Chapter 4

Determining the effect of rhizobia overproducing glutathione on abiotic stress mediated damage in fenugreek seedling

4.1 Introduction

4.1.1 Fenugreek and its agricultural importance

Fenugreek (*Trigonella foenum-graecum* L.) is a Fabaceae family crop which have a very high nutritive and medicinal properties.[1] It is mainly cultivated on the eastern shore of the Mediterranean, Africa, India, Pakistan and Bangladesh.[2] The name of genus, *Trigonella* is derived from a Greek name, which means "three angled" and that probably denotes triangular flowers. The first recorded use of fenugreek is seen in ancient Egyptian papyrus, which could be dated back to 1500 BC. And the term fenugreek comes from "*foenum graecum*" which is a Greek hay and it was used to scent inferior hay.[3]

From the ancient times fenugreek is regularly used in India for cooking. Description about its medicinal properties can be found in many Ayurvedic texts. Many active metabolites such as saponins, steroids, alkaloids and flavonoids are found in its leaves, stem and seeds. The plant leaves and seeds are regularly consumed in the Indo-Pak subcontinent as a spice. In ancient time this plant was widely used to treat aliments like digestive issues, kidney problems, ulcers, arthritis, bronchitis and urinary infections. Also due to the presence of high number of flavonoids, it is an excellent anti-inflammatory medicine. [4]

Botany

Kingdom: Plantae
Division: Magnoliophyta
Class: Magnoliopsida
Order: Fabales
Family: Fabaceae
Genus: Trigonella
Species: foenum-graecum Linn.

Fenugreek plant generally grows up to 3 ft in height. It has a long slender stem with Gray/green leaves. Leaves are tripartite, toothed and obliviate in shape. Average length leaves are between 20-25 mm. The flowering season of this plant is generally mid-summer (June/July) and the pods of the plants are hairy with persistent brakes in between. Each sword shaped pod bears 10-20 seeds and its average length is about 15 centimetres. Fenugreek seeds are small, hard, brown-yellow in colour and about 5 mm long. The plant radiates spicy and pleasant odour whenever touched by hand. In India it as an annual crop and its mainly cultivated in semi-arid

regions. It is best grown in winter season and it also fairly tolerates frosty climate. It requires full sunlight and requires well drained soil for optimal growth.[4]

4.1.2 Heavy metal stress in legumes and its mitigation by PGPR

Heavy metals exert negative effects on all plants including fenugreek. It disrupts its normal growth of plant by affecting it at the molecular levels by binding with proteins. Heavy metals like Cadmium, lead and Chromium cause deleterious effects by alteration of an antioxidant enzyme profile in fenugreek plant in response to ROS. It was reported that these metal ion contamination in soil can significantly increase the expression levels of antioxidant enzyme encoding genes in fenugreek.[5] Heavy metal like Cobalt can significantly reduce the fresh and dry weight of shoots and roots in fenugreek. When treated with *R. tibeticum*, the fresh and dry weight significantly increased compared to the untreated.[6] Cadmium is one of most toxic metal responsible for contamination of the agricultural soils. Fenugreek growing in cadmium contaminated soil was reported to have a poor growth and increased oxidative parameters like MDA, but when treated with Arbuscular mycorrhiza fungus, the cadmium stress was alleviated and the MDA accumulation in the plant parts significantly decreased.[7] *Vicia faba* L. growing under copper stress showed a decreased biomass and accumulation of copper inside the edible parts of the plant, but when treated with a mixture of rhizobium and other PGPR, it not only improved the growth of the plant but also reduced the copper accumulation in plant. This finding suggests that rhizobium not only helps in mitigating the heavy metal stress effects but it also reduces the bioaccumulation by sequestering the heavy metals in the soil and not allowing them to accumulate in plants.[8] These PGPR can be helpful for plants growing in other abiotic stress like pesticide contamination. It was reported that the rhizobium isolated from the pesticide contaminated soil showed the PGPR effect and improved the plant growth characteristics such as shoot length, root length and germination ability in fenugreek to maximum 1%. [9] Rhizobial consortium made of *S. meliloti* and *P. fluorescence* have been reported to mitigate drought stress in fenugreek seedlings. Inoculation of these bacteria significantly increased the crop yield, all visible growth parameters, total N content and K content in fenugreek plant. [10] All reports suggests that inoculating PGPR in legumes and especially fenugreek growing in heavy metal contaminated soil can be beneficial. Behind this ability, are many capabilities of a PGPR and especially a rhizobium. They produce biofilms in the rhizosphere which can retain the moisture as well as trap the heavy metals and reduce its translocation inside the plants, produce organic acids which can solubilize the insoluble

phosphate and make it available to the plants, their interaction with plants releases cytokines and gibberellins which helps in overall plant development, produces ABA which helps in reduction of oxidative stress and produce siderophores which chelates heavy metal ions. [11] Glutathione is one of the most important siderophore produced by majority of the gram-negative bacteria including rhizobium. [12–14] It is reported that exogenous supplementation of glutathione to the rice plants growing in Arsenic contaminated soil alleviated arsenic induced oxidative damage and restored the ascorbate-glutathione homeostasis.[15] Similarly exogenous supplementation of glutathione also alleviated cadmium induced oxidative stress by modulating antioxidant scavenging activity. [16] It has also been reported that an exogenous application of the mixture of glutathione, thiourea and melatonin significantly reduced the lead induced oxidative stress in fenugreek.[17] These findings suggests that the PGPR and rhizobium are the best candidate for protecting crops growing in heavy metal contaminated soil.

4.1.3. Plan of work

This study constitutes plant experiments. In this study we coated the fenugreek seeds (obtained from local market) with the wild type and genetically modified rhizobium bacteria. After coating the seeds with bacteria, they were sown in loamy soil (obtained locally), spiked with heavy metals and allowed to grow till seedling stage. Plants were harvested and all the morphological and biochemical parameters were recorded and compared between the seeds coated with wild type and genetically modified rhizobium bacteria. In all the experiments seeds coated with *P. fluorescens* were used as a positive control. Soil was spiked with Arsenic and Cadmium heavy metals. Experiments were performed with individual bacteria as well as bacterial consortium.

4.2 Materials and Methods

4.2.1 Bacterial growth and colony morphology under the influence of heavy metal stress

Bacterial growth and colony morphology under the influence of heavy metal stress were observed by growing them on YEM agar plates spiked with increasing concentration (0,30, 50 and 100 ppm) of $NaAsO_2$ and $CdCl_2$. [18] Bacteria were cultured in nutrient media broth to an OD_{600} of 0.8 at 30 °C and 120rpm in an incubator shaker. They were pelleted down and washed twice with phosphate buffer (Ph 7.2) and resuspended in phosphate buffer. 10ul volume of

bacterial suspension was spotted onto the media plate and incubated at 30 °C for 3 days and observations were made.

4.2.2 Seed radicle emergence test

Seed radicle emergence under the influence of Arsenic and Cadmium metals was monitored. Aqueous solution of Arsenic and Cadmium was prepared from Sodium meta Arsenite ($NaAsO_2$) and Cadmium chloride ($CdCl_2$) respectively. 0, 15, 30, 60, 120,300 ppm solution of Arsenic and 0,15, 25,50 and 100 ppm of Cadmium were prepared in sterile distilled water and used for the experiment. Fenugreek seeds were first surface sterilized by 0.1% $HgCl_2$ followed by 70% ethanol and subsequently rinsed with sterile distilled water twice. Ten surface sterilized seeds of uniform colour, weight and size were placed on a Petri dish (9 cm diameter) on double-layered Whatman filter paper No. 1. The filter paper was sprayed with the varying concentration of heavy metal solution, 5ml followed by 2 ml on alternate days till 5 days. After 5 days measurements of seeds were recorded and photographs were taken.[19] Seed germination test under the influence was done to check the heavy metal tolerance capacity of the seeds, so that we do not exceed the heavy metal concentration in soil during plant experiment.

4.2.3 Spot assay

Spot assay was performed to check the tolerance limit of individual bacteria towards Arsenic and Cadmium metals. Bacteria were cultured in the nutrient broth to an OD_{600} of 0.5 at 30 °C and 120 rpm in an incubator shaker. They were pelleted down and washed twice with phosphate buffer (pH 7.2) and again resuspended in phosphate buffer. Bacterial suspension was serially diluted (10^{-1}, 10^{-2} and 10^{-3}), and 2ul of each dilution was spotted on TYE agar plates containing $CdCl_2$ (0, 25 and 50 ppm) and $NaAsO_2$ (0,15 and 30 ppm). These plates were incubated at 30 °C for 48 hours and checked for any evident differences.[20]

4.2.4 Soil selection and analysis

A rich well drained loamy soil was used for the experiments. The soil was procured from the local nursery and ensured that any kind of chemical or biological fertilizers were not added. Basic soil analysis was done from Anand agriculture university and the parameters are mentioned in Table 4.1.

Table 4.1 Result of Soil analysis

Sr. No.	Parameters	Value
1)	Organic carbon (%)	0.09
2)	Available phosphorous (mg/kg)	5640
3)	Available potassium (mg/kg)	88000
4)	Soil pH	7.93
5)	Soil EC (dS/m)	0.13

4.2.5 Bacterization of seeds and pot experiments

Bacterization refers to the coating of seeds with bacteria. Surface sterilized seeds were allowed to dry at room temperature for 1 hour. Bacteria were grown in flasks containing 100 ml enriched medium to an OD_{600} of 0.8 at 30 °C and 120rpm in an incubator shaker. Cells were pelleted down and washed with phosphate buffer (pH 7.2) followed by resuspending them in a flask containing 100 ml 0.03% Carboxymethyl cellulose prepared in sterilized distilled water. Seeds were added in the flask and incubated at 30 °C for 1 hours at 20 rpm for bacterization, followed by 1 more hour without shaking. [21] Bacterized seeds were collected by draining of the solution and allowed to partially dry in Laminar air flow for 5-6 hours and sown in the pots containing loamy soil spiked with heavy metals. [22]

Autoclaved soil was used for the experiments conducted with study group mentioned in Table 4.2 and unautoclaved soil was used for the experiments conducted with the study group mentioned in Table 4.3. Table 4.2 shows the study groups prepared to check the effect of individual PGPR on fenugreek seedlings growing in heavy metal contaminated soil and the Table 4.3 shows the study groups prepared to monitor the effect of PGPR consortium on fenugreek seedlings growing in heavy metal contaminated soil. Consortium C1 and C2 were made by mixing equal number of individual bacteria, followed by coating them on surface sterilized seeds. Consortium 1 (C1) is made up of *Pseudomonas fluorescence* (NAIMCC-B-00342), *Sinorhizobium fredii* NGR 234 and *Sinorhizobium meliloti* (NAIMCC-B-00863). Consortium 2 (C2) is made up of *Pseudomonas fluorescence* (NAIMCC-B-00342), *Sinorhizobium fredii* NGR 234 (pPAT), *Sinorhizobium meliloti* (NAIMCC-B-00863) (pPAT). All the bacteria were mixed in 1:1:1 ratio. Pot experiments were conducted in a make shift greenhouse of the biochemistry department of The M. S. University of Baroda. Sterile plastic

pots were filled with 2 kg soil and the bacterized seeds (Twenty bio-primed seeds) were sown in single pot. Pots were placed in green house under natural photoperiod of 12-13 hours and temperature 25 ± 4 °C. Each treatment was replicated three times. Uncoated seeds and unspiked soil were used as a control for the experiments.

Table 4.2 Individual PGPR study groups, to monitor their effect of fenugreek growing with and without heavy metal stress

Sr. no.	Fenugreek seeds coated with PGPR
1)	Uncoated seeds
2)	*Pseudomonas fluorescens* (NAIMCC B 00342)
3)	*Sinorhizobium fredii* NGR234
4)	*Sinorhizobium fredii* NGR234 (pPAT)
5)	*Sinorhizobium meliloti* (NAIMCC B 00863)
6)	*Sinorhizobium meliloti* (NAIMCC B 00863) (pPAT)

Table 4.3 PGPR consortium study groups, to monitor their effect of fenugreek growing with and without heavy metal stress

Sr. no.	Fenugreek seeds coated with PGPR consortium
1)	Uncoated seeds
2)	C1 (Consortium 1)
3)	C2 (Consortium 2)

4.2.6 Growth measurements

After 16 days (individual PGPR treatment) and 25 days (PGPR consortium treatment) plants were uprooted from the pots carefully (to avoid breaking) and rinsed under running tap water several times. The length of the shoots and roots of seedlings were manually measured. Total number of leaves on each seedling were manually counted. Fresh weight and dry weight of entire seedling was considered. Fresh weight and dry weights were recorded by electronic balance. Seedlings were dried in a hot air oven at 80 °C for 2 days for the measurement of dry weight. [23]

4.2.7 Chlorophyll and carotenoids estimation

Chlorophyll (a, b and total) and carotenoids were estimated from the fresh leaves after 16 days (individual PGPR treatment) and 25 days (PGPR consortium treatment). 200 mg of leaves were collected and crushed in 5 ml 80% (v/v) acetone using a mortar pestle. 2 ml was collected from that and centrifuged at 5000 rpm for 15 minutes. Supernatant was collected and used for colorimetric estimation at 663nm (Chl a), 647nm (Chl b) and 470nm (Car).[24]

4.2.8 Estimation of Hydrogen peroxide

Hydrogen peroxide levels were estimated form fresh shoots and roots after 16 days (individual PGPR treatment) and 25 days (PGPR consortium treatment). H_2O_2 was estimated by homogenising 250 mg tissue in 2.5 ml TCA (0.1%). The homogenate was centrifuged a 12000g for 15 minutes followed by the addition of 250ul of potassium phosphate buffer (10mM, pH 7) and 500ul potassium Iodide (1M). The absorbance of the mixture was recorded at 390 nm.[25]

4.2.9 Estimation of Lipid peroxidation

MDA levels were estimated form the fresh shoots and roots after 16 days (individual PGPR treatment) and 25 days (PGPR consortium treatment). The level of peroxidation in plant tissue was determined by measuring the concentration of 2- thiobarbeturic acid (TBA) reactive metabolite, which is malondialdehyde (MDA). 250 mg plant tissue was homogenised in 2 ml TCA (0.1%) and centrifuged at 12,000 g for 15 minutes. 250ul of supernatant was collected from that and mixed with 500ul of TBA (0.5%, prepared in 20% TCA). The mixture was boiled for 30 minutes followed by its incubation on ice for 5 minutes. The absorbance of mixture was recorded at 532 nm.[25]

4.2.10 Antioxidant enzyme estimation from roots and shoots

200 mg plant tissue (root/shoot) were homogenised by a mortar pestle in 1.2 ml ice cold extraction buffer (50mM phosphate buffer, pH 7; 1% PVP40 and 0.2 mM EDTA). The homogenate was centrifuged at 13000g for 30 minutes at 4 °C and supernatant was collected. 70 ul supernatant was aliquoted for protein estimation by Bradford method and rest was stored at -80 °C for antioxidant enzyme activity determination. All the estimations were carried out within 40 days of storage. Glutathione reductase, Superoxide dismutase, Ascorbate peroxidase and Catalase enzyme activity was determined form the supernatant. For determination of Ascorbate peroxidase, extract was supplemented with 5 mM Ascorbate. Methods for the

determination of SOD and GR activity were adjusted from the originally published methods to facilitate the use of 96 well plates and a plate reader. **Glutathione reductase (GR)** was determined according to Foyer and Halliwell [27]. Of the reaction mixture containing 30 mM potassium phosphate buffer, pH 7.6, 0.2% BSA, 5 mM EDTA, 2.4 mM GSSG, and 0.19 mM NADPH, 160 ul was pipetted to 20 ul sample extract, and the measurement started immediately. Absorbance at 340 nm was recorded for 180 s. GR activity was calculated from the slope of absorbance readings over time based on 1.0 umol of oxidized glutathione reduced per minute at pH 7.6 at 25 C defined as one unit (U).[26] **To determine the SOD activity**, a reaction mixture containing 0.05 M sodium carbonate, 13 mM methionine, 1.3 uM Riboflavin, and 21 uM nitro blue tetrazolium (NBT) in a total volume of 180 ul, 160 ul was pipetted to 20 ul of the sample extract. The 96 well plate was carefully shaken and placed under a strong light source at a distance of 15 cm. After 60 s, initial absorbance was measured at 560 nm. The plate was exposed to the light treatment another 5 min before absorbance was measured again. The presence of SOD inhibits the reduction of NBT, and 1 unit of enzyme is responsible for 50% inhibition.[26] **To determine Ascorbate peroxidase activity,** the reaction mixture was prepared using 50mM potassium phosphate buffer, 0.5 mM Ascorbate, 1 mM H_2O_2 and 0.1 mM EDTA in a total volume of 900 ul, and add 100 ul plant tissue lysate to initiate the reaction. The mixture was taken in a clean 1 ml quartz cuvette, and the absorbance at 290 nm was recorded for 130s. Activity of ascorbate peroxidase was determined by the extinction coefficient of ascorbate (2.8 mM^{-1} cm^{-1}). One unit of APX activity can be defined as the amount of enzyme that can oxidize 1 umol of ascorbic acid/ min/ mg protein. [26,27] **Catalase activity** was determined by monitoring the disappearance of H_2O_2 which was measured as decrease in absorbance at 240 nm (extinction coefficient of 39.4 mM^{-1} cm^{-1}) of a reaction mixture containing 50 mM potassium phosphate buffer, 30 mM H_2O_2 and plant tissue extract.[25,27] Data (n=3) was analysed by GraphPad prism 8.0 software using Two-way ANOVA test and $p<0.05$ was considered to be statistically significant.

