Genetic modification strategies in Rhizobia to combat abiotic stress in legumes

1. Introduction

1.1Background

Ancient humans adapted agriculture practices somewhere between 12000-10000 years ago, which totally transformed their lifestyle from hunter-gatherers to a permanent settler. Global agriculture land area was estimated around 5 billion hectares, which is 38% of global land mass. India holds second largest agriculture land in the world after United states of Baroda, which is estimated around 157 million hectares. Around 60.3 % land in India is an agriculture land, but its percentage is declining due to anthropogenic factors.[1] Agriculture is a recognized global driver of economic growth and is essential to tackling any socioeconomic agenda for the twenty-first century.[2]

But a rapid increase in population has put a pressure on our agriculture which has forced majority of the farmers to use different tools and techniques to enhance crop yield irrespective of its impacts or hazards. Not all tools and techniques used in modern day agriculture are good for the soil and environment. Now a days, an extensive use of chemical fertilizers, herbicides, pesticides and insecticides has damaged our agriculture land and significantly reduced its fertility. Herbicides when applied are only used from 5 to 40 percentage, while the rest of it remains in the environment for a long time due to its high stability and do not undergo any physiological or biochemical degradation. Herbicides are known to cause chromosomal aberration in plant cells and are responsible for the alteration of apoptotic characters of plant cells. They are also responsible for increasing the oxidative stress in plant cells and ultimately cause oxidative stress related damage in whole plant. Cytogenetic effect of herbicides like Gesgard, Zenkor, Semeron, Simazine and Terflan are well established in many studies.[3] Application of pesticides in the agricultural fields can cause a significant reduction in chlorophyll and protein content in plants and is also linked with increase in the oxidative stress in plants. Such plants are reported to have a reduced photosynthetic efficiency. [4] Pesticides contaminates soil, water, turfs and other vegetation. Besides killing target pests, it is also harmful for other birds and insects (which are not pests) which comes in contact to these chemicals. Insecticides can be called as most acutely toxic class of pesticides.[5] Excessive use of chemical fertilizers has degraded the agriculture lands and many problems such as reduction of soil organic carbon, loss of soil carbon, soil compacting and nitrogen leaching have come up. [6] There are varieties of chemical fertilizers such ass Urea, ammonium nitrate, ammonium phosphate, ammonium chloride and ammonium sulphate which are used by farmers on daily basis and these fertilizers could have an indirect or direct negative effect on plants. In such study of Urea exposure (greater that 1000 ppm) to with *Allium cepa L.* reported that higher concentration of urea for only 72 hours can cause a heavy clastogenic effects in plant cells. [7] Another important concern is the acidification of the soil linked with the use of acidifying nitrogen fertilizers or incomplete cycling of nitrogen species in soil.[6] It is also reported that the application of insecticides and pesticides significantly reduces the number of beneficial fungi and nitrifiers from the rhizosphere of legume crops and possess a high biocidal activity against soil bacteria.[8] As we are aware of the fact that rhizospheric microorganisms play an important role in cycling of organic matter between soil-plant ecosystem, fix atmospheric nitrogen and providing nutrition to the crops/plants, their healthy population in rhizosphere is very important from any crop to thrive.[9] Also, these friendly microorganisms play a very important role in protecting the plants from soil borne infection and other biotic stress which crops face in the field. If there is any alteration in the population of friendly soil bacteria then the soil will gradually lose its fertility and hinder the healthy growth of a plant.

Besides this, Industrialization has also impacted the soil quality in a negative way. Industries seldom release their effluents either in soil or in water in an unregulated way. Due to this, many organic, inorganic and heavy metal containing chemicals percolates in soil and mixes with the ground water. This is really a very dangerous situation in which a very little amount of effluent can contaminate a very large source of ground water below. Same ground water is later used for drinking as well as irrigation purpose, which leads to toxicity of different chemicals in humans as well as crops. Based on the CEPI ranking for the Comprehensive Environmental Pollution Index, the Central pollution control board (CPCB) has listed down some critically polluted industrial areas. It was reported that 43 polluted zones in 16 states had a CEPI rating higher than 70, which is really an alarming situation. 21 sites from 43 are present in Maharashtra, Uttar Pradesh, Gujarat and Tamilnadu states [10]. If the final CEPI score for any region/zone is 70 or above than that region is considered as critically polluted area. In Gujarat areas like Ankleshwar, Vapi, Vatva, Bhavnagar etc. have a CEPI score above 70. CEPI is an overall score given to any polluted area for land, water and air pollution. These chemicals either pesticide, insecticides or effluents from Industries are harmful for crops as well as humans. If humans or other animals consume the crops grown on polluted land than these chemicals will be biomagnified and bioaccumulated within the body. They are known to cause numerous dermatological, gastroenterological, neurological, respiratory, reproductive, endocrine and carcinogenic issues.[11] Due to the lack of proper regulation regarding the disposal of industrial effluents in some developing countries, these harmful chemicals eventually find its way to an open land or nearby water source where they are discharged. Reports from a rice field of Bangladesh which came in contact with industrial effluent indicated an increase in electrical conductivity and presence of heavy metals such as cadmium, Lead and Nickle above permissible range in water of field.[12] Consumption of crops grown in heavy metal contaminated soils will gradually accumulate heavy metals in human body which can cause neurological diseases like Minamata. These heavy metals are also related to many birth defects and are associated with numerous defects of liver, heart and kidneys. [13] Also the crops grown in polluted lands have reduced nutritive value and low overall yield which is a main concern for farmers. Lower yield and nutritive value significantly reduce farmer's profit and compelled many to shift to organic farming instead of using harmful chemicals in the field. Industrial pollution is on a large scale and cannot be tackled by an individual, as it needs an ample number of legal resources and government permissions, but what we can minimize the use of artificial fertilizers and chemicals for farming. Lately, the concept of organic farming has been picked up by many farmers and is promoted as a best alternative for harmful chemicals used in agriculture. Also, a self-awareness has led people form urban and rural areas to consume organically grown grains, vegetable and fruits. Besides this advancement in science has also developed so many products containing plant growth promoting microorganisms to restore the fertility of barren soil as well as introducing the useful microbes in contaminated soils to protect that crop. These are called as biofertilizers commonly. Plant growth promoting microorganisms used in these biofertilizers can be genetically modified or wildtype. These excellent tools can be used to delivers any metabolite or byproduct of our wish into the soil to make it available to the plants and enhance soil fertility and increase crop production.[14] Plant growth promoting microorganisms used is biofertilizers can also provide protection to the crops from various abiotic stresses such as drought, photooxidative damage, heavy metals, salinity, cold and flood.[15] These technologies have really been promising and has given a satisfactory result. Biofertilizers and organic farming is the future of modern-day agriculture.

