show a considerable reduction in root length on 65 days in Cd soil.

Shoot length have highest mean value in T1 and lowest mean value in T2. There is highly significant difference between the T1 and T2 however, T5, T6, T7 and T8 show no significant with T1 under 65^{th} days period. On 30^{th} day timeframe T2, T3, T4 show significant result toward T1, whereas T8 show equivalent mean values as T1. This confirm T8 combination show growth enhancement in Brassica juncea.

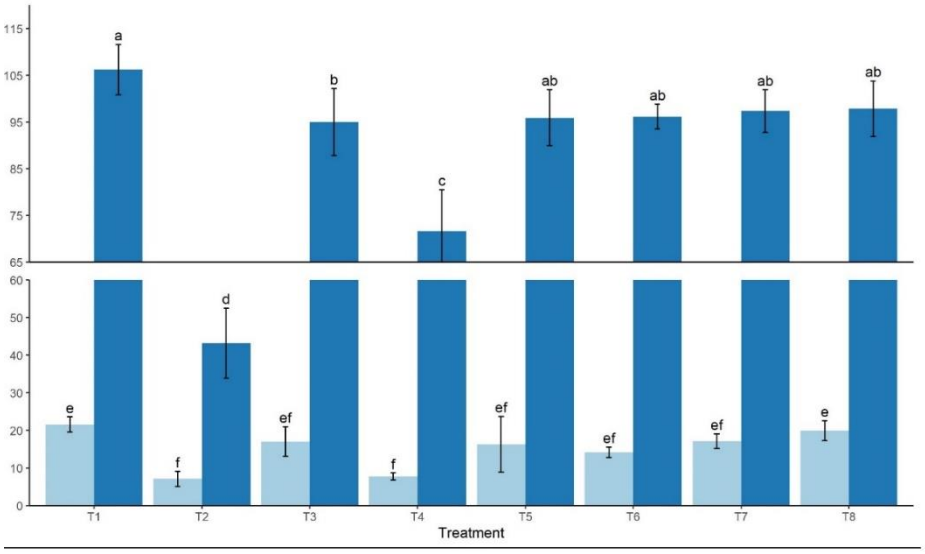

Figure 5 Shoot length of the plant determine the results shows only T2 and T4 decline in shoot growth compare with other treatments. Data shown as interaction of different combination mixture as treatments applied to plants. Significance inferred with Two-way ANOVA under the Tukey's HSD post-hoc test for normalizing the data distribution (Honest Significant Detection $p < 0.001$).

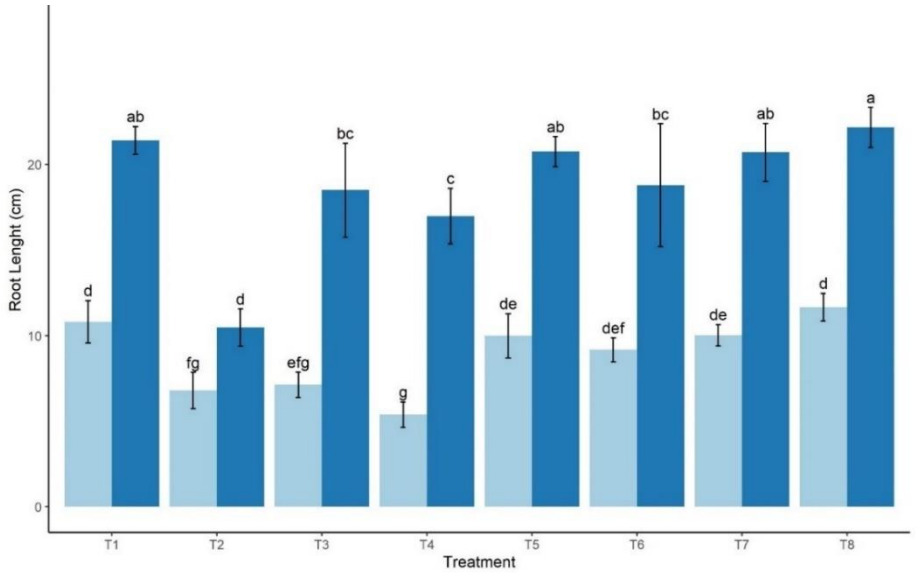

Figure 4 show the root length of mustard plant under cadmium stress. Root length results show the presence of Cd effect root length on T2 compare with other treatments. Data is shown as interaction of different combination mixture as treatments applied to plants. Significance was inferred with Two-way ANOVA under the Tukey's HSD post-hoc test for normalizing the data distribution (Honest Significant Detection $p < 0.001$).

4.1.3 Total Length

The Total length of the mustard plant has measured with measuring scale on 30th and 65th day for each treatment (T1 =BJ, T2 = BJ+ Cd, T3 = BJ +Cd+ PGPR, T4 = BJ+ Cd+ Biochar, T5 = BJ +Cd+ PGPR+ Biochar, T6 = BJ+ Cd+ Co-plant, T7 = BJ+ Cd+ PGPR+ Co-plant, T8 = BJ+ Cd + PGPR+ Biochar+ Co-plant). Their mean values, standard deviation (SD), and p-value have calculated with R studio.

The results show on a *figure 6* massive reduction in T2 and T4 for other treatments. It concludes that the presence of Cd negatively impacts the plant length compared with other treatments having different combinations of biochar, PGPR and co-planting.

The total length of plant results also shows variations among 30^{th} and 65^{th} days treatments. It shows that the 30th-day timeframe, length has reduced up to 40% on T2 and T4 with the comparison T1, while other treatments that include the compositions of Biochar, co-planting and PGPR show non-significant results with T1. However, in the 65th-day timeframe, a 61% reduction has been noticed in T2 and a 30% reduction in T4 compared to T1. On the other hand, T5, T7, and T8 non-significance results compare with T1 on 65 days, showing meaningful results with on T3 and T6.

In total length of plant its shows highest significance values have been noticed in T2 and T4 at 65^{th} day timeframe. Although, significant differences only observed T2 and T4 under 30-day period. Through these results it can be concluded that these biological mixture compositions help to boost the plant growth parameters specifically plant height.

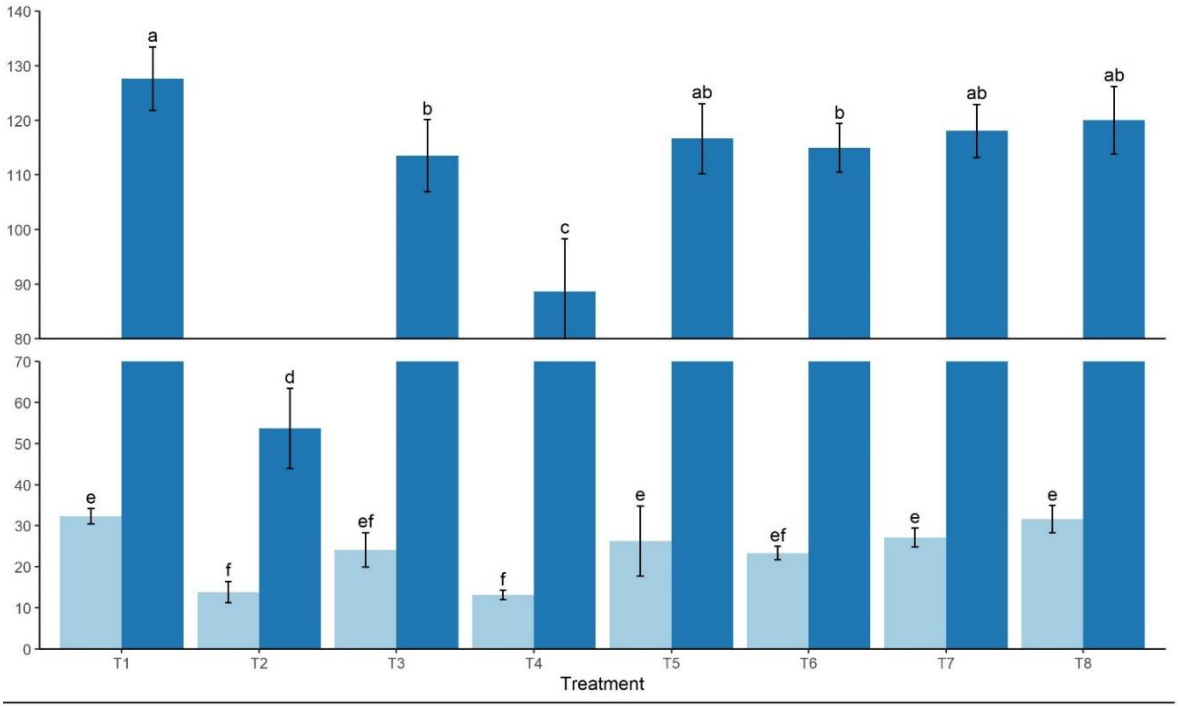

Figure 6 The total length of the plant includes shoot and root length it demonstrates the total length of the plant. It shows that the 30th-day timeframe size has reduced up to 40% on T2 and T4 with the comparison T1, while other treatments that include the compositions of Biochar, co-planting and PGPRs show growth results with T1. However, in the 65th-day timeframe, a 61% reduction has been noticed in T2 and a 30% reduction in T4 compared to T1. On the other hand, T5, T7, and T8 non-significance results compare with T1 on 65 days, showing meaningful results with on T3 and T6. Data is shown as interaction of different combination mixture as treatments applied to plants. Significance was inferred with Two-way ANOVA under the Tukey's HSD post-hoc test for normalizing the data distribution (Honest Significant Detection $p < 0.001$).