4.3 Results and Discussion

4.3.1 Effect of arsenic and cadmium on the growth and colony morphology of bacteria

Bacteria M1; *Pseudomonas fluorescence* (NAIMCC B-00342), M2; *Sinorhizobium fredii* NGR 234, M3; *Sinorhizobium fredii* NGR 234 (pPAT), M4; *Sinorhizobium meliloti* (NIAMCC B-00836), M5; *Sinorhizobium meliloti* (NIAMCC B-00836) (pPAT), C1 and C2 were grown on YEM plates supplemented with Arsenic and Cadmium (Figure 4.1.a and 4.1.b). C1 and C2 are bacterial consortia. (C1) is made up of *Pseudomonas fluorescence* (NAIMCC-B-00342), *Sinorhizobium fredii* NGR 234 and *Sinorhizobium meliloti* (NAIMCC-B-00863). (C2) is made up of *Pseudomonas fluorescence* (NAIMCC-B-00342), *Sinorhizobium fredii* NGR 234 (pPAT), *Sinorhizobium meliloti* (NAIMCC-B-00863) (pPAT). All strains were able to tolerate Arsenic stress until 100 ppm. At 100 ppm M2 grew very sparsely. Unlike M2, its GMO counterpart M3 was able to grow well at 100 ppm Arsenic stress (Figure 4.1.a). This could be due to its ability to produce enhanced levels of glutathione (1.377 folds more compared to M2 in YEM broth) and it is known that GSH plays a very important role in alleviating the heavy metal induced oxidative stress. [28] M4 and M5 were easily tolerated 100 ppm arsenic stress, but M4 colony was darker and bigger compared to M5. As M5 is a GMO (contains pPAT plasmid), it could be facing plasmid induced metabolic stress, [29] which might be responsible for its retarded growth in 30 ppm -100 ppm Arsenic stress compared to M4. At 0 ppm the growth of all the bacteria was almost similar. Consortium C1 and C2 were able to tolerate 100 ppm Arsenic stress and their colony on the plate appeared largest and darkest with lobate margins. In Cadmium stress, M1 showed diminished growth from 30 ppm to 100 ppm. M4-C2 grew well till 100 ppm Cadmium stress, while M2 and M3 grew properly until 50 ppm. M2 showed retarded growth at 100 ppm Cadmium stress while M3 was unable to grow. At 50 ppm Cd stress M2 colony was bigger and darker compared to M3 (Figure 4.1.b), which could be due to the plasmid induced metabolic stress[29] added up with the cadmium metal stress. Consortium C1 and C2 were able to tolerate 100 ppm Cd stress and their colony on the plate appeared largest and darkest with an entire margin. This implies that Cadmium is more toxic than Arsenic for the PGPRs used in our study. Observations of this experiment were used to determine the upper limit of the arsenic and cadmium stress for further experiments. Also, it is very important to note that the growth rate of bacterium in liquid and solid growth media can vary. It was also noted earlier (Chapter 3, growth curve) that the GMO showed a retarded

growth initially in liquid broth (YEM, M9) in log phase (which might be due to plasmid induced metabolic load), but soon its growth rate increased significantly compared to its wild type counterpart. Similar results cannot be expected for an identical bacterium growing on a plate. Unlike liquid broth, the scope of obtaining the nutrition is limited (form the colony periphery only) for the bacterial colony growing on plates (figure). The liquid broth is constantly shaking which provides a wider scope for a bacterium to obtain nutrition form different points while revolving in a liquid medium.[30]

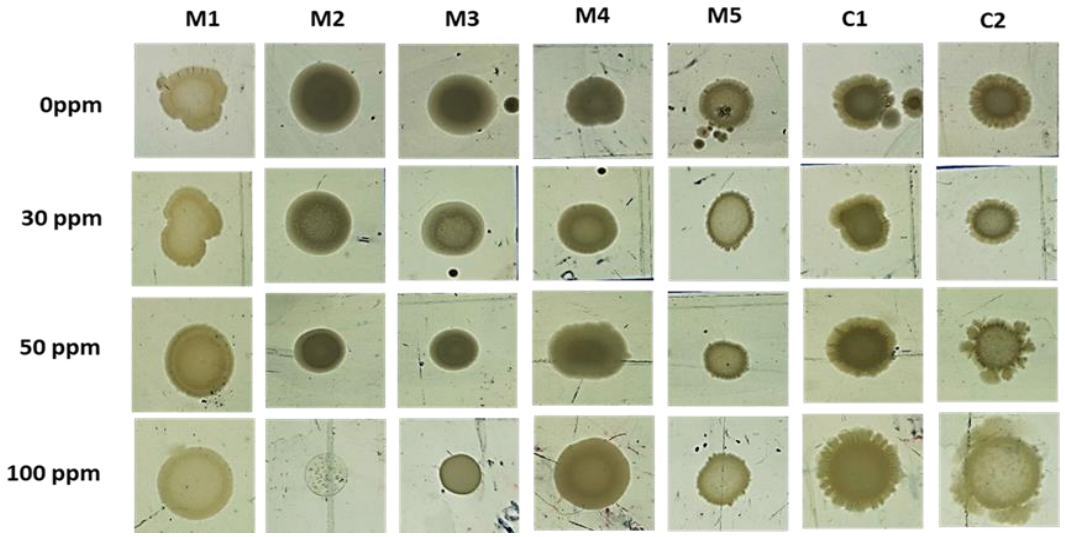


Figure 4.1.a Bacterial colony morphology under the influence of Arsenic stress

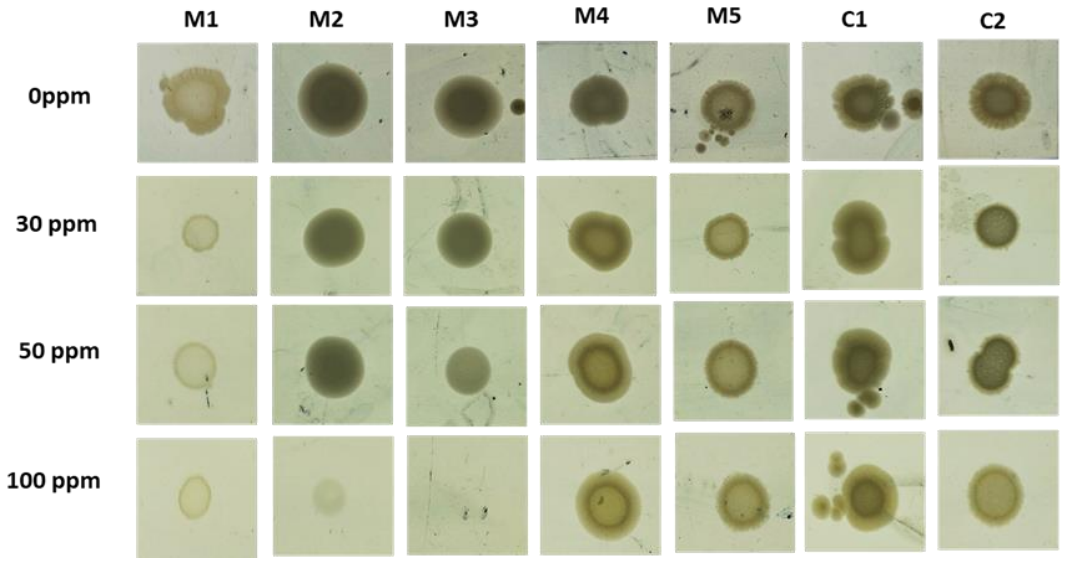


Figure 4.1.b Bacterial colony morphology under the influence of Cadmium stress

4.3.2 Effect of Arsenic and Cadmium on seed radicle emergence

The radicle is the first organ to emerge from the seed and contact the soil as well as PGPR, so it is necessary to check the effect of heavy metal stress of seed radicle emergence.[31] Treatment with varying concentration of Arsenic and Cadmium showed a significant effect on the radicle emergence of fenugreek seeds. Seeds under arsenic stress showed a significant progressive decrease in radicle length till 60 ppm. While at 120 ppm and 300 ppm the seed were unable to germinate, hence no radicle emergence was seen (Figure 4.2.a). At 15 ppm and 30 ppm the colour of the tip darkened significantly compared to 0 ppm. Arsenic is responsible for damaging the root epidermal cells and aerenchymatous cortex by increasing lipid peroxidation, [32] H2O2 and proline,[33] which results in improper growth of radicle, fewer root hair development and the roots tip becomes dark-brown and brittle. Similarly, under cadmium stress the length of the radicle significantly decreased until 100 ppm. Maximum reduction in radicle length was seen at 50 ppm and 100 ppm, while the darkening of radicle tip was noted 15 ppm onwards (Figure 4.2.b). The darkening of root tip is well observed phenomena with many species growing in cadmium contaminated soils.[34] Cadmium chloride is known to inhibit the proliferative activity in root cells and cause chromosomal aberrations, [35] which decreases the mitotic index. Also, high concentrations of cadmium can significantly increase oxidative stress by accumulating hydrogen peroxide and superoxide radicles in a very short period which causes damage. [36] These results were used to determine the upper limit of Arsenic and Cadmium stress for further experiments.

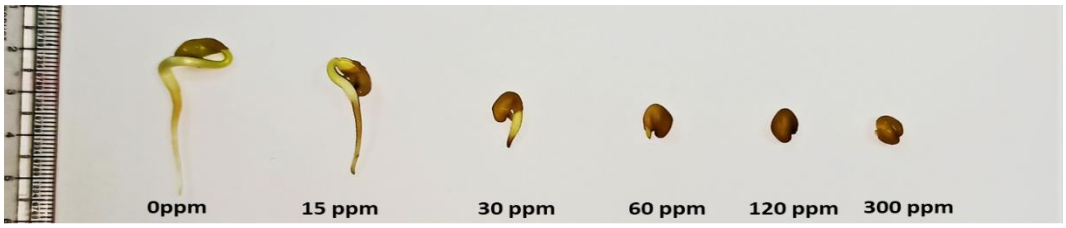


Figure 4.2.a. Effect of Arsenic on radicle emergence in fenugreek seed

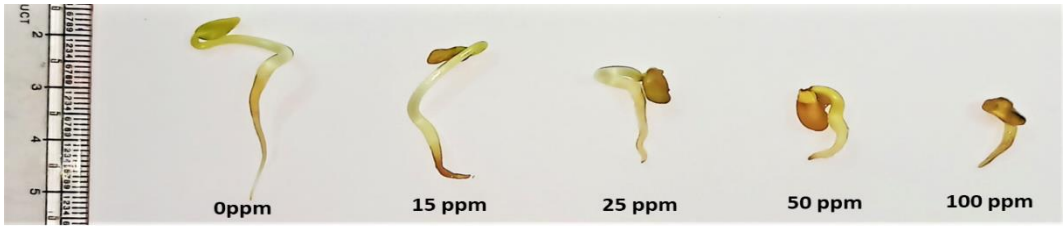


Figure 4.2.b. Effect of Cadmium on radicle emergence in fenugreek seed

Looking at the results of section 4.3.1 and 4.3.2, concentration of arsenic and cadmium were determined for the further experiments. In Arsenic stress radicle was not able to emerge beyond 30 ppm concentration (figure 4.2.a) and PGPR showed optimal growth up to 50 ppm concentration. Since the seeds were not be able to germinate properly beyond 30 ppm, 30 ppm concentration was fixed as the upper limit for plant experiments. In cadmium stress the radicle was able to develop properly up to 50 ppm concentration, at 100 ppm the emerged radicle was smaller and unhealthy and would not develop in plants probably. And the PGPR showed proper growth up to 50 ppm cadmium stress, so 50 ppm concentration was determined as the upper limit for cadmium stress. GMO has been successful in producing higher levels of glutathione and has shown enhanced growth rate in the nutrient broth as well as minimal media broth (Chapter 3), so there is a confirmation that the growth rate of GMO bacteria has been enhanced due to its ability to produce more glutathione compared to their wild type counterpart. It is also important to note that the translocation rate of heavy metals, availability of nutrients and the mobility of bacteria will differ in soil, solid nutrient media and liquid nutrient broth.

4.3.3 Spot analysis to determine the sensitivity of bacteria towards arsenic and cadmium

Glutathione is a chelating agent and it plays a very important role in alleviating the metal induced oxidative stress. To compare the sensitivity of the GMO rhizobium (producing more glutathione) harbouring *ybdK* gene and wild type rhizobium, spot analysis was performed.

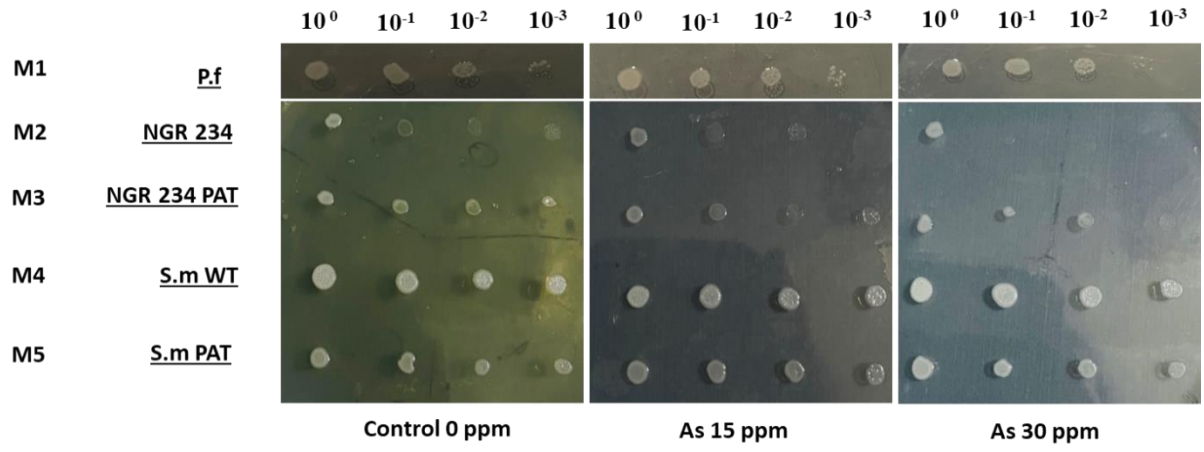


Figure 4.3.a Spot assay to determine bacterial sensitivity towards Arsenic induced stress

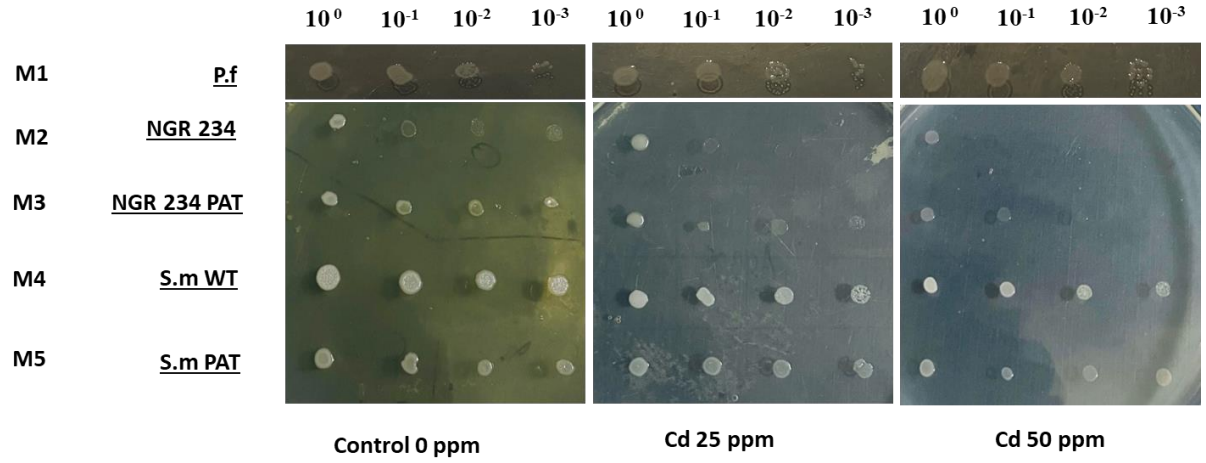


Figure 4.3.b Spot assay to determine bacterial sensitivity towards Cadmium induced stress

Figure 4.3.a and figure 4.3.b shows the spot analysis of all strains under arsenic and cadmium stress respectively. All strains grew properly according to their growth rates (growth curve, chapter 3) at 0 ppm As and Cd stress, and showed the growth till 10^{-3} dilution. M3 proved to be less sensitive to arsenic (15 and 30 ppm) and cadmium (25 and 50 ppm) the heavy metals, compared to M2, while no significant difference in sensitivity was recorded between M4 and M5. Only at cadmium 50 ppm stress M5 showed enhanced sensitivity as compared to M4. This might be due to plasmid induced metabolic load (explained in section 4.3.1) which translates into a development of slightly smaller colonies of GMO bacteria. But it is also important to note that the growth pattern of GMO and wild type will differ in liquid broth and solid media (explained in section 4.3.1) and the GMO has been successful in producing higher levels of glutathione in nutrient broth as well as minimal media broth (Chapter 3). In Arsenic 15 ppm and 30 ppm stress, M2 showed growth till 10^{-2} and 10^{-1} dilution respectively, while M3 showed growth till 10^{-3} and 10^{-3} dilutions respectively. In cadmium 25 ppm and 50 ppm stress, M2 showed growth till 10^{-1} and 10^{-0} dilution respectively, while M3 showed growth till 10^{-3} and 10^{-2} dilutions respectively. These results suggests that M1, M3, M4 and M5 are more resistant to arsenic and cadmium compared to M2.