1.2 Soil Pollution: a threat to agriculture

1.2.1 Contribution of soil in agriculture sustainability

It was estimated that 85.9% of agriculture land in India was under the threat of pollution by human activities.[16] As a developing nation about 60% population of our country is dependent on agriculture and its related products for survival. Besides this the revenue generated by agriculture significantly contributes in national GDP. To maintain this cycle properly, it is very important that the regenerative capacity of the biosphere is sustained [17], which can only be achieved by keeping a proper check on soil pollution and taking a responsibility as an individual to track, trace and stop the pollution. A healthy biosphere microenvironment ultimately provides a healthy agriculture ecosystem which can benefit maximum population of our country.[17] Since 1980, human needs for commodities and services have greatly outpaced the biosphere's potential for regeneration. This can be quantified by an analysis made in a study which suggests that humanities load for usage of biosphere increased to 120 % in year 1999 compared to 70% in year 1960. [10] It is also estimated that the contribution of biosphere reserve in agriculture production during pre-green revolution was much more compared to post green revolution period which had all the technological advancements like fertilizers, pesticides and agrochemicals which clearly indicates a significantly larger role of agrochemicals in soil pollution.[10]

1.2.2 Threats to soil and environment

India has a broad variety of soil types, terrain, and climate, which contributes to its rich biodiversity. India was regarded as one of the 17 megadiverse countries harboring the bulk of earth species and endemic species, according to the UNEP-affiliated World Conservation Monitoring Centre. The ability of soil to affect the surrounding plants, fauna, and microorganisms makes it by far the most complicated ecosystem. In addition to this, it filters water, manages the bulk of greenhouse gases, and governs the earth's temperature. Heavy metals and other agrochemicals accumulate more in the soil and water due to more anthropogenic activity in urban regions compared to rural and forest settings. In the presence of heavy metals, which should ideally only be present in small amounts, it denotes a negative balance.[18] Each state lost 1% of its total agricultural land between 2001 and 2010 owing to urban pressure, and this number is still rising today.[19] Increasing pressure on soil and agroecosystem is a cause of rapidly increasing population and its needs, besides this there is also a socio-economic factor for a developing country like India which puts a significant amount of pressure on usage of agriculture land. For a developing country like India there is a need to keep a balance between Industrial development and environment protection.[20] There is a considerable amount of research done by Indian council of agriculture research and different state agriculture research institutes to address the pollution of soil and its declining quality, still the efforts are insufficient to address the harmful effects of uncontrolled use of agrochemicals, pesticides, herbicides and insecticides to soil ecosystem. Indian institute of soil science reported that excessive usage of fertilizers contaminates the ground water by Nitrates.[21] Also, a properly balanced, integrated and controlled way of using chemical fertilizers for crop production doesn't harm the soil. These type of contradictory research results and estimates are still a matter or discussion and are always based on the policies of state, but we cannot ignore the fact that agrochemicals have more cons than pros. In the last 70-80 years we have seen a rapid expansion in urban centers and a rapid decline of rural, forest and agriculture lands. These urban centers and its ecosystem have put different types of pressure on soil ecosystem such as land surface sealing by concrete or by road constructions, excessive emptying of ground water in urban setups, low or negligible ground water recharge, uncontrolled dumping of plastic and other non-biodegradable wastes. These activities contribute in excessive drying of soil and leaves the land dead with very low organic content. [10] Apart from these problems of excessive land consumption by urban setup, main problem is of soil contamination by various industrial activities. Most common chemicals involved in soil contamination are heavy metals, petroleum hydrocarbons, pharmaceutical products and solvents. Impacts of these chemicals is quiet alarming. There is a complex intertwining between agriculture and industrial/ anthropogenic activities with nature and humans,[22] which can be visualized by Figure 1.1.

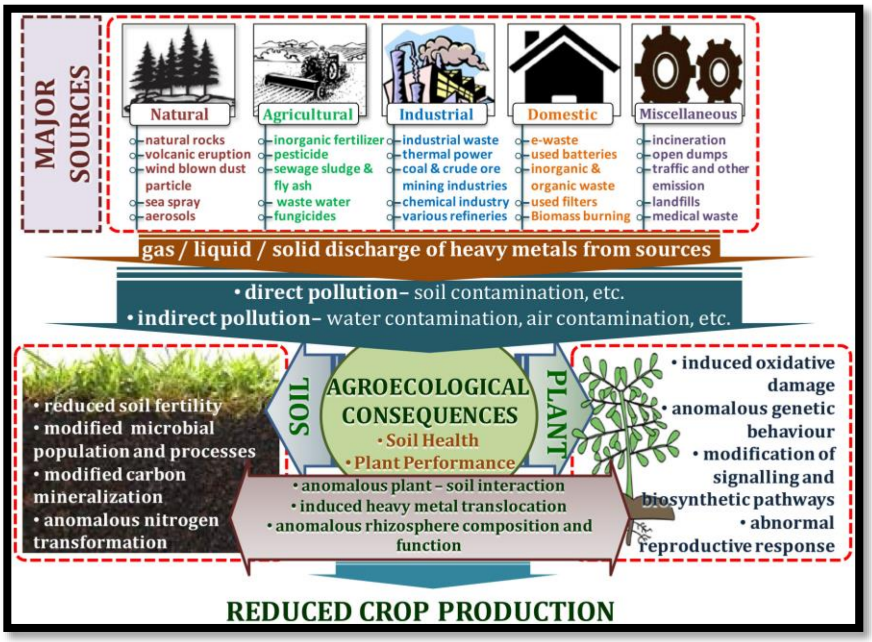

Figure 1.1 Intertwining interactions of Agriculture, Industries and humans with Nature (Srivastava *et.al.*, 2017, Frontiers in environmental Science)

