Unraveling the Molecular Mechanisms in *Brassica juncea* under Cadmium stress

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Islamabad, Pakistan

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A thesis submitted to the National University of Sciences and Technology Islamabad in partial fulfillment of the requirements for the degree of Master of Science in Plant Biotechnology

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THESIS ACCEPTANCE CERTIFICATE

Certified that the contents and form of the thesis entitled "**Unraveling the Molecular Mechanisms in *Brassica juncea* under cadmium stress** " submitted by Rimsha Azhar found satisfactory for the requirement of the degree.

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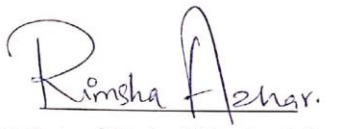

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Dedication

To My MOM & DAD

(Who blossomed me into a flower at their own expense)

I am because you are

IN THE NAME OF

ALLAH,

THE MOST

BENEFICENT,

THE MOST

GRACIOUS,

THE MOST

MERCIFUL!

Acknowledgment

"My Success is Only by Allah"

Initially, I would like to thank my supervisor, ***Dr. Rabia Amir,*** for her inspiration, coaching, and enthusiasm throughout my MS. None of this would have been accomplished without her guidance. I can't express my gratitude for her kindness love and support. She consistently believed in me by motivating me that "YOU CAN DO"

I must express my profound gratitude to my constant support my **MOM & DAD,** and my siblings for their support and courage throughout the journey. A special thanks to my Dearest teacher ***Syed Muhammad Babar Hussain*** as this achievement was not possible without his encouragement. I thank and appreciate Qurat***-ul-Ain Ali Hira, Midhat Mahboob***, for helping to complete this project. I am also very grateful for the presence of my best friend, ***IJAZ-ul-Haq*** throughout my two years tenure for his continuous support, and encouragement, in my thick and thin. This accomplishment would not have been possible without all of them. Thank you.

Rimsha Azhar

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List of Abbreviation

µl	Microlitter
^{0}C	Degree Celsius
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
MgCl2	Magnesium chloride
NCBI	National Center for Biotechnology Information
NF H2O	Nuclease Free Water
NaCl	Sodium Chloride
NARC	National Agriculture Research Center
MYB1	Myeloblastosis1
PCR	Polymerase Chain Reaction
TAE Buffer	Tris-Acetate-EDTA Buffer
TE Buffer	Tris Ethylenediaminetetraacetic acid Buffer
TF	Transcription factor
UV	Ultra-Violet
%	Percentage
Nm	Nanometer
Ng	Nanogram
CAX4	Cation Exchanger4
GSH	Glutathione

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Abstract

Indian mustard (Brassica juncea L. Czern.) is a potential plant for the aim of phytoextraction of cadmium (Cd) from metal-contaminated soils since it tolerates high concentrations of heavy metals. The degree to which metal sequestering systems are in charge of this tolerance is examined in this study. To achieve this, Indian mustard seedlings were grown in 10mM Cd for 30 and 70 days. According to earlier studies, a number of organic amendments stop cadmium ions in plants from moving about. As a consequence, the planning and implementation of this project included the use of several combinations of biological components, including biochar, PGPR bacteria, and co-planting. The primary objective is to ascertain which elements combine to prevent cadmium ions from translocating inside the mustard plant. A progressive Gene expression analysis, which is crucial in many biological research disciplines, was caused by rising Cd concentrations. Understanding the targeted genes' expression patterns is a handy method for examining the various expression patterns of complicated regulatory networks. Researchers have shown that combining biochar, PGPR bacteria, and intercropping (T8) yields outcomes that are competitively equivalent to a negative control (T1). Additionally, under Cd stress, glutathione and phytochelatin concentration in leaves rose noticeably, although biological combinations offered a great way to boost the mustard plant's phytoremediating effectiveness. This shows that non-hyperaccumulator plants, particularly crops that thrive under cadmium stress, may benefit in the future if a particular combination may enhance the features of hyperaccumulator plants.

Chapter 1: Introduction

1.1 Heavy Metals Occurrence, effects & toxicity to Plants

The primary factor limiting crop growth and yield globally is abiotic stress. Plants must contend with a number of abiotic stressors throughout their lives. The adverse environmental circumstances are something that plants must constantly deal with (such as soil salinity, drought, heat, cold, flooding and heavy metal contamination). One of the main abiotic stressors causing dangerous health consequences in animals and plants is heavy metal poisoning. They may directly affect the processes of growth, senescence, and energy synthesis due to their high reactivity. For ecological, evolutionary, nutritional, and environmental reasons, heavy metals are major environmental contaminants, and their toxicity is an issue that is becoming more and more important. Any metallic element that has a relatively high density and is hazardous or deadly even at low concentrations is referred to as a "heavy metal."(Maksymiec, n.d.; Nagajyoti et al., n.d.) As rare elements that naturally exist in soil, heavy metals are distributed throughout the ecosystem via agricultural activities, waste disposal, metallurgy, and industrialization. In contrast to their density, the heavy metals' chemical characteristics have the greatest impact. Lead (Pb), cadmium (Cd), nickel (Ni), cobalt (Co), zinc (Zn), chromium (Cr), iron (Fe), arsenic (As), silver (Ag), and the platinum group element are examples of heavy metals.(Bot et al., 2013) Elaborating the role of heavy metals their stress &interactions to the plants the Cadmium is a metal disturbing natural sources in a very effecting way. Cadmium is also regulating in ecosystem by anthropogenic activities & different other sources.

1.2 Cadmium Stress & Soil Interactions

Two of the most significant soil components that affect Cd availability are pH and organic matter (Kirkham, 2006) Soil pH was thought to be the most significant of the several soil characteristics that were known to influence the availability of Cd.

Numerous studies revealed a linear relationship between soil pH and Cd uptake: if other soil parameters are constant, a decrease in soil pH causes an increase in the concentration of Cd in plants. Under field circumstances, a wide range of changeable meteorological and soil factors may have an impact on how much Cd plants absorb.(Li et al., 2005) Field tests with rice plants grown in China's acidic red soil revealed that the Cd content of the grain was 0.36 mg kg-1 at a soil pH of 4.95 and 0.43 mg kg-1 at a pH of 6.54. Results from greenhouse research are difficult to apply to actual field settings. The findings of several greenhouse and pot tests also demonstrated that soil pH had an impact on plant absorption of Cd. The genotype of the plant also affects how much Cd accumulates.(Li et al., 2005) The biological activity of cadmium is mostly controlled by the soluble complexes it produces when combined with chlorides, hydroxyl, sulfhydryl, and thiol groups. Because soil is such a complicated system, it is difficult to draw broad conclusions about how ligands in solutions affect the sorption of Cd and, therefore, the availability of Cd. Various studies, however, indicate that the bioavailable percentage of Cd in soil declines with time and with increases in pH, clay content, and organic matter levels.(Vig et al., 2003)

1.3 Cadmium Uptake in Plants & their biological Functions

Cadmium (Cd) is regarded as one of the most phytotoxic heavy metal contaminants.

The heavy metal Cd, which has been listed No. 7 among the top 20 poisons, is often discharged into the arable soil by industrial operations and agricultural practices1.

2 Except for Cd-hyperaccumulators, which can withstand Cd concentrations of 100 g Cd g-1 leaf dry weight, most plants are poisonous to Cd at concentrations more than 5-10 g Cd g-1 leaf dry weight. 4-6. Power plants, heating systems, metal-working enterprises, waste incinerators, urban traffic, cement factories, and as a by-product of

phosphate fertilisers all discharge large amounts of the common heavy metal cd (density=8.6 g cm3) into the environment. Due to its high solubility in water, it is quickly absorbed by plants, representing the primary entry channel into the food chain and seriously affecting human health. Even at low concentrations, mineral nutrition and homeostasis in plant shoot and root growth and development are negatively impacted by root absorption and transport to the vegetative and reproductive organs. Cd reduces the amount of carotenoid and chlorophyll and harms the photosynthetic machinery. Since it is not present in nature in a pure form, cd is a rather uncommon element. Cadmium quickly transforms into cadmium oxide in the air. Cadmium carbonate, hydroxide, sulphide, or chloride are the products of its simple reactions with carbon dioxide, water vapour, sulphur dioxide, sulphur trioxide, or hydrogen chloride. To carbon and other atoms that are more electronegative, Cd may form weak bonds. Volcanic eruptions and rock weathering are caused by changes in the earth's crust. Different mechanisms within the biosphere cause the Cd to be translocated. Around 25,000 t of Cd are naturally discharged into the environment each year. Despite having a high phytotoxicity, Cd is readily absorbed by plant roots, transferred to tissues above ground, and enters the food chain where it may pose major risks to human health. Due to its high mobility in the phloem, 16 Cd can accumulate in all plant parts, resulting in stunted growth, chlorosis, and leaf epinasty, as well as changes to the chloroplast ultrastructure, photosynthesis inhibition, inactivation of CO2 fixation enzymes, induction of lipid peroxidation, inhibition of pollen germination and tube growth, as well as disruptions to the nitrogen (N) and sulphur (S) metabolism (Gill & Tuteja, 2011). Rock mineralization processes have the potential to release Cd in places with little human pressure (Sanità Di Toppi & Gabbrielli, 1999). Cd is often ingested or inhaled by people and is then absorbed into the body. The majority of Cd that is ingested and enters

the body originates from foods that are grown on land. According to estimates, 98% of the Cd that is consumed comes from eating terrestrial foods, 1% from eating aquatic foods like fish and shellfish, and 1% from drinking water that contains Cd. It functions as a mitogen and encourages the growth of cancer in certain tissues. Additionally, it promotes cell division, prevents DNA repair, and prevents apoptosis. On the one hand, it causes cell death, which causes renal tissue damage. Cadmium causes apoptosis in cell culture systems at low concentrations, while necrosis is seen at higher concentrations. When cadmium is exposed to the environment, it also impairs renal function.

1.3.1 Uptake mechanisms, translocation, and sequestration of cadmium

Depending on the species and cultivar of the plant, the physico-chemical makeup of the soil, and the amount of Cd absorbed by the soil, different plants will respond differently to elevated soil Cd levels (Benavides et al., n.d.). Cd is efficiently transported inside the plant in the form of metalloorganic complexes. The temperature, redox potential, concentration, pH, and concentration of different components in the soil all affect how bioaccessible Cd is .(Hasan et al., 2009a) Mechanisms involving Cd uptake by plant roots often include competition with other nutritional minerals with comparable chemical properties for absorption sites (Clemens et al., n.d.; Hasan et al., 2009b; Wong & Medrano, 2005). Zn and Cd's active absorption have a negative association with each other in lettuce roots. (Dalcorso et al., 2010). Cd first penetrates plant roots where it destroys the root structure and morphology of the plant. Electrochemical potential differential between Cd activity in the cytosol and in the root's apoplasts controls cadmium absorption across the plasma membrane of root cells.(*Hasan: Cadmium-Induced Changes in the Growth and... - Google Scholar*, n.d.) While the functional groups in the root cell walls, such as carboxyl groups, are gradually de-protonating with an increase in

soil pH, cations accumulate in the plant root apoplast, which is controlled by cell wall exchange properties and is pH-dependent at the initial level (i.e., metal ion adsorption from the soil solution). (Ismael et al., n.d.-a). The symplastic route is substantially slower than the apoplastic pathway and is an active process that depends on metabolic activity. (Abedi & Mojiri, 2020; Begum et al., n.d.). However, the significance of each step changes depending on the type and concentration of the metal and other ions. Cadmium travels by the apoplastic channel across the cell membrane of the root cell (Ismael et al., n.d.-a). The sequence of the Cd particles in a plant is normally roots > leaves > fruits > grains, with only a tiny amount reaching the plant's above-mentioned components (i.e., leaves, stems, and reproductive organs). Cd absorption in roots may take place as either inorganic compounds (such as Cd2+SO4, CdCl+, and CdCl2) or organic forms (i.e., complexes of phytometallophore). Due to its great mobility and assimilability, cadmium enters plants from the roots and is subsequently transported by transporters or ascent of sap through shoots and into vascular bundles (i.e., phloem and xylem). Cadmium enters plant xylem vessels by the symplastic route.(Dong et al., n.d.). In essence, a number of variables, such as plant species, agronomic techniques, environmental factors, and soil qualities, influence Cd absorption in various plant sections(Yang et al., n.d.)

1.4 Phytochelatins (PCs) and their role in Cadmium Decontamination

There are number of Mechanisms through which Plant respond to toxicity of Heavy Metal. Such Response contain Immobilization, exclusion, chelation, and compartmentalization. In all these defensive Systems there is one remarked known as the chelation Of Heavy Metals. This has done by number of Ligands of peptide Family called Phytochelatins. In Molecular biology & genetics methodologies several results are shown to appreciate the advancement in Biosynthesis of Phytochelatin. Specifically,

those genes which encodes for phytochelatin synthase enzyme have been split up from Yeast & Plant species. There are number of retorts which have been comprehensively reviewed for plants subjected to cadmium stress. A pervasive mechanism for heavy-metal decontamination is the chelation of the metal ion by a ligand. Various metal-binding ligands have been stated in plants which show their exact role in detoxification of heavy metals stress. For example, organic acid, amino acids, peptides & polypeptide etc.