Plant experiment results

Section 4.3.4: Investigating the effect of genetically modified *Sinorhizobium fredii* NGR 234 & *Sinorhizobium meliloti* (NAIMCC-B-00863) on the growth of fenugreek seedlings in Arsenic contaminated soil.

Section 4.3.5: Investigating the effect of genetically modified *Sinorhizobium fredii* NGR 234 & *Sinorhizobium meliloti* (NAIMCC-B-00863) on the growth of fenugreek seedlings in Cadmium contaminated soil.

Section 4.3.6: Investigating the effect of rhizobial consortium on the growth of fenugreek seedlings in Arsenic & Cadmium contaminated soil.

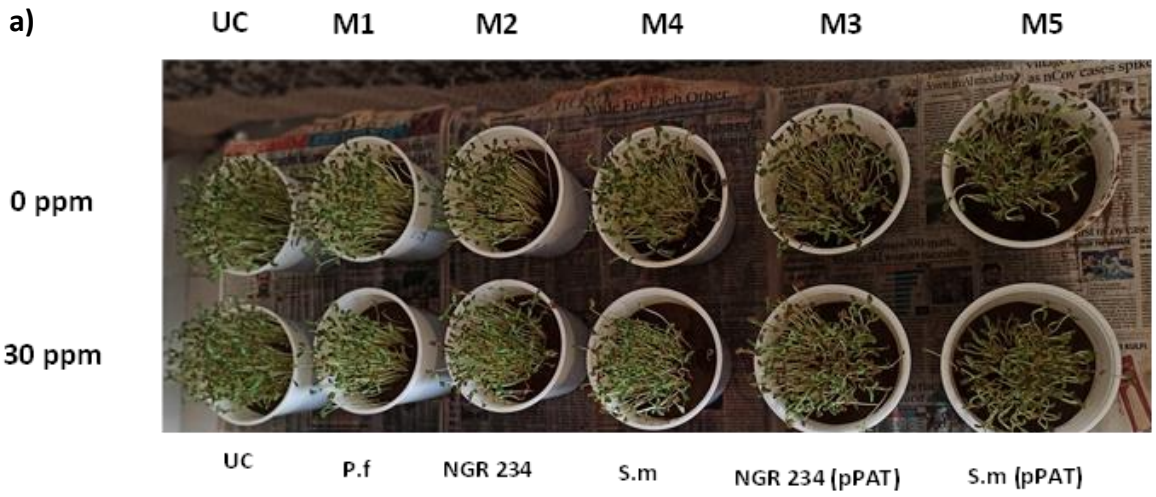
Section 4.3.4: Investigating the effect of genetically modified *Sinorhizobium fredii* NGR 234 & *Sinorhizobium meliloti* (NAIMCC-B-00863) on the growth of fenugreek seedlings in Arsenic contaminated soil.

4.3.4.1 Assessment of growth and chlorophyll production in fenugreek seedlings treated with GMO and wild type bacteria growing in Arsenic contaminated soil

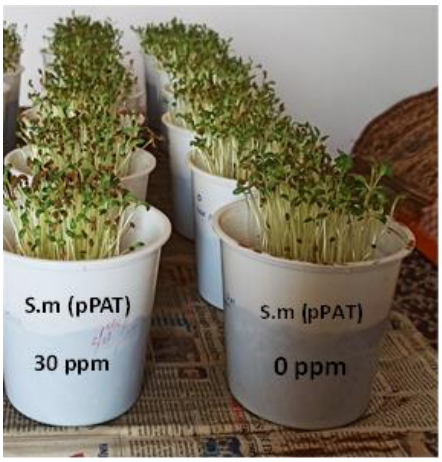
Seeds were coated with PGPR (mentioned in Table 4.2) and sown in the soil spiked with Arsenic (30 ppm). Unspiked soil (0 ppm) and uncoated seeds were the controls of the experiment. Seedlings were allowed to grow for 16 days and photographs were taken. Figure 4.4.a and 4.4.b shows the fenugreek seedlings (treated with different PGPR) growth in pots in control and stress conditions. Seedlings were carefully uprooted from the soil and photographed on a white paper (Figure 4.4.c and 4.4.d) and the observations were recorded. Root length and shoot length were measured by scale. Figure 4.4.e, 4.4.f and 4.4.g shows the graph of shoot length, root length and total length respectively, which describes the seedlings treated with different PGPR at 0 ppm stress (grey colour) and the seedlings treated with different PGPR at 30 ppm stress (light green colour) on x axis and the length (in centimetres) on y axis. Name of the PGPR is described by a single letter M in the upcoming results and discussions. Bacteria used in the experiment are, M1; *Pseudomonas fluorescence* (NAIMCC B-00342), M2; *Sinorhizobium fredii* NGR 234, M3; *Sinorhizobium fredii* NGR 234 (pPAT), M4; *Sinorhizobium meliloti* (NIAMCC B-00836), M5; *Sinorhizobium meliloti* (NIAMCC B-00836) (pPAT) and the uncoated seeds were denoted as UC. It was observed that the total

length of seedlings treated with GMO bacteria was significantly greater compared to the seedlings treated with wild type bacteria.

a)



b)



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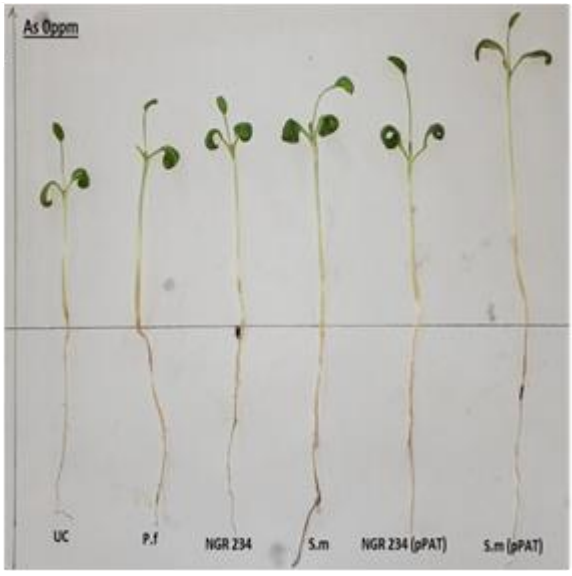

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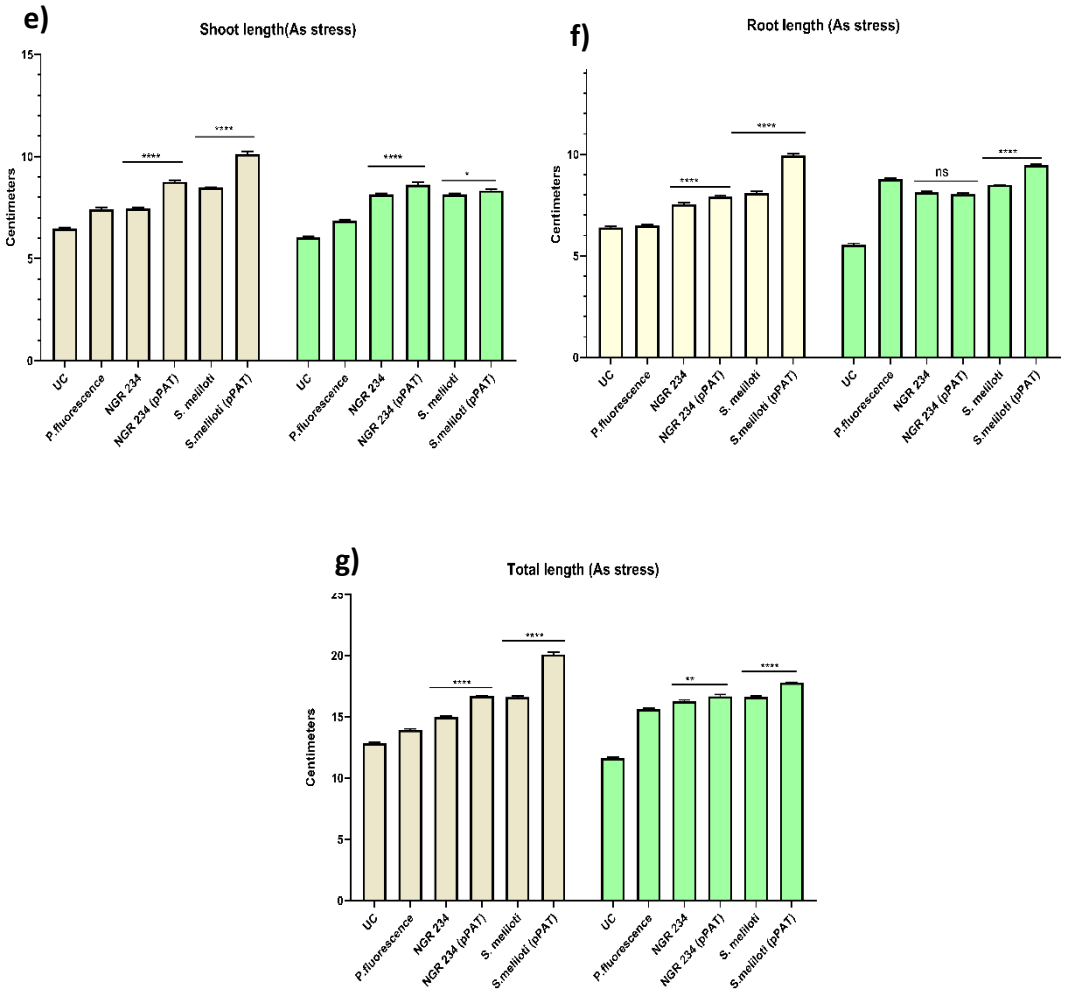


Figure 4.4. a) Growth of fenugreek seedlings treated with PGPR in Arsenic stress (Top view) b) Growth of fenugreek seedlings treated with PGPR in Arsenic stress in Arsenic stress (Side view) c) Seedlings morphology at 0 ppm arsenic stress d) Seedlings morphology at 30 ppm arsenic stress e) Shoot length of fenugreek seedlings treated with PGPR growing in arsenic stress f) Root length of fenugreek seedlings treated with PGPR growing in arsenic stress g) total length of fenugreek seedlings treated with PGPR growing in arsenic stress.

At 0 ppm the total length of seedlings treated with M3 was 10 % greater than M2, while M5 was 17.42% greater than M4. At 30 ppm the total length of seedlings treated with M3 was 2.34 % greater than M2, while M5 was 6.52 % was greater than M4 (Figure 4.4.g). Similar trend was observed with the individual shoot and root length, Figure 4.4.e and 4.4.f). Uncoated seeds/ untreated seedlings reported the lowest growth. Since it is known that the exogenous application of glutathione can enhance the plant height in normal and stressed conditions [37], our results are in accordance with this fact. M3 and M5 capable to produce more glutathione

compared to the wild type bacteria, which would release GSH in the rhizosphere, hence increase the plant height.

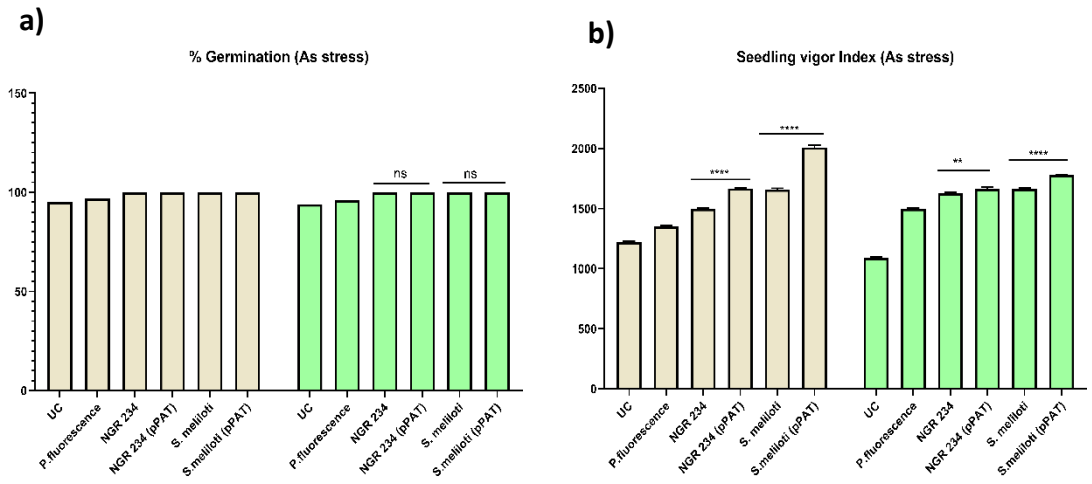


Figure 4.5.a) Germination % of the seeds coated with PGPR sown in Arsenic stress. b) SVI of seedlings treated with PGPR sown in Arsenic contaminated soil.

Heavy metal stress can significantly reduce the plant growth [38] and seed germination percentage, which could result in decrease in the seedling vigor index.[39] But it is also known that coating seeds with bacteria (PGPR), antioxidants (Glutathione, Ascorbate) [40] and other chemicals [41] could reverse this condition by protecting the seed form heavy metal induced ROS and enhance the seed germination. It was observed that the seeds coated with PGPR showed higher germination percentage in 0 ppm as well as 30 ppm Arsenic stress as compared to the uncoated seeds. At 0 ppm, uncoated seeds and the seeds coated with M1 reported 95% and 97 % germination respectively and the seeds coated with M2- M5 reported 100 % germination. Similarly at 30 ppm, uncoated seeds and seeds coated with M1 reported 94 % and 96 % germination respectively and the seeds coated with M2-M5 reported 100 % germination (Figure 4.5.a). This implies that priming of the seeds do have a beneficiary effect as the primed seeds did not report a fall in germination percentage in 30 ppm arsenic seeds, while there was a decrease in germination percentage in the case of uncoated seeds. Also, the seedling vigor index of the seedlings treated with GMO bacteria, was significantly higher than the seedlings

treated with wild type bacteria at 0 ppm and 30 ppm arsenic stress. SVI of the untreated/ uncoated seedlings was lowest at 0 ppm and 30 ppm arsenic stress (Figure 4.5.b).