1.2.3 Extent of soil degradation in India

There are various agencies in different countries which handle the issues related to land degradation and soil pollution for the purpose of protection of environment and organisms. Among these agencies, United states environmental protection agency operating in USA has developed a program called as ‘superfund’ where they clean up the most degraded and polluted land of the country along with the oil spills of oceans. [23]Similarly, Europe has also devised an organization which tackles the land pollution throughout the member countries. In India attention towards pollution has been driven by laws like Environmental protection act,1968 and through various campaigns and awareness programs. Unlike any developed country, most of the attention in our country has been given to air and water pollution and land pollution is generally ignored. This could be due to higher mobility of toxicants in air and water compared to soil and its higher impact on living beings.[23] In most of the studies done by Environmental impact association (EIA) on different industrial/mining/infrastructure development projects, an impact of these anthropogenic activities on land resource and agroecosystem in generally ignored. Unlike developed countries, developing countries always face a shortage of funds to carry out proper research in specific fields which could require complex instruments and setup, but for India scenario is rapidly improving since a decade as the government is trying its best to provide maximum support to research related to land resources. Still the information related to impacts of pollution on soil and its interrelation with effect on human health is sparse. About 60 percent of Indian geographical area consists of agricultural land and most of it is under some or other kind of degradation. According to National academy of Agricultural Science, India different degradation factors and the area of agriculture land affected[23] is summarized in Table 1.1.

Table 1.1 Causes of soil degradation in India

Type of degradation	Area under arable land (million ha)	Area under open forest with <40% canopy (million ha)
Water erosion (>10 t/ha)	73.27	9.30
Wind erosion (Aeolian)	12.40	–
Physical degradation		
(a) Mining and industrial wastes	0.19	–
(b) Permanent surface inundation	0.88	–
Chemical degradation		–
(a) Exclusively salt-affected soils	5.44	–
(b) Salt-affected and water eroded soils	1.20	0.10
(c) Exclusively acidic soils (pH <5.5)	5.09	–
(d) Acidic (pH <5.5) and water eroded soils	5.72	7.13
Total degraded area	104.19	16.53

1.3 Heavy metal pollution

When there are excessive amounts of heavy metals in the environment, it is referred to as heavy metal pollution or heavy metal contamination. Heavy metals have the characteristics of being poisonous, non-biodegradable, persistent, and bio accumulative. Elements with a high atomic weight and at least five times the density of water are considered heavy metals. Heavy metals include elements like cadmium, lead, mercury, and arsenic. These elements are present in the crust of the planet naturally. However, human activities including industrial operations, the use of pesticides and fertilizers, and the burning of fossil fuels can also cause them to be released into the environment.[24] To safeguard the environment and people, heavy metal pollution must be effectively managed and reduced. This can be achieved through the implementation of regulations and guidelines, the use of control technologies, and the proper disposal of waste. This can have negative effects on both the ecosystem and human health.

1.3.1 Sources of heavy metal pollution

There are several sources like mines, smelters, thermal power plant, metallurgical industry, electrical industry, textile industry, industrial sludge, municipal solid waste, and phosphatic fertilizers that are responsible for the release of heavy metals in our environment [10,25] as shown in Table 1.2. Many thermal power plants[26] are responsible to contaminate the environment with the heavy metals like Cr, Hg, Ni, V, and Se, while copper production[27] and zinc production industries are responsible for contaminating the environment with As, Cd, Cu and Zn heavy metals. Vehicular exhaust releases the highest amount of Pb in the atmosphere.[28] After being emitted from the source, most of it is deposited on the ground in the nearby vicinity of the source, while some of it is transported a very long distance through winds. These trace particles are very tiny and they travel so far that they cross the boundaries of countries or sometimes continents.[29] Any particle size less than 10 micrometer, are known as respirable particulate matter or PM_{10} that remains suspended in air and can be easily adsorbed by the alveolar sac in lungs and the heavy metals fall into the same category. While, some of the heavy metals that are deposited on land are in very minute quantity that they do not exhibit any toxic effects to plants or animals initially. However due to their immobility and persistent presence in the soil, it becomes toxic in the long run which affects plants, plant growth promoting microorganisms and overall soil-plant ecosystem.[10] Compost is the best natural fertilizer for increasing the crop production, but the compost made from municipal sludge can contain many heavy metals in very high concentration.[30] Similarly, many phosphatic fertilizers/chemical fertilizers have the contamination of Cadmium in it, because the cadmium comes from the raw material used to prepare this fertilizer.[31] Pesticides also contains heavy metals like Zn, Cu, Hg and Sn which are responsible for contaminating the rhizosphere, if used without any regulation repeatedly.[32]

Table 1.2 Source of heavy metal pollution (Source- Gautam SP, CPCB, Delhi)

Metal	Industry
Chromium (Cr)	Mining, industrial coolants, chromium salts manufacturing, leather tanning
Lead (Pb)	lead acid batteries, paints, E-waste, Smelting operations, coal- based thermal power plants, ceramics, bangle industry
Mercury (Hg)	Chlor-alkali plants, thermal power plants, fluorescent lamps, hospital waste (damaged thermometers, barometers, sphygmomanometers), electrical appliances etc.
Arsenic (As)	Geogenic/natural processes, smelting operations, thermal power plants, fuel burning
Copper (Cu)	Mining, electroplating, smelting operations
Vanadium (Va)	Spent catalyst, sulphuric acid plant
Nickel (Ni)	Smelting operations, thermal power plants, battery industry
Cadmium (Cd)	Zinc smelting, waste batteries, e-waste, paint sludge, incinerations & fuel combustion
Molybdenum (Mo)	Spent catalyst
Zinc (ZN)	Smelting, electroplating