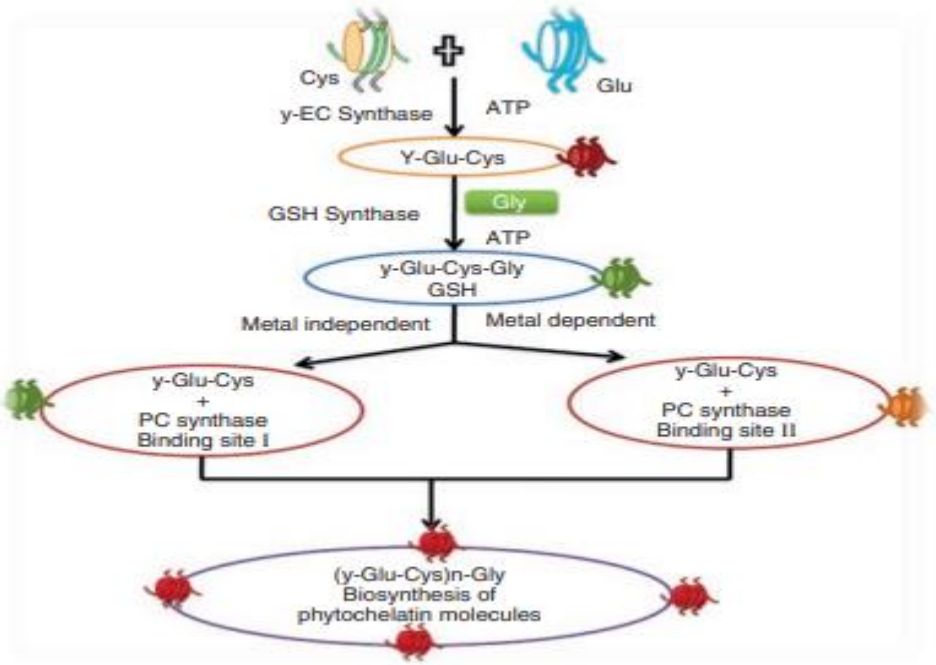

Figure 1: Biosynthesis Pathway for PCs Molecules under stress Conditions(Ahmad et al., 2019)

A particular enzyme called g-glutamylcysteine synthase catalyses the first step of PC biosynthesis, the production of g-glutamylcysteine from L-glutamate and L-cysteine. GSH for mation takes place when g-glutamylcysteine is given more glycine moieties. In the presence of adenosine triphosphate (ATP), glutathione synthase enzymes perform this process. The creation of PCs in the presence of the PC synthase enzyme is

the last stage in PC biosynthesis after GSH generation (Fig). This enzyme is known as g-Glu-Cys dipeptidyl transpeptidase because it transpeptides a g-Glu-Cys moiety of GSH. Glycine moieties are, however, separated from GSH before to the transpeptidation activity, and the resulting transpeptidation reaction then forms a peptide link with either PC2 or GSH to generate np1 oligomer.(Ahmad et al., 2019)

Phytochelatins (PCs) were firstly identified as the Cd-binding peptides in Schizosaccharomyces pombe and particularly seen to play similar function in Plant species. Early analyses demonstrated PCs consisted of only the three amino acids: Glu, Cys and Gly with the Glu, and Cys residues linked through a g-carboxylamide bond. In Plants Phytochelatins are heavy metal binding peptides with structure (γ-Glu-Cys)n-Gly structure ($n = 2–11$). (g-Glu-Cys)n-Gly (g-Glu-Cys)n-Gly (g-Glu-Cys)n-Gly (g-Glu-Cys)n-Gly (g-Glu-Cys)n-Gly (g-Glu-Cys)n-Gly (g-Glu-Cys).It has been speculated that the number may be as high as 11, although it is more likely to be in the tens of thousands.Range of 2–5 points Though, in a few plants, the C-terminal Gly can be substituted serine as (γ-Glu-Cys)n-Ser, glutamine as (γ-Glu-Cys)n-Gln, glutamate as (γ-Glu-Cys)n-Glu and alanine as (-γ-Glu-Cys)n-β-Ala. Plants and microbes have both been found to contain PCs. They are an enzyme manufactured from GSH by phytochelatin synthase (PCS). (Kühnlenz et al., 2014) The Combination of these organic compounds and Plant is very Valuable tactic for the removal of contamination in environment using Plants. Scientist also have reported some significant organic chelation in plant by citrate and malate as a response to aluminum stress. From all the organic and amino acids an important one that is “His” was also reported as a chelating element within cell and its xylem sap as well.(van den Berg et al., 1998). Notwithstanding the detection and purification of PC synthase over a decade ago, the estrangement of the associated gene and, as a result complete knowledge of the process

of PC biosynthesis remained tenuous until recently. Depending on the interaction and chemistry of certain metals, many metals may be poisonous to plants at once and may act independently, antagonistically, or synergistically (Chung et al., 2021) Wheat plants exposed to Cd and Pb had their phytochelatin and glutathione production closely watched as a sign of heavy metal stress (Chibuike & Obiora, 2014). These results imply that the performance of PCs and GSH as significant stress indicators depends on the mutual interactions of metals. When compared to other metals, Cd has a higher contact with PCs; for instance, the T-DNA line and Tos 17 mutants of rice accumulated less Cd and more As, demonstrating that these two metals interact with PCs via distinct methods. PCs are created and amassed underground, together with the components of aerial plant life. They are created and accumulated initially in the roots, according to several studies (Pollard et al., 2009) shown that exposure to Cd increased the expression of the PC synthase gene, which was largely identified in the roots of the Brassica parachinensis cultivars Lvbao-701 and Chixin-4. Lvbao-701 roots showed a considerably higher induction than Chixin-4 roots. Because of this, it is possible that Lvbao-701 cultivars exhibit less Cd translocation to shoots and greater resistance to Cd stress than Chixin-4 cultivars. This might be because to the increased Cd accumulation and overexpressed PCs production in the roots. There has been much research on phytochelatins and antioxidant systems as mediators of cd detoxification in plants (Pollard et al., 2009; Raj & Maiti, 2021). Previously, Chen et al. (2008) In his investigation, he confirmed the interaction between phytochelatins and antioxidant mechanisms. They discovered that Brassica chinensis had higher PC production and antioxidant activity, both of which improved the plant's ability to withstand Cd stress. The higher levels of GSH under Cd stress may be explained by an increase in PC biosynthesis enzymes as well as GSH-related enzymes including glutamylcysteine synthetase, glutathione

synthetase, and glutathione reductase. The peroxidases, which include ascorbate peroxidase (APX), glutathione peroxidase (GPX), and guaiacol peroxidase (GOPX), are gifted and fortunate antioxidant enzymes that play a particular role in metal stress tolerance and assist in determining the sublethal metal toxicity in plants (Ismael et al., n.d.-b)

1.5 Foundation of Soil clean-up strategy "the Phytoremediation & Phytoremediator Plants"

When HMs present in excess, they might become poisonous. Pb, Cd, As, and Hg are non-essential heavy metals that are extremely toxic and have no known biological purpose in plants (Fasani et al., 2018) These substances may also cause environmental pollution, adversely affect a number of physiological and biochemical processes in crop plants, and lower agricultural productivity (Fox & Guerinot, 1998)They pose a serious hazard to human health because they may accumulate in the human body via biomagnification and infiltrate the food chain through crops (Rimm et al., 2017). Therefore, it is essential to implement remediation strategies to reduce the amount of polluted land while also preventing the entry of heavy metals into terrestrial, atmospheric, and aquatic habitats(Hasan et al., 2009a). To date, several different remediation techniques have been created to restore soil that has been polluted with heavy metals. The majority of these actions rely on mechanical or physio-chemical methods, such as soil incineration, excavation and landfilling, soil washing, solidification, and electric field application (DalCorso et al., 2019a). There are reported drawbacks to these physicochemical techniques, including their high cost, inefficiency when contaminants are present in low concentrations, irreversible changes to the physicochemical and biological properties of soils, which deteriorate the soil ecosystem, and the introduction of secondary pollutants (Amin et al., n.d.; DalCorso et

al., 2019b). The ecological impact of HM is modified by soil microbes and plants in a naturally occurring process known as remediation of HM in soils (Park et al., 2011). However, two main scientific methods—the washing method and HM reduction using in-situ techniques—have been utilized to remove or extract HM from soil. The latter method has been used to lessen the toxicity of HMs in various soils(Duan et al., 2009). Researchers from all over the globe have worked hard to develop different organic amendments or additives, and/or phytoremediation, either alone or in combination, for HM remediation. Therefore, there is a need to create remediation methods that are economical, effective, and environmentally friendly in order to recover soil that has been polluted by heavy metals. The phytoremediation strategy as in situ HM remediation is believed to be affordable and environment-friendly (Adediran et al., 2015)

1.6 Phytoremediation

A method known as phytoremediation uses plants to absorb and eliminate toxic elements from the environment or to reduce the bioavailability of those contaminants in the soil(*Chelate-assisted Phytoextraction of Lead from Contaminated Soils,* 1999). Through their root systems, plants are capable of absorbing ionic substances from the soil, even at low quantities. In order to collect heavy metals and control their bioavailability, plants extend their root systems into the soil matrix and create rhizosphere ecosystems, which stablize soil fertility and allow for the reclamation of contaminated soil(Ali et al., 2013). Even at low quantities, ionic substances in the soil may be absorbed by plants via their root systems. In order to absorb heavy metals and control their bioavailability, plants stretch their root systems into the soil matrix and create rhizosphere ecosystems, recovering the contaminated soil and maintaining soil fertility (Ali et al., 2013; Cooper et al., 1999)

1.6.1 Hyperaccumulator & Non yperaccumulator Plants

Plants known as hyperaccumulator plants are those that absorb and tolerate more metal ions without displaying any outward symptoms.(Pollard et al., 2009). Some 450– Thlaspi is one of 500 plants that have been discovered as hyperaccumulators. Pb, Ni, Cd, Zn, and *caerulescens* accumulate; Arabidopsis halleri accumulate *Alyssum bertolonii* can absorb Ni and Co, Cd and Zn but not Pb, and certain other plants from other families, such *Caryophyllaceae, Fabaceae,* and *Poaceae,* Plants belonging to the *Lamiaceae, Asteraceae, Cunoniaceae, Cyperaceae,* and many more may also take part in the accumulation of heavy metals. Plants absorb metal particles by the roots and root hairs that generate forces (cohesive and adhesive) through which pollutants can be absorbed from contaminated soil and water. Various agricultural plants, including *Zea mays, Pteris vittata, Astragalus bisulcatus, Eichhornia crassipes, Euphorbia macroclada, Berkheya coddii, Alyssum and Thlaspi,Euphorbia macroclada, Phragmites australis, Phytolacca americana, Astragalus bisulcatus, Cardamine hupingshanensis, Sesbania drummondii, Sedum alfredii*.According to reports, *Iberis intermedia's* shoots are particularly good in phytoextraction of Cu, Ni, Cd, Zn, Cr, As, Mg, Se, and Ti. *Salix daphnoides* (Bleu), *Salix purpurea*, *Salix triandra,* and *Salix dasyclados* (Loden) are the five willow tree species that accumulate Zn and Cd at a higher concentration in their shoots. *Salix schwerinii* (Christina), *Salix fragilis* (Belgisch Rood), *Salix triandra* (Noir de villaines), and *Salix triaddra* (Bleu)(van Ginneken et al., 2007). A plant called *Lolium multiflorum* was recently discovered and is utilized for the phytoremediation of Mn, Cu, Pb, and Zn (van Ginneken et al., 2007). A excellent accumulator of Cr and Cd from ponds is the aquatic plant *Hydrilla verticillata,* which has a high potential for heavy metal absorption(Brown et al., 2004; van Ginneken et al., 2007). Due to its great capacity for hazardous metal buildup, the

perennial plant Sauropus androgynus has the potential to phytoremediate soil and may be exploited economically(Adenot et al., 2006). Another plant called *Pannicum virgatum* (switchgrass) is known to accumulate Zn, Cr, and Cd in heavy metals. According to reports, *Pannicum virgatum* (switchgrass) may accumulate heavy metals including Zn,Cd and Cr.

Among all the phytoremediator plants "*Brassica juncea*" is a very Potential candidate for Phytoremediation & Hyper accumulation of Cadmium stress. Due to its characteristics like faster growth, higher biomass, and hype tolerance to heavy metals, it is also regarded as a potential candidate for the easy and affordable removal of heavy metals, particularly lead (Pb, Cd, Zn, etc.), from contaminated aquatic and terrestrial areas. As a result, it plays a crucial role in phytoremediation (Singh & Fulekar, 2012). This crop recently surpassed other brassica crops in terms of area under cultivation and industrial relevance thanks to its capacity to withstand a range of biotic and abiotic challenges.

1.7 *"Brassica Junceae*" an Excellent Candidate for removing the Heavy metal toxicity from Soil

With more than 338 genera and 3,079 species, the family Brassicaceae is primarily one of the most economically significant families in the world. Its uses range from providing food, fodder, and medicine to providing a variety of environmental functions. *Brassica juncea,* also known as the Indian or Brown mustard, is one such member of this family that originated as a result of interspecific crosses between Brassica nigra and Brassica rapa, with Central Asia serving as the primary centre of diversity. From this region, this amphidiploid crop was primarily introduced to other parts of the world by humans.