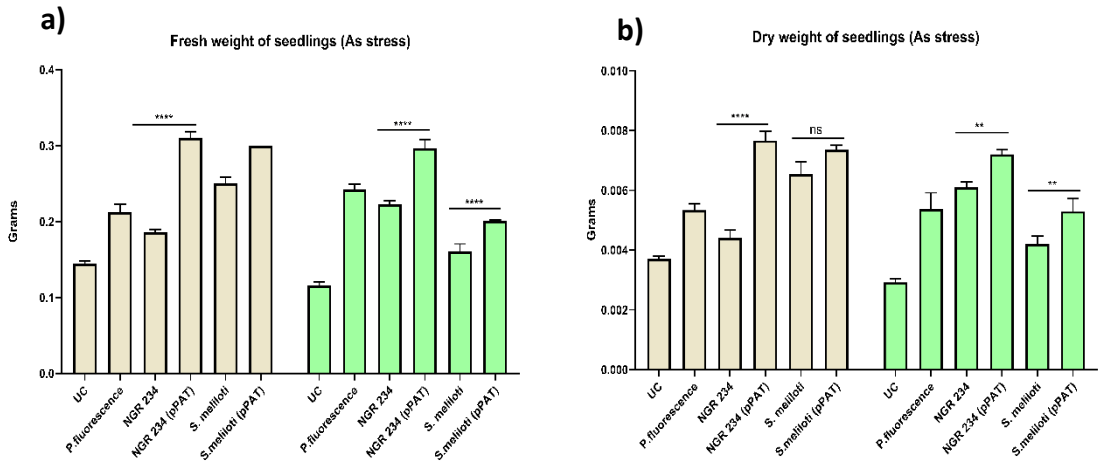


Figure 4.6.a) Fresh weight of seedlings treated with PGPR grown in Arsenic contaminated soil. b) Dry weight of seedlings treated with PGPR grown in Arsenic contaminated soil.

Similarly fresh weight and dry weight of the seedlings treated with GMO bacteria was greater compared to the seedlings treated with wild type bacteria, at 0 ppm and 30 ppm arsenic stress. At 30 ppm arsenic stress the fresh weight of the seedlings treated with M3 was 25.25 % greater than M2 and M5 was 19.90 % greater than M4 (Figure 4.6.a). At 30 ppm arsenic stress the dry weight of the seedlings treated with M3 was 14.28 % greater than M2 and M5 was 20 % greater than M4 (Figure 4.6.b). GSH is an antioxidant and it alleviates the arsenic induced oxidative stress which improves the physiological conditions of a plant cell [15]and protects it from genetic aberrations which could have resulted in increased SVI, germination percentage and plant weight for the seedlings treated with GMO PGPR compared to the seedlings treater with wild type PGPR.

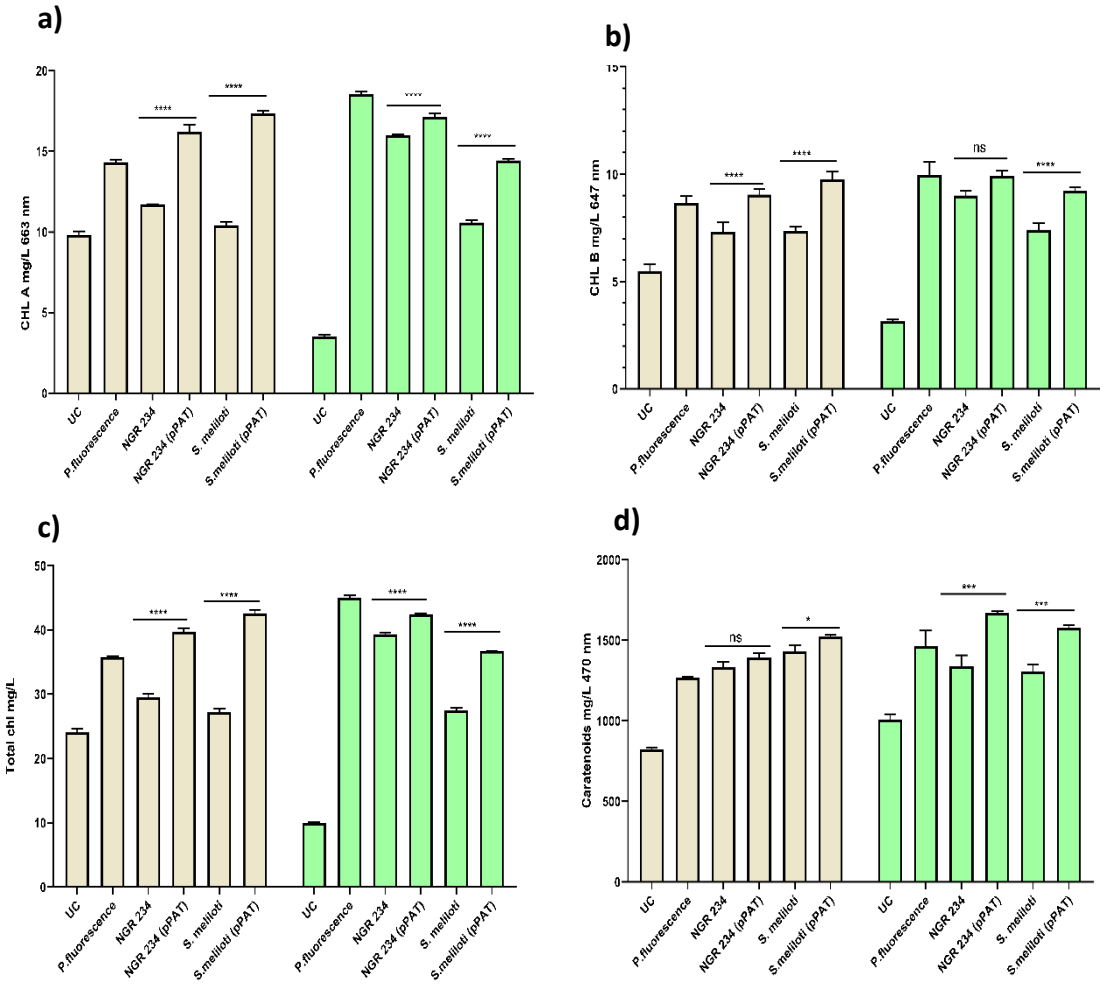


Figure 4.7.a) Chlorophyll A content in the leaves of seedlings treated with PGPR growing in arsenic contaminated soil b) Chlorophyll B content in the leaves of seedlings treated with PGPR growing in arsenic contaminated soil c) Total Chlorophyll in the leaves of seedlings treated with PGPR growing in arsenic contaminated soil d) Carotenoids content in the leaves of seedlings treated with PGPR growing in arsenic contaminated soil.

Heavy metals get absorbed by the roots and are transported to various parts of the plants including the leaves. They can get accumulated in the leaves and generate enormous amount of ROS, which damages lipids and proteins of various cellular components including chloroplasts. Several studies also reported that heavy metals can determine the rate of release of proteins and lipids from the thylakoid membrane, which causes damage to light harvesting complexes and photosystem II. Any damage to chloroplast will decrease the chlorophyll content which results in lowered photosynthetic rate. Moreover, these heavy metals can also replace the Mg in chlorophyll.[42] It is known that an exogenous application of glutathione can improve the photosynthetic rate in the plants growing in heavy metal contaminated soil. [43]

Similar results were obtained during the assessment of chlorophyll and carotenoids content from the leaves of fenugreek growing in arsenic contaminated soil. There was a significant increase in the Chlorophyll A, B, total chlorophyll and carotenoids content in the leaves of the seedlings treated with M3 and M5 compared to M2 and M4 respectively. At 30 ppm arsenic stress, seedlings treated with M3 had 7.30 % more total chlorophyll compared to M2 and M5 had 25.04 % more total chlorophyll compared to M4. At 0 ppm similar significant increase was noted. Among the rhizobia, the highest amount of total chlorophyll at 30 ppm arsenic stress was accumulated in the leaves of the seedlings treated with M3, while at 0 ppm it was M5. Similar observations were noted for carotenoids content in the leaves.

4.3.4.2 Changes in the production of H_2O_2 and MDA in fenugreek seedlings treated with GMO and wild type PGPR growing in Arsenic contaminated soil

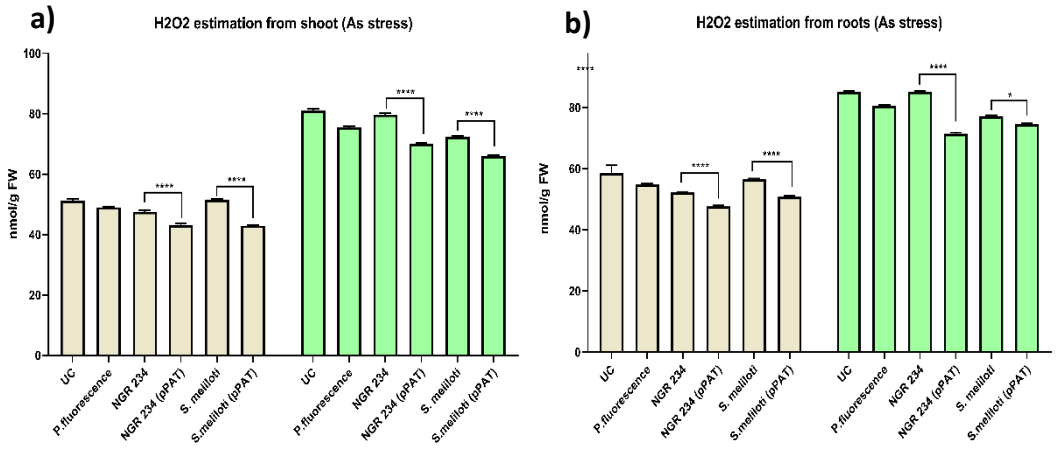


Figure 4.8 H2O2 estimation from, a) shoots and b) roots of the seedlings growing in Arsenic contaminated soil.

H2O2 and MDA were estimated from the shoots and roots after 16 days. Fresh tissue was used for the estimation. Significant reduction of H2O2 content was observed in shoots and roots of the seedlings treated with GMO rhizobia compared to the seedlings treated with wildtype bacteria (At 0 ppm and 30 ppm Arsenic stress). In shoots, at 0 ppm M3 treated seedlings accumulated 9.27 % less H2O2 compared to M2 treated seedlings, while M5 treated seedlings accumulated 16.67 % less H2O2 compared to M4 treated seedlings. Similarly at 30 ppm, M3 treated seedlings accumulated 12.14 % less H2O2 compared to M2 treated seedlings, while M5 treated seedlings accumulated 8.80 % less H2O2 compared to M4 treated seedlings (Figure

4.8.a). In roots, at 0 ppm M3 treated seedlings accumulated 8.40 % less H2O2 compared to M2 treated seedlings, while M5 treated seedlings accumulated 10.02 % less H2O2 compared to M4 treated seedlings. Similarly at 30 ppm, M3 treated seedlings accumulated 16.19 % less H2O2 compared to M2 treated seedlings, while M5 treated seedlings accumulated 3.14 % less H2O2 compared to M4 treated seedlings (Figure 4.8.b).

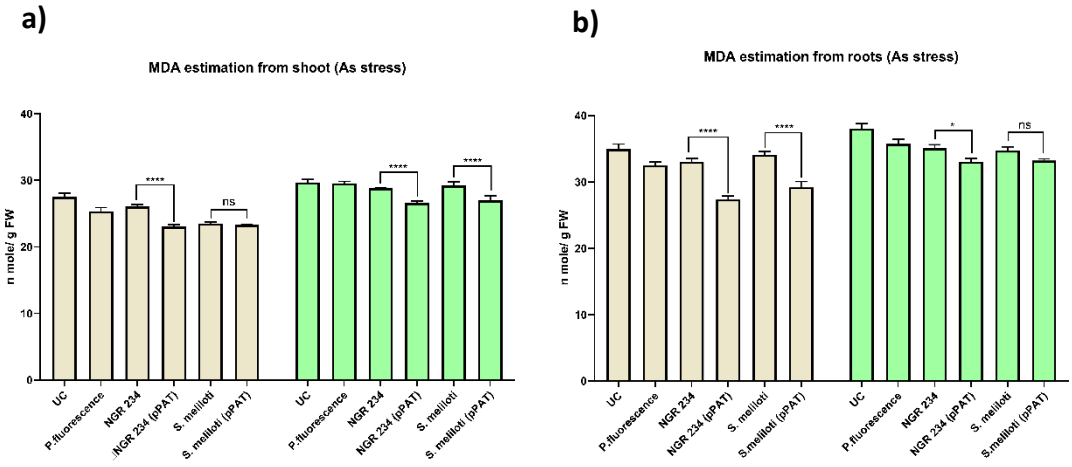
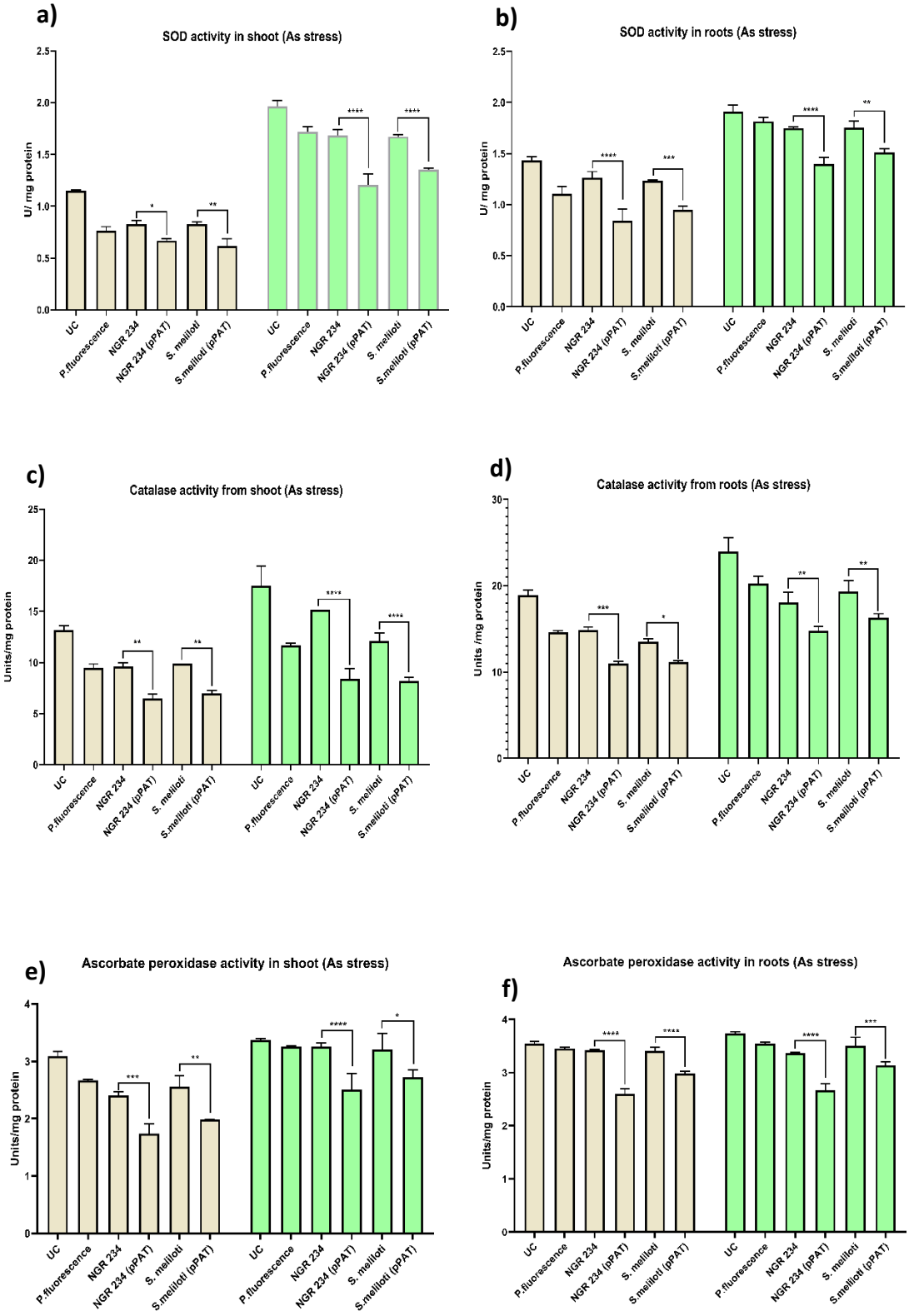


Figure 4.9 MDA estimation from, a) shoots and b) roots of the seedlings growing in Arsenic contaminated soil.