1.3.2 Negative effects of heavy metal pollution

Excess exposure to the heavy metals can have a range of negative effects on all the living beings. In soil bacteria they can cause disruption of cellular metabolism, DNA damage, interfere with the uptake and utilization of essential nutrients, alter the bacterial community structure. For instance, cadmium found in pesticides and industrial waste are reported to inhibit the activity of enzymes involved in nitrogen metabolism in soil bacteria, which results in decreased nitrogen availability for plants, low soil fertility and reduced crop yields.[33] Lead, a common heavy metal, has been reported to cause DNA damage in soil bacteria.[34] In addition to their direct toxic effects, heavy metals can also have indirect negative effects on soil bacteria through their ability to interfere with the uptake and utilization of essential nutrients. For example, zinc is an essential trace element for many living organisms, but excessive levels of zinc can inhibit the uptake and utilization of other essential nutrients such as iron and copper in soil bacteria.[35] Heavy metal pollution can also affect the diversity and abundance of soil bacteria. Some heavy metals, such as lead and cadmium, can selectively inhibit the growth of certain types of bacteria while promoting the growth of others. This can lead to changes in the overall bacterial community structure and potentially alter the ecosystem functions that rely on these bacteria.[36] Disruption of the PGPR- soil ecosystem can have cascading consequences on health and productivity plants and the animals that rely on them. The presence of heavy metals above permissible concentration affects the edaphological environment. Heavy metals can bind to enzymes and other proteins in the cell, disrupting their function and leading to a range of negative physiological effects.[37] Heavy metals are toxic to plants and can have a range of negative effects at the molecular level. For example, heavy metals can bind to and inhibit enzymes involved in important cellular processes such as photosynthesis and respiration.[38] This can lead to decreased energy production and impaired growth and development. Heavy metals can also interfere with the normal function of ion channels in the cell membrane, leading to changes in cell signaling and communication. Plants have evolved various mechanisms to cope with heavy metal stress, such as the production of metal-binding compounds called phytochelatins and the sequestration of heavy metals in specialized cell types called vacuoles.[39] However, high levels of heavy metal exposure can overwhelm these protective mechanisms and result in significant damage to plant cells. Overall, the toxic effects of heavy metals on plant cells can be severe and can result in impaired function, DNA damage, and even cell death.[40] Heavy metals are toxic to animals, including humans. Heavy metals can interfere with the normal function of the mitochondria, which are the powerhouses of the cell responsible for generating energy. This can lead to decreased energy production and impaired cellular function.[41] Heavy metals can also alter the expression of certain genes, leading to changes in protein production and potentially resulting in the development of diseases such as cancer. Heavy metals can accumulate in the tissues of animals, leading to negative impacts on their growth, reproduction, and survival. In humans, heavy metal exposure can cause a range of health issues, including damage to the nervous system and kidneys. [42]

1.3.3 Records of soil pollution by heavy metals throughout India

Contamination of heavy metals has been recorded throughout India. According to the report of WHO, there is a heavy contamination of Arsenic in the ground water of the states like Assam, Chhattisgarh, Uttar Pradesh, West Bengal, Bihar and Manipur.[43] Total 718 districts in our country face to problem of ground water pollution by heavy metals like Cadmium, Arsenic, Chromium and Lead. There are numerous sites in India, where the agricultural lands are also polluted by heavy metals. For instance, the north-west areas in India shows high concentration of selenium in the farm soil as well as ground water and many districts in Punjab shows the highest contamination. In a study of selenium contamination in Punjab agriculture soil and water, conducted by German scientists, it was reported that ground water as deep as 76 m showed up to 341ug/L concentration of selenium, whereas the farm soil showed up to 11.6 mg/kg of selenium contamination. The recommended dietary intake of selenium is 55ug/d for a person above 14 years, while its upper tolerable limit is 400 ug/d.[44] Selenium enters the food chain mainly through soil-plant ecosystem, but it is still unclear that whether selenium has any importance in plant growth.[45] Basically the ground water pollution translates to the soil pollution, as majority of the farmers in rural India uses ground water for irrigation.[46]

1.3.3.1 Arsenic pollution and prevalence in India

Arsenic is also one of the most toxic heavy metals which is responsible for the pollution of ground water, and its presence in drinking water and soil is a global phenomenon. In India this problem is more prevalent in Ganga-Brahmaputra-Meghna plains, Karnataka and Gujarat region as seen in Figure 1.2. According to BIS (2012) and WHO (2011), arsenic levels exceeding 10 ug/L is considered as an Arsenic pollution. Permissible amount of Arsenic in drinking water till 2008 in India was 50 ug/L, but it was reduced to 10 ug /L in accordance with BIS and WHO. CGWB published a report in 2008 which mentioned only 10 states that contained arsenic polluted ground water, but in 2018 during reassessment the number of states increased to 20, and 9 states from them were identified which has more than 50 ug/L contamination of Arsenic in ground water. [47] Most of the rural population in India uses ground water for drinking and irrigation.[46] Besides this these rural arear are also densely packed and they are most vulnerable due to their low education level and poor economic conditions. Arsenic can be taken up by vegetable if the contaminated water is used in irrigation. Also, arsenic can be taken up by human body if these contaminated vegetables are consumed, which can result in varieties of skin related issues as well as bladder and kidney dysfunction.[48]

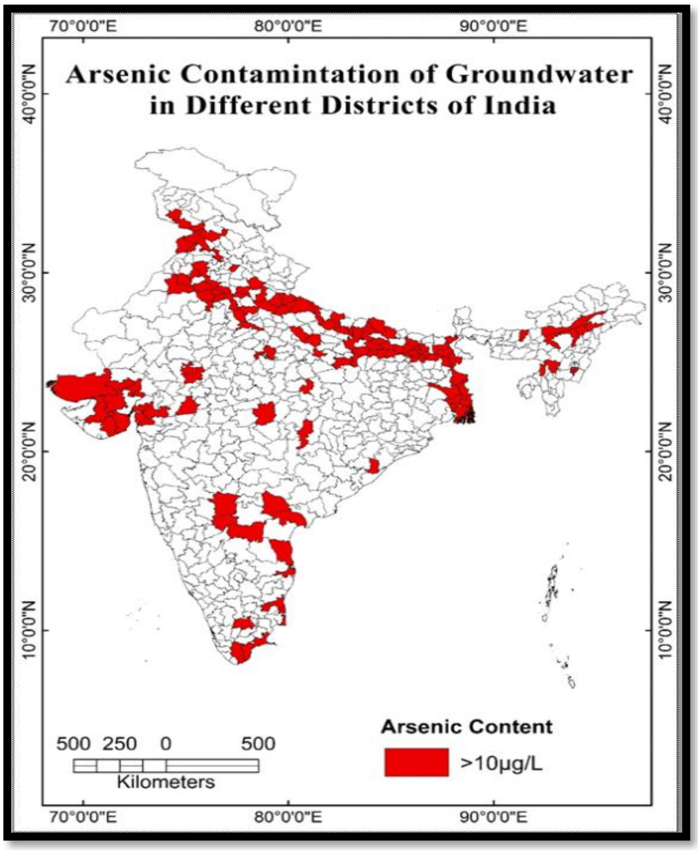

Figure 1.2 Arsenic contamination in India (Source- CGWB,2018)