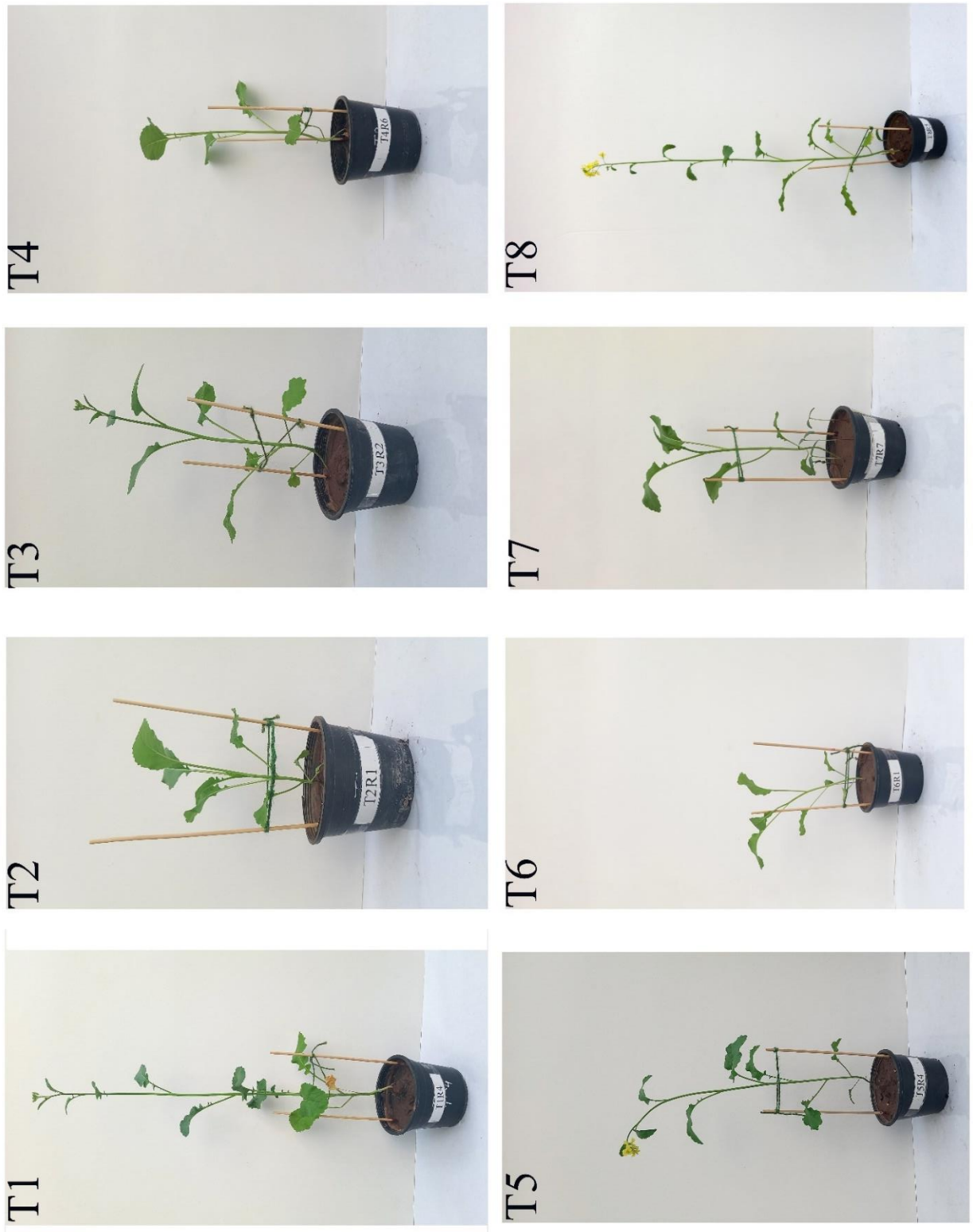

Figure 7 Mustard plant picture has been captured on 45-day period. It shows plant height, growth, and leaves.

4.2 Weight

4.2.1 Fresh and dry root weight

The results in *figure 8* show the root fresh weight a huge reduction has been noticed under cadmium stress in T2, T3, and T5 with the comparison to T1 in 65 days however, other treatments show non-significant results with toward T1. Via comparison with T1 the percentage of reduction rate of dry weight indicates that T2, T3, T4, T5, T6, T7, and T8 reduce to 68%, 63%, 45%, 66%, 42%, 27.2%, and 22.7%, respectively on 65^{th} day period. On the other hand, the result of the 30-day timeframe shows fresh root weight reduction as follows to T1, as T2, T3, T4, T5, T6, T7, and T8 reduced at 66.6%, 33.3%, 37.5%, 41.1%,37.5%,33.3%, and 16.6% respectably.

The results in *figure 9* show the dry root biomass the reduction of biomass has also observed, via comparison with T1, it also indicates that T2, T3, T4, T5, T6, T7, and T8 reduce to 68%, 63%, 45%, 66%, 42%, 27.2%, and 22.7%, respectively under 65^{th} day period. The result of the 30-day timeframe shows fresh weight reduction as follows to T1, as T2, T3, T4, T5, T6, T7, and T8 reduced at 66.6%, 33.3%, 37.5%, 41.1%,37.5%,33.3%, and 16.6% respectably.

It concluded that Cd negatively impacts the fresh shoot weight, but the different combinations of biochar, PGPR, and co-planting help reduce its effectivity; moreover, the less reduction rate observed under T7, T8 elaborate that these treatments composition work positively than other. It concludes that the presence of Cd negatively impacts the root weight, but the different combinations of biochar, PGPR, and co-planting help reduce its effectivity.

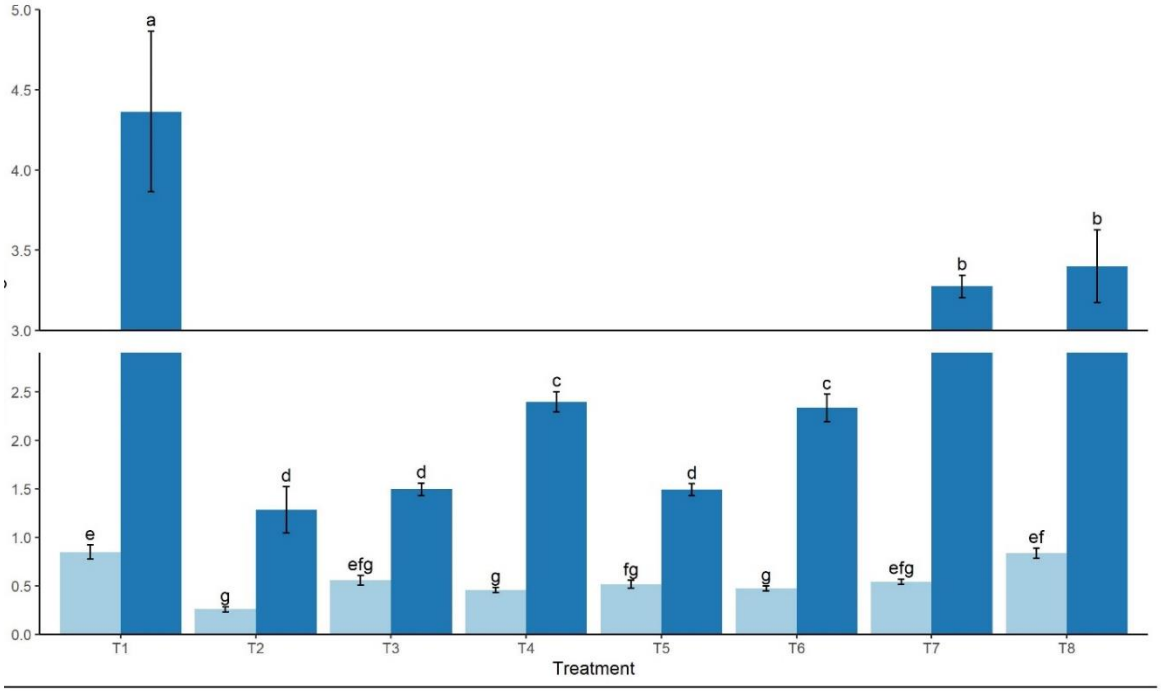

Figure 9 Plant fresh root weight results demonstrate highly reduced in the presence of Cd. Data is shown as interaction of different combination mixture as treatments applied to plants. Significance was inferred with Two-way ANOVA under the Tukey's HSD post-hoc test for normalizing the data distribution (Honest Significant Detection $p < 0.001$).

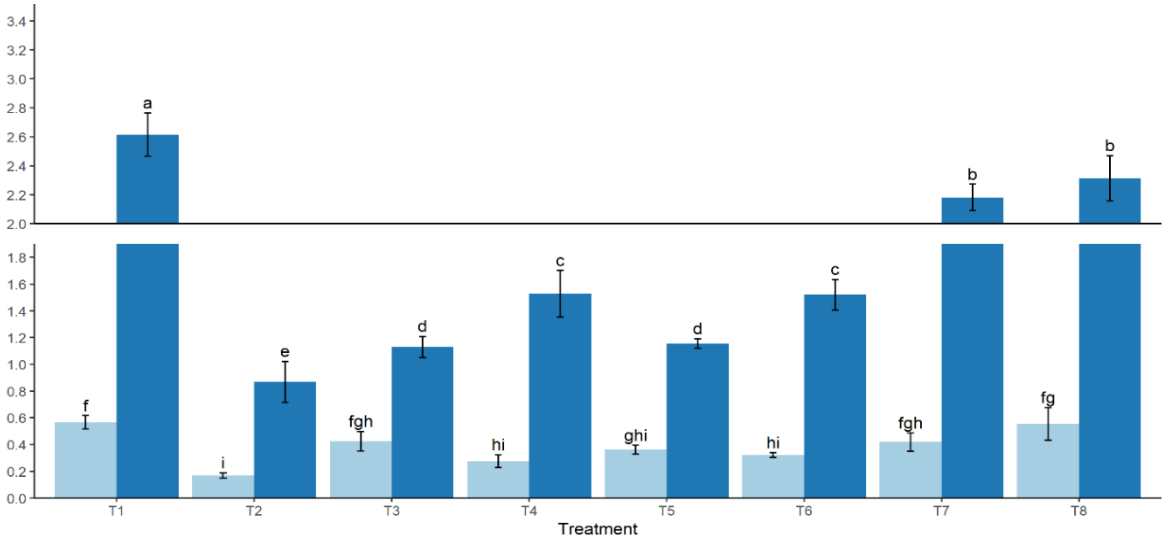

Figure 8 Dry root weight result confirm the reduction in dry matter in the presence of Cd stress. Data is shown as interaction of different combination mixture as treatments applied to plants. Significance was inferred with Two-way ANOVA under the Tukey's HSD post-hoc test for normalizing the data distribution (Honest Significant Detection $p < 0.001$).

4.2.2 Fresh Shoot weight

The results show on *figure10 an* enormous reduction in T2 and T5 for T1 in 65 days. Via comparison with T1, it also indicates that T2, T3, T4, T5, T6, T7, and T8 reduce to 75.5%, 57.7%, 56.6%, 66.8%, 63.1%, 53.3% and 4%, respectively. It concluded that Cd negatively impacts the plant's fresh weight, but the different combinations of biochar, PGPR, and co-planting help reduce its effectiveness.

The result of the 30-day timeframe shows fresh weight reduction as follows to T1, as T2, T3, T4, T5, T6, T7, and T8 reduced at 75%, 53.1%, 62.5%, 55.6%, 43.75%, 33.11%, and 0% respectably. It concludes that the presence of Cd negatively impacts the plant's fresh weight in a 30-day timeframe, but the different combinations of biochar, PGPR, and co-planting help reduce its effectiveness. Moreover, the T8 combination shows a 0% reduction in dry weight, indicating that this combination works quite effectively against cadmium.

The results show on *figure 11* an enormous reduction in T2, T3 and T5 for T1 in 65 days. Via comparison with T1, it also indicates that T2, T3, T4, T5, T6, T7, and T8 reduce to 77.7%, 64%, 62.9%, 66.6%, 64.2%, 55.5% and 7.03%, respectively. It concluded that Cd negatively impacts the shoot weight, but the different combinations of biochar, PGPR and co-planting help reduce its effectivity.

The result of the 30-day timeframe shows fresh weight reduction as follows to T1, as T2, T3, T4, T5, T6, T7, and T8 reduced at 77.5%, 50%, 56.25%, 51.2%, 47.5%, 27.5%, and 0% respectably. It concludes that the presence of Cd negatively impacts the shoot weight, but the different combinations of biochar, PGPR and co-planting help reduce its effectivity.