In shoots at 0 ppm MDA content decreased about 11.53% in M3 treated seedlings compared to M2 treated seedlings, while no significant difference in MDA content was recorded between the seedlings treated with M4 and M5. At 30 ppm stress, it was observed that the seedlings treated with M3 and M5 accumulated 7.70% and 7.59% less MDA compared to M2 and M4 respectively (Figure 4.9.a). In roots at 0 ppm, it was observed that the seedlings treated with M3 and M5 accumulated significantly lower amounts of MDA compared to M2 and M4 respectively. The difference was 17.14 % and 14.18 % respectively. Similar trend was observed at 30 ppm stress in roots. The difference was 5.93 % and 4.36 % respectively, but the later was non-significant (Figure 4.9.b). The uptake of arsenic promotes the production of ROS such as O_2^- , H_2O_2 and OH^- which increases the oxidative stress, thus disturbs redox homeostasis and metabolic pathways. Further this can cause lipid peroxidation, which can damage the plant by withering the cell membrane. Significant increase in oxidative stress was observed by overproduction of H_2O_2 and MDA on application of arsenic stress, but the application of GMO PGPR capable of secreting enhanced levels of GSH significantly reduced the stress. These results were similar to the previous findings. [15]

4.3.4.3 Changes in antioxidant enzymes in Arsenic stressed fenugreek seedlings treated with GMO and wild type rhizobium bacteria.



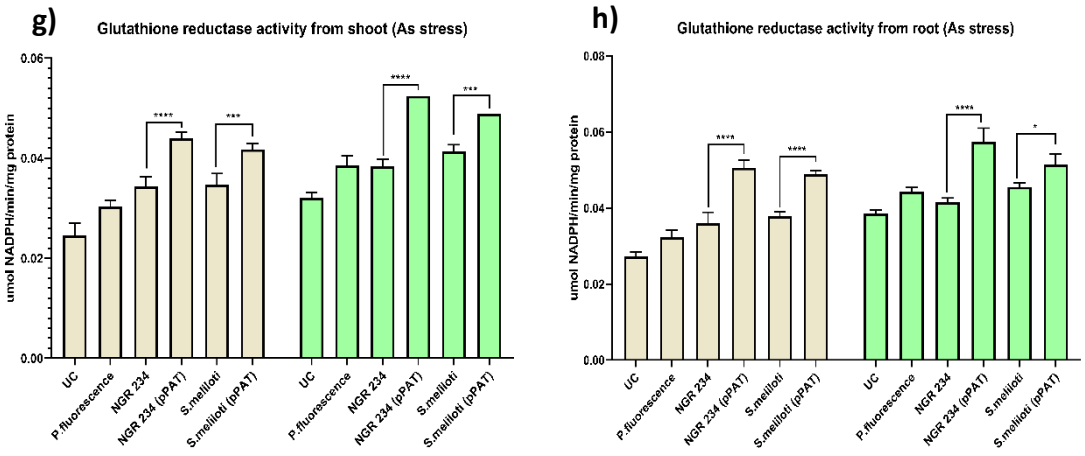


Figure 4.10 SOD activity in shoots (a) and roots (b) of the seedlings growing in Arsenic contaminated soil, CAT activity in shoots (c) and roots (d) of the seedlings growing in Arsenic contaminated soil, APX activity in in shoots (e) and roots (f) of the seedlings growing in Arsenic contaminated soil, GR activity in shoots (g) and roots (h) of the seedlings growing in Arsenic contaminated soil.

ROS generated in response of arsenic stress needs to be cleared out and for the same antioxidant enzyme system exists which scavenges or converts reactive species into less toxic form. They help in maintaining the redox homeostasis and protects the structure and function of cells, thus reducing stress. In the enzymatic antioxidant or redox enzymes, SOD plays a critical role as it catalyses the dismutation of O2•− to H2O2.Subsequently, CAT and APX convert H2O2 to H2O and O2 for scavenging of the oxidative damages. It was observed that Superoxide dismutase (SOD), Catalase (CAT), Ascorbate peroxidase (APX) activities significantly decreased in the seedlings treated with GMO PGPR compared to the seedlings treated with wild type PGPR, while Glutathione reductase (GR) activity increased. These observations were recorded at 0 ppm and 30 ppm in shoots as well as roots. The results of our study match with the previous findings.[15] Also, it is known that an exogenous application of GSH significantly increases the activity of glutathione reductase (GR) in rice plant under arsenic stress, which also holds true for our study.[44]

In shoots, at 0ppm 19.28% decrease in SOD activity was observed in the seedlings treated with M3 compared to the seedlings treated with M2, while 25.63 % decrease was observed in the seedlings treated with M5 compared to the seedlings treated with M4. At 30 ppm arsenic stress, 28.39 % decrease in SOD was observed in the seedlings treated with M3 compared to the seedlings treated with M2, while 19.17 % decrease was observed in the seedlings treated with

M5 compared to the seedlings treated with M4 (Figure 4.10.a). In roots, at 0 ppm 33.30 % decrease in SOD was observed in the seedlings treated with M3 compared to the seedlings treated with M2, while 23.26 % decrease was observed in the seedlings treated with M5 compared to the seedlings treated with M4. At 30 ppm arsenic stress, 20.05 % decrease in SOD was observed in the seedlings treated with M3 compared to the seedlings treated with M2, while 13.94 % decrease was observed in the seedlings treated with M5 compared to the seedlings treated with M4 (Figure 4.10.b).

At 0 ppm, Catalase activity in shoots decreased by 32.47 % in M3 treated seedlings compared to M2 treated, while it decreased by 29.33 % in M5 treated seedlings compared to M4 treated seedlings. At 30 ppm, Catalase activity in shoots decreased by 44.80 % in M3 treated seedlings compared to M2 treated, while it decreased by 32.29 % in M5 treated seedlings compared to M4 treated seedlings (Figure 4.10.c). And in roots, at 0 ppm Catalase activity decreased by 26.20 % in M3 treated seedlings compared to M2 treated, while it decreased by 17.56 % in M5 treated seedlings compared to M4 treated seedlings. At 30 ppm, Catalase activity in shoots decreased by 18.19 % in M3 treated seedlings compared to M2 treated, while it decreased by 15.98 % in M5 treated seedlings compared to M4 treated seedlings (Figure 4.10.d).

In shoots at 0 ppm, Ascorbate peroxidase activity decreased by 27.74% in M3 treated seedlings compared to M2 treated seedlings, while it decreased by 22.26 % in M5 treated seedlings compared to M4 treated seedlings. At 30 ppm it decreased by 23.05 % in M3 treated seedlings compared to M2 treated seedlings and by 15.03 % in M5 treated seedlings compared to M4 treated seedlings (Figure 4.10.e). In roots at 0 ppm, Ascorbate peroxidase activity decreased by 24.04 % in M3 treated seedlings compared to M2 treated seedlings, while it decreased by 12.48 % in M5 treated seedlings compared to M4 treated seedlings. At 30 ppm it decreased by 20.71 % in M3 treated seedlings compared to M2 treated seedlings and by 12.41 % in M5 treated seedlings compared to M4 treated seedlings (Figure 4.10.f).

In shoots at 0 ppm, Glutathione reductase activity significantly increased by 28.28 % in M3 treated seedlings compared to M2 treated seedlings and it increased by 20.28 % in M5 treated seedlings compared to M4 treated seedlings. At 30 ppm, it increased by 36.90 % in M3 treated seedlings compared to M2 treated seedlings and it increased by 18.09 % in M5 treated seedlings compared to M4 treated seedlings (Figure 4.10.g). In roots at 0 ppm, Glutathione reductase activity significantly increased by 40.76 % in M3 treated seedlings compared to M2 treated seedlings and it increased by 29.09 % in M5 treated seedlings compared to M4 treated

seedlings. At 30 ppm, it increased by 38.09 % in M3 treated seedlings compared to M2 treated seedlings and it increased by 13.07 % in M5 treated seedlings compared to M4 treated seedlings (Figure 4.10.h).

Section 4.3.5: Investigating the effect of genetically modified *Sinorhizobium fredii* NGR 234 & *Sinorhizobium meliloti* (NAIMCC-B-00863) on the growth of fenugreek seedlings in Cadmium contaminated soil.

4.3.5.1 Assessment of growth and chlorophyll production in fenugreek seedlings treated with GMO and wild type bacteria growing in Arsenic contaminated soil

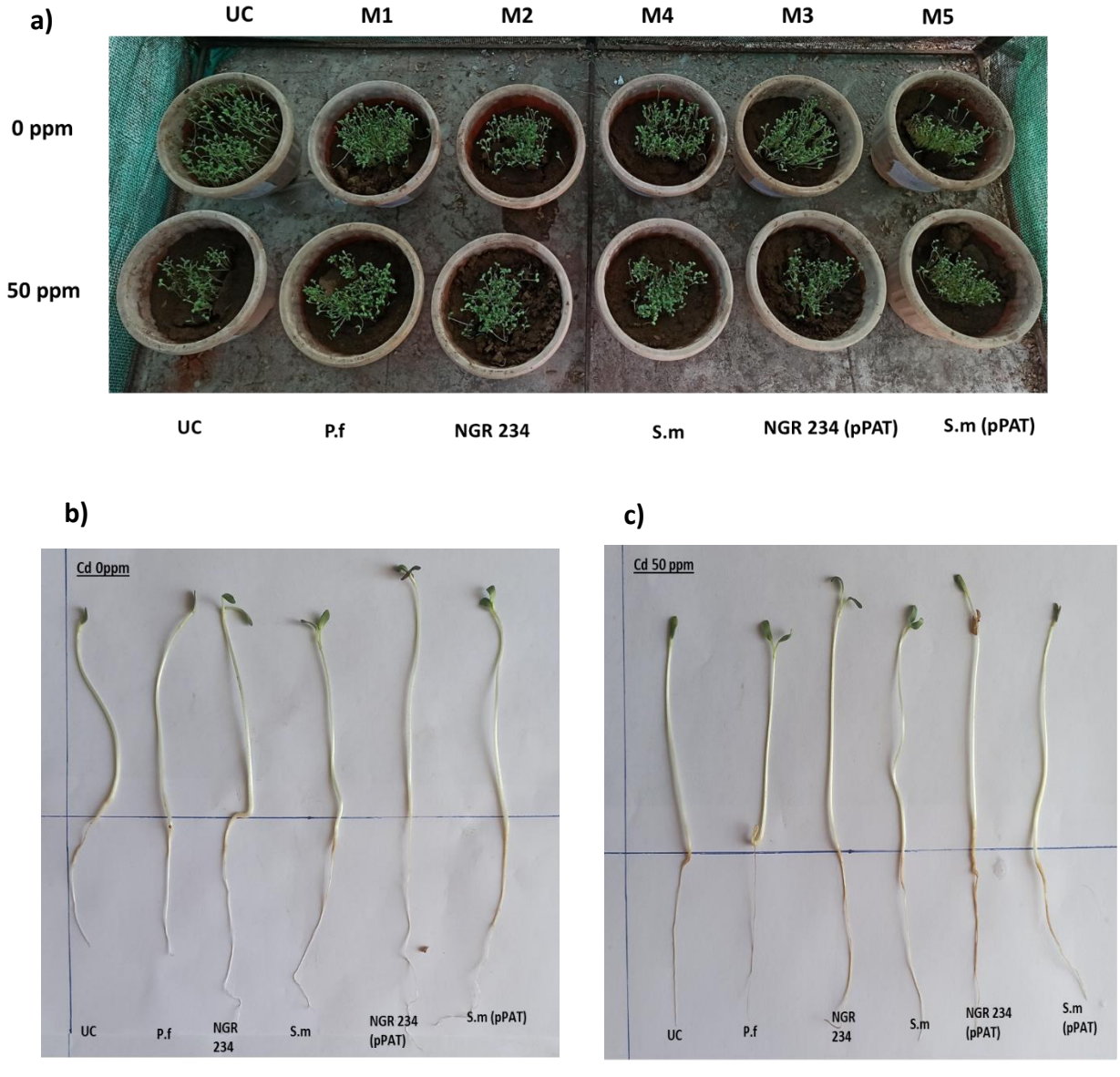


Figure 4.11 a) Growth of fenugreek seedlings treated with PGPR in Cadmium stress (Top view) b) Seedlings morphology at 0 ppm Cadmium stress c) Seedlings morphology at 50 ppm Cadmium stress

Figure 4.11.a shows the growth of fenugreek seedlings (treated with different PGPR) in pots of control and stress conditions after 16 days. After 16 days the seedlings growing in 0 ppm and 50 ppm cadmium stress were uprooted carefully from the pots and rinsed gently under the tap water and photographed (Figure 4.11.b and 4.11.c). Root length and shoot length were measured by scale. Figure 4.12.a, 4.12.b and 4.12.c shows the graph of shoot length, root length and total length respectively, which describes the seedlings treated with different PGPR at 0 ppm stress (grey colour) and the seedlings treated with different PGPR at 500 ppm cadmium stress (Orange colour) on x axis and the length (in centimetres) on y axis. Name of the PGPR is described by a single letter M in the upcoming results and discussions. Bacteria used in the experiment are, M1; *Pseudomonas fluorescence* (NAIMCC B-00342), M2; *Sinorhizobium fredii* NGR 234, M3; *Sinorhizobium fredii* NGR 234 (pPAT), M4; *Sinorhizobium meliloti* (NIAMCC B-00836), M5; *Sinorhizobium meliloti* (NIAMCC B-00836) (pPAT) and the uncoated seeds were denoted as UC.

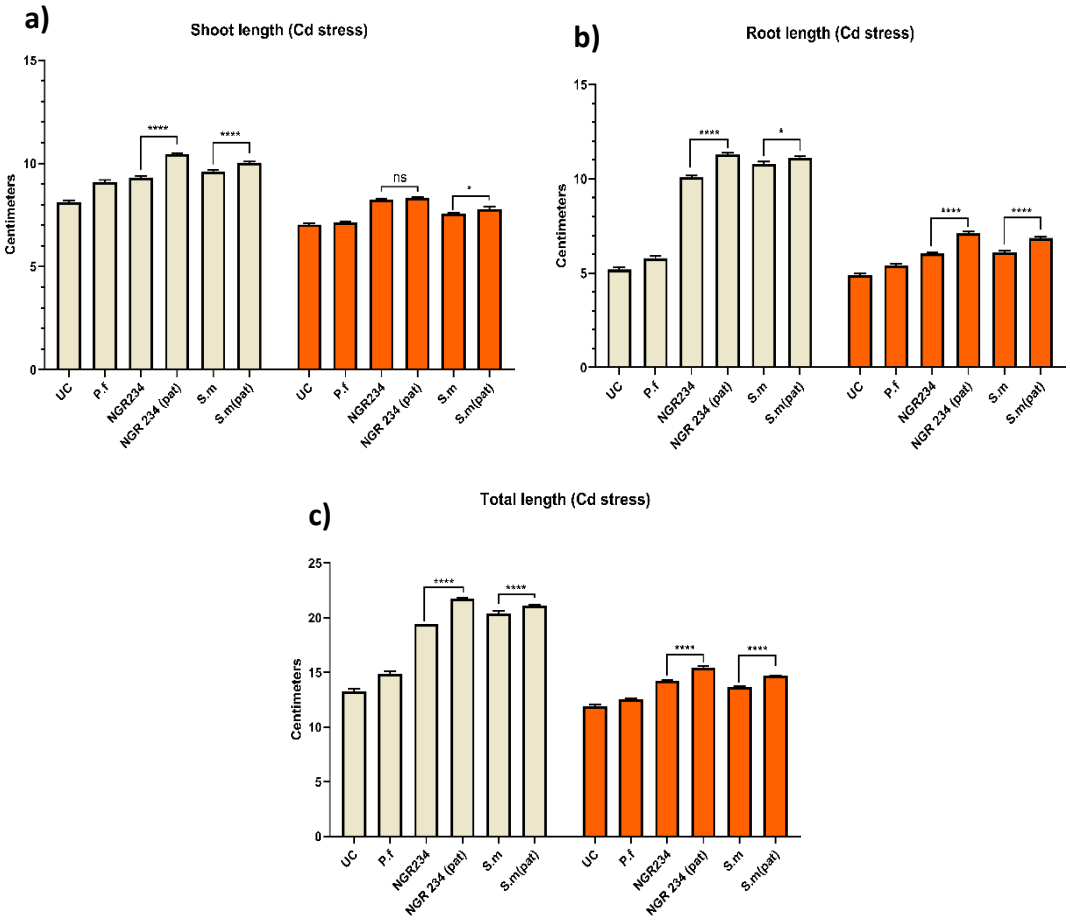


Figure 4.12.a) Shoot length of fenugreek seedlings treated with PGPR growing in Cadmium stress b) Root length of fenugreek seedlings treated with PGPR growing in Cadmium stress c) Total length of fenugreek seedlings treated with PGPR growing in Cadmium stress

It was observed that the total length of seedlings treated with M3 and M5 was significantly greater compared to the seedlings treated with M2 and M4 respectively (Figure 4.12.c). PGPR biopriming showed a significant positive effect on germination of seeds and seedling vigor index. It was observed that at 50 ppm cadmium stress, the germination percentage significantly increased for the seeds coated with M5 compared to M4. Also, the germination percentage increased for M3 compared to M2, but it was non-significant. At 0 ppm the difference was non-significant, but the seeds coated with PGPR were able to germinate more efficiently compared to uncoated seeds (Figure 4.13.a). SVI significantly increased for the seedlings treated with GMO bacteria compare to the seedlings treated with the wild type bacteria. It was observed that at 50 ppm cadmium stress, the seedlings treated with M5 showed a significant increase of about 12.6% in SVI compared to M4, and the seedlings treated with M2 showed a significant increase of about 11.5 % compared to M2. Similar trend of increase in SVI was noted at 0 ppm (Figure 4.13.b).