1.3.3.2 Cadmium pollution prevalence in India

Cadmium is a non-essential trace element that is widely distributed in the environment. Its anthropogenic as well as natural sources can elevate its presence. According to WHO guidelines for drinking water, the permissible range of cadmium is 3ug/L. Cadmium is the most mobile and toxic element present in the atmosphere and due to its similar ionic radius, similar chemical behavior and similar identical charge it can replace calcium in minerals. Due to this reason Cd can enter human body and accumulate in many organs.[49] All heavy metals including cadmium are not degradable and the cadmium can remain present in the soil for 15-1100 years.[50] Ground water contamination by cadmium can mainly be observed in west Uttar Pradesh regions [51] and major metro cities of India.[52]Average values of Arsenic and Cadmium in all soil types in India has exceeded their permissible limit. The mean value of the As and Cd contamination in Indian soil is 148.70 ug/g and 14.16 ug/g respectively.[53]

1.4 Heavy metal remediation

Several studies have demonstrated that of reactive oxygen species and elevation in oxidative stress by heavy metals are the main factors for the generation of toxicity and carcinogenicity. These heavy metals are Arsenic, Cadmium, Chromium, Lead and Mercury. [54] There are various remediation techniques ranging from physical, chemical, biological or electrical techniques [55]which are summed up in Figure 1.3. But of all these techniques biological methods of remediation are most economic and effective.

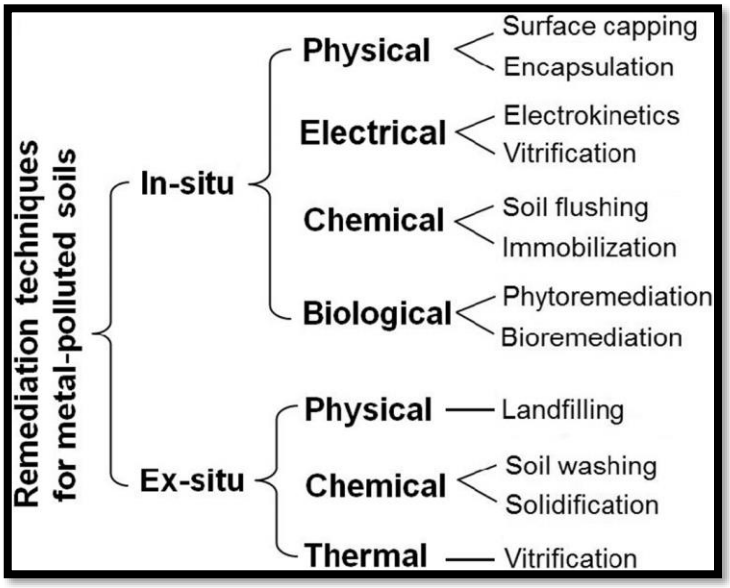

Figure 1.3 Various methods of heavy metal remediation from soil (Liu *et.al.*, 2018)

1.4.1 Phytoremediation

The use of plants to remove or neutralize the heavy metals present in the soil is called as Phytoremediation method. Generally, the plants which have a very dense root system as well as are capable of producing high biomass are used for this purpose. Both terrestrial and aquatic plants are used. Commonly terrestrial crops like sunflower and Indian mustard are used for remediation of agricultural lands, while for the remediation of wetlands, plants like duckweed, cattail, hyacinth and azolla are used. Terrestrial crops have longer and more hairy root system compared to the aquatic plants, which cover more area of the rhizosphere. These crops absorb the heavy metals present in the soil through their roots and neutralize them. The first level of defense are the root exudates like organic acids and amino acids which acts as the heavy metal ligand and forms a stable complex with them. Some root exudates can change the pH of the rhizosphere, resulting in the precipitation of heavy metals, thus their bioavailability is reduced.

Secondly cell wall pectin consists of negatively charges carboxylic groups which can act as the cationic exchanger thus binding the heavy metal and immobilizing it on the cell wall itself.[56] If these defenses fails and the heavy metal enters the cell cytoplasm, plants adapt different strategies such as inactivation, chelation and compartmentalization to tackle heavy metal stress. There are many organic and inorganic ligands present in the cytosol of plants. Organic compounds like phytochelatins, metallothionines, glutathione and cell walls (pectin, polyphenols and proteins attached on them) present in cytosol chelates the heavy metals. After chelation these ions are moved to vacuoles where they remain stored away from normal cytosol. In this way these ions would not interfere with normal function of cells. This is known as compartmentalization. Accumulation of heavy metals in plant cells could greatly increase oxidative stress which damages DNA and proteins by generating reactive oxygen species (ROS). [56] To cope up with the stress and neutralize oxidative species, plants have various enzymatic (Catalase, Superoxide dismutase, Ascorbate peroxidase, glutathione reductase and peroxidase) as well as non-enzymatic (Glutathione, flavonoid, carotenoids, ascorbate and tocopherols) antioxidant compounds. The description of these mechanisms can be visualized in Figure 1.4.