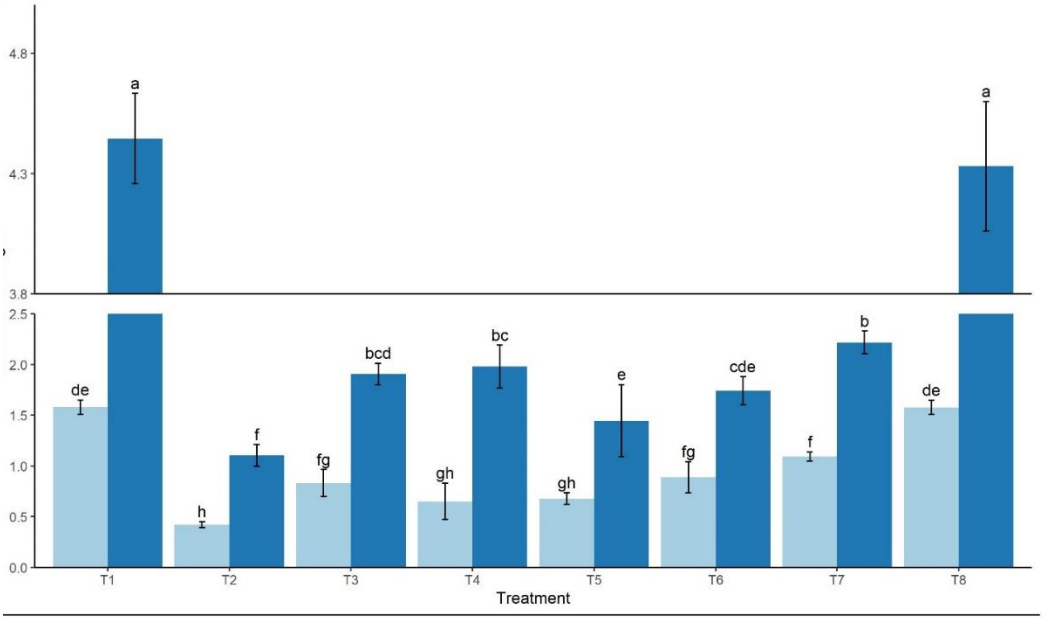

Figure 11 Fresh Shoot weight also show decline in the presence of Cd stress. However different combination of Biochar, co-plant, and PGPR try to reduce the effect of Cd and overcome the stress. Data is shown as interaction of different combination mixture as treatments applied to plants. Significance was inferred with Two-way ANOVA under the Tukey's HSD post-hoc test for normalizing the data distribution (Honest Significant Detection $p < 0.001$).

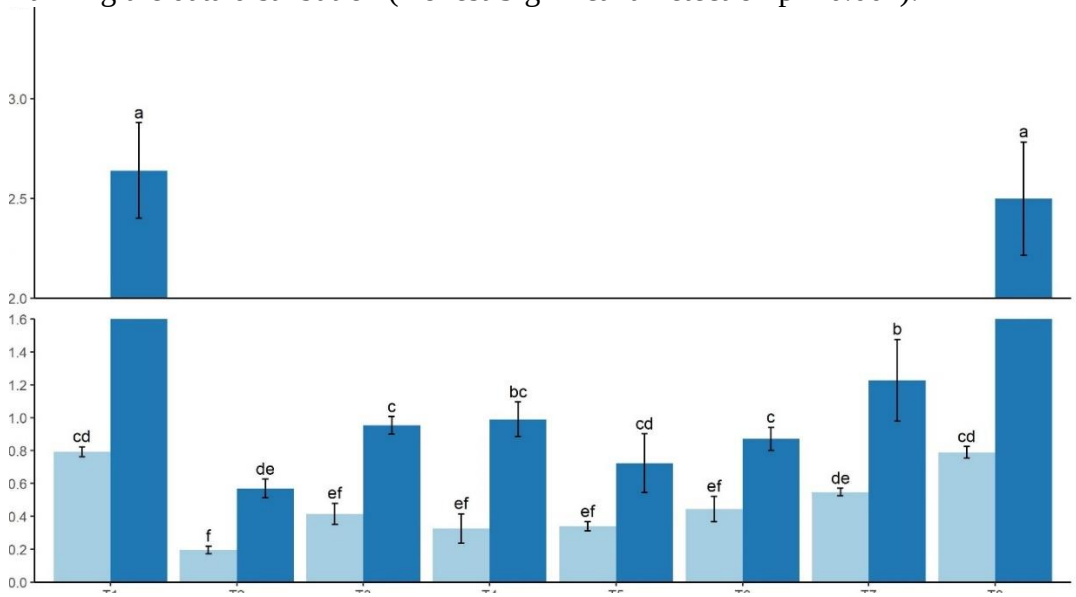

Figure 10 Dry shoot weight also show reduction in the presence of Cd stress. T2 as positive control have less dry mass compare with other treatments. Different combination mixture as treatments applied to plants. Significance was inferred with Two-way ANOVA under the Tukey's HSD post-hoc test for normalizing the data distribution (Honest Significant Detection $p < 0.001$).

4.2.3 Fresh and dry weight of leaves

The results of leave fresh weight show in *figure 12* an enormous reduction in T2, T3, and T5 for T1 in 65 days. Via comparison with T1, it also indicates that T2, T3, T4, T5, T6, T7, and T8 reduce to 74.8%, 46%, 42.4%, 17.2%, 8.27%, 1%, and 0.71%, respectively. The result of the 30-day timeframe shows the fresh weight of the leaves. It shows decrease as follows to T1, as T2, T3, T4, T5, T6, T7, and T8 reduced at 83.05%, 32.2%, 56.25%, 61.8%, 65.25%, 1.5%, and 0.77% respectably.

The results of leave dry weight show in figure *13* show reduction in T2, T3 and T5 for T1 in 65 days. Via comparison with T1, it also indicates that T2, T3, T4, T5, T6, T7, and T8 reduce to 75%, 50%, 45%, 25%, 15%, 5% and -2%, respectively. The result of the 30-day timeframe shows the dry weight of the leaves. It shows that under the Cd stress, the dry weight of leaves reduced to 81.25%, 31.2%, 62.5%, 61.8%, 60%, 11.2%, and 0%. with the comparison of T1, to T2, T3, T4, T5, T6, T7, and T8, respectively.

These results help us to analyze that biomass of leave is highly effective with cadmium, the reduction in fresh leave weight in T2 as (74.8%), and dry biomass of leave as (75%) on 65^{th} day period identified that how adversely cadmium inhibit the plant growth. moreover, in different compositions of biological methods T7, and T8 show non significance results confirm that plant leave biomass was not affect under cd stress in these two treatments. These two are considered as helpful for plant growth.

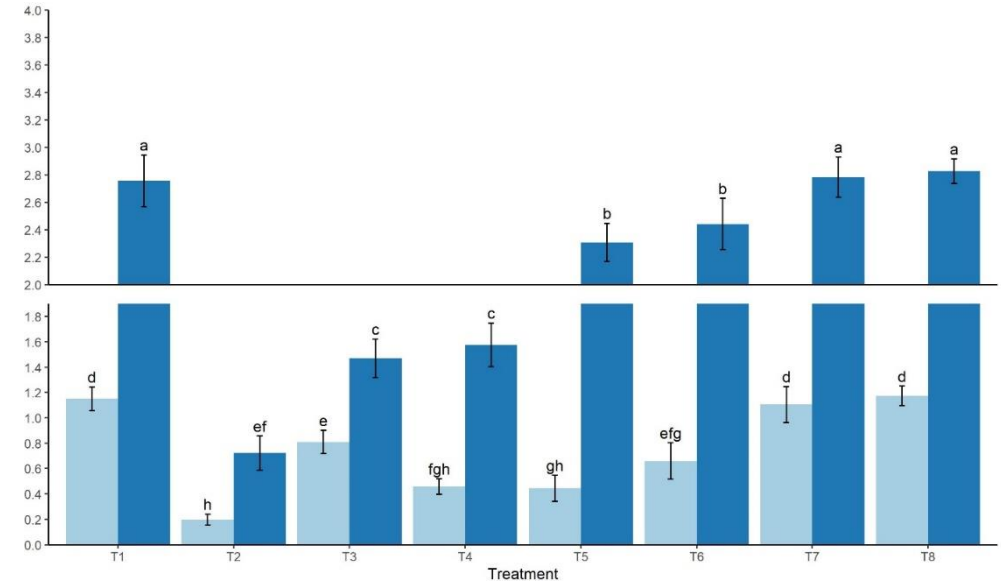

Figure 13 Leave weight also show the decline in mass under the presence of Cd. Data is shown as interaction of different combination mixture as treatments applied to plants. Significance was inferred with Two-way ANOVA under the Tukey's HSD post-hoc test for normalizing the data distribution (Honest Significant Detection $p < 0.001$).

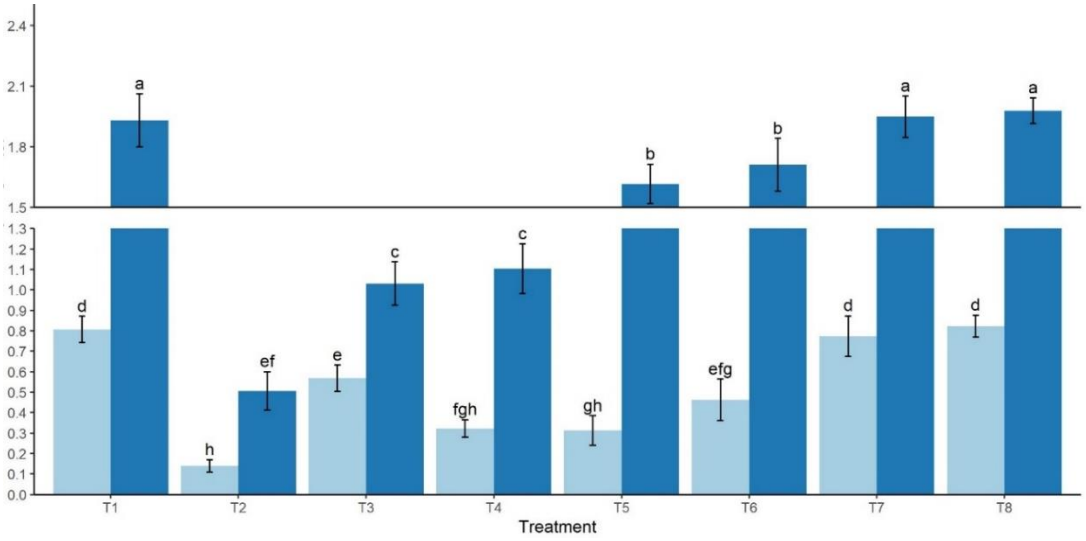

Figure 12 Dry weight of leaves decline in Cd stress also indicate that the presence of Cd reduces dry mass of plants. Different combination mixture as treatments applied to plants. Significance was inferred with Two-way ANOVA under the Tukey's HSD post-hoc test for normalizing the data distribution (Honest Significant Detection $p < 0.001$).

4.2.1 Pods fresh and dry weight

The results in *figure 14* show fresh pod weight on 65 days. Via comparison with T1, other treatments do not show a significant result other than T2 and T4. It shows that T2 and T4 pod daily reduce pod weight due to cadmium while other treatments have similarities in pod fresh and dry weight with T1.