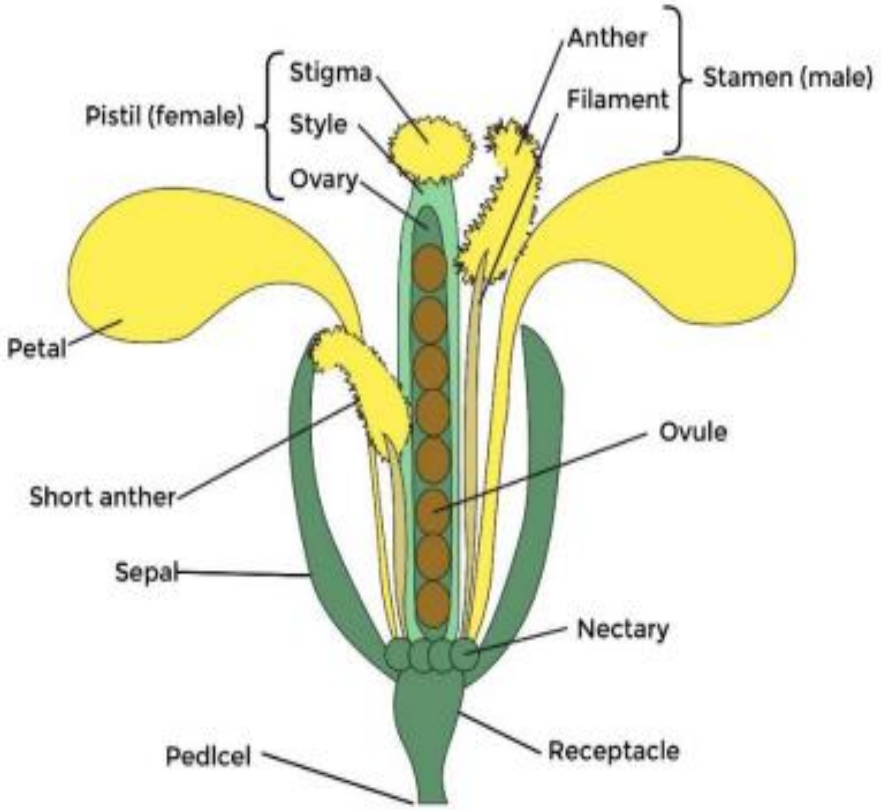

Figure 2: Morphology of Mustard Plant

Due to its resilience for a variety of biotic and abiotic stress conditions, this annual to biennial plant with light green leaves has naturalized in numerous settings. In terms of world oil output, the main oil-producing crop is in third place, after palm and soya beans, but along with B. rapa and B. napus, it is a significant source of canola oil. The primary chemical of this plant, glucosinolates, not only gives its seed oil a distinct pungent flavour and functions as a biocontrol agent for weeds and illnesses carried by the soil, but it also has a chemopreventive character and limits malignant growths. Additionally, a significant number of folk medicines, particularly in the ancient systems of medicine of China and India, are heavily dependent on this plant crop.Due to its hypertolerance and hyperaccumulative power, which aids in the removal of hazardous

heavy metals like Cd, Pb, Zn, and others from highly polluted soils, this plant is also useful in maintaining a clean environment. Scientists are using techniques like intra- or inter-hybridization and metabolic engineering to further increase its commercial value. In terms of world oil output, the main oil-producing crop is in third place, after palm and soya beans, but along with B. rapa and B. napus, it is a significant source of canola oil. The primary chemical of this plant, glucosinolates, not only gives its seed oil a distinct pungent flavor and functions as a biocontrol agent for weeds and illnesses carried by the soil, but it also has a chemopreventive character and limits malignant growths. Additionally, a significant number of folk medicines, particularly in the ancient systems of medicine of China and India, are heavily dependent on this plant crop. Due to its hypertolerance and hyperaccumulative power, which aids in the removal of hazardous heavy metals like Cd, Pb, Zn, and others from highly polluted soils, this plant is also useful in maintaining a clean environment. Scientists are using techniques like intra- or inter- hybridization and metabolic engineering to increase its economic value.

The potential for hyperaccumulators to accumulate considerable amounts of toxicants, such as heavy metals and pesticides, exists (Diana et al. 2007).Heavy metals, herbicides, and other pollutants from contaminated soils may be removed, sequestered, and neutralised by Brassica juncea.Brassica juncea's capacity for sequestering substances depends on the mobility of hazardous substances, plant characteristics, and factors of crop management. Aspects of crop management include intercropping, improving plant development and soil metal dissipation, adding organic matter, and including legumes for enhanced phytoextraction through Indian mustard.

***Table 1**:Taxonomy of Brassica juncea*

KINGDOM	**PLANTAE – (VEGETAL)**
SUBKINNGDOM	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)

With the exception of northern and polar regions, where the average temperature is below 6°C, this plant is extensively distributed. This crop is more prevalent in subtropical parts of Asia because of its strong heat and drought tolerance ability, as opposed to the other two brassica oilseed crops, Brassica napus and Brassica rapa, which are more frequent in temperate locations. Two varieties of this crop, the vegetable type, and the oilseed type, are often produced depending on their intended use. The majority of vegetable varieties with edible root, stem, and leafy components are grown in Asian nations, particularly China, which is also recognized as the key sscenter of varietal differentiation of this crop since the largest degree of variety is found there.(Lim, 2015; *Therapeutic Potential of Mustard Crop | Request PDF*, n.d.)

1.7.1 Cadmium Stress and *Brassica Juncea*

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 (Reeves et al., 2018). It is also observed that in hyperaccumulator plants, the concentration of chlorophyll increases and decreases in typical plants.(Sevugaperumal R, 2015)

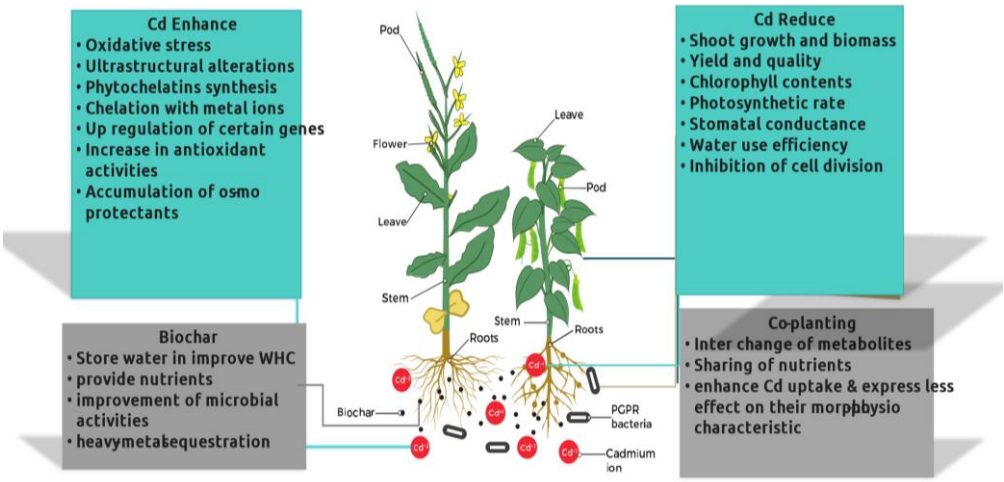

Figure 3: Showing the effects of Biochar & co-planting that's aids in the mechanisms of Phytoremediation.

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)

1.7.2 Changes at Molecular Level

Chlorosis, growth inhibition, water imbalance, phosphate and nitrogen insufficiency, impaired manganese transfer, and accelerated senescence are only a few of the phytotoxic symptoms caused by cadmium. Due to its strong affinity for sulfur-containing peptides and proteins, cadmium induces the generation of oxygen free radicals or reduces enzymatic and non-enzymatic antioxidants, which both result in oxidative stress. Cope up system of the plant to reactive oxygen species constitutes enzymes like SOD, CAT, APX, GPX, GR and other antioxidant compounds such as GSH, carotenoids etc. Moreover, *Brassica juncea* being an excellent hyperaccumulator plant increase its defensive mechanism against the heavy metal stress particularly cadmium stress. Growth factors showed stunted growth and at gene level different types of genes are regulated either helping the plant to excellently activate their defensive mechanisms. PCs do their chelation, by activating the hormones transcription factors and all other metabolic process in response to cadmium stress.(Mishra et al., 2006)

Aims and Objectives

This study aims to explore the effect of Cadmium stress in *Brasssica juncea* along with biological combination of biochar, PGPR, and Co-planting with the following Objective:

- To investigate the Gene Expression of Identified cadmium stress responsive genes in *Brassica juncea* under cadmium stress with the aid of biological combinations biochar, co-planting, PGPR bacteria

Chapter 2: Literature Review

(Pallavi Sharma & Dubey, 2006) investigate that, the amount of harmful heavy metals in soil steadily rises over time. High quantities of HMs have the potential to impair plant development and metabolism. Diverse techniques have been developed to remove these metals from soil, but the most of them are pricy, harmful to the environment, or sluggish. In the meanwhile, soil remediation has been done using chemical, physical, and biological techniques. There are several substances used in chemical cleanup. Fortunately, not all HMs can be destroyed by a single chemical (Chaney & Oliver, 1996).

Chemical cleanup is both difficult and dangerous for soil-dwelling plants and bacteria. Physical procedures need a lot of time and equipment for this since they are now an affordable kind of rehabilitation. The scientist described a novel technique called "bioremediation." According to A. J. Baker, McGrath, Reeves, and Smith (2020), bioremediation cleans the environment and recovers polluted places.

According to research done by (Viehweger, 2014), the rate of bioremediation is directly related to plant Biomass of the plants is correlated with growth and overall remediation. The increased transfer of Cd from root to shoot in Brassica juncea greatly lowers the amount of metal in the soil. When plants acquire a lot of Cd, it significantly affects their development and metabolism.

Certain unique metabolites are created in hyperaccumulation plants by genes that have been activated in the presence of Cd ions. These substances are referred to as chelators. On a cytosolic level, they aid in metal detoxification.

(A. Baker, McGrath, Sidoli, & Reeves, 1994) attest that the buildup of HMs often occurred in the shoot as opposed to the root. (2013) (Ucer, Uyanik, & Kutbay). Their research plant has a 100 mg kg-1 Cd accumulation capability. While (Thijs, Langill, & Vangronsveld, 2017) claimed that some proteins and metabolites that function as chelators improve the high accumulation capacity of plants, in line with the study of the overexpression transport system. Around 720 plant species have been identified as hyperaccumulator plants, which are able to collect several heavy metals simultaneously. The research that follows proposes seven plants that may accumulate cadmium (Reeves et al., 2018). According to (Hörger, Fones, & Preston, 2013), the buildup of metal ions triggers defence mechanisms against infections and herbivores. the movement of Cd via the phloem transport pathway from roots to shoots, fruits, seeds, and leaves (Turgeon & Wolf, 2009).

Plants have low molecular masses for metal ions linked to ligands and proteins, while a single minute. There are several free metal ions present. Numerous metal-binding ligands are produced by hyperaccumulator plants, including the following thiol group compounds: nicotianamine (NA), glutathione, Phyto-chelators and Metalothioneins (MTs) (PCs). Histidine (His) is an amino acid that contains hyperaccumulation by functioning as nitrogen donor ligands in the roots, according to research by (Krämer, Cotter-Howells, Charnock, Baker, & Smith, 1996). A core compound including cadmium (Cd), nickel (Ni), and zinc has been discovered (Zn).

Another research finds that nicotinamide (NA) is a metal chelator (Stephan & Scholz, 1993). present in all plants. S-adenosylmethionine (SAM) is trimerized under the supervision of the enzyme NA synthase (NAS) (NAS). Nicotianamine is involved in the transfer of micronutrients in plants that help the body utilise iron. According to other findings, NA binds differently to Cu and Cd plants. The plant's NAS expression

determines how much Cd will accumulate. Plant growth, photosynthesis, food production, oil quality, morphology, and physiology are all negatively impacted by cadmium stress.

Metallothionins (MTs), which are found in all plants and animals, are the other most important metal detoxifying ligands. Under typical physiological circumstances, their major job is to keep the equilibrium of metal ions in hyperaccumulator and non-hyperaccumulator plants. The three most significant forms of the MTs family (MT-I, MT-II, and MT-III) express when various HMs are present. Under the influence of Cd ion stress, MT-III has been demonstrated to operate as an activator (Jack et al., 2007).

Numerous studies have shown that glutathione (GSH) is essential for maintaining cellular ROS equilibrium and that it also plays a role in the detoxification of plant metals. Thiol ligands are known to have a small involvement in hyperaccumulation, according to earlier research. 2007 (Freeman & Salt) His research revealed enhanced assimilatory sulphur pathway activity, mitochondrial serine acetyltransferase (SATm), and excessive GSH synthesis. Arabidopsis that expressed TgSATm also shown enhanced metal resistance (van de Mortel et al., 2008). They discovered that exposure to Cd boosted the formation of foliar and root GSH in metal hyperaccumulators as well as sulphate synthesis and GSH metabolism in the plant T. caerulescens (also known as N. caerulescens).