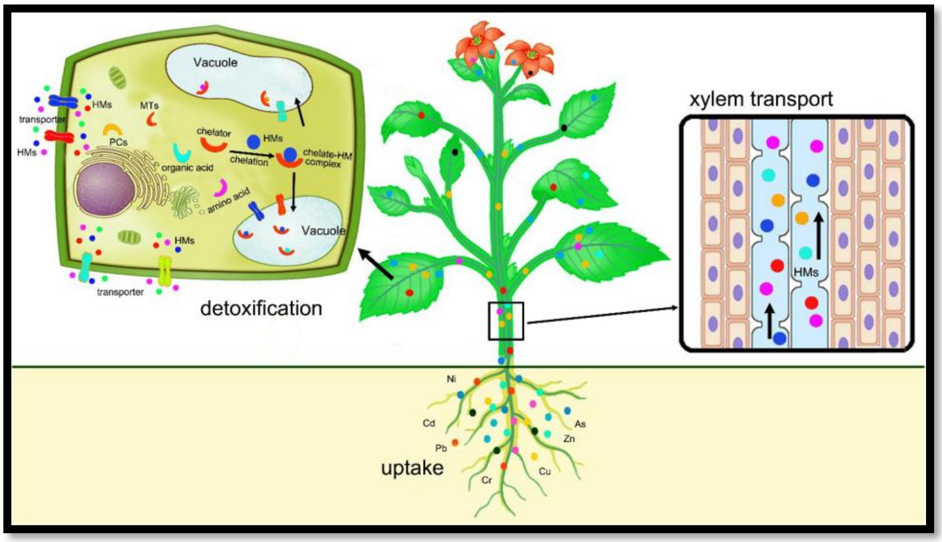

Figure 1.4 Schematic diagram for uptake, translocation and sequestration of heavy metals by plants (Yan *et.al.*,2020)

1.4.2 Bioremediation

The use of living microorganism for the neutralization of heavy metals is known as bioremediation. Generally, plant associated rhizospheric microbes are used in this technique.

These microbes are also broadly known as Plant growth promoting rhizobacteria (PGPR). PGPR's are capable of synthesizing various enzymes, organic acids, siderophores, antibiotics and phytohormones.[57] Organic acids and siderophores can act as an efficient chelator which can help remove heavy metals.[58] PGPR can synthesize ACC deaminase, which degrades ethylene precursor which helps in promotion of the plant growth.[59] PGPR also secrete IAA, which helps in root hair development, thus prompting plant growth.[60] Generally these microorganisms release high concentration of organic acids and siderophores, which changes the pH of the rhizosphere thus modifying the bioavailability of heavy metals.[61] Besides this they also solubilize insoluble phosphate in soil and fixes atmospheric nitrogen. It is also known that the plants inoculated with PGPR significantly decreases the antioxidant enzymes and ROS, thus increasing the nutritive value and biomass of the plant. General PGPR activities are summarized in Figure 1.5.

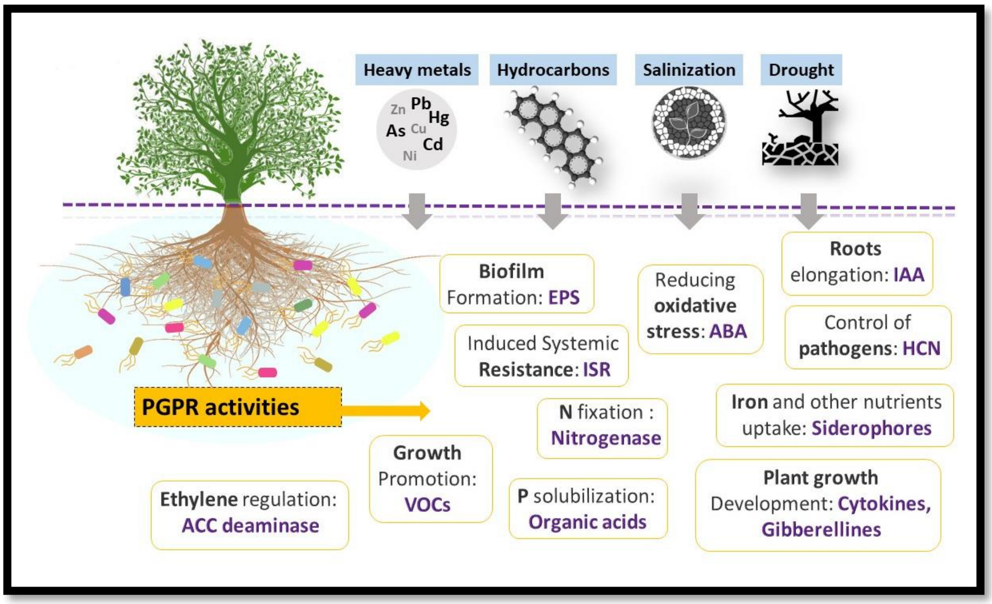

Figure 1.5 Schematic representation of the PGPR activities (Vocciante *et.al.*,2022)

1.4.2.1 Mechanisms employed by bacteria/PGPR to combat heavy metals

Bacteria present in the metal contaminated soil faces enormous stress, as the heavy metals present in the rhizospheric soils are mobile. Their mobility is due to their capacity to develop ligands with the organic matter present in the rhizosphere. The heavy metals are present in the form of oxides, sulfides, phosphates, silicates or hydroxides. This mobility leads to their leaching from total soil mass to aqueous medium. Generally, they are taken up by the rhizospheric bacteria but it is very important to maintain the internal concentration of heavy metals to reduce their toxicity. Bacteria has developed a plethora to mechanisms to tackle this problem. Mechanisms include intracellular sequestration, precipitation on cell wall, binding with proteins, reduction of metals into less toxic form and efflux pumps. Cysteine rich peptides like metallothionines which are present in the cytoplasm captures and neutralizes the heavy metals.[62] Mechanisms employed by bacteria are summarized in Figure 1.6.