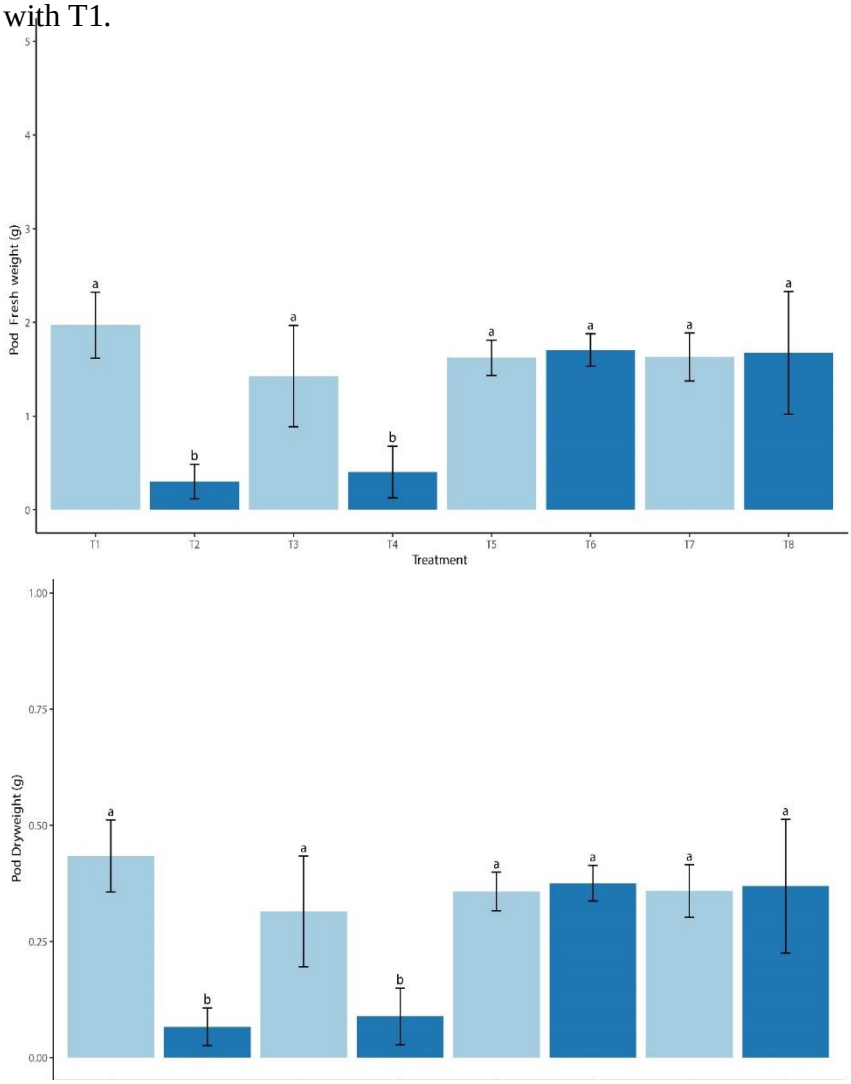

Figure 14 pod fresh and dry weight demonstrate the decrease in T2 and T4 which show that these two treatments have high accumulation of Cd and delay in plant flowering and pods formation. Data is shown as interaction of different combination mixture as treatments applied to plants. Significance was inferred with Two-way ANOVA under the Tukey's HSD post-hoc test for normalizing the data distribution (Honest Significant Detection $p < 0.001$).

4.2.2 Total fresh and dry weight

The results in *figure 15* show total fresh weight of plant upon analysis it is indicated that T2, T3, T5 have significant result according to T1 in 65th day period. Percentage comparison show that T2, T3, T4, T5, T6, T7, and T8 reduce to 74.8%, 46%, 42.4%, 17.2%, 8.27%, 1%, and 0.71%, respectively. On the 30th days fresh weight results elaborate the significant difference between the T2, T4, T5, and T6 is high as compared to other treatments. Upon the analysis of percentage, reduction had me noticed in results of as follows; T1, as T2, T3, T4, T5, T6, T7, and T8 reduced at 83.05%, 32.2%, 56.25%, 61.8%, 65.25%, 1.5%, and 0.77% respectably. The major reduction found in T2 and T4 however, other treatments cope up with the cadmium stress. These results conclude that the presence of different biological mixtures helps in plant overcome the cadmium stress and maintain its biomass.

The results in *figure 16* show the root dry mass in this figure it is explained the decline of dry mass of a plant on 65th period. The major reduction in dry mass has been noticed in T2 (74.3%), T3 (53.7%), T4(51.2%) these treatments show high significance towards the comparison with T1, while the other treatments T5(49.2%), T6(44.8%), T7 (25.6%) and T8 (3.8%), show reduction less than 50% under cadmium stress. we can conclude that T5, T6, T7, and T8 biological mixture help in plant growth and improve the biomass quantity ask compared to other biological mixtures. On the analysis of 30-day dry biomass it showed that T2, and T4 are highly significant as compared to other treatments. T2 (77.2%), and T4 (54.5%) reduction in dry mass while, T3(31.8%), T5 (45.4%) T6 (31%), T7 (13.6%) and T8 (9%). Biological mixtures boost the plant biomass under Cd stress.

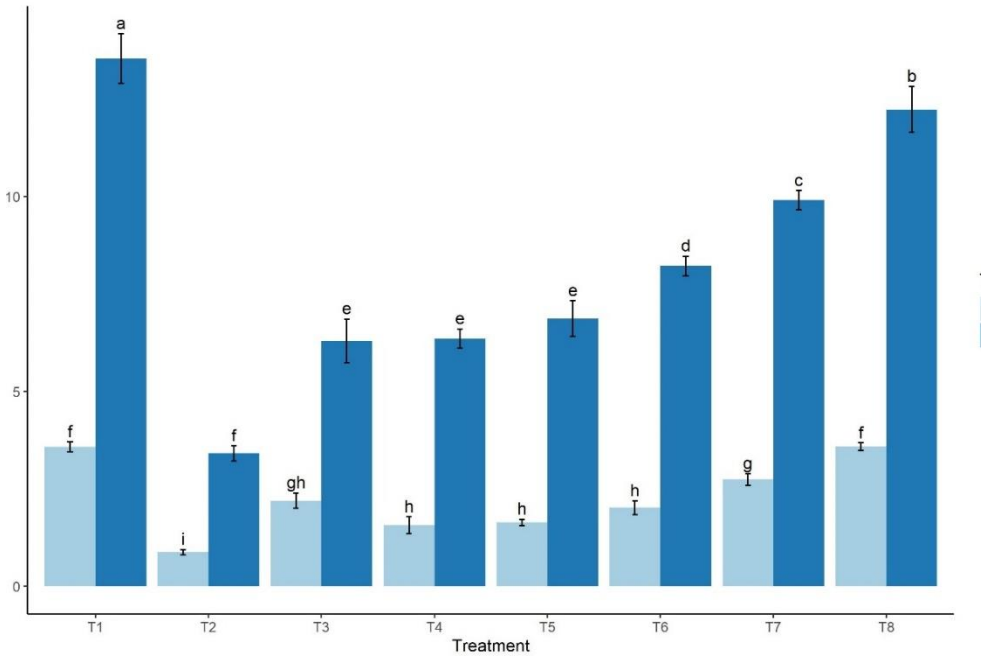

Figure 16 show that total fresh weight of the plant decrease in the presence of Cd. Data is shown as interaction of different combination mixture as treatments applied to plants. Significance was inferred with Two-way ANOVA under the Tukey's HSD post-hoc test for normalizing the data distribution (Honest Significant Detection $p < 0.001$).

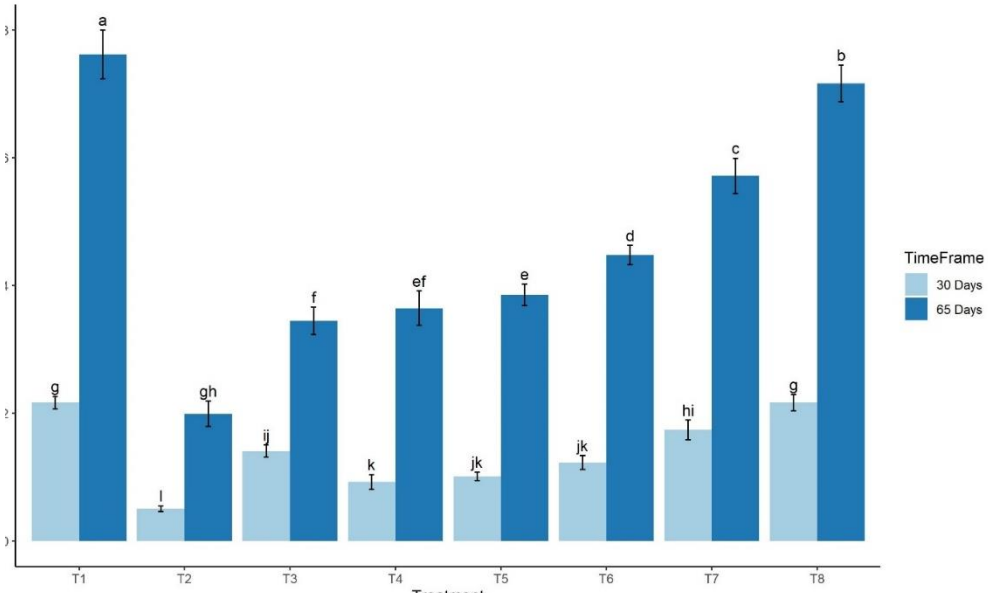

Figure 15 total dry weight of plant show that different combination enhanced plant growth. Data is shown as interaction of different combination mixture as treatments applied to plants. Significance was inferred with Two-way ANOVA under the Tukey's HSD post-hoc test for normalizing the data distribution (Honest Significant Detection $p < 0.001$).

4.2 Number of Leaves

The number of leaves also found reduced in the presence of cadmium stress in the *figure 17* the number of leaves reduced and go T2 (62%) and T3 (44%). However, the reduction in other treatment is non-significant. On percentage analysis it concludes that T4 (16%), T5 (12%), T6 (24%), T7 (24%) and T8 (-4%) on 65^{th} day timeframe. Whereas, on 30^{th} day only T2 show significant result then other.

4.3 Leave Area

Reduction in leave area causes a decrease in photosynthesis and respiration activities. Cadmium induces the drop of leave area in plants. Results showed in *figure 18* indicate that positive control treatment T2 leave area reduced on 65-days (46.2%) and 30-days (41.6%) compared with negative control T1. However, via comparison with T1, it also indicates that T2, T3, T4, T5, T6, T7, and T8 reduce to 46.2%, 25.4%, 7.7%, 15.4%, 15.4%, 20% and 6.2%, respectively. On 30-day leave area reduction as follows to T1, as T2, T3, T4, T5, T6, T7, and T8 reduced at 41.67%, 25%, -5%, 13.33%, 5%, 20%, and -3.33% respectably.

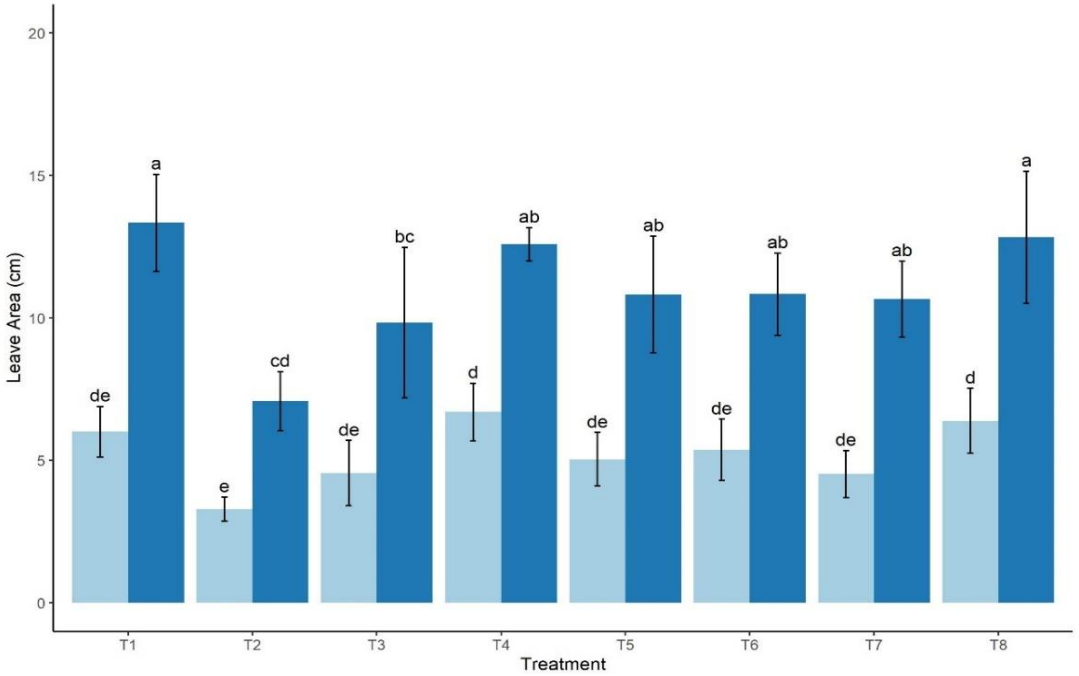

Figure 17 Leave area reduction show that plant on 65 days has increases compare with 30 day time period. Data is shown as interaction of different combination mixture as treatments applied to plants. Significance was inferred with Two-way ANOVA under the Tukey's HSD post-hoc test for normalizing the data distribution (Honest Significant Detection $p < 0.001$).