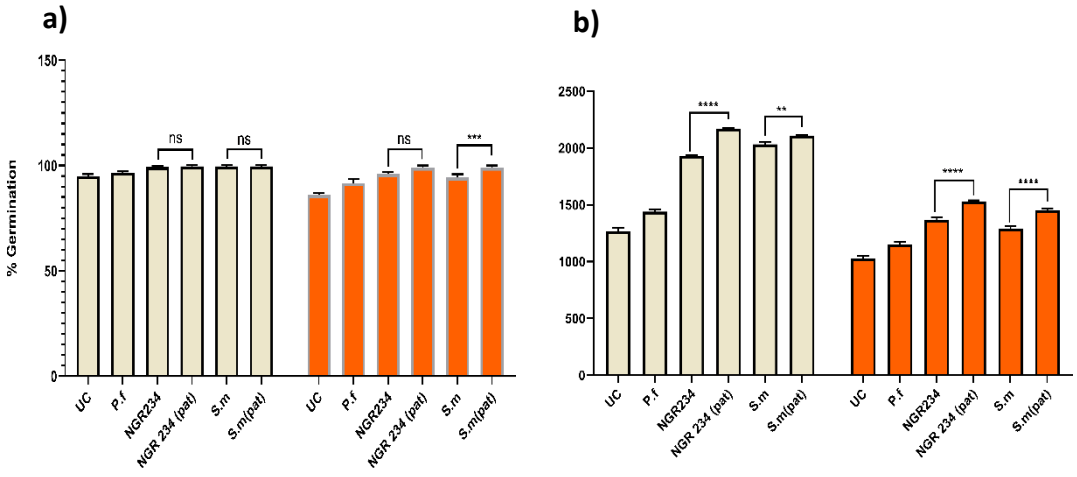


Figure 4.13.a) Germination % of the seeds coated with PGPR in Cadmium stress b) SVI of seedlings treated with PGPR growing in Cadmium contaminated soil

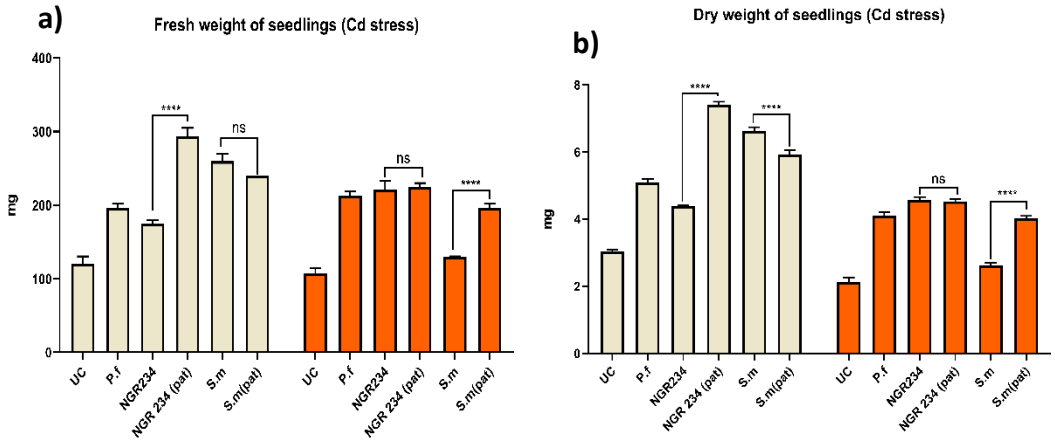


Figure 4.14.a) Fresh weight of the seedlings treated with different PGPR grown in cadmium contaminated soil. b) Dry weight of the seedlings treated with different PGPR grown in cadmium contaminated soil.

At 50 ppm the fresh weight of the seedlings treated with M5 was 51.6 % greater compared to the seedlings treated with M4, while there was no notable difference in fresh weight between M3 and M2. Similarly, there was no notable difference in the dry weight of seedlings treated with M3 and M2 at 50 ppm stress, but the dry weight of the seedlings treated with M5 was 53.5% greater compared to the seedlings treated with M4. At 0 ppm the dry weight and fresh weight significantly increased for the seedlings treated with M3 compared to M2, while no notable difference in weight of the seedlings treated with M5 and M4 was observed (Figure 4.14).

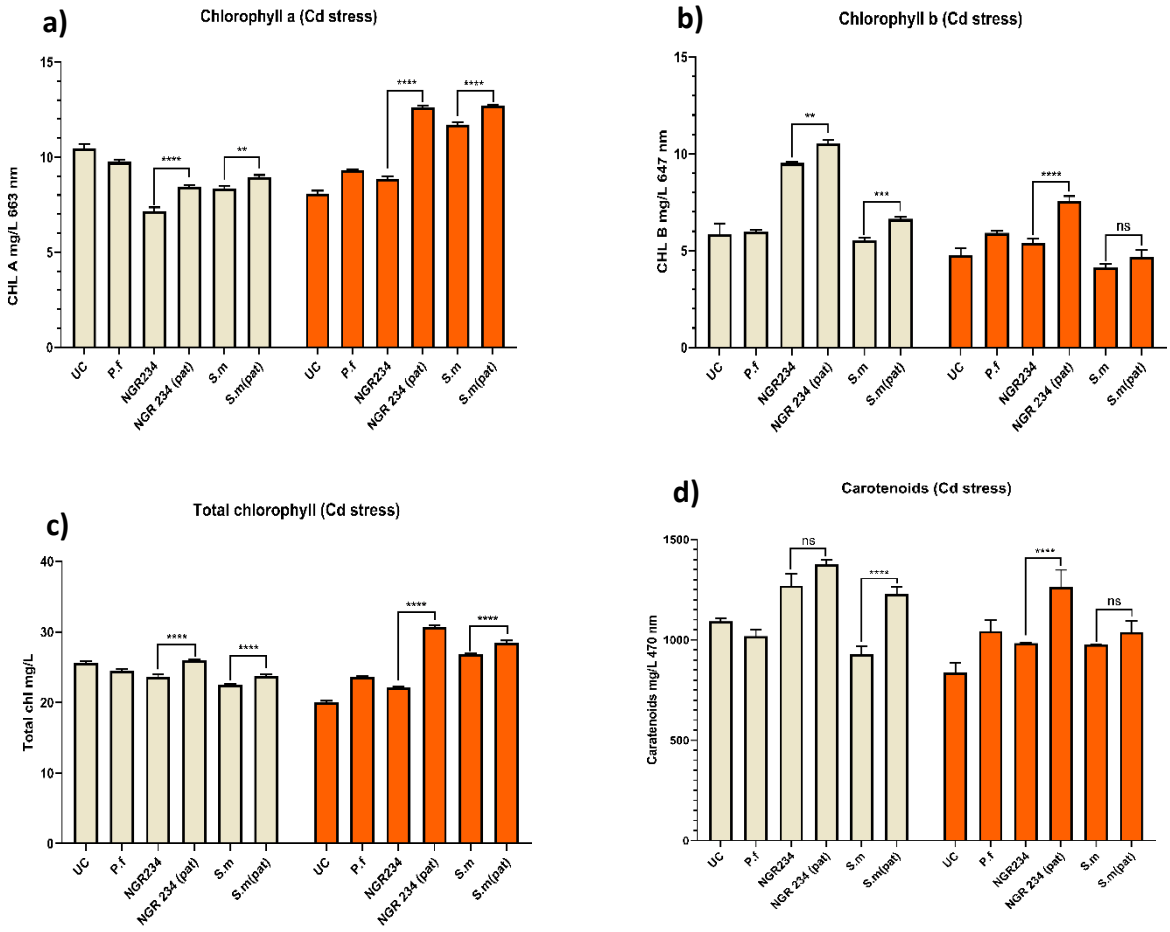


Figure 4.15.a) Chlorophyll a content in the leaves of seedlings treated with PGPR growing in cadmium contaminated soil b) Chlorophyll b content in the leaves of seedlings treated with PGPR growing in cadmium contaminated soil c) Total Chlorophyll content in the leaves of seedlings treated with PGPR growing in cadmium contaminated soil d) Carotenoids content in the leaves of seedlings treated with PGPR growing in cadmium contaminated soil.

At 50 ppm cadmium stress, the total chlorophyll in the leaves of M5 treated seedlings was 5.98% more compared to M4, while it was 38.44 % more in M3 treated seedlings compared to M2 (Figure 4.15.c). At 50 ppm, Carotenoids content in the leaves of M5 treated seedlings increased by 6.27 % compared to M4, while it increased by 28.4 % in the leaves of M3 treated seedlings compared to M2. But the increase in M5 compared to M4 was non-significant (Figure 4.15.d). At 0 ppm total chlorophyll and carotenoids showed the significant increase in the seedlings treated with GMO bacteria compared to the wild type bacteria. Similar trend of increase in chlorophyll a and chlorophyll b was observed (Figure 4.15.a and 4.15.b). At 50 ppm M3 treated seedlings accumulated highest levels of chlorophyll a, b, total and carotenoids compared to other seedlings treated with PGPR.

4.3.5.2 Changes in the production of H_2O_2 and MDA in fenugreek seedlings treated with GMO and wild type PGPR growing in Cadmium contaminated soil

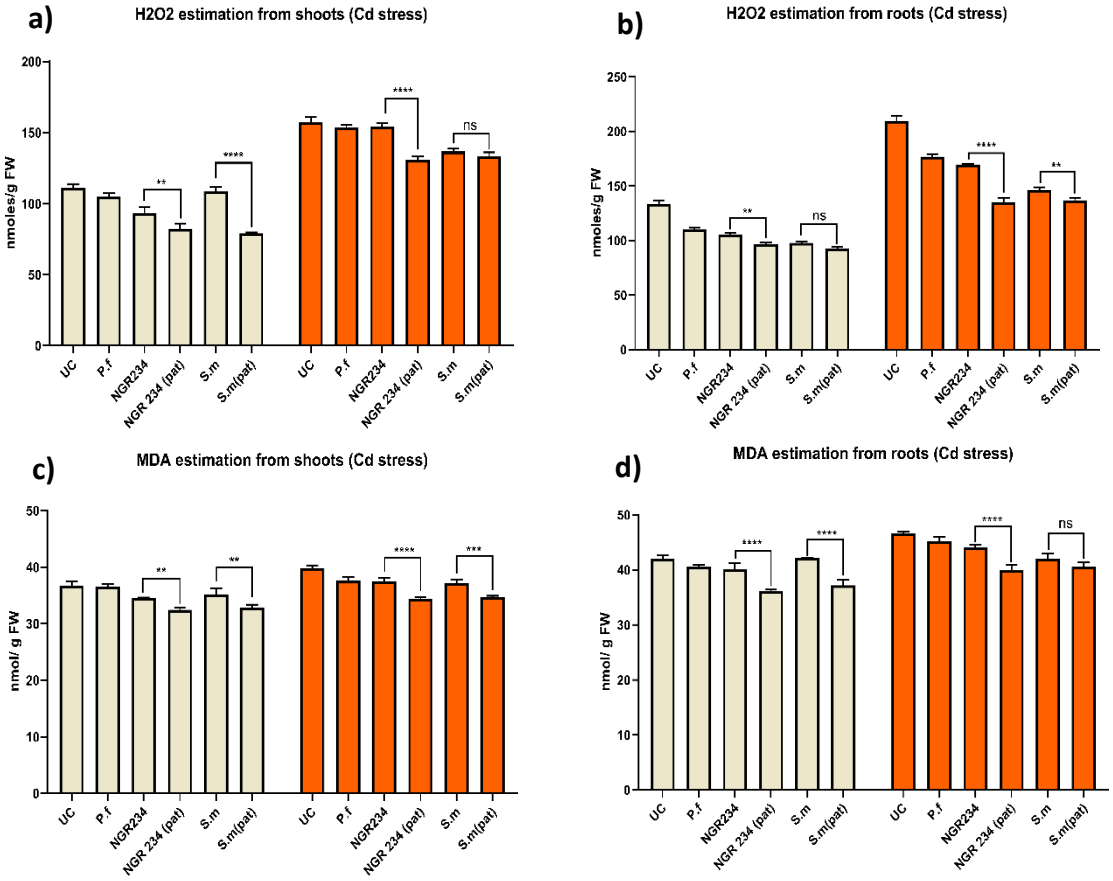


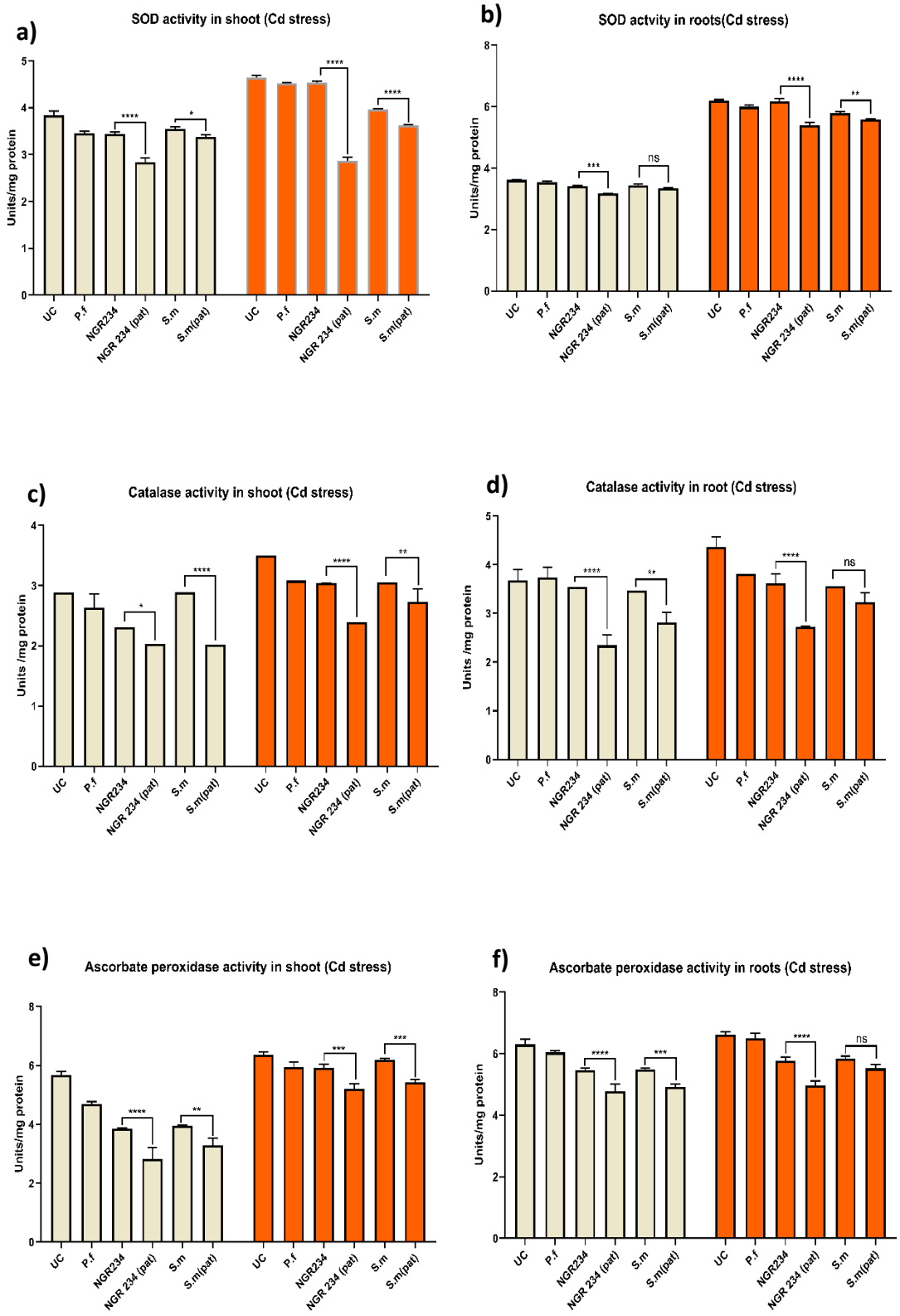
Figure 4.16.a) H2O2 estimation from shoots and b) H2O2 estimation from roots c) MDA estimation from shoots d) MDA estimation from roots.