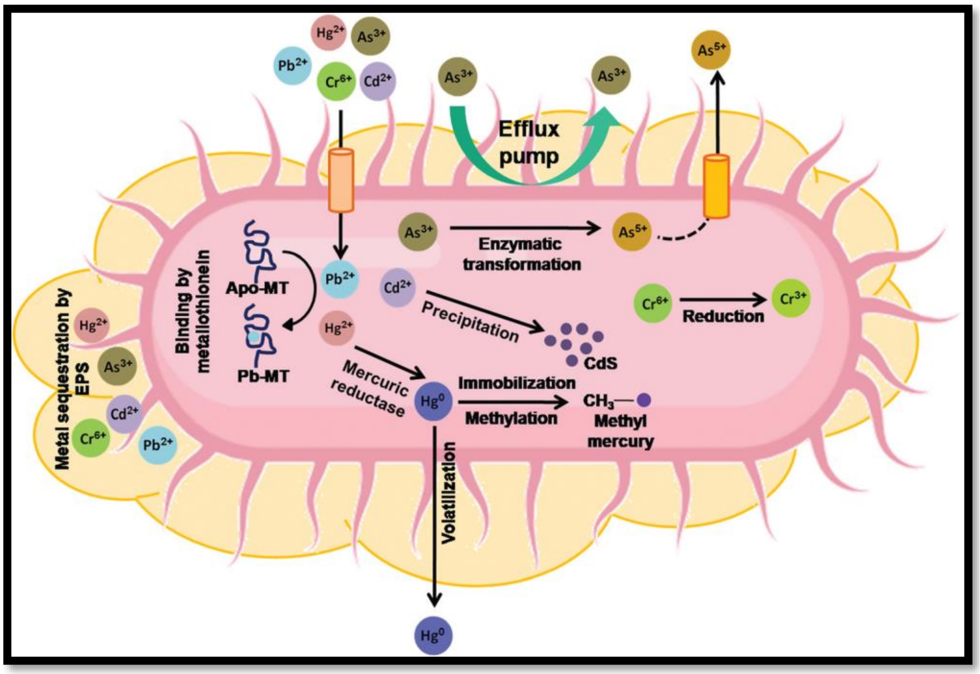

Figure 1.6 Mechanisms employed by bacteria to neutralize heavy metal stress (Chatterjee *et.al.*, 2020)

One of the most important traits of bacteria is the precipitation of heavy metals, which results in the formation of nanoparticles. This essentially converts the elemental heavy metal ion into a heavy nano particle whose mobility is reduced in the rhizosphere, thus this process is capable of decreasing the bioavailability of heavy metals. Cysteine desulfhydrase, an important enzyme present in the cytoplasm of bacteria is responsible for generating sulphide from cysteine. Whenever the bacteria absorb cadmium metal ion, the sulphide is generated from cysteine by the enzyme which binds with cadmium to form cadmium sulphide. This cadmium sulphide is stabilized with glutathione or metallothionein and stored inside the vacuole. Besides this there is also an extracellular precipitation method in which the sulphide inside the cells when the bacteria are challenged with heavy metals. This sulphide molecules are transported outside the cells where they precipitate metal ion and form metal sulphide (Figure 1.7).[63] Metal sulphide are stabilized by the proteins and peptides released by the bacteria.[64]

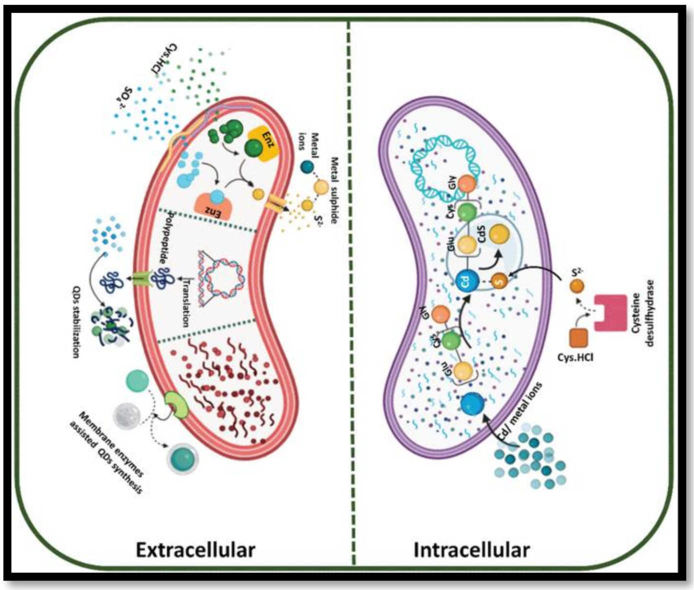

Figure 1.7 Schematic representation of formation of metal sulphide by bacterial cells exposed to heavy metal stress (Mahale *et.al.*, 2021)

1.5 Symbiosis- heavy metals- glutathione relation and interplay

1.5.1 Rhizobium, a PGPR capable of symbiosis with legumes

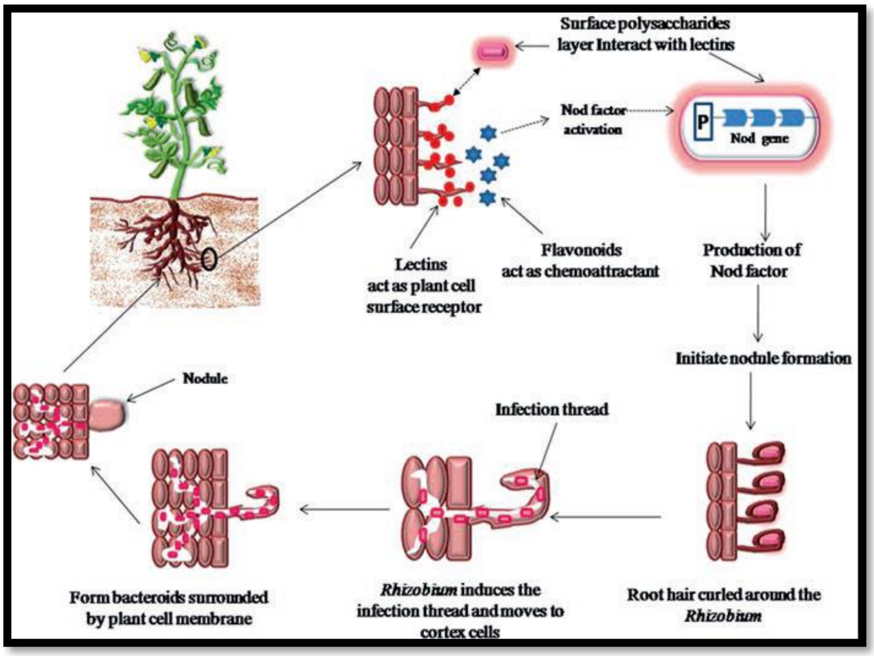

Figure 1.8 Schematic representation of symbiosis mechanism by rhizobium (Singh *et.al.*, 2019, Book chapter)