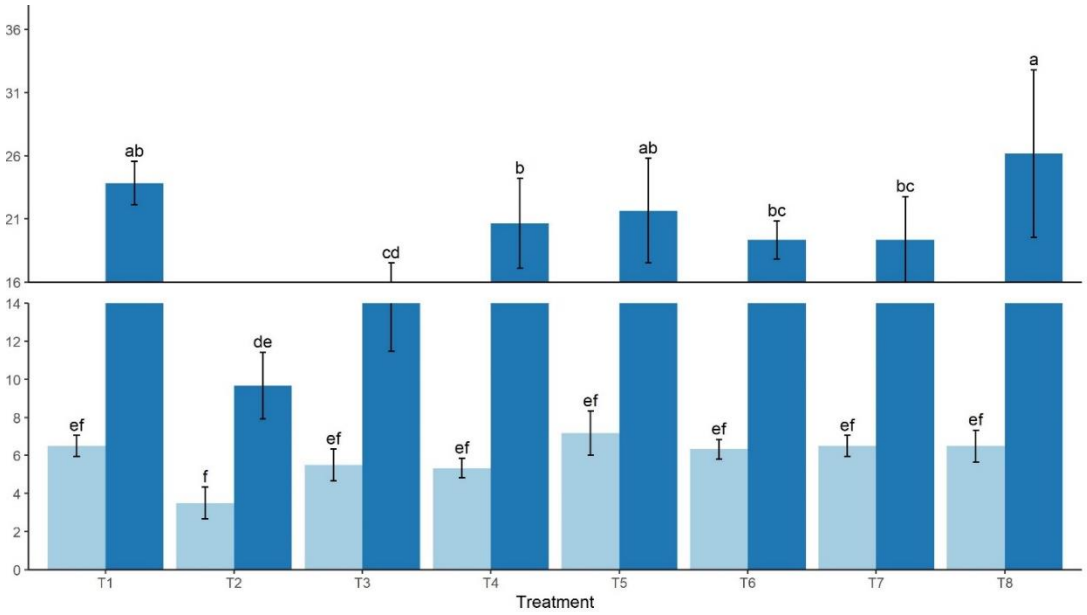

Figure 18 Number of leave of the plant show us the reduction in positve control where as different mixture of combination boost the plant growth different combination mixture as treatments applied to plants. Significance was inferred with Two-way ANOVA under the Tukey's HSD post-hoc test for normalizing the data distribution (Honest Significant Detection $p < 0.001$).

4.5 Number of Pods

The number of pods per treatment shows that the quantity is only reduced in T2. However, the other treatments have the same number as the control group.

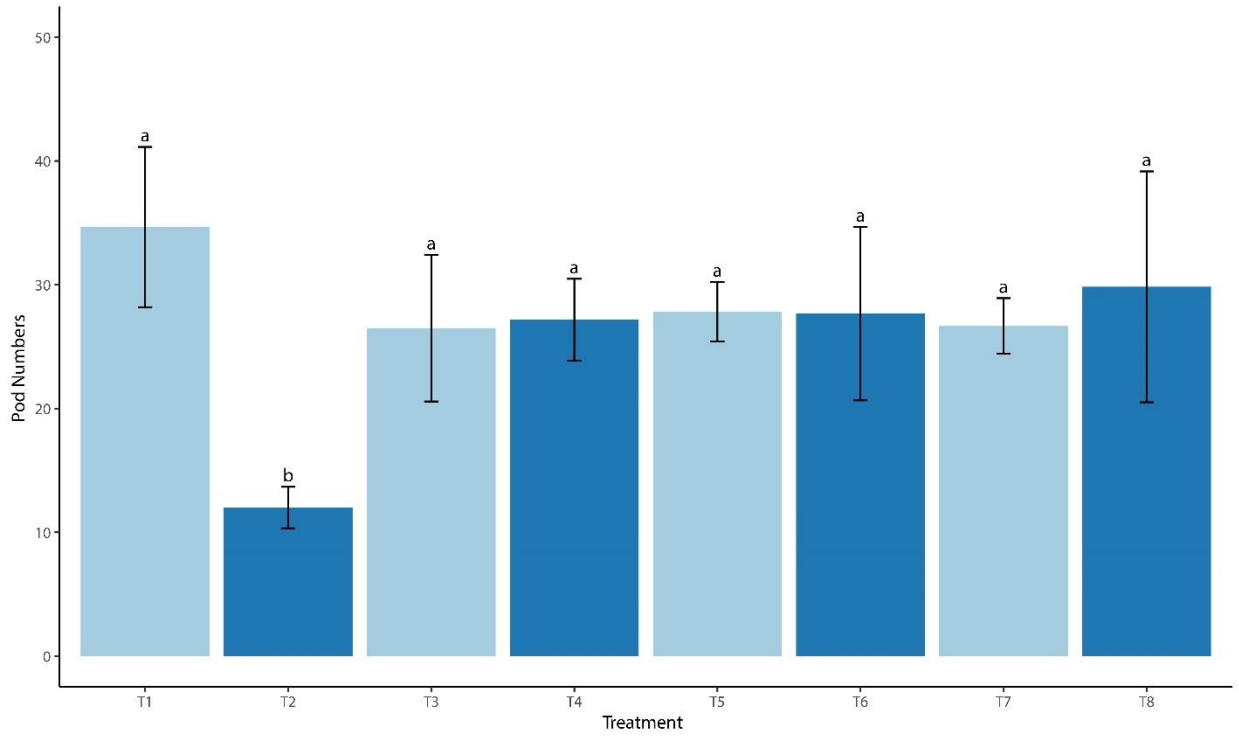

Figure 19 Number of pod in T2 show decline with the compairison to T1 and other treatmeents. Data is shown as interaction of different combination mixture as treatments applied to plants. Significance was inferred with One-way ANOVA under the Tukey's HSD post-hoc test for normalizing the data distribution (Honest Significant Detection $p < 0.001$).

4.6 Pigments

4.6.1 Chlorophyll

The Chlorophyll content was measured by the following method (Arnon, 1949). The absorbance of the supernatant was collected at 645 and 663 nm for chlorophyll a and chlorophyll b, respectively. The chlorophyll level was highly reduced in T2 with the comparison of T1. T1 shows an increased value of chlorophyll a and chlorophyll b presence. Moreover, their treatments show a high deviation in chlorophyll content.

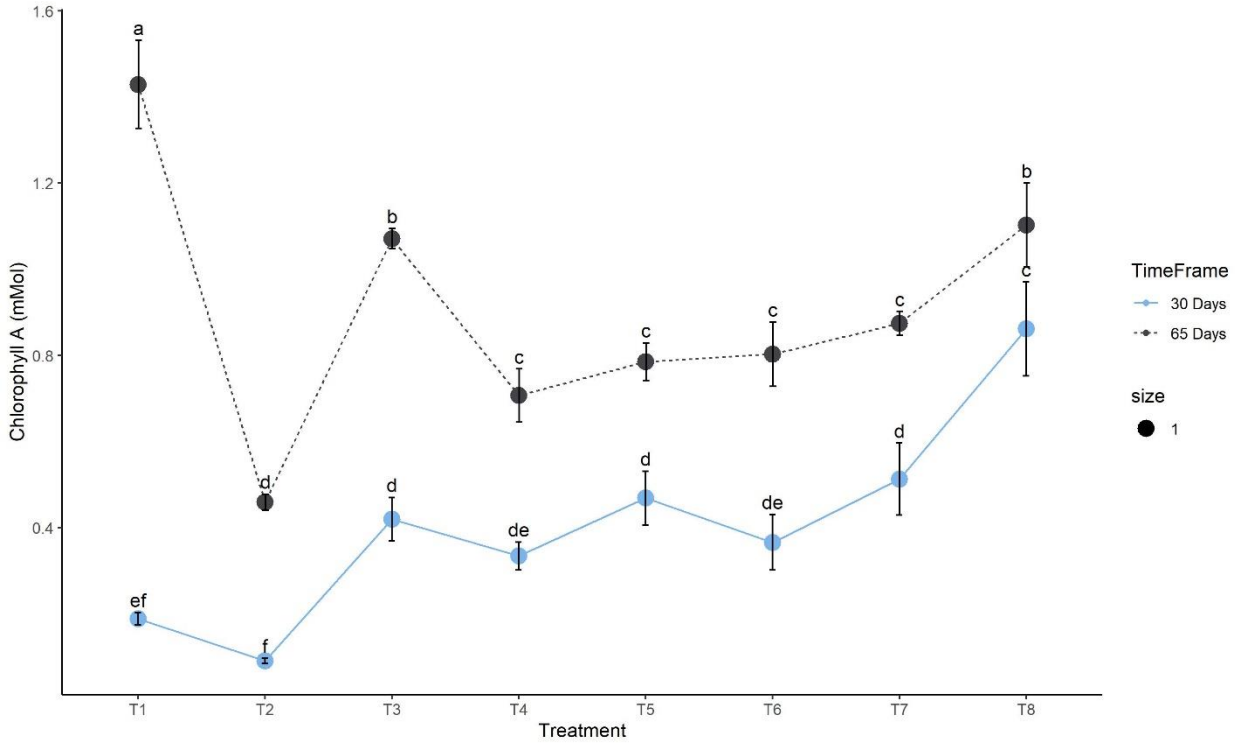

Figure 20 Chlorophyll A graph confirms the reduction of photosynthesis pigments in T2. Data is shown as interaction of different combination mixture as treatments applied to plants. Significance was inferred with Two-way ANOVA under the Tukey's HSD post-hoc test for normalizing the data distribution (Honest Significant Detection $p < 0.001$).