Biochar supplies nutrients to soil (carbon, nitrogen, calcium, and phosphorus). Due to its porous nature, which keeps the plant hydrated for longer than anticipated, it also aids in water storage. Different forms of biochar exist based on physicochemical characteristics (pore structure, surface area, amount of phosphorus, and functional groups). Compared to biochar generated at a carbonization degree, high pyrolysis

temperature biochar needs a big surface area, enormous porosity, normal pH, and low cation exchange capacity (CEC). According to Trampczynska, Küpper, Meyer-Klaucke, Schmidt, and Clemens (2010), lignin, cellulose, and moisture are some other factors that affect the characteristics of biochar. As a nutritional enhancer for young plants, biochar is created by heating various plant materials, such as palm kernel shells, maize cobs, cocoa pod husks, rice husks, and wheat husks, to temperatures between 350°C and 650°C.

To safeguard the public health against cadmium-contaminated food, several biological adjustments are needed. Cadmium has an impact on both plants and people. World Health Organization and Food and Agriculture Organization (FAO, 2017). According to studies, the new maximum Cd limit for vegetables is 0.05–0.2 mg kg 1. Previous investigations have shown that Cd buildup is not affected by acidic soil or pH values of 3–5.5. (Huang et al., 2017). Limiting acidic soil will lessen Cd uptake by plants (Zhipeng Wu et al., 2014; Zhichao Wu et al., 2016). The use of biochar enhances the characteristics of the soil and reduces its acidity. The activation of biochar's functional groups, such as carboxylic acid (-COOH), -C=O, and inorganic ionic PO4 -, has also been proven to reduce the mobility of harmful heavy metals in soil.

The effects of various biochar features on Cd and Pb phytoavailability in hazardous metal-polluted soil types and their influence on metal absorption by vegetable crops have not yet been fully understood by study. This research sought to determine how soil phytoavailable Cd concentrations and plant uptake in three distinct soils were impacted by biochars produced from three different feedstocks. We predicted that different biochar feedstocks could change its physiochemical properties, leading to different modes of action in both acidic and alkaline soils. Plant uptake and soil Cd and

Pb phytomobility may be impacted by biochar's potential to shift soil pH. (Houssou et al., 2022).

While Cd stress also has a deleterious impact on soil microorganisms, rhizosphere soil has a high concentration of bacteria that are resistant to Cd stress. Various Cd-resistant microorganisms have the ability to increase the absorption of Cd by plants. Another theory is that Cd-resistant bacteria influence plant Cd absorption by increasing or decreasing it. Numerous organic acids that aid in the solubilization of minerals have been found in diverse plants, including indole-3-acetic acid (IAA) and 1-aminocyclopropane-1-carboxylate (ACC). In the top portion of the plant where microorganisms are present, these organic acids aid in the absorption of Cd (Z. Deng & Cao, 2017; Zhipeng, Weidong, Shenglu, & Shaohua, 2016).

Microbes primarily lower the pH of the soil and decrease the absorption of Cd (Jing et al., 2014). Additionally, by assisting each other to decrease ROS generation in plants, microbes improved Cd tolerance in Brassica species (Panwar et al., 2011). Microbes known as plant growth-promoting rhizobacteria (PGPR) are widely utilised in the removal of heavy metals (Pramanik, Mitra, Sarkar, & Maiti, 2018). Plants and PGPRs work together symbiotically to increase plant growth, competition for nutrients and space, and tolerance to environmental stresses. These bacteria get their nutrition from root exudates and assist plants in a variety of ways, including by producing growth hormones (Miransari & Smith, 2014).

Because they can effectively transfer heavy metals from roots to shoots and tolerate high soil metal concentration, brassicales have been widely investigated in phytoremediation. Additionally, they practise rapid growth and generate a lot of biomass (Marchiol, Assolari, Sacco, & Zerbi, 2004).

According to empirical study, certain Brassica plants accumulate more heavy metals than others, which shows that they have a high tolerance for heavy metal stress. The sole issue with utilising brassica vegetables for phytoremediation is the possibility that brassica oil might become polluted owing to high quantities of metal accumulated in the seed, posing a threat to the food chain and the environment (Park, Kim, & Kim, 2012).

The danger of contamination in the food chain is nonexistent, however, according to experimental study (Park et al., 2012), which shows that heavy metals are not incorporated into the oil during the extraction process. A research has also been done on the use of PGPRs to enhance accumulator plant heavy metal absorption. This study attempted to determine how Cd built up in B. oleraceae under controlled circumstances. Additionally, the plants were exposed to PGPR strains and cultivated at various Cd concentrations to see how microbial strains impacted plant absorption of heavy metals. In a controlled setting, experimental plants can withstand the effects of Cd poisoning (Asad, Rehman, Ahmad, & Umer, 2018).

An old agricultural technique known as intercropping or co-planting involves two or more crop species growing together and coexisting for a certain amount of time (Brooker et al. 2015). Co-planting is a term used to describe how plants work together to increase each other's capacity for phytoremediation by sharing resources such nutrients, water, soil space, and light (Wu et al., 2007; Sun et al., 2018; Zeng, Guo, Xiao, Peng, Feng, et al., 2019). Previous research has mainly concentrated on the patterns of coplanting of crops and hyperaccumulators in the soil to control the levels of heavy metal pollution and lessen heavy metal accumulation in crops. These studies on Thlaspi caerulescens and Ryegrass (Jiang et al., 2010), Sedum plumbizincicola and "welsh" onion (Allium cepa) (S. Wang, Wei, Ji, & Bai, 2015), and Solanum nigrum and

"welsh" onion (Allium cepa) (L. Deng et al., 2016) demonstrate some advantages for the environment against heavy metals.

The study also discovered that co-planting with hyperaccumulator plants reduces the accumulation of HMs, which helped in these tests with the remediation of polluted soil and safe agricultural output.

The metal (loid)-tolerant plants Morus alba and Broussonetia papyrifera may be co-planted with Pteris vittata to enhance planting structure, remediate polluted soil at different depths, and boost phytoremediation effectiveness (Zeng et al., 2019).

Co-planting is also said to have major impacts on competition and facilitation, which are always present at the same time (Kutrowska et al., 2017). Different co-planting patterns have different effects on plant development and metal accumulation depending on the kind of metal, its amount, and how it interacts with different plant species (Ling, Shen, Gao, Gu, & Yang, 2007).

Arbour trees provide greater ecological and monetary advantages than bushes do. The phytoremediation of co-planting in arbour trees and its hyperaccumulators capabilities toward metal-polluted soil are now little understood. According to Rizwan et al. (2016), Solanum nigrum, a perennial weed that can withstand high concentrations of Cu, Pb, Ni, and Zn, is categorised as a Cd-hyperaccumulator. In recent decades, Quercus nuttallii and Quercus pagoda have been widely imported and used in greening in China's subtropical zones. These tall, straight arbour trees have beautiful crown shapes and fall leaf colours, and they are also extremely resilient to abiotic stressors brought on by heavy metals (Suresh Kumar, Dahms, Won, Lee, & Shin, 2015). It is yet unknown how well heavy metals can be removed by phytoremediation when Q. nuttallii and Q. pagoda are co-planted.

Chapter 3: Materials and Methods

3.1. Soil and seed collection

At the National Agriculture Research Center (NARC) in Islamabad, Pakistan, the department of research in oil and seed provided the fresh seeds of B. Juncea that were needed for the experiment. The sandy loamy soil was also taken from NARC's peanut farms for use in plant development.

3.2. Germination of seeds

The surfaces of the seeds were sterilised for one minute with 70% ethanol, then washed with distilled water. Then, inside the safety cabinet, seeds were spread out on filter paper to allow the most ethanol to evaporate. The seed was aligned on UV-sterilized germination paper in a germination box and wrapped in aluminum foil after it had completely dried from the ethanol. This limited the interaction with light and moved the seed to a dark location at a temperature of 25°C-28°C for 48 hours in order to break seed dormancy. B. Juncea seeds successfully germinated after three days, and they were prepared for soil transformation. Equally likely to germinate seeds were moved into pots.

3.3. Soil examination

At the National Agriculture Research Center (NARC) in Islamabad, soil analysis was done. The soil has a sandy loam texture, a pH of 7.78, and a 0.49 percent organic matter content. The respectable saturation percentage (SP) is 32. 2013 (Estefan)

3.4. Prepared soil

The soil was prepared for plant development utilising eight distinct treatments. The first treatment, which does not include cadmium stress, was chosen as the control group. The other seven treatments, which do contain cadmium stress, also contain other organic variables, such as the bacterial strain known as plant growth-promoting rhizobacteria (Rhizobium leguminosarum), Biochar (Wheat Husk), and co-planting (Vigna radiata), which is shown in the given table. Variables provide the concentrations listed below: - 1.2% of biochar, 0.1% of rhizosphere, and one legume plant (Vigna radiata) as a co-plant variable. There were two batches; the first batch had seven replicas, and the second batch had ten. Each pot included one kilogramme of soil. To the prepared 10 millimole Cadmium chloride (CdCl2) solution, 10 millilitres of solution were added to each pot for the necessary treatments. Following the addition of the cadmium stress solution, water is added to the soil and the solution each day for 10 days at a temperature of 25 °C to mix the two together. After 10 days, seedlings were planted in the ground, and growth conditions were maintained at 25°C to 28°C with light.

Table 2: Experimental Design

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

3.5. Water Holding Capacity of Soil

We use 6 pots with 1 kilogramme of soil each, 3 with plain soil (control group), and 3 with a combination of biochar and bacterial strain to test the soil's ability to retain water (experimental group). Each pot has a number of holes at the bottom that were filled with tissue paper. In one water tub were the three control pots, while in the other tub were the three experimental pots. All six pots received water until it began to drip from the bottom. All pots are weighed once again with an electrical balance gauged as W1 after four hours. The dirt was then dried in the dried oven for two hours at 100 C, and its weight was once again determined as (Wa). At room temperature, the pot was air-dried, and its weight was calculated as (Wb). The formula W2=, Wa + Wb and water holding capacity are used to get the W2 value (D. Y. Wang, Yan, Song, & Wang, 2014).

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

Table 3: 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

3.6. Total RNA Extraction from Leaves

3.6.1. Sampling for RNA Extraction

In the leaf samples of Brassica plants from all the examined groups, expression analysis of cadmium sensitive genes was done. With the use of sterile forceps, tissue samples from each plant totaling around two to three leaves weighing less than 0.2 g each were gathered into appropriately labelled, autoclaved DNase and RNase free 1.5 mL Eppendorf tubes before being instantly frozen in liquid nitrogen. From each of the plants under study, three to five tissue samples were taken. Either tissue samples underwent RNA extraction immediately, or they were kept at -80°C until RNA extraction. It was strongly advised against thawing tissue samples to preserve the integrity and purity of the RNA that was extracted.

3.6.2. The TRIzol Method for Extracting RNA

Total RNA was extracted from a sample of leaves using the TRIzol/Tri reagent, commonly known as guanidinium thiocyanate-phenol-chloroform extraction (Jaakola et al., 2001). Using a sterile, autoclaved mortar and pestle, fresh or frozen tissue samples were crushed to a fine powder. As soon as samples were taken out of the -80°C freezer, they were immediately transferred to liquid nitrogen to prevent thawing and remained there until grinding. Each eppendorf tube containing a tissue sample was treated separately. In order to create a slurry that was put into a 1.5 mL Eppendorf tube, 1 mL of Invitrogen TRIzol Reagent was placed straight into the mortar after grinding. Slurry was manually homogenised for 10s, then allowed to sit on ice for 10 minutes. The mixing was not vigorous. The material that had been incubated was centrifuged at 14,000 rpm for 10 min at 4 °C. A 1.5 mL Eppendorf tube was filled with the recovered supernatant and 200 L of chloroform. The mixture was gently stirred for 15 seconds by

rotating the tube up and down. The mixture was centrifuged at 14,000 rpm for 5 min at 4°C after being incubated on ice for 5 min. A intermediate white layer of protein was generated between the two stages. The top aqueous layer was collected into a fresh 1.5 mL Eppendorf tube, and then 500 mL of ice-cold isopropanol was added. Samples were centrifuged at 14,000 rpm for 10 minutes at 4°C after being incubated at -20°C for two hours. A white pellet was produced, and the supernatant was carefully disposed without damaging the particle. The pellet was cleaned with 1mL of 75% ethanol. By centrifuging the pellet at 9500 rpm for one minute at 4°C while the supernatant was once again discarded, the pellet was recovered. To eliminate any remaining ethanol, the pellet was air dried in a fume hood that had been surface sterilised. The pellet was dissolved in 50 L of TE buffer, which contains 1 mM EDTA and 10 mM Tris-HCl, and it was then tested using gel electrophoresis and spectrophotometry. In order to be used later, RNA was kept at -80°C.