About 1400 million metric tons of atmospheric nitrogen is fixed annually out of 10^{15} tons of gaseous atmospheric nitrogen. About 90 % of the fixed nitrogen is biological and rest is fixed by lightening. The prokaryotes so far are the only known source of nitrogen fixation. Total 87 species in 38 genera of bacteria, 2 in archaea and 20 in cyanobacteria have the capacity to fix atmospheric nitrogen. They can be free living as well as symbiotic. Symbiotic nitrogen fixation in Leguminosae family is associated with the class alphaproteobacterial, family *Rhizobaiceae.* Rhizobium is a genus of gram-negative motile bacteria which is also known as root nodule bacteria (RNB), as it establishes the symbiosis with legume roots, enters them and forms nodules where they can fix the atmospheric nitrogen. Schematic representation of the mechanism of symbiosis establishment is shown in Figure 1.8.[65]

1.5.2 Prokaryotic glutathione secretion mechanism and the importance of rhizobial glutathione in symbiosis

Genetically engineered bacterial species that are capable of overexpressing glutathione synthesis related enzymes can significantly increase the precipitation of heavy metals.[63] Although the transport of glutathione in bacteria is poorly studied, it is known that some gram-negative bacteria including *E. coli* are capable of secreting glutathione.[66] Besides this *S. typhimurium* LT-20[67] and *Proteus mirabilis*[68] are also reported to excrete glutathione. In spite of the poorly studied transport system of glutathione in prokaryotes, an ABC type transporter CydDC has been identified in *E. coli* [69] which is responsible for secretion of glutathione. Also, there are other ABC peptide transporters which can possibly secrete glutathione outside of the gram-negative bacterial cell.[70] One of the most important soil bacteria is rhizobium, which is a class of PGPR responsible for fixing nitrogen by formation of root nodules in the legume plants. Free living rhizobium also fixes nitrogen as well as promotes growth in the plants. Legumes growing in heavy metal polluted soil shows decreased biomass, poor nutritive value as well as depleted rhizospheric PGPR bacteria. It is also known that glutathione plays an important role in growth as well as symbiotic capacity of rhizobium. *S. meliloti* mutant strain having disrupted gshA and gshB gene showed no growth phenotype and delayed growth (with reduced nodulation and 75 % reduction in nitrogen fixation) respectively in legume.[71] In a study it was also proved that the glutathione produced by rhizobium prevents early senescence of nodules in common bean legume plant.[72] These points proves that the glutathione is not only important in bioremediation of heavy metals, but it is equally important in legume symbiosis of rhizobium bacteria. A strategy of overexpression of glutathione in a rhizobium bacterium and inoculating it with a legume growing in heavy metal polluted soil could help in bioremediation as well as improvement in symbiosis between rhizobium and legume.

1.6 Effect of heavy metal stress on fenugreek and alleviation of stress by PGPR

Fenugreek (*Trigonella foenum-graecum*) is an important legume consumed throughout the world. It is known for its medicinal and nutraceutical properties. Besides this it is also used in food processing industry as a stabilizer, adhesive or emulsifying agent. [73] It is known that the abiotic stress significantly decreases the biomass of fenugreek and increases the ROS levels which in turn increases the enzymatic and non-enzymatic antioxidant levels. Heavy metal stress also affects the germination of fenugreek seeds.[74,75] Similar results were observed when the fenugreek was allowed to grow under lead stress, but when glutathione and melatonin were added externally, the biomass increased, oxidative parameters and antioxidant enzyme levels decreased, thus nutrition increased.[76] Besides the direct application of compounds, treatment/inoculation of the legumes with PGPR is also a very beneficial and economical option. Many studies have suggested the use of PGPR including rhizobium for different medicinal plants like fenugreek growing in abiotic stress. They have alleviated the abiotic stress induced oxidative damage and improved the biomass as well as nutritional quality of these plants.[77] Therefore, we can comment that employing a PGPR for a crop growing in heavy metal polluted soils is beneficial. Not only wild type but genetically modified PGPR are also used to alleviate abiotic stress in crops. [78]

1.6.1 Fenugreek- an important culinary microgreen

Traditionally fenugreek seeds are used as a dry spice and in varieties of culinary preparations throughout India. Seeds have a great nutritional value due to its high antioxidant properties, as it contains high amounts of flavonoids and polyphenols.[79] Besides this fenugreek leaves and seeds are rich source of proteins, important lipids and minerals.[80] Consumption of fenugreek leaves is very common in India. It has multiple benefits and also have a great medicinal importance. A study reported that fenugreek consumption by diabetic patients significantly improve their lipid profile in comparison to the control patients. Patients fed with fenugreek leaves significantly showed lower LDL and TG, while their HDL levels increased.[81] Fenugreek is an annual herb and generally grows about up to 3 feet in height in 120-150 days. After 120 days its ready to harvest, when the leaves and pods become yellowish, and the leaves starts to fall off. [82] At this time the plants are harvested, seeds are collected and stored. Fresh leaves of fenugreek are the better nutrition source than seeds as it has a capability to retain the nutrition in a better way. After 30-40 days of sowing the leaves are ready to harvest and consumed. While there is another better option of microgreen fenugreek which can be harvested in seedling stage for consumption.[83] Microgreens are an emerging functional food of this century. A study showed that the micronutrients are more nutrient dense compared to the mature edible leaves, and a great source of antioxidants. Also, the most interesting thing about microgreens is that it has a very less amount of oxalate, which is an anti-nutrient.[84] It is also known that the microgreens of fenugreek contained higher amount of dietary iron compared to the mature fenugreek.[85]

1.7 Goal of this study

As stated above, microgreens are superior in nutrition value than the mature plants, Also, they are easily cultivable and ready to eat in very less time. It is very important to protect the edible crops from the heavy metal stress as it could result in the decrease of nutritional quality and increase in antinutrients. Heavy metal stress increases the ROS levels which generates lots of oxidative stress which results in decreased in biomass. The primary objective of our study is an employment of PGPR (GMO/wildtype) for the protection of fenugreek seedlings growing in heavy metal stress. (Chapter 4). We are not looking at levels of heavy metals in microgreens or in leaves of fenugreek. Our study was undertaken to exploit the role of PGPR (Wild/genetically modified) in alleviation of effects of heavy metal stress on a nutritionally important plant, Fenugreek.

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