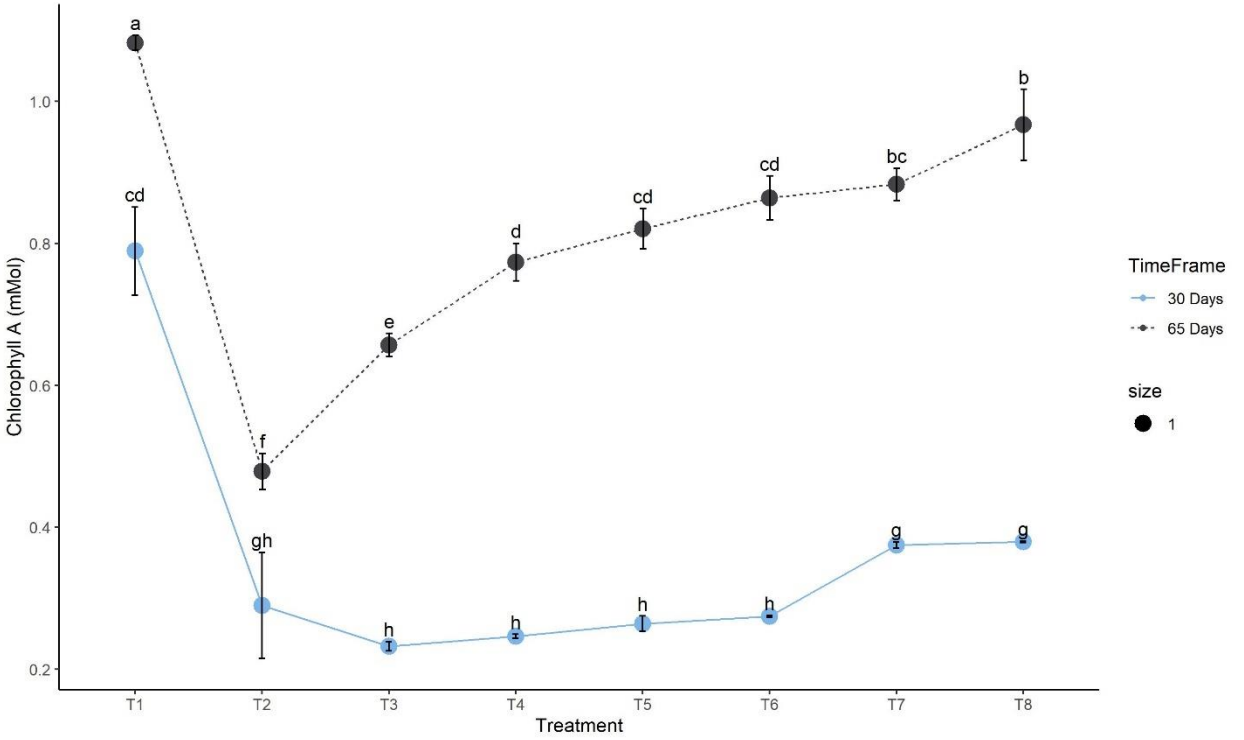

Figure 21 Chlorophyll B graph confirms the reduction of photosynthesis pigments in T2 concluded that Cd presence reduces the chlorophyll content. Data is shown as interaction of different combination mixture as treatments applied to plants. Significance was inferred with Two-way ANOVA under the Tukey's HSD post-hoc test for normalizing the data distribution (Honest Significant Detection $p < 0.001$).

4.7 Biochemical Assay

4.7.4 SOD

The Antioxidant assay of SOD was measured by the method of (Kono, 1978). UV spectrophotometer analysis was done at 560nm. The SOD value was found to be increasing under Cd stress. The control plant T1 shows a lower level of SOD compared to T2.

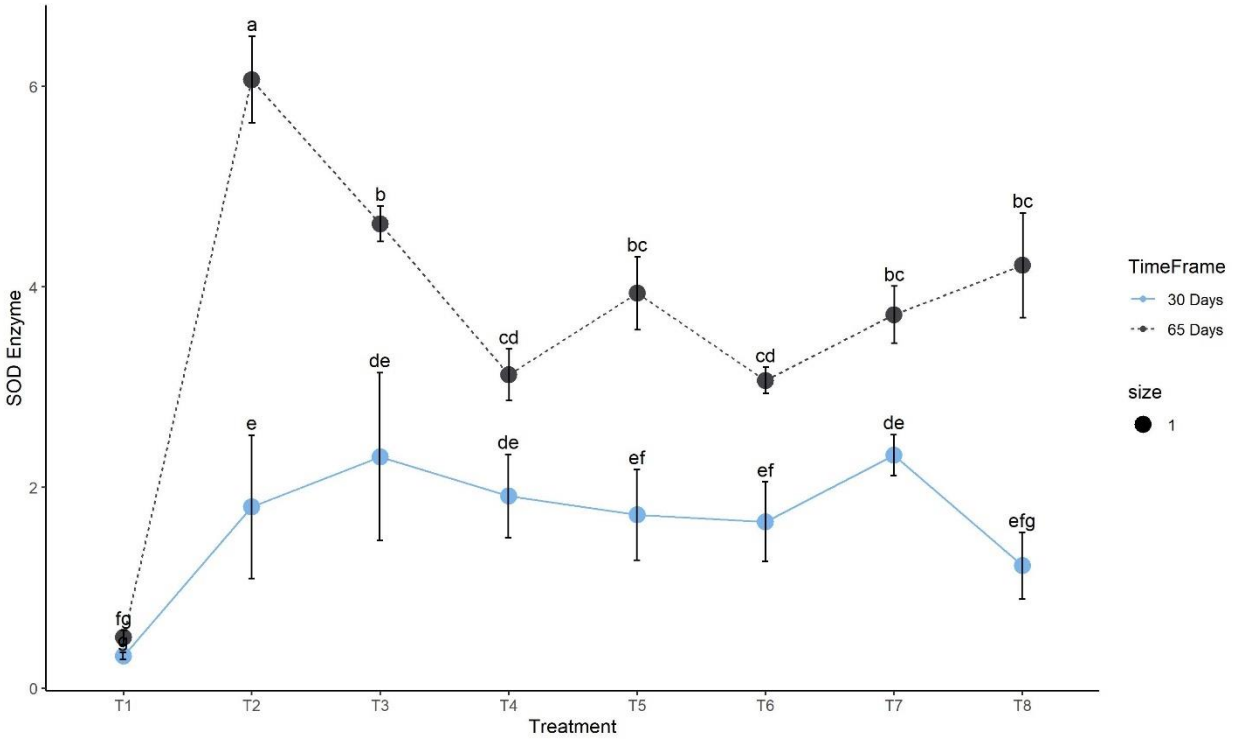

Figure 22 SOD enzyme activates when the ROS production in plant cells increases due to stress. SOD enzyme converts the reactive O2- ions to hydrogen peroxides. The result concludes that SOD is extremely present at T2 as compared to other. Data is shown as interaction of different combination mixture as treatments applied to plants. Significance was inferred with Two-way ANOVA under the Tukey's HSD post-hoc test for normalizing the data distribution (Honest Significant Detection $p < 0.001$).

4.7.5 POD

The Antioxidant assay for peroxidase (POD) was measured by following the method given by (Bergmeyer, 1974). UV spectrophotometer analysis was done at 270nm. The POD value was found to be increasing under Cd stress. The control plant T1 shows a lower level of POD compared to T2.

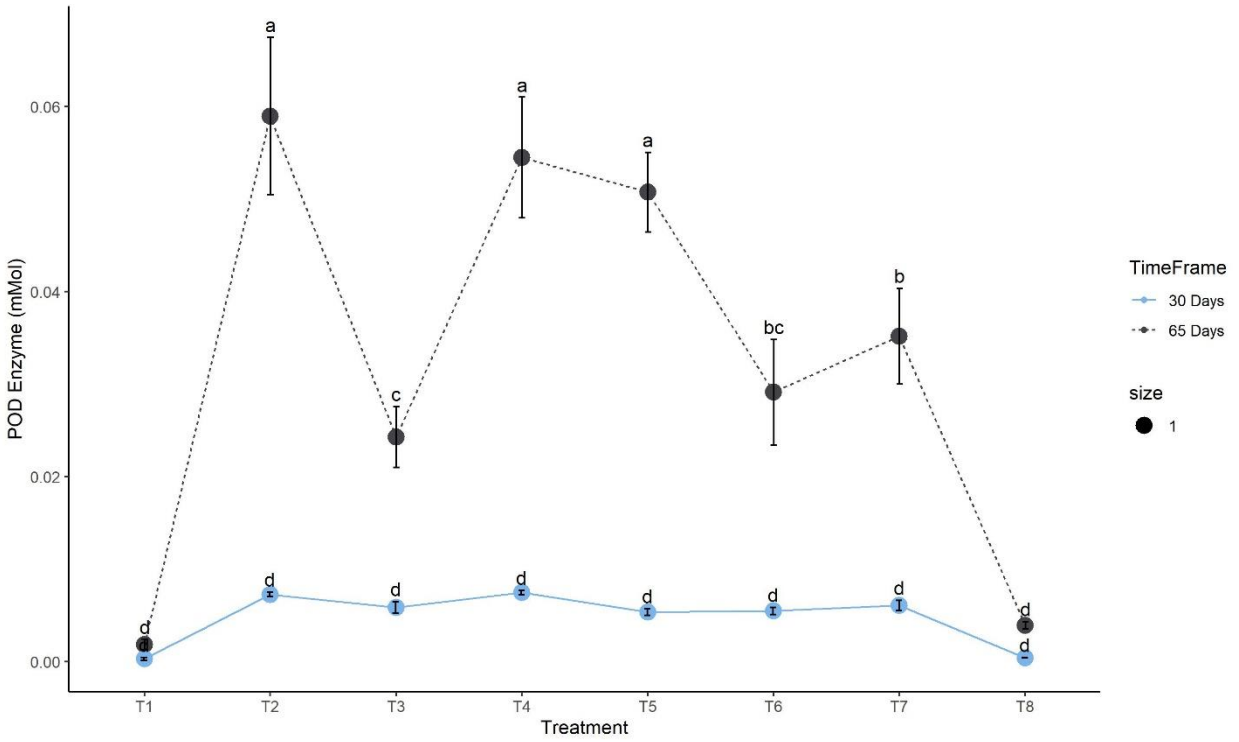

Figure 23 POD enzyme activates when the ROS production increase in the plant due to stress. POD enzyme converts H2O2 to H2O. the result shows that POD concentration was high at T2. Data is shown as interaction of different combination mixture as treatments applied to plants. Significance was inferred with Two-way ANOVA under the Tukey's HSD post-hoc test for normalizing the data distribution (Honest Significant Detection $p < 0.001$).

4.7.6 CAT

The Antioxidant assay for Catalase (CAT) was measured by the following method (Aebi, 1984). The reading was counted using a UV spectrophotometer at 240nm with a time point of 10 seconds. The CAT level was found highly increased in T2 with the comparison of T1 as a control. T1 shows a decline in CAT presence. However, other treatments show the presence of CAT.