3.6.3. Electrophoresis of Gel

RNA extraction integrity and quality were assessed using agarose gel electrophoresis. 0.7g of Agarose 1-Biotechnology grade from bioWORLD was dissolved in 70 mL of 1X Tris base-acetic acid-EDTA buffer to create 1% agarose gel (TAE buffer). Agarose was entirely dissolved in the microwave. By setting spacers and combs, the casting tray was prepared. The liquid was thoroughly mixed with the addition of 4 L of ethidium. The mixture was poured into the pre-set caster and given time to set. The gel/gel well was not damaged during the removal of the combs and spacer. A medium-sized gel tank was filled with 1X TAE buffer and filled with gel. The buffer was fully submerged in the gel. By Thermo Fisher Scientific, 3 mL of RNA and 0.5 mL of blue 6X loading dye were combined. The intended well was carefully filled with the mixture. Positive control (verified RNA sample), negative control (TE buffer used to degrade RNA), and

Quick-Load® 1 kb DNA Ladder-NEB were each loaded into the appropriate well. Gel electrophoresis was carried out using a 90-volt electrical potential for 25 minutes. The UV-transilluminator Biotop® was used to evaluate the gel in order to check for the presence/absence, size, intensity, and quality of RNA. A picture was taken for documentation.

3.6.4. Analysis via Spectrophotometry

Thermo Fisher Scientific's NanoDropTM 2000/2000c Spectrophotometer was used to perform a spectrophotometric analysis to determine the concentration of RNA and impurities in the extracted sample. The spectrophotometer was blanked with 1 L of the TE buffer used to dissolve the RNA before 1 L of the sample was utilised for analysis. In order to determine the sample concentration in ng/L, 260/280 ratio, and 260/230 ratio, sample absorbance was measured. The purity of nucleic acids was assessed using the 260/280 absorbance ratio (for DNA, the approved 260/280 ratio is 1.8, whereas for RNA, it is 2). The existence of contamination in the sample, which was anticipated to be in the range of 2 to 2.2, was checked using the 260/230 absorbance ratio.

3.7. mRNA to single-stranded DNA conversion (cDNA)

Thermo Fisher Scientific's RevertAid First Strand cDNA Synthesis Kit and oligo dT primers were used to convert mRNA into single stranded complementary DNA. 1 g of total RNA with 260/280 ratios of 2 and 260/230 ranges of 2-2.2 was utilised for a 20 L reaction. Table 3.2 lists the chemicals and how much of each one was utilised to synthesize cDNA. In a labelled, sterile 0.2 mL Eppendorf tube, NF water, primer, and RNA were added. The tube was spun down and incubated in an Applied Biosystems thermal cycler at 65°C for 5 min before being chilled on ice for at least 2 min. Following the addition of RNase inhibitor, dNTPs, 5X buffer, and RT-enzyme (Reverse

transcriptase), the tube was once again put in the thermal cycler for 60 min at 42°C before the reaction was stopped for 5 min at 70° C. After polymerase chain reaction (PCR) validation of cDNA synthesis using actin-housekeeping gene primers, reaction mixtures were stored in tubes and preserved at -20°C. All ingredients other than RNA were used in the reaction's negative control.

Table 4:Reagents and their quantity used to synthesis cDNA

Sr.#	**Ingredients**	**Quantity**
1.	Nuclease Free water (NF water)	To 12.5 µL
	10 µM Oligo (Duan et al.)$_{18}$ primer	1
	RNA	1 µg
	5X reaction buffer	4 µL
	10 mM dNTPs	2 µL
	RiboLock RNase inhibitor (20U/µL)	0.5 µL
	RevertAid 200U/µL (*Reverse tanscriptase*)	1 µL
	Total Volume	**20 µL**

3.7.1. Actin PCR verification of cDNA synthesis

Using primers for the actin housekeeping gene, reverse transcription polymerase chain reaction (RT-PCR) was carried out to verify the synthesis of cDNA. All of the PCR described in Table 3.3 was added to a labelled, 0.2 mL, PCR-graded tube that had been sterilised or autoclaved and spun down. Figure 3 shows the set settings for the Applied Biosystems thermal cycler while Supplementary Table 1 contains the primer sequences

for the reverse and forward actin primers. Tubes holding the reaction mixture for the relevant cDNA were then put in the thermal cycler. While the positive control contains the cDNA of the verified sample, the negative control included all PCR reagents other than cDNA. Amplification was discovered upon reaction completion using 2% agarose gel electrophoresis.

Table 5: PCR reagents

Sr.#	**Ingredients**	**Quantity**
	NF water	14.5 µL
	10X $(NH_4)_2SO_4$ buffer	2.5 µL
	25 mM $MgCl_2$	2.5 µL
	2.5 mM dNTPs	2 µL
	10 µM Actin forward primer	1 µL
	10 µM Actin reverse primer	1 µL
	cDNA	1 µL
	Taq DNA *Polymerase* (5 U/ µL) Thermo Fisher Scientific	0.5 µL
	Total Volume	**25 µL**

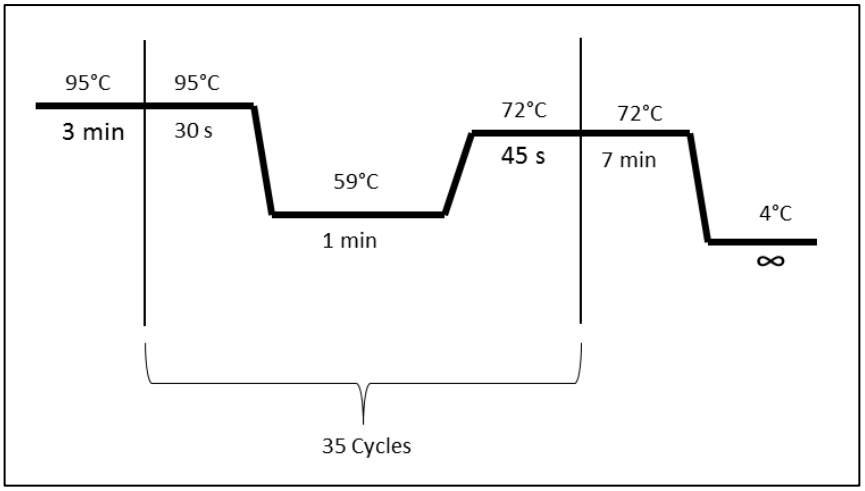

Figure 4: Conditions for PCR

3.7.2. Gel Electrophoresis

2% agarose gel, which was made by dissolving 1g of agarose in 50 mL of 1X TAE solution, was used to examine amplification results, including positive and negative controls. When making the gel, 4 L of ethidium bromide was employed. For gel electrophoresis, an electric potential of 120 volts was established for 25 minutes. Gel was examined in the UV, and a record-keeping photo was taken.

3.8. Expression Profiling of Cd stress responsive Genes

Due to its sensitivity and repeatability, real-time PCR has emerged as the technique of choice for measuring both absolute and relative gene expression. To determine the impact of intercropping, biochar, and PGPR on the regulation of Cd sensitive genes in Brassica leaves, relative gene expression studies were conducted. All of the study's confirmed cDNA samples underwent real-time PCR analysis.

3.8.1. Designing & Optimization of Primers

From a conserved area, primer sets for three genes were created, including actin, BjCAX4, BjGSH1, and BjMYB1. In Supplementary Table, all primer set sequences are listed. To increase real-time PCR efficiency, primers were created to provide an

amplification of no more than 200 base pairs. Through RT-PCR, the Tm for each pair of primers was tuned.

3.8.2. Real-time PCR

Fluorescent reporters, either particular or non-specific, are used in the real-time PCR technique to track the development of the process in real time. SYBR® Green, a non-specific DNA binding dye, was employed in real-time PCR to measure gene copy number as the junction of the threshold line and amplification curve, or Ct-value. The master mix was made in an Eppendorf tube on ice using all of the reagents listed in Table 3.4, with the exception of cDNA. Each designated PCR tube for the 7300 Real-Time PCR System received 13.5 μL of master mix. In each tube, 1.5 μL of diluted cDNA in NF water was added in a 1/5 ratio. The tubes were sealed, and the Applied Biosystems 7300 Real-Time PCR System was used to analyse the samples under the predetermined conditions shown for each gene in Figure 3.2. The Livak technique was used to examine and record the Ct-value with dissociation and amplification curve for relative gene expression. Each sample's real-time PCR was carried out three times. Utilizing an actin primer, standard curve analysis was used to adjust the working cDNA dilution for real-time use.

Table 6: Reagents and their quantities used in real-time PCR

Sr.#	Reagents	Quantity for 1X
	NF water	5 µL
	Maxima SYBR Green/ROX qPCR Master Mix	7.5 µL
	Forward Primer	0.5 µL
	Reverse Primer	0.5 µL
	1/5 dilution of cDNA	1.5 µL
	Total Volume:	**15 µL**

3.8.3. Livak Approach

The expression of a gene of interest relative to an internal control gene is referred to as relative gene expression (Schmittgen and Livak, 2008). Using the 2-CT approach, sometimes referred to as the Livak method (Livak and Schmittgen, 2001), it has been possible to determine how closely a gene's expression to actin expression corresponds. The methods below were used to determine the relative gene expression of each group that received treatment:

Step #1: Measurement of the Mean and Variance

This stage included calculating the mean with variance of the Ct-value of the target gene and the corresponding actin of the treatment group and control group. Standard deviation (S.D.) was used for triplicate samples while standard error mean (S.E.M.) was used for duplicate or more samples per group.

Step #2: CT calculation

The difference between the CT-values of the target gene and actin in the same sample is CT (Gene of interest – actin). The square root of (S12-S22), where S1 represents the variance of the target gene and S2 represents the variation of actin, was used to determine variance.

Step #3: CT calculation

The difference between the CT values for the treatment group and the control group, or CT (Treatment group – Control group). Variance was seen as same to that of CT.

Step #4: Determine the Mean Fold

Using the 2-CT algorithm, the mean fold, a measure of relative gene expression, was obtained. A mean fold for the control group is thought to be 1. Variance was seen as same to that of CT.

3.8.4. Statistical Analysis

Excel 2010 from Microsoft® Office was used to organise and organise the data. Utilizing GraphPad Prism® version 5.01, USA, inferential statistics were used to determine the importance of the data that had been gathered. The difference between the control and treatment groups was determined using the Student t test, and the total variation was determined using the Analysis of Variance (ANOVA test). In the analysis that was conducted, a 0.05 p-value was regarded as significant.

Chapter 4: Results

4.1. RNA Extraction and Polymerase Chain Reaction

RNA was successfully extracted for the given time points of Flowering stage i.e 30days and 60days for *Brassica juncea* with distinct bands of 28SrRNA, 18SrRNA and 5SrRNA. RNA was converted to cDNA and the PCR product amplification was confirmed with actin on 2% agarose gel electrophoresis.

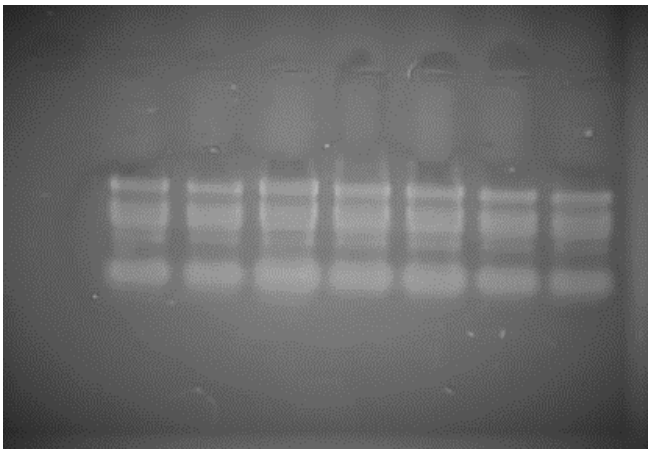

Figure 5: 1% agarose gel showing integrity of RNA extracted from leaves of *Brassica juncea.*

Lane 1-8 represents genomic DNA, 18S and 5S RNA band with mRNA band below 18S.

4.2. Confirmation of Single Stranded DNA Synthesis

cDNA synthesis from RNA samples of both control and treated groups were used as template in RT PCR using primer of actin (housekeeping gene) for confirming cDNA synthesis. 2% agarose gel was used to evaluate amplification of actin gene. Band of approximately 200 bp was observed in positive control while no band was appeared in negative control (Figure 4.6. The samples which have shown distinct band of approximately 200 bp were considered as positive and while their respective cDNA template were selected for real time PCR analysis.

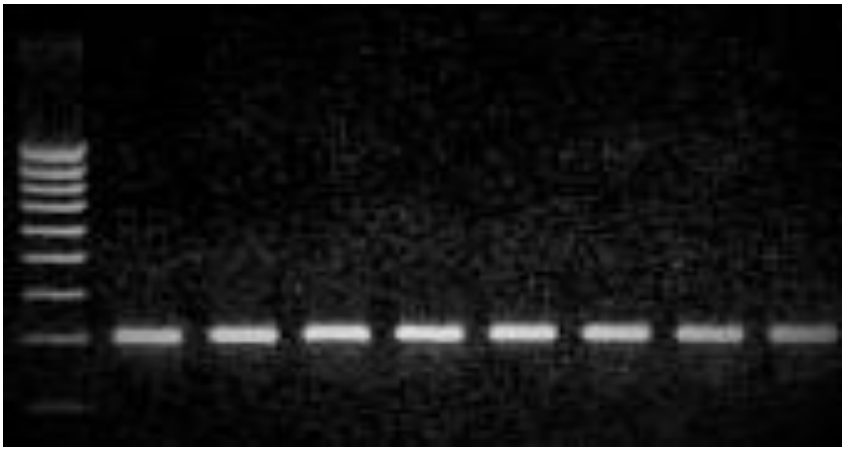

Figure 6: 2% agarose gel confirming first strand DNA synthesis from total RNA through RT-PCR of housekeeping gene i.e. Actin Lane 1 contains 100 bp ladder, Lane 2,3,4,5,6,7,8 are representing successful amplification of actin and confirm cDNA synthesis.