H2O2 and MDA contents were estimated from the fresh plant tissues (roots and shoots) immediately after 16 days. In shoots at 50 ppm stress, it was observed that the seedlings treated with M3 has about 15 % less H2O2 compared to M2 treated seedlings, while the difference between the H2O2 content in the seedlings treated with M5 and M4 was non-significant. At 0 ppm both M3 and M5 treated seedlings accumulated significantly lower amount of H2O2 in shoots compared to M2 and M4 respectively (Figure 4.16.a). In roots at 50 ppm stress, it was observed that the seedlings treated with M3 had about 20.2 % less H2O2 compared to M2 treated seedlings, while M5 treated seedlings had about 6.4 % less H2O2 compared to M4 treated seedlings. The difference was significant. At 0 ppm M3 treated seedlings accumulated

significantly lower amount of H2O2 in roots compared to M2, while M5 treated seedlings also reported a decrease in H2O2 in roots compared to M4, but the difference was non-significant (Figure 4.16.b).

In shoots at 50 ppm stress, it was observed that the seedlings treated with M3 has about 8.80 % less MDA compared to M2 treated seedlings, while the seedlings treated with M5 had about 6.59 % less MDA compared to the seedlings treated with M4. At 0 ppm both M3 and M5 treated seedlings accumulated significantly lower amount of MDA in shoots compared to M2 and M4 respectively (Figure 4.16.c). In roots at 50 ppm stress, the seedlings treated with M5 accumulated 3.28 % less MDA compared to the seedlings treated with M4, while the seedlings treated with M3 accumulated about 9.27 % less MDA compared to the seedlings treated with M2. At 0 ppm stress, the seedlings treated with M5 and M3 accumulated significantly less MDA compared to M4 and M2 treated seedlings (Figure 4.16.d).

4.3.5.3 Changes in antioxidant enzymes in cadmium stressed fenugreek seedlings treated with GMO and wild type rhizobium bacteria.



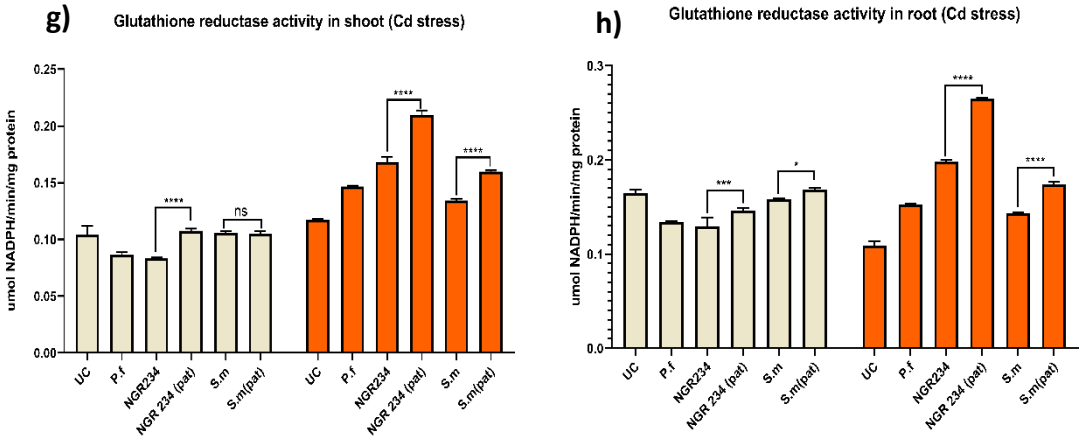


Figure 4.17) SOD activity in shoots (a) and roots (b) of the seedlings growing in Cadmium contaminated soil, CAT activity in shoots (c) and roots (d) of the seedlings growing in Cadmium contaminated soil, APX activity in in shoots (e) and roots (f) of the seedlings growing in Cadmium contaminated soil, GR activity in shoots (g) and roots (h) of the seedlings growing in Cadmium contaminated soil.

In shoots at 0 ppm and 50 ppm stress, a significant decrease in SOD activity was seen in the seedlings treated with GMO bacteria compared to wildtype bacteria. M3 reported the least SOD activity at 0 and 50 ppm cadmium stress. At 50 ppm M5 treated seedlings reported 8.33 % decrease in SOD activity compared to M4, while the M3 treated seedlings reported 36.81 % decline in SOD activity compared to M2 (Figure 4.17.a). Similar observations were noted for roots at 50 ppm cadmium stress. M5 treated seedlings reported 3.48% decline in SOD activity compared to M4 treated seedlings, while the M3 treated seedlings reported 12.74 % decline in SOD activity compared to M2 treated seedlings. At 0 ppm in roots, a significant decline in SOD activity was only noted in M3 compared to M2, while no significant change in SOD activity was noted between M3 and M2 at 0 ppm in roots (Figure 4.17.b).

At 0 ppm, Catalase activity in shoots decreased by 12.06 % in M3 treated seedlings compared to M2 treated seedlings, while it decreased by 30.10 % in M5 treated seedlings compared to M4 treated seedlings. At 50 ppm, Catalase activity in shoots decreased by 21.15 % in M3 treated seedlings compared to M2 treated seedlings, while it decreased by 10.66 % in M5 treated seedlings compared to M4 treated seedlings (Figure 4.17.c). And in roots, at 0 ppm Catalase activity decreased by 33.8 % in M3 treated seedlings compared to M2 treated seedlings, while it decreased by 19 % in M5 treated seedlings compared to M4 treated seedlings. At 50 ppm, Catalase activity in shoots decreased by 24.5 % in M3 treated seedlings

compared to M2 treated seedlings, while it decreased by 9.3 % in M5 treated seedlings compared to M4 treated seedlings. The decline in the CAT activity in the later in non-significant (Figure 4.17.d).

In shoots at 0 ppm, Ascorbate peroxidase activity decreased by 26.71 % in M3 treated seedlings compared to M2 treated seedlings, while it decreased by 16.46 % in M5 treated seedlings compared to M4 treated seedlings. At 50 ppm it decreased by 12.15 % in M3 treated seedlings compared to M2 treated seedlings and by 12.22 % in M5 treated seedlings compared to M4 treated seedlings (Figure 4.17.e). In roots at 0 ppm, Ascorbate peroxidase activity decreased by 12.46 % in M3 treated seedlings compared to M2 treated seedlings, while it decreased by 10.21 % in M5 treated seedlings compared to M4 treated seedlings. At 50 ppm it decreased by 13.79 % in M3 treated seedlings compared to M2 treated seedlings and by 5.42 % in M5 treated seedlings compared to M4 treated seedlings. The decline in APX activity in the later in non-significant (Figure 4.17.f).

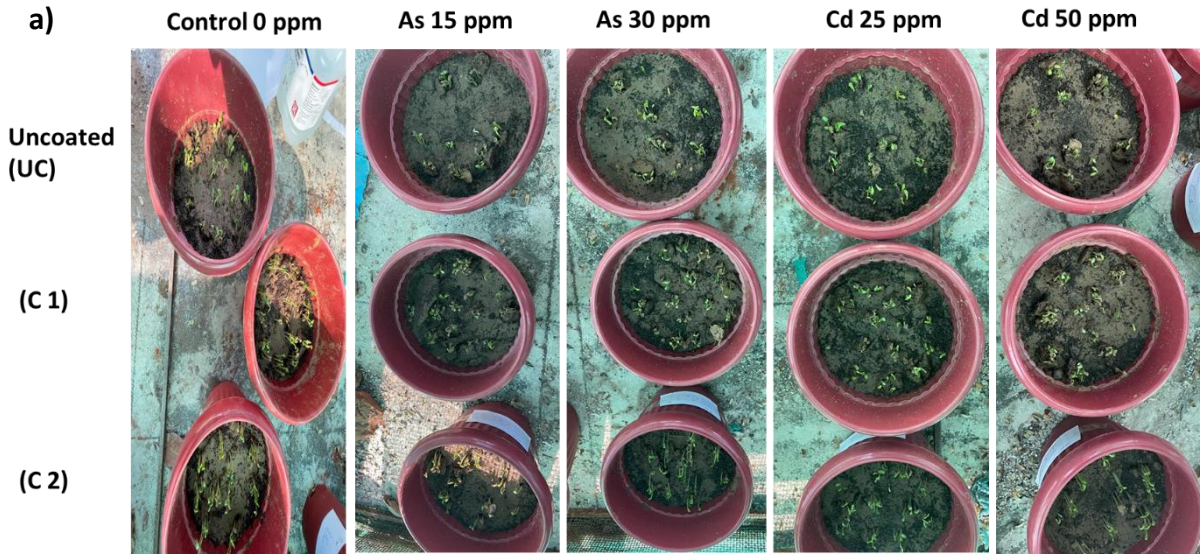
In shoots at 0 ppm, Glutathione reductase activity significantly increased by 29 % in M3 treated seedlings compared to M2 treated seedlings, while there was no significant difference in GR activity of M5 and M4 treated seedlings. At 50 ppm, it increased by 24.5 % in M3 treated seedlings compared to M2 treated seedlings and it increased by 19.3 % in M5 treated seedlings compared to M4 treated seedlings (Figure 4.17.g). In roots at 0 ppm, Glutathione reductase activity significantly increased by 12.6 % in M3 treated seedlings compared to M2 treated seedlings and it increased by 6.3 % in M5 treated seedlings compared to M4 treated seedlings. At 50 ppm, it increased by 33.5 % in M3 treated seedlings compared to M2 treated seedlings and it increased by 20.9 % in M5 treated seedlings compared to M4 treated seedlings (Figure 4.17.h).

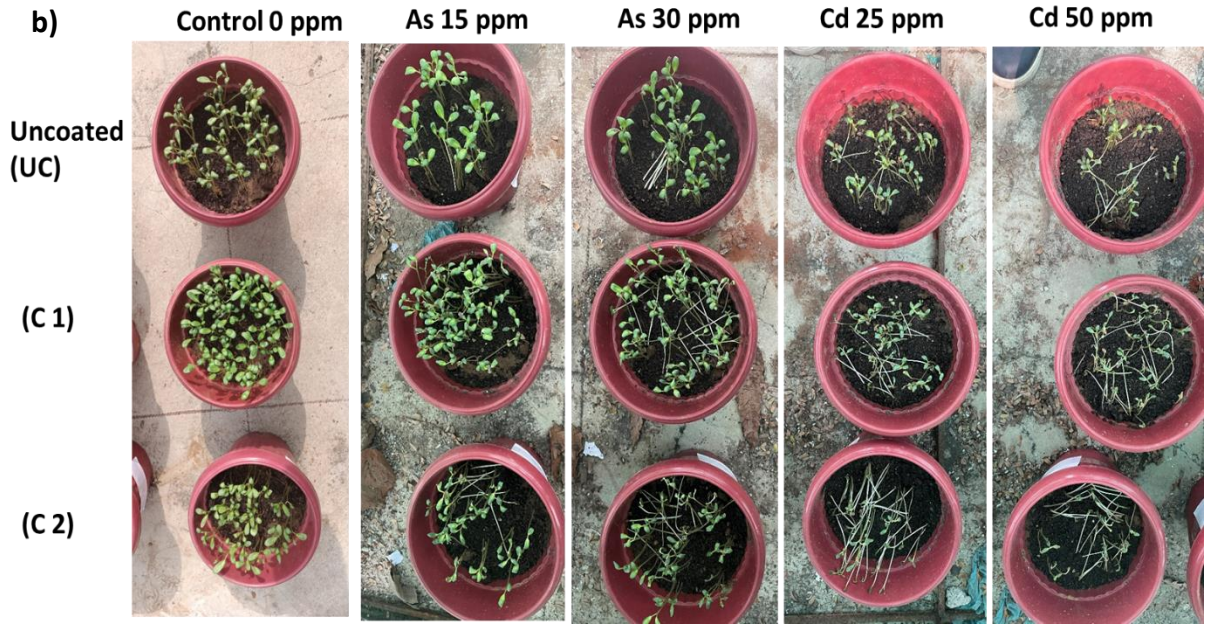
4.3.5.4 Discussion

The results with Cadmium were similar to that of Arsenic. Results were in accordance with the previous findings, where a plant growing in cadmium showed diminished growth characters,[16] increased ROS levels and enhanced antioxidant enzyme levels, but when supplied with exogenous GSH the ROS and antioxidant enzyme levels are also lowered. Instead of exogenous GSH, GMO rhizobia capable of secreting more glutathione are inoculated/coated on seeds.

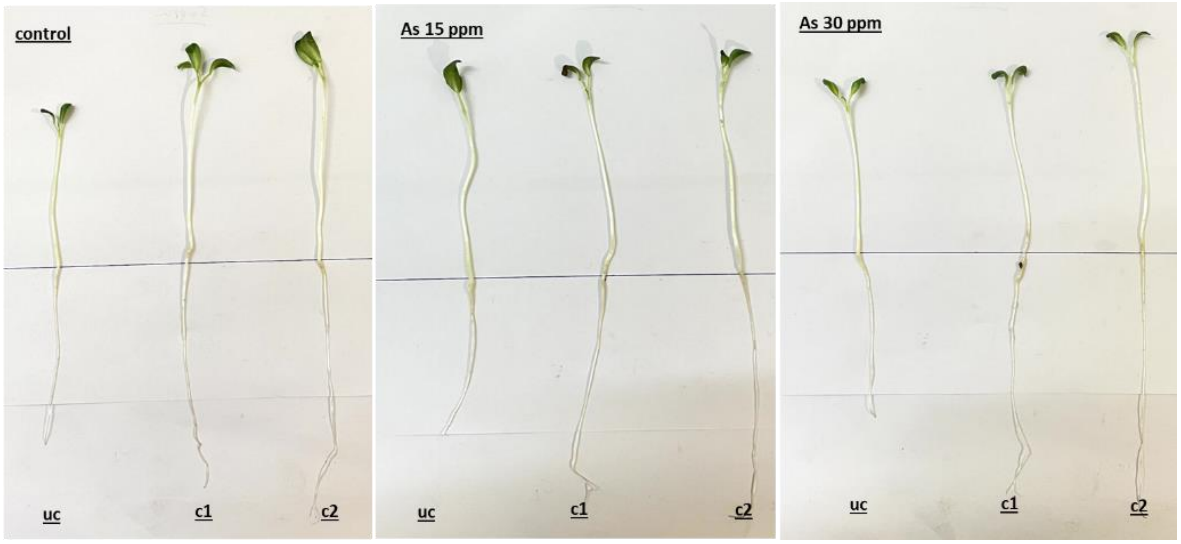
Section 4.3.6: Investigating the effect of rhizobial consortium on the growth of fenugreek seedlings in Arsenic & Cadmium contaminated soil.