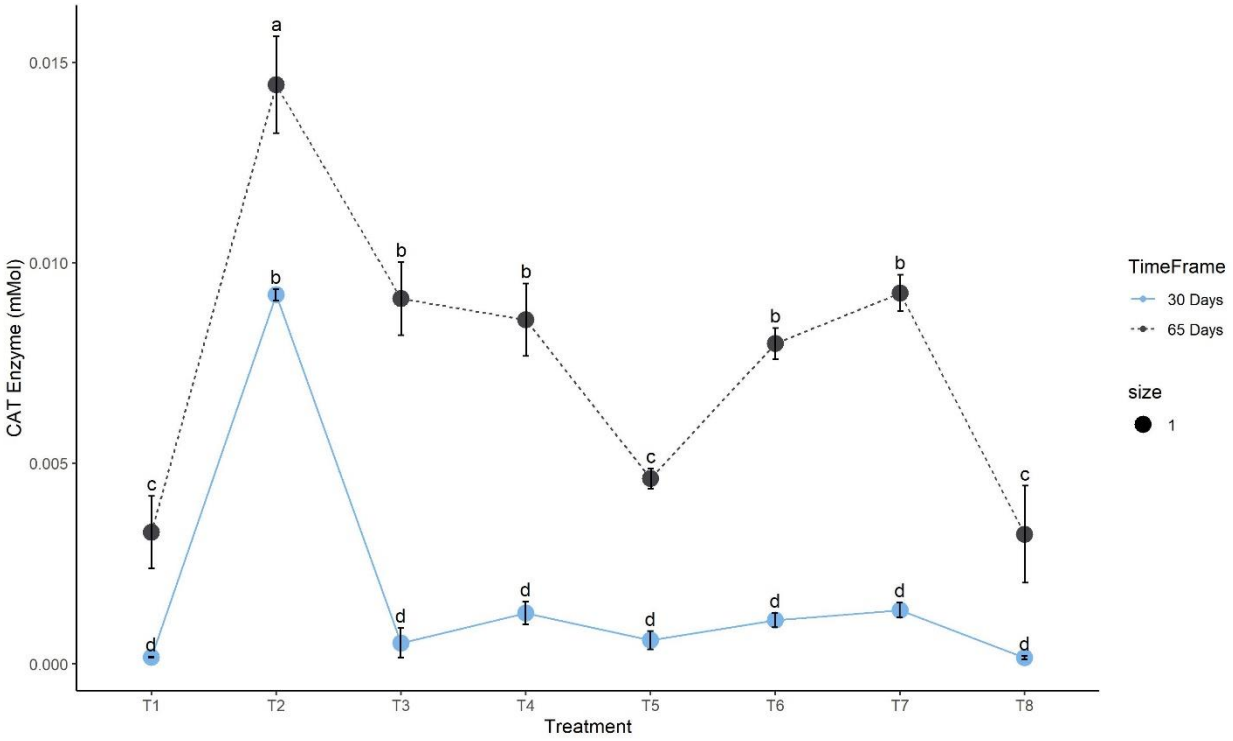

Figure 24 CAT enzyme activates when the ROS production increase in the plant due to stress. CAT enzyme converts H2O2 to H2O. The result shows that CAT concentration was high at T2. Data is shown as interaction of different combination mixture as treatments applied to plants. Significance was inferred with Two-way ANOVA under the Tukey's HSD post-hoc test for normalizing the data distribution (Honest Significant Detection $p < 0.001$).

4.7.7 APX

Ascorbate peroxidase (APX) was measured by the method (Habib et al., 2014). UV spectrophotometer readings were recorded on 290nm at 10-second intervals. APX increased Cd concentration in T2 compared with T1 and other treatments.

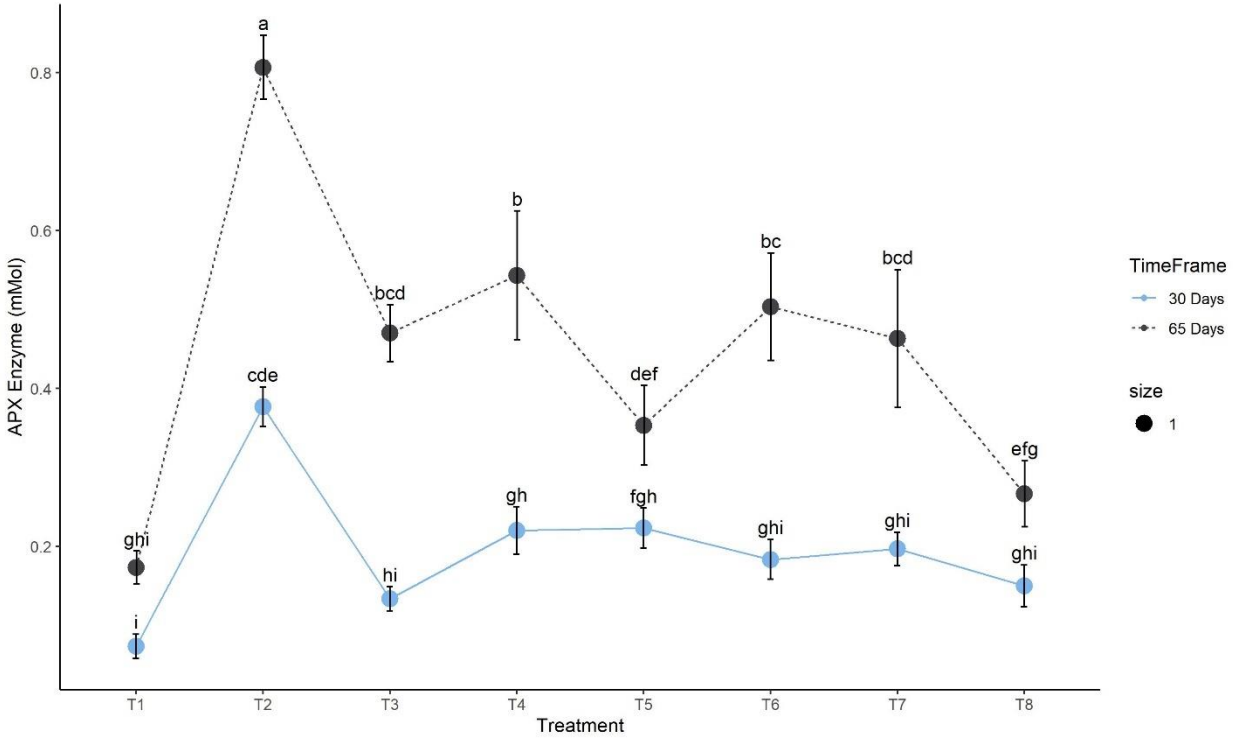

Figure 25 APX Enzyme activates when the ROS production increases due to stress. APX enzyme converts H2O2 to H2O. Results show that APX concentration was high at T2. Data is shown as interaction of different combination mixture as treatments applied to plants. Significance was inferred with Two-way ANOVA under the Tukey's HSD post-hoc test for normalizing the data distribution (Honest Significant Detection $p < 0.001$).

4.7.8 MDA

The Melo-dialdehyde content (MDA) was measured by the following method (T. Chen & Zhang, 2016). The reading counted using a UV spectrophotometer at 532 nm and 600 nm with a time point of 10 seconds. The MDA level found highly increased in T2 with the comparison of T1 as a control. T1 shows a decline in CAT presence. However, other treatments show the presence of MDA.

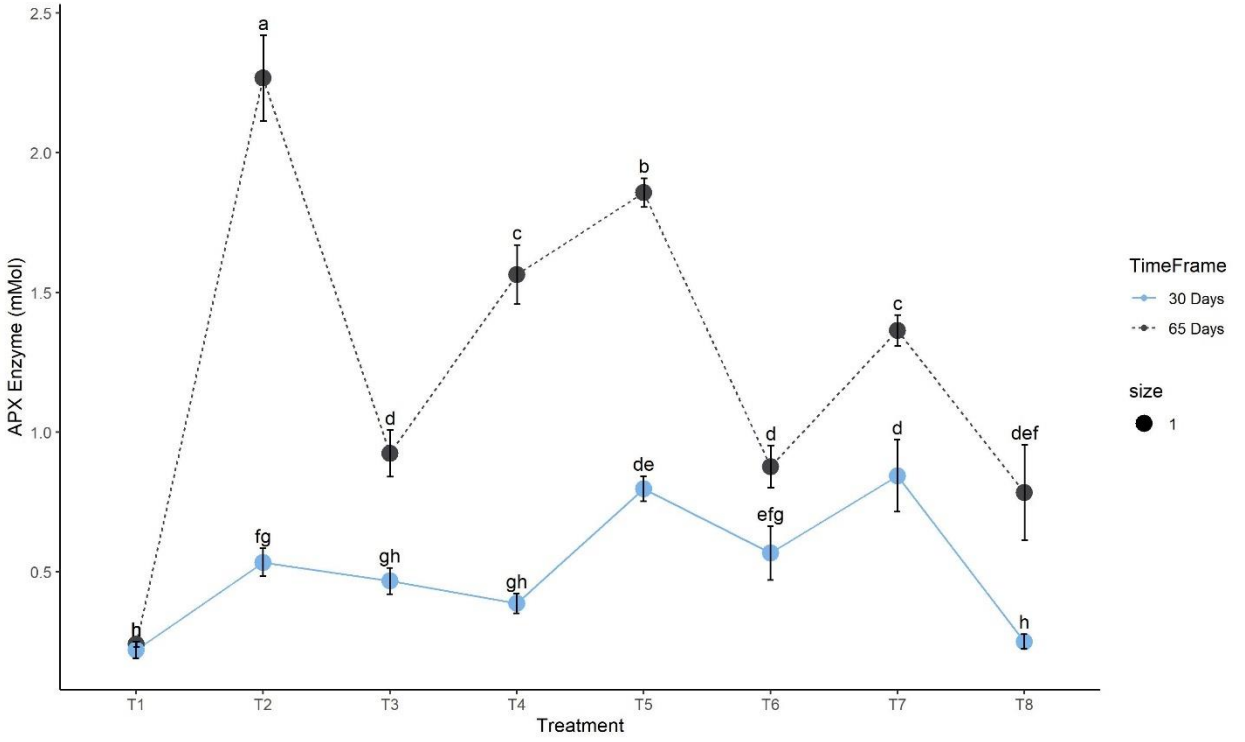

Figure 26 MDA s a widely used parameter measuring lipid peroxidation in plant tissue that increases under oxidative stress. Its show that T2 has high MDA content than other. Data is shown as interaction of different combination mixture as treatments applied to plants. Significance was inferred with Two-way ANOVA under the Tukey's HSD post-hoc test for normalizing the data distribution (Honest Significant Detection $p < 0.001$).

4.8 Scanning Electron Microscopy

SEM and EDX results confirm the accumulation of Cadmium ions in plant leaves. The height of peaks defines the concentration of Cd at different points in a sample. These images explain that T2 and T4 have a high accumulation of cadmium in leaves; moreover, other morphological parameters show a reduction in T2 other than T4. It indicates that T4 decreased in different morpho-physio aspects of T4 due to Cd concentration. However, other treatments also confirm the presence of Cd in them.