4.3. Expression Analysis of Cadmium Responsive Genes

Expression analysis of Cadmium responsive stress genes in leaves of *B. junceae* has revealed differential expression of genes at flowering stage and maturity stage. Real-time PCR analysis has, also, shown that expression of fthese genes is depend upon time duration and intensity of stress in addition to the biological combinition i.e biochar, PGPR, and coplanting with mungbean.

4.3.1. Relative Expression of genes at Flowering stage

At flowering stage, behaviour of three genes i.e cation excahnger CAX4, MYB1, and GSH1 was observed at 30-day time point. These genes show very prominent expressuon in eachtreatmentThe RT-PCR results shown in graphs.

4.3.2. Expression of Cation Exchanger CAX4 at Flowering stage

BjCAX4 showed a prominent up-regulation in treatment with the cd stress along with combination of Biochar, PGPR. In treatment 4 bacteria helped the phytoremediator plant to mitigate the effect of cadmium stress. The graph shows a prominent upregulation of the gene in treatment 7 and treatment 8 as compared to the control.

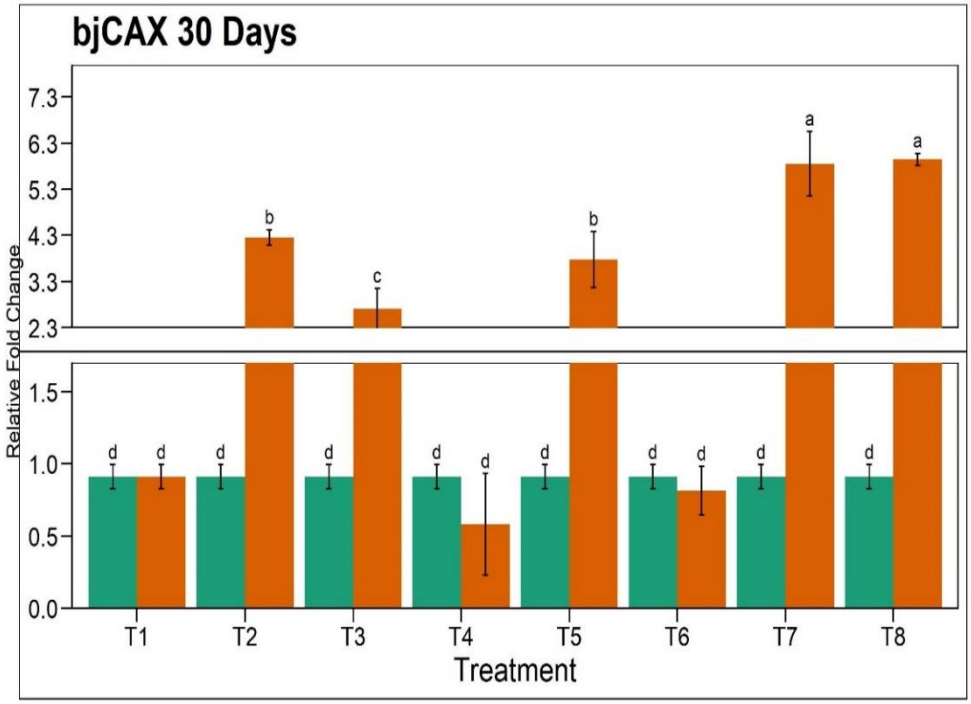

Figure 7: The graph depicts the expression of CAX4 under stress condition time period 30 days. As the 30 day is the flowering stage of the mustard plant so CAX4 showed a prominent reduction of expression in treatment 4 & 6 only. The combination of biochar + stress and Co-planting + stress helped the phyto-remediator plant to mitigate the stress in early defensive pathway in B.juncea. 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 $p < 0.001$). Different letters on the graphs indicate that mean values of treatments are significantly different at $p < 0.5$ according to Tukey's multiple comparison test.

4.3.3. Expression of Cation Exchanger BjGSH at Flowering stage

Glutathione GSH is basically the antioxidant enzyme which activates defense system in plants. Being a hyperaccumulator activated its cellular activities in bare cadmium treatment. But in other treatments GSH mitigated the effect of cadmium as biochar, Bacteria & co-planting helped the phytoremediator to mitigate the negative impact of Cadmium. GSH Positively upregulated the defensive mechanism in the control treatment as shown the expression level in graph. Treatment 3, 5 and 8 showed minimum expression of GSH level under cadmium stress. GSH maximum expression in treatment 2 (negative control) and Treatment 7 to decrease the toxicity of Stress in early 30 days of time period.

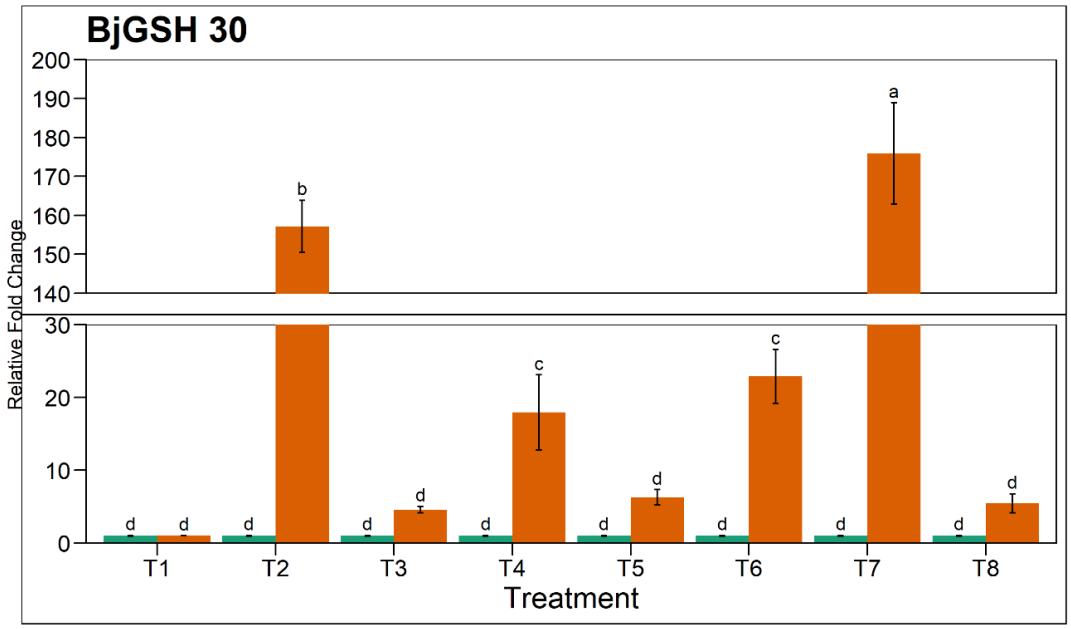

Figure 8: Expression Analysis of BjGSH in various treatments of B. juncea under cadmium stress at the flowering stage. Expression analysis for each group has been given as mean ± standard error mean.

Group with Treatment 2 has been designated as control group while all other groups considered as treated group 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 $p < 0.001$) Different letters on the graphs indicate that mean values of treatments are significantly different at $p < 0.5$ according to Tukey's multiple comparison test.

4.3.4. Expression of BjMYB1 at flowering stage

MYB1 is transcription factor acting In the stress signaling in B.juncea whose expression was found to be up-regulated in all treated group of B.junea as compared to control group at 30 day, its expression became more regulated in treatment 3,5, and lower in all other treatments with biological combination of Biochar, PGPR and Co-planting. BjMYB1 showed minimum regulation of expression in treatment 4,6 and 8. This shows that MYB mitigated the effect of cadmium itself in Treatent 2 as there was no biological aid was used, but in treatment 3 and Treatment 7 BjMYB is significantly upregulated by showing maximum of expression as depicting in graph.

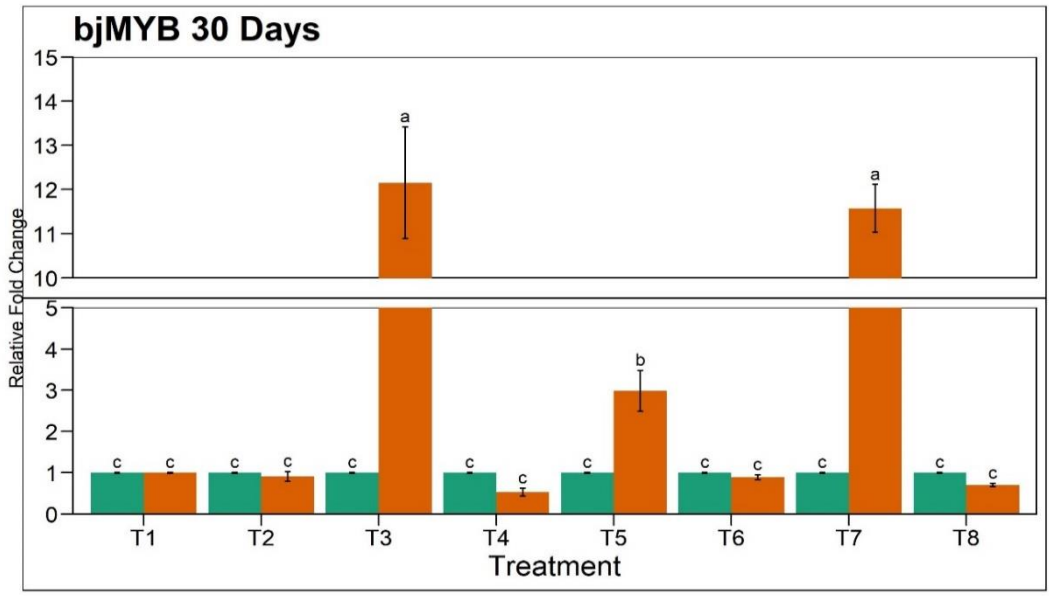

Figure 9: Expression analysis of bjMYB1 gene under Cd treatment in B. Juncea showing maximum and minimum upregulation in different treatments. Significance was inferred with Two-way ANOVA under the Tukey's HSD post-hoc test for normalizing the data distribution (Honest Significant Detection $p < 0.001$) Different letters on the graphs indicate that mean values of treatments are significantly different at $p < 0.5$ according to Tukey's multiple comparison test.

4.4. Relative Expression at Maturity Stage

Cadmium Responsive which are targeted here showed some late expression at maturity stage of plant. All the genes showed very prominent decrease in their expression except some of the treatments. So this experiment reveals that in B.juncxea all genes started their defense at maturity & exogeneous aids given to them helped a lot to mitigate the cadmium effect.

4.4.1. Expression of BjCAX at maturity stage

CAX4 was positively upregulated in treatment 7 as in this treatment the B.juncea was exogenously treated with biochar and assisted with a leguminous plant i.e mungbean.In all other treatments BjCAX4 expression was negively regulated as compared to the control treatment. This show that at 60 days of stress, phhytoremedior plant increasingly mitigatd the negative effect of cadmium with the help of Biochar, PGPR & a leguminous plant.

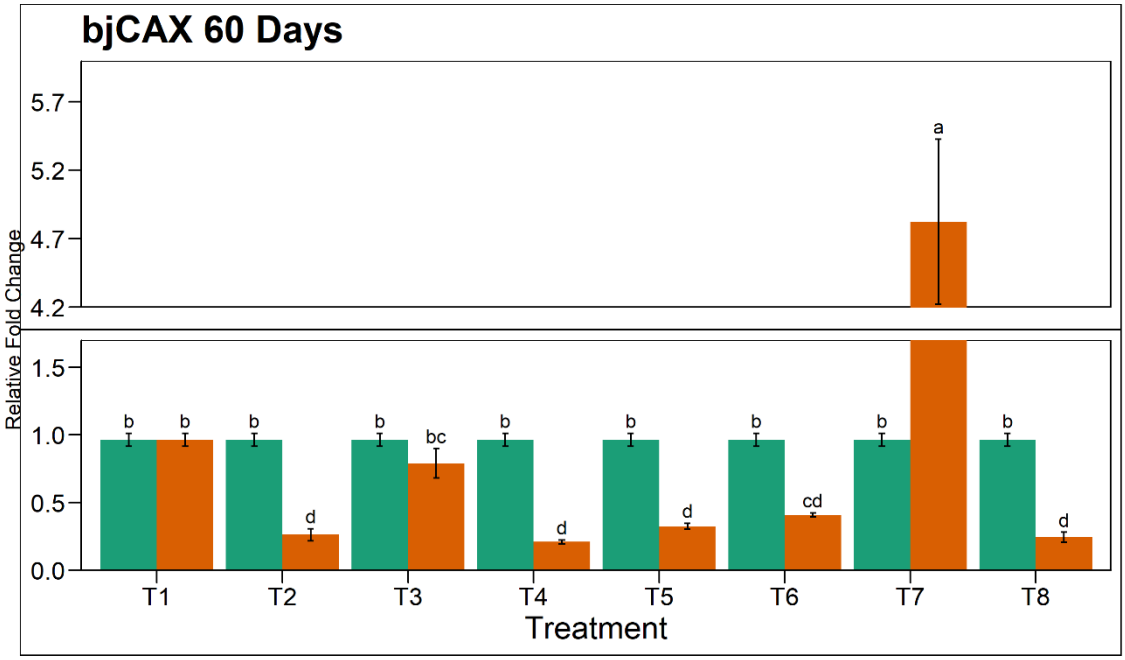

Figure 10: Relative Expression is shown at the interaction of different combination mixture as treatments applied to plants. Significance was inferred with Two-way ANOVA under the Tukey's HSD post-hoc test for normalizing the data distribution (Honest Significant Detection $p < 0.001$)

4.4.2. Expression of BjGSH at Maturity stage

At the 60 day time period of stress induction in B. juncea the gene BJGSH showed maximum upregulation in various treated plant. Treatment 3 production and activity of GSH is observed at maximum epression as shown in graph. While in treatment 8 The reduced expression of bjGSH was observed as the biochar, mungbean and PGPR bacteria significantly reduced the toxicity of cadmium and assisted the hyperaccuulator plant to increase it metal accumulation property. A significance Upregulation of GSH content in all treatment was observed as compared to the control groups. Different letters on the graphs indicate that mean values of treatments are significantly different at $p < 0.5$ according to Tukey's multiple comparison test.

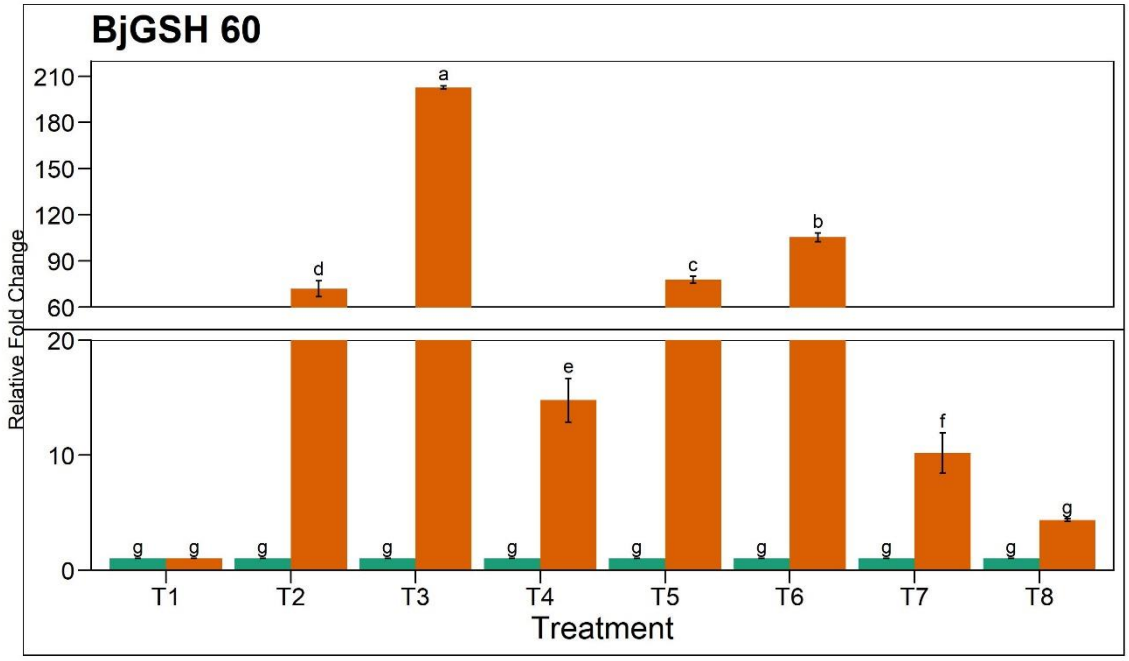

Figure 11: Relative Expression of bjGSH at maturity stage of B.juncea in various treatments. Data shows Significance was inferred with Two-way ANOVA under the Tukey's HSD post-hoc test for normalizing the data distribution (Honest Significant Detection $p < 0.001$). Different letters on the graphs indicate that mean values of treatments are significantly different at $p < 0.5$ according to Tukey's multiple comparison test.

Graph depicts the maximum expression of Gene in all treatments as compared to the control except T8 which shows that Coplantig, PGPr & biochar gave combined aid to phytoremediator plant to mitigate the negative impact of cadmium.

4.4.3. Relative Expression Of BjMYB in leaves of B.juncea at Maturity stage

The Activity of BjMYB is importantly considered in Abiotic stress condition as it works as transcription factor. An increase expression of BjMYB was observed in T5 and T2 as compared to the control group i.e. T1. But at the time period of 60 days being stressed, T8 showed a prominent reduction in its expression. This shows that Biological combinations which are used have combined effect in mitigating the negative impact of cadmium. T5 shows the plant is very negatively stressed by Cd.

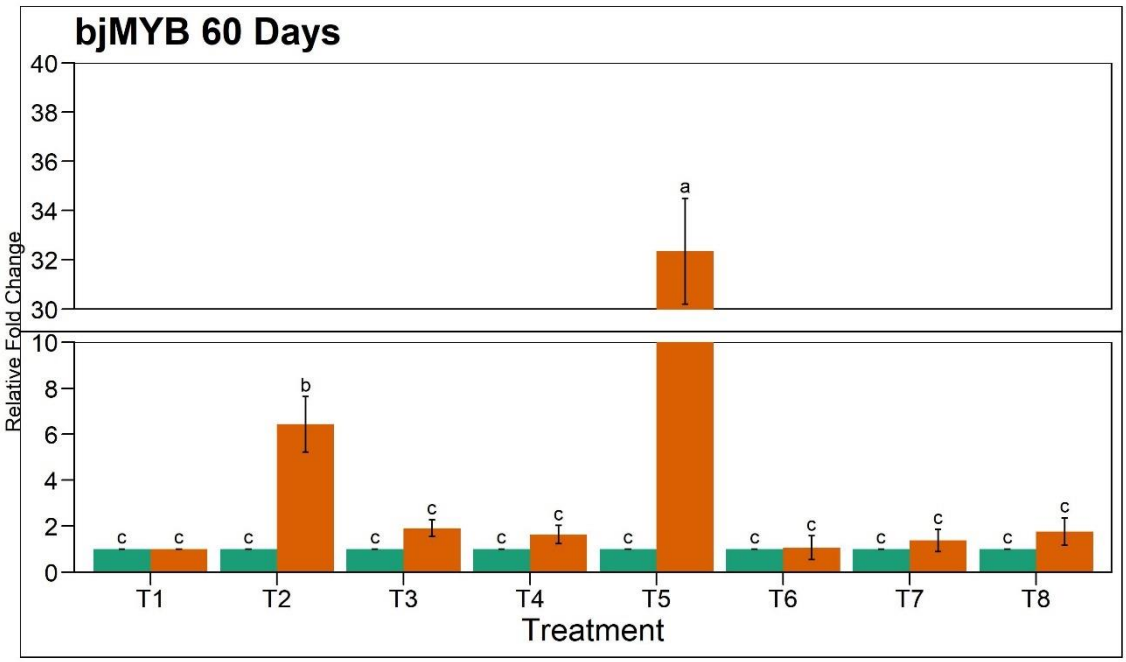

Figure 12: The above graph illustrates the expression of BjMYB I various treatment. A Prominent increase in the expression of BjMYB is observed in T5 which was treated with cadmium, Biochar And rhiospher bacteria as compared to the control plant. Significance was inferred with Two-way ANOVA under the Tukey's HSD post-hoc test for normalizing the data distribution (Honest Significant Detection $p < 0.001$). Different letters on the graphs indicate that mean values of treatments are significantly different at $p < 0.5$

All other treatment positively decreased the effect of cadmium in B.juncea as minimum expression of MYB is seen in entire treatments.

4.5. A comparative explanation of cd-Responsive targeted gene in leaves of B. juncea at flowering And Maturity Stage

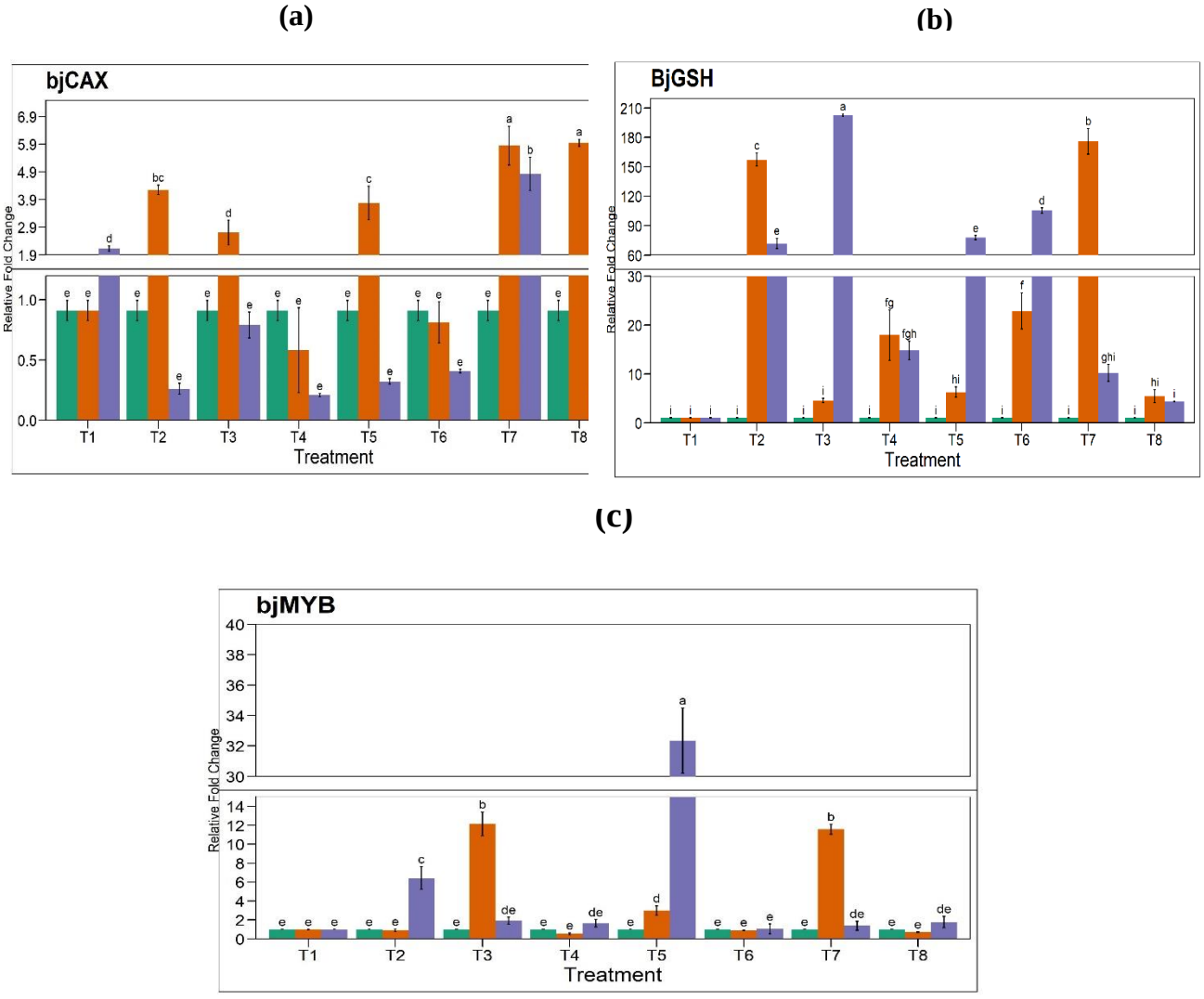

Figure 13:Relative expression of all targeted genes at flowering and maturity stage of B.juncea.

(a) Illustrates the expression of CAX , which indicates the late response of the gene in T8 at 60 days of induction of stress, while T8 expression was reduces by Biochar, Co-planting & PGPR increased the ability of accumulation of Cd in B.junea (b) A significant increase & decrease in the up-regulation of GSH level was observed at 30 and 60 days of stress. T8 is prominently reduces the expression as all out aids helped

the plant to mitigate the effect of Cadmium both at flowering & Maturity stage (c) Significant difference in the upregulation of BjMYB is observed at day 30 & 60 of stress. In T3, T5 & T7 a positive upregulation is observed s at 30 & 60 day respectively. This shows that sole PGPR, PGPR+biochar and PGPR+Co-plantinng didn't aided the phytoremediator plant to alleviate cd stress. Moreover, a combined positive effect of all these biological combinations helped the plant to mititgate the impact of cd on B.juncea. Significance was inferred with Two-way ANOVA under the Tukey's HSD posthoc test for normalizing the data distribution (Honest Significant Detection $p < 0.001$). Different letters on the graphs indicate that mean values of treatments are significantly different at $p < 0.5$ according to Tukey's multiple comparison test.