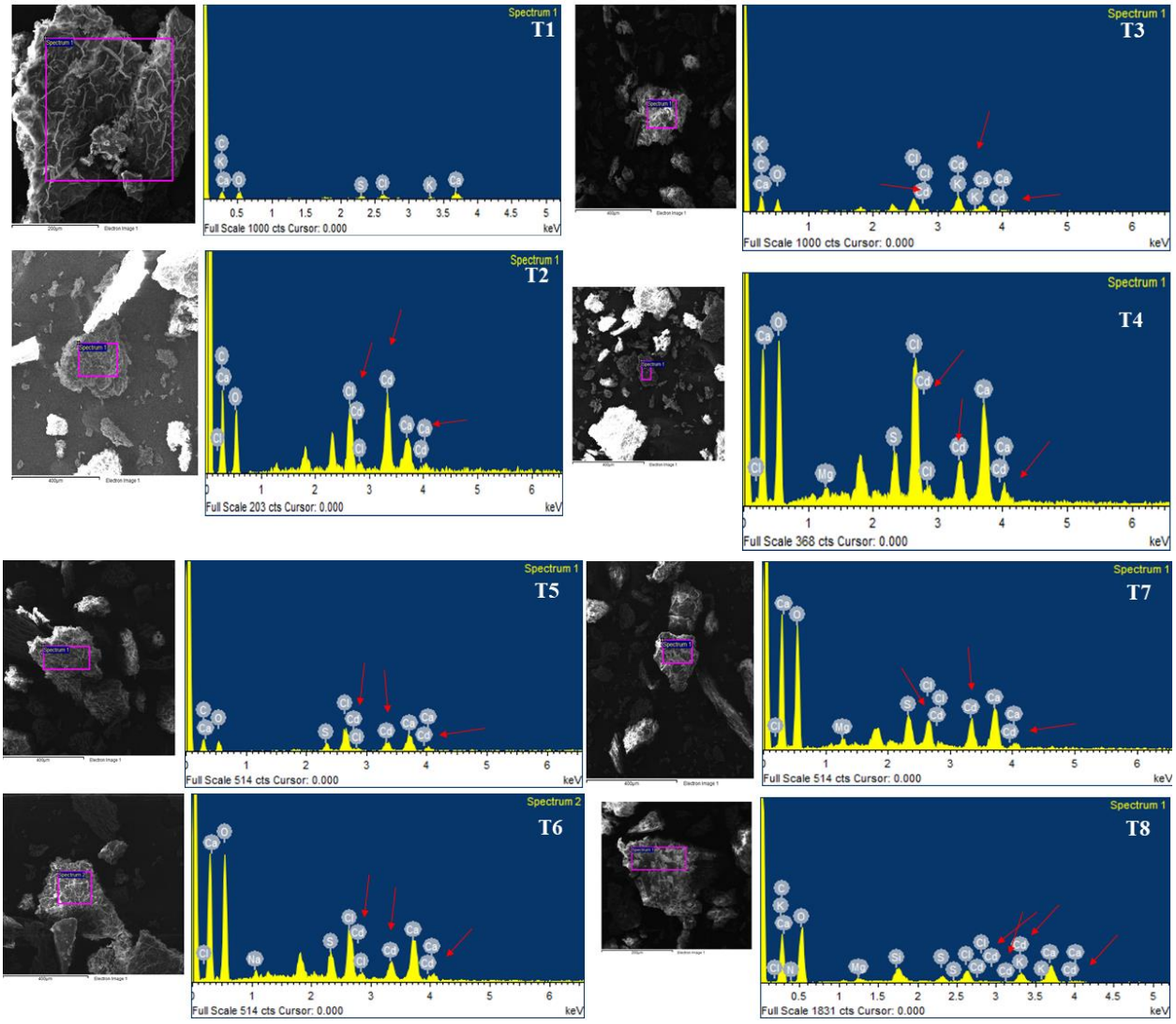

Figure 27 SEM /EDX result confirm the accumulation of Cd in plant leaves. It shows T2 and T4 has high accumulation compare with other treatments.

Chapter 5: Discussion

Brassica Juncea (Mustard Plant) used for food and edible oil. This plant has substantial consumables every year throughout the world. Moreover, the main concern about the mustard plant as it has hyperaccumulator properties that store heavy metal ions such as cadmium ions in a different part of the plant (Agriculture, 2019; FAO, 2017; Mutlu et al., 2012). Previous studies conclude that cadmium ions adversely affect plants' morphological, physiological and biochemical processes (Kapoor, Kaur, & Bhardwaj, 2014). The conformation changes in this aspect also observed in our research. Meanwhile, different composition of biochar, PGPR and inter-cropping boost plant growth in our study cases.

Previous study conducted by conclude that 5 mM of Cd reduce the plant Biomass till 75% (Houssou et al., 2022). In our study we use 10mM CdCl2 solution show also reduction in biomass 75%. The morphological traits also affected under cadmium of Brassica juncea (Zhichao Wu, Zhao, Sun, Tan, Tang, Nie, Qu, et al., 2015).

Cadmium affects plants at morpho-physio, genetic and enzymatic levels. Antioxidative defence systems include plant growth regulators and antioxidative enzymes (J. Chen & Yang, 2012). Enzymes like SOD, POD, CAT, APX, and MDA help scavenge free radicals and protect against specific stress. Protective proteins like heat shock proteins protect plants against oxidative damage. Due to heavy metal toxicity, several types of defence responses are produced in plants, but their action depends upon the doses, type of plant species, and so forth (Arora, Sairam, & Srivastava, 2002). The ability of plants to ameliorate the heavy metal toxicity or to bear the stress makes them survive in those conditions (Hall, 2002). Exposure to heavy metals activates the antioxidative defence system.

Similarly, in the present work, increased SOD, POD, CAT, APX and MDA enzymes were stimulated with metal treatment and thus helped scavenge free radicals like DPPH. These results are in coherence with the findings of Doganlar et al. (Doganlar, Cakmak, & Yanik, 2012). The antioxidative potential of the plant enhanced in a dose-dependent manner. Another mechanism of plant defence involves the secondary metabolites and plant growth regulators. But with the help of organic amendments, including PGRP Bacteria, Biochar, and Co-planting with legume plants, reduce the translocation of cadmium in Brassica Juncea. These amendments improve plants' growth, yield, and biomass (P Sharma & Bhardwaj, 2007).

Chapter 6: Conclusion

The combination of PGPR, Biochar, and co-planting helps in plant growth and provides resistance against Cd stress. Solitary biochar did not display improving results as it shows in different combinations of PGPR+ co-plant+ biochar. EDX results confirm the hyperaccumulation of Cd in all treatments but expressively do not show as a reduction in morpho-physiological aspects as T2 present. Cd stress causes a reduction in plant length by 60% and Biomass up to 75%. Activation of the different antioxidant enzymes has noticed (SOD, POD, CAT, APX, and MDA). On T2 the antioxidant enzyme activity was higher as compared with other treatments. Detection of antioxidant confirms the activation of ROS species.

Here we have investigated the consequence of cadmium in mustard plant and helped to overcome it with biochar, co-planting, and PGPR bacteria. These combinations boost the morpho-physiological aspect of hyperaccumulator plant thus, this combination can be used to enhance the growth of non-hyperaccumulator plants. However, future studies should also consider different plants and bacteria to understand the process and features to overcome the Cd and other heavy metals toxicity. This study can used for further investigation for other heavy metals stresses with this combination.

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APPENDIX

REGENT	PREPARATION
PBS BUFFER	• 200 mM Na2HPO4 stock solution • Dissolve 53.65 g Na2HPO4.7H2O in 1,000 ml ddH2O • Dissolve 27.8 g NaH2PO4.H2O in 1,000 ml ddH2O • Keep it four °C before using **100 mM PBS (pH 7.0)** • 61 ml 200 mM Na2HPO4 stock solution • 39 ml 200 mM NaH2PO4 stock solution • 100 ml ddH2O **100 mM PBS (pH 7.8)** • 91.5 ml 200 mM Na2HPO4 stock solution • 8.5 ml 200 mM NaH2PO4 stock solution • 100 ml ddH2O **100 mM PBS (pH 6.5)** • 31.5 ml 200 mM Na2HPO4 stock solution • 68.5 ml 200 mM NaH2PO4 stock solution • 100 ml ddH2O
TEN PERCENT TCA	• Dissolve 10 g TCA in 100 ml ddH2O
0.25% TBA	• Dissolve 0.125 g TBA in 5 ml 1 mol/L NaOH • Add into 45 ml 10% TCA • Keep it four °C before using
1 MM EDTA-2NA	• Dissolve 0.037 g EDTA-2Na in 100 ml ddH2O
130 MM METHIONINE	• Dissolve 0.970 g methionine in 50 ml 100 mM PBS (pH 7.8) • Keep it four °C before using
750 MM NBT	• Dissolve 0.031 g NBT in 50 ml 100 mM PBS (pH 7.8) • Keep in the dark
TWENTY MM RIBOFLAVIN	• Dissolve 0.007 g riboflabin in 100 ml ddH2O • Keep in the dark

0.2% GUAIACOL	• Dissolve 0.1 g guaiacol in 0.5 ml ethanol • Add into 50 ml 100 mM PBS (pH 7.0) • Keep in the dark
5 MM GSH	• Dissolve 0.077 g GSH in 50 ml 100 mM PBS (pH 6.5)
CdCl2	**250 mM CdCl2 (pH 7.0)** • 45.8g in 1000 ml ddH20 **100 mM CdCl2 (pH 7.0)** • 18.332g in 1000 ml ddH2O **10 Mm CdCl2 (pH 7.0)** • 1.83g in 1000 ml ddH2O
SEVENTY PERCENT ACETONE	• Dissolve 70 ml acetone in 30 ml ddH2O
FeCL3	• 3g of FeCl3 in 97ml of ddH2O
BIPYRIDYL	• 4g of bipydiryl in 96ml ddH2O

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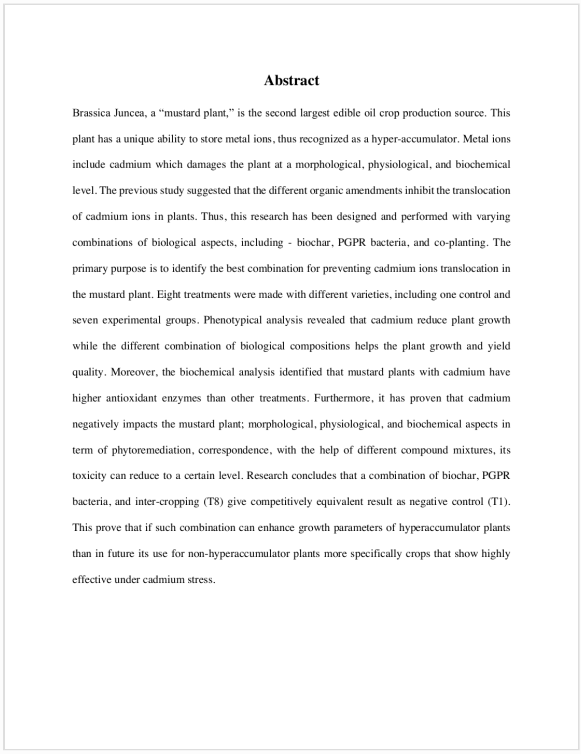

Abstract

Brassica Juncea, a "mustard plant," is the second largest edible oil crop production source. This plant has a unique ability to store metal ions, thus recognized as a hyper-accumulator. Metal ions include cadmium which damages the plant at a morphological, physiological, and biochemical level. The previous study suggested that the different organic amendments inhibit the translocation of cadmium ions in plants. Thus, this research has been designed and performed with varying combinations of biological aspects, including - biochar, PGPR bacteria, and co-planting. The primary purpose is to identify the best combination for preventing cadmium ions translocation in the mustard plant. Eight treatments were made with different varieties, including one control and seven experimental groups. Phenotypical analysis revealed that cadmium reduce plant growth while the different combination of biological compositions helps the plant growth and yield quality. Moreover, the biochemical analysis identified that mustard plants with cadmium have higher antioxidant enzymes than other treatments. Furthermore, it has proven that cadmium negatively impacts the mustard plant; morphological, physiological, and biochemical aspects in term of phytoremediation, correspondence, with the help of different compound mixtures, its toxicity can reduce to a certain level. Research concludes that a combination of biochar, PGPR bacteria, and inter-cropping (T8) give competitively equivalent result as negative control (T1). This prove that if such combination can enhance growth parameters of hyperaccumulator plants than in future its use for non-hyperaccumulator plants more specifically crops that show highly effective under cadmium stress.

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