4.6. Heat-map based expression approach target genes

The percentage relative expression of each gene in each treatment is shown at maturity and flowering stage of stress induction. Relative expression of genes is shown in the form of heat map which represents the maximum and minimum expression in the form of colors. In Figure Blue color is providing the information about maximum expressed gene in treatments.

4.6.1. Heat-map of flowering stage gene Expression

A percentage expression of all the targeted gene in entire experimental treatments is represented in the graph. While data shows that GSH 7 MYB showed 40% expression in T2 and T3 respectively, While GSH1 & MYB1 giving 45% & 38% expression in T7 and T8 respectively. Moreover, CAX showed maximum of expression in T7 & T8 i.e. 24%. (Figure)

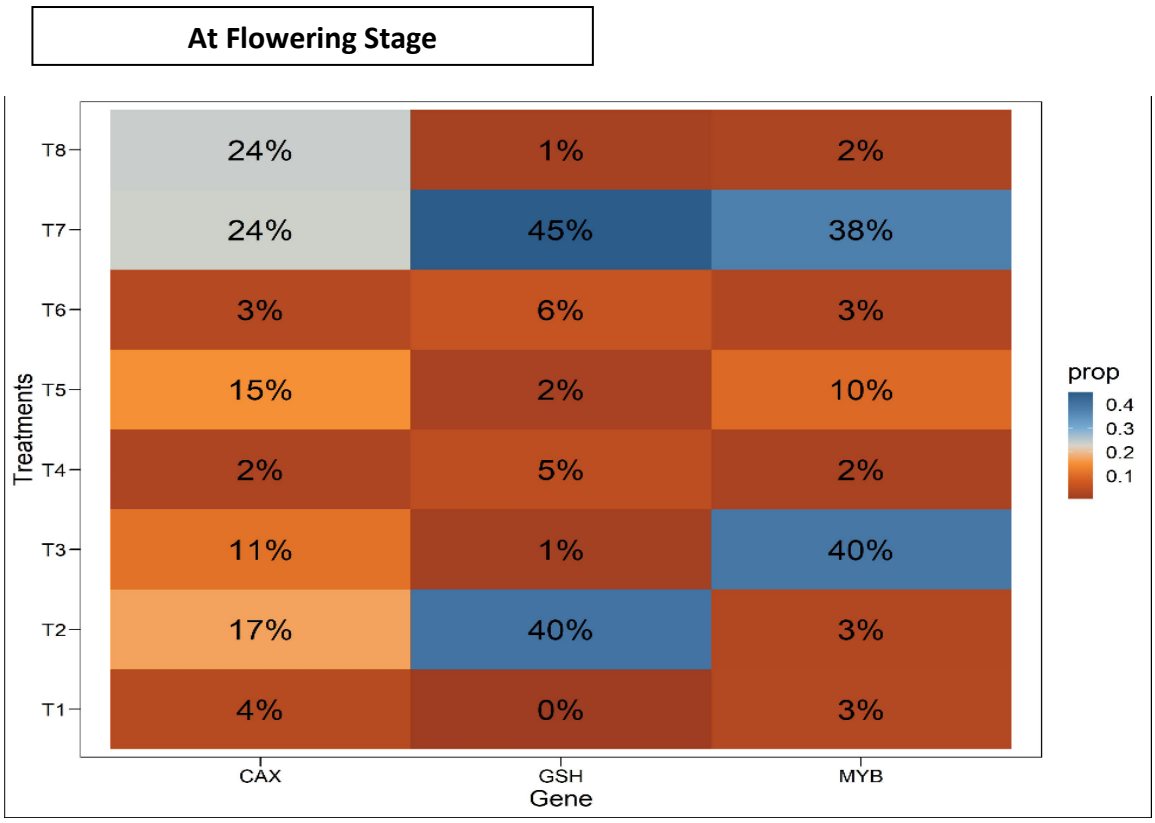

Figure 14: At the 30day stage of B. juncea's development, heat map diagrams showing the relative expression levels of the differentially expressed Cd-Responsive genes were created. The relative expression value of the genes was used to create the heat map. b.juncea whereas T1 to T8 stands for therapy provided to the bjCAX4, bjGSH1, and bjGSH1

4.6.2. Heatmap of Maturity stage gene Expression

A percentage expression of all the targeted gene in entire experimental treatments is represented in the graph. While data shows that all gene expressed differently at the maturity stage i.e 60 day of stress. CAX4 showed 66% expression in T7 and MYB1 showed 66% expression in T5, While GSH1 went at 41% at T3. Experiment was aimed to check the minimum expressions we aided the B. juncea with biological combinations

so, a combined effect of Copalnting, PGPR & Biochar can be seen in T8 as 3%, 1%, and 3% in CAX4, GSH1 and MYB1 respectively.

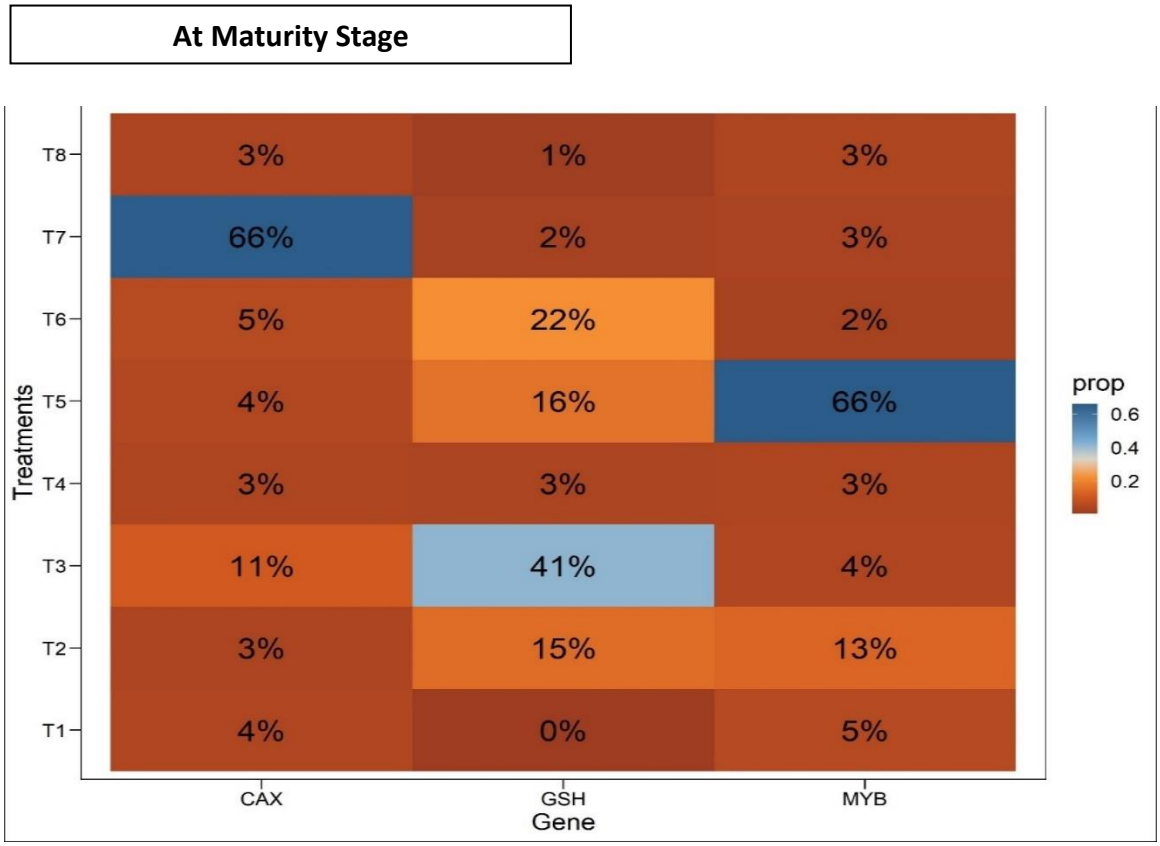

Figure 15: Heat map diagrams displaying the relative expression levels of the differentially expressed Cd-Responsive genes were made during the 60-day stage of B. junncea's development. The heat map for the genes B. juncea was made using their relative expression values, and T1 to T8 represents the treatments given to bjCAX4, bjGSH1, and bjGSH1.

Chapter 5: Discussion

Plants can generally be divided into four groups based on how they respond to metal stress: metal-sensitive species, metal-resistant excluder species, metal-tolerant non-hyperaccumulator species, and metal-hypertolerant, hyperaccumulator species. Each of these groups uses a different molecular mechanism to achieve its resistance or tolerance to metal stress or lessen the negative effects brought on by metal toxicity (Lin and Aarts, 2012).

Mustard plant, *Brassica juncea*, used for food and edible oil. Every year, this plant produces a sizable amount of consumables all over the world. Additionally, the primary issue with the mustard plant is that it has the ability to hyperaccumulate heavy metal ions like cadmium ions in a separate area of the plant (Mutlu et al., n.d.). According to earlier research, cadmium ions have a negative impact on the morphological, physiological, and biochemical processes of plants (Kapoor, Kaur, & Bhardwaj, 2014). We also saw conformational alterations in this element of our investigation. Meanwhile, in our research situations, varied biochar, PGPR, and intercropping compositions all promote plant growth.

Brassica species are well known as metal accumulators and have been investigated for many years for the discrepant accumulation ranges of heavy metals (Wu et al., 2015) The present study also concludes about the metal accumulation property of the mustard plant. Conflicting results have been reported on the Cd tolerance of *Arabidopsis thaliana* and *Nicotiana tabacum* over-expressing the PC biosynthetic gene, (Guo et al., 2008)possibly due to different experimental conditions and the use of different constructs. Present study relates with the as gene expressed them differently under 10mM CdCl2 solution as previously given 5mM. 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 Physiological And molecular level also affected under cadmium of Br*assica juncea* (Wu et al., 2015)

The previous investigation sought to determine the effects of plant growth-promoting bacteria (PGPR) on plant growth and development as well as the gene expression of Cadmium responsive gene and in switch grass exposed to cadmium stress. Genes showed maximum of the expression & PGPR helped the plant to cope up the effect of cadmium, but gene expression was maximum (Zheng et al., 2018). According to our deign of project, we treated Phytoremediation plant with Biological combination of PGPR, biochar and Co-planted with mung bean (leguminous plant). Sole treatment of cadmium, biochar & Mung bean was also given to brassica to check out the phytoremediation capacity of the plant. A positive combined effect of all these combinations was seen in the plant. Stress was also mitigated 7 plant defensive mechanism was also aided by this combination. As a result, the low regulation of gene in treatment was seen. Both at flowering and Maturity stage, all the targeted gene sh0wed different expression. Late response of these gene during gene expression is also reported in our study. Moreover, Antioxidant assay & Defensive mechanism were activated through this. CAX protein take part in the mitigation of abiotic stress responsive mechanisms and also act as pH regulator. As it is a housekeeping component, it has direct effect on the physiological & Molecular processes. With the aid of all other combination under cadmium stress, the CAX showed a variable expression at maturity and early stage. But its expression was seen in the treatment where PGPR, Co-planting and Biochar was exogenously given to cadmium treated plant showed reduces upregulation in leaves at the 60- days' time span. So it tells about

the late response of all the treatment of this genes. Moreover, the other two genes, MYB1 and GSH1 expression was seen reduced at both stages of growth that concludes that this combination i.e., biochar, Bacteria and Co-planting combined provided a better solution to negate the cadmium toxicity to the soil and plant as well. This study relates with the (Mohamed et al., 2012) in which the expression of roots was observed under cadmium stress and the phyto-chelatins were upregulated. We have also seen the upregulation of all these genes in leaves samples at two growth stages Maturity and Flowering stage. But PGPR bacteria, Mung bean co-planting helped the plant to mitigate the cd stress by showing reduce expression in leaves, Promoted growth and presence of antioxidant enzymes.

Conclusion

The plant's capacity to sequester the metal and strengthen the antioxidant defense may account for Indian mustard's tolerance to high Cd concentrations. Both systems use a lot of energy, which might be the reason for the sharp growth decline seen at the root and shoot levels. Growth decrease might be attributed to the metal's toxicity toward biomolecules in addition to a switch in metabolic resources from growth to defense (either direct or ROS-mediated). Co-planting, PGPR, and Biochar together promote plant development and give resistance to Cd stress. As it appears in various combinations of PGPR+ co-plant+ biochar, solitary biochar did not demonstrate increasing outcomes. Gene Expression result showed the effectiveness of the combination PGPR+ co-plant+ biochar to reduce the negative effect of cd in soil & in plant phytochemical, growth and cellular parameters as well. Usually, Gene Expression data is evaluated at Hours of time point but our study illustrates the behaviour of genes under cd stress in Two growth stages i.e Flo9wering (30day) and Maturity (60 day).

Here, we looked at the effects of cadmium on the mustard plant and discussed how biochar, co-planting, and PGPR bacteria may assist. The development of non-hyperaccumulator plants may be accelerated by using these combinations since they improve the morphophysiological characteristics of hyperaccumulator plants. However, future research should also take into account various Understanding the mechanism and characteristics to combat Cd and other heavy metals from plants and bacteria Metals are poisonous. This paper may be used for more heavy metal stressors research using such an amalgamation**.**

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