4.3.6.1 Assessment of growth parameters, chlorophyll production, oxidative parameters and antioxidant enzyme levels in fenugreek seedlings treated with GMO and wild type bacteria growing in Arsenic contaminated soil





c)



d)

Figure 4.18 a) Growth of fenugreek seedlings treated with PGPR consortia in Arsenic and Cadmium contaminated soil (5 days) b) Growth of fenugreek seedlings treated with PGPR consortia in Arsenic and Cadmium contaminated soil (25 days) c) Morphology of the seedlings treated with PGPR consortia growing in Arsenic stress (0,15 and 30 ppm) d) Morphology of the seedlings treated with PGPR consortia growing in Cadmium stress (0,25,50)

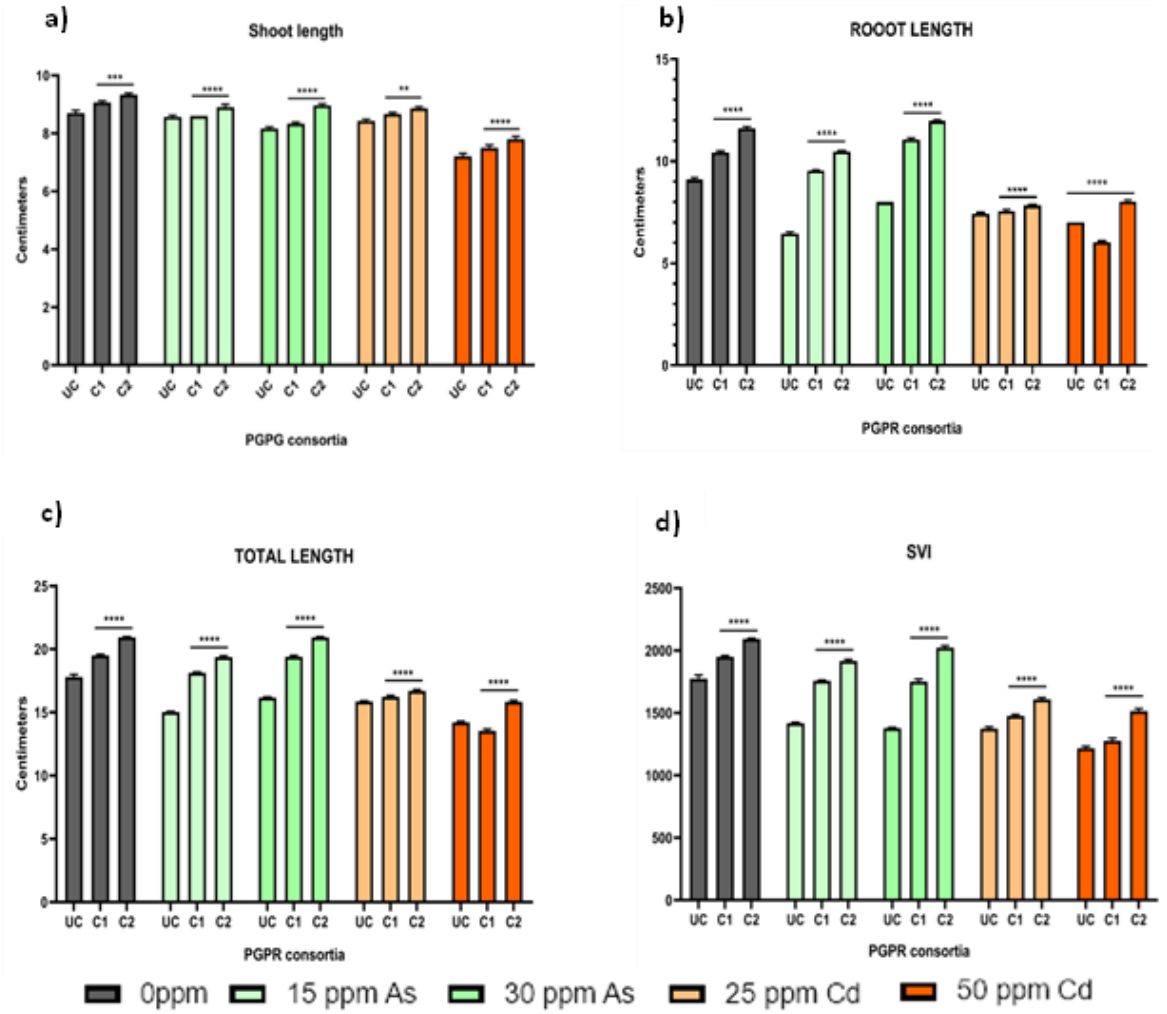


Figure 4.19 a) Shoot length b) Root length c) Total length d) SVI of the seedlings treated with PGPR consortium growing in Arsenic stress (0,15 and 30 ppm) & Cadmium stress (0,25,50)

Seeds were coated with PGPR consortia (mentioned in Table 4.3) and sown in the soil spiked with Arsenic (0,15,30) ppm and (0,25,50) ppm cadmium. Unspiked soil (0 ppm) and uncoated seeds were used as a control. Seedlings were allowed to grow up to 25 days. Photographs of the seedlings growing in pots were taken of 5th (Figure 4.18.a) and 25th (Figure 4.18.b) day. After 25 days seedlings were carefully uprooted from the soil, rinsed under tap water and photographed on a white paper (Figure 4.18.c and 4.18.d). Root length and shoot length were measured by scale. Figure 4.19.a, 4.19.b and 4.19.c shows the graph of shoot length, root length and total length respectively. Y axis values denotes the length in centimetres and X axis values denotes different PGPR treatments and heavy metal stress. Colour codes are assigned to different stress values, which is shown below the figure of every graph. Seedlings under every

stress are given three different treatments which are UC (uncoated), C1 (consortium 1) and C2 (consortium 2) and compared.

In heavy metal stress conditions, it was observed that the shoot length (Figure 4.19.a) and the root length (Figure 4.19.b) of the seedlings treated with C2 increased significantly compared to the seedlings treated with C1, therefor the total length (Figure 4.19.c) also increased. Because of the significant increase in the length of the C2 treated seedlings, their SVI (Figure 4.19.d) also significantly increased compared to the SVI of the seedlings treated with C1. We can say that the seedlings treated with C2 would have received more GSH compared to the seedlings treated with C1, as the consortium 2 is made up of GMO bacteria, which secretes more glutathione compared to wild type bacteria (Chapter 3,5). ROS generated due to heavy metals not only cause DNA damage but also interferes with the signalling molecules which helps in normal growth and development of plants [45], but the GSH released by C2 will be absorbed by the seedlings which helps in alleviating the heavy metal generated ROS, as GSH is directly involved in detoxification of ROS. [28] Also, the GSH released in rhizosphere will complex with arsenic[46] As $(GS)_3$ and cadmium[46,47] Cd $(GS)_2$. These complexes can be intra cellular[48] as well as extracellular, which will ultimately decrease the bioavailability of that heavy metal ions. Due to this phenomenon less heavy metal ions are likely to get transported to the seedlings treated with C2 compared to C1 and UC. This will generate less metal induced ROS in C2 treated seedlings. Due to these reasons the C2 treated seedlings showed enhanced growth compared to the C1 treated and untreated seedlings. These results are in accordance to the results of a study in which the application of exogenous GSH in stress conditions enhanced the growth of plant in terms of length.[49]

In stress condition, the fresh weight of the seedlings treated with C2 increased significantly compared to the seedlings treated with C1 and the untreated seedlings (Figure 4.20.a), while the opposite was observed in dry weight (Figure 4.20.b). It was observed that in stress conditions (As 15ppm, Cd 25 ppm and Cd 50 ppm) the dry weight of the untreated seedling was significantly high compared to C1 and C2 treated seedlings. While at 30 ppm arsenic stress, the dry mass of the seedlings treated with C2 was highest. On comparing only C2 and C1, the dry weight of C2 was significantly higher at all stress points (Figure 4.20.b). The treated seedlings (coated with 3 different PGPR) are capable of releasing siderophores, other chelating compounds and EPS. PGPRs and the biomolecules released by them in response to heavy metal stress are known to act as an efficient barrier[50] between the heavy metals and the root hairs in rhizosphere. These PGPRs have several mechanisms of entrapping the heavy metals (as

explained in chapter 1). Also, the glutathione and other thiols released by PGPR might have formed complex with the heavy metal ions and precipitated them. Both mechanisms in synchroneity are known to reduce the bioavailability of heavy metals for plants.[51] As the untreated seedlings (UC) had no PGPR coating, they had less protection compared to the treated seedlings (C1 and C2), thus more metal ions were absorbed by untreated seedlings (UC) which might have increased their dry weight. Also, it is important to note that untreated seedlings (uncoated seeds/ UC) relied completely on the PGPR ability of the bacteria which were already present in the soil (soil in this study was unautoclaved), while the treated seedlings had double protection, one from the coated PGPRs and second from the PGPRs already present in the soil.

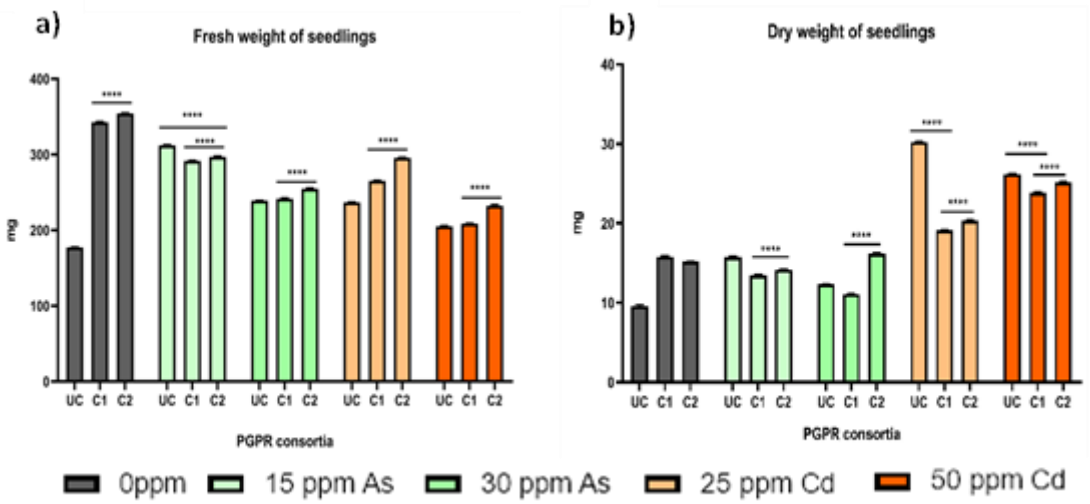


Figure 4.20 a) Fresh weight b) Dry weight of the seedlings treated with PGPR consortium growing in Arsenic stress (0,15 and 30 ppm) & Cadmium stress (0,25,50)

It is also important to note that GSH protects the plants form ROS injury and enhances its growth,[52] which could also increase the dry mass (as observed in 4.3.4 and 4.3.5). In the earlier sections treatment consisted of the individual PGPR and the soil was autoclaved, which made the plants totally dependent of the coated PGPR only. Whereas the uncoated seeds in the earlier sections had zero PGPR protection, which resulted in very less fresh weight and dry weight of the seedlings (as observed in 4.3.4 and 4.3.5). In this experiment (4.3.6) the soil in unautoclaved and the consortium of bacteria is coated on the seeds instead of single bacteria. The consortium is capable of producing higher amounts of glutathione and other protective biomolecules compared to single type of PGPR. Due to higher amounts of protective biomolecules, we are observing a drastic difference in dry mass between the untreated and treated, which could be

only due to higher efficiency of a consortium to reduce the bioavailability of heavy metals compared to the single type of PGPR. It is already known that the PGPR consortium is better than the single type PGPR.[53] Also, the fenugreek is known to accumulate heavy metals (used for phytoremediation). In a study, it was shown that the increasing concentration of tannery sludge (heavy metal contaminated) resulted in increase in dry mass of fenugreek,[54] which matches with our results of dry mass in the consortium experiment. The untreated seedlings had a minimum PGPR protection (PGPRs present in unautoclaved soil), so it becomes obvious that they will absorb more heavy metals compared to the treated seedlings which had a heavy barrier due to coating of 3 different PGPRs, therefor the dry mass of untreated seedlings (UC) was higher at all stress points compared to treated seedlings (C1 and C2).While comparing only C2 and C1, there was a significant increase in the dry mass of C2 treated seedlings compared to C1 treated seedlings at all stress points. This is due to the antioxidant property[15,16] of enhanced glutathione secretion by GMO rhizobia of C2 compared to wildtype rhizobia of C1.

The amount of chlorophyll and carotenoids were estimated from the fresh leaves. C2 treated seedlings had the highest amount of total chlorophyll (Figure 4.21.c) at all stress points. Similarly, C2 treated seedlings had the highest carotenoids level at all the stress points (Figure 4.21.d), except 15 ppm arsenic stress. It is known that any abiotic stress decreases the chlorophyll and carotenoids level but the supplementation of exogenous GSH is known to elevate chlorophyll content in the leaves in comparison to control, so we can say that our results are consistent with previous studies.[49] The chlorophyll a (Figure 4.21.a) and Chlorophyll b (Figure 4.21.b) level were highest in the seedlings treated with C2 compared to C1 and untreated seedlings.

Figure 4.22 (a, b) represents the H2O2 levels in shoots and roots respectively, while the Figure 4.22 (c, d) represents the MDA levels in shoots and roots respectively. The H2O2 levels in shoots and roots increase with increasing heavy metal concentration. It was observed that the seedlings treated with C2 had the lowest H2O2 content compared to the seedlings treated with C1 and untreated seedlings in shoots (Figure 4.22.a) as well as roots (Figure 4.22.b) at all stress points. Similar observations were recorded for MDA levels (Figure 4.22.c & 4.22.d). Our results matched with the previous findings where the exogenous application of GSH reduces the oxidative damage and lowers the H2O2 and MDA levels.[15,16]

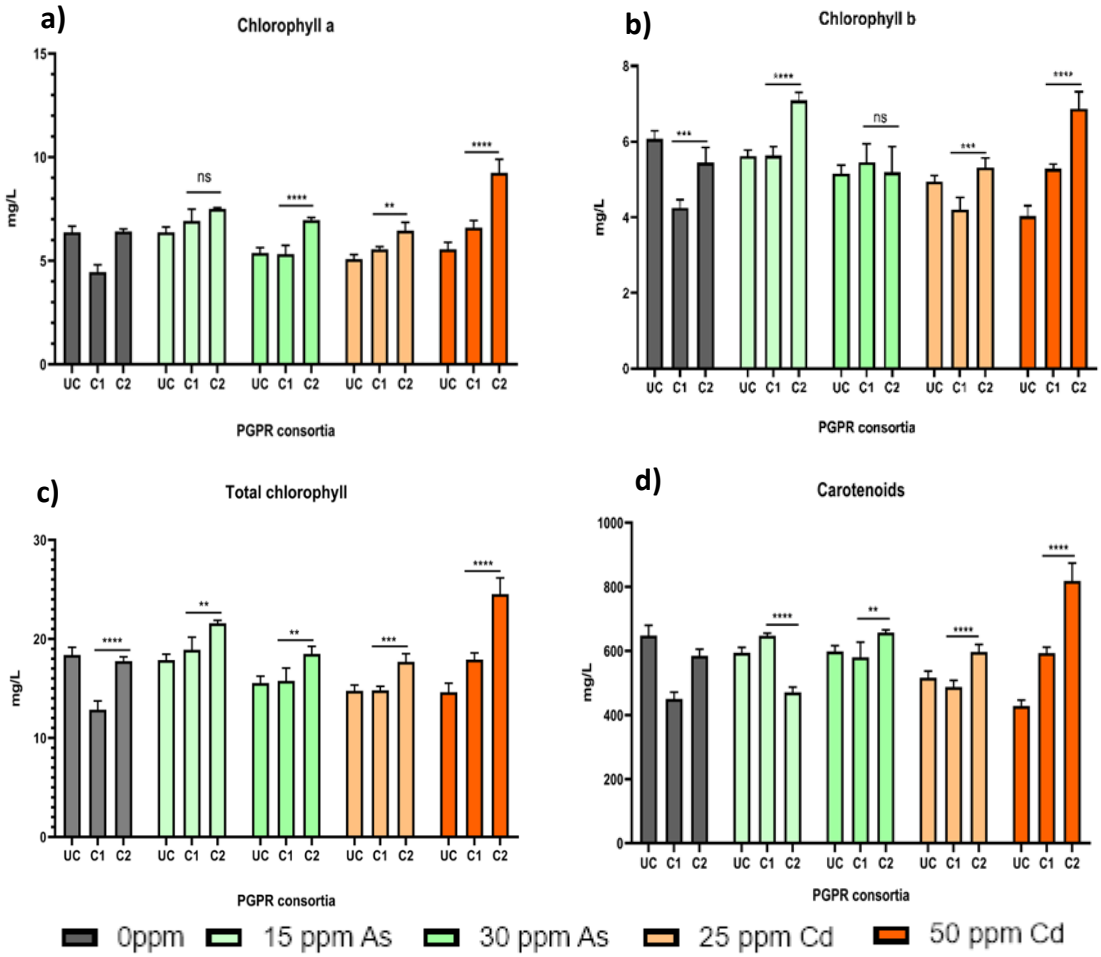


Figure 4.21 a) Chlorophyll a b) Chlorophyll b c) Total chlorophyll d) Carotenoids levels in the seedlings treated with PGPR consortium growing in Arsenic stress (0,15 and 30 ppm) & Cadmium stress (0,25,50)

In shoots at 0ppm, 15ppm and 30ppm arsenic stress (Figure 4.23.a), the C2 treated seedlings had the lowest SOD levels, whereas for cadmium stress, C1 treated seedlings had the lowest SOD levels. Untreated seedlings showed the highest SOD levels at all stress points, except arsenic 30 ppm stress. In roots at 0 ppm, C1 treated seedlings had the lowest SOD levels and at 15 ppm arsenic stress C2 had the lowest SOD levels, while at 30 ppm arsenic stress there was no significant difference in SOD levels between C1 and C2, but UC had the highest level of SOD. At 25 ppm and 50 ppm cadmium stress, the C2 treated seedlings had significantly higher SOD as compared to the C1 treated seedlings (Figure 4.23.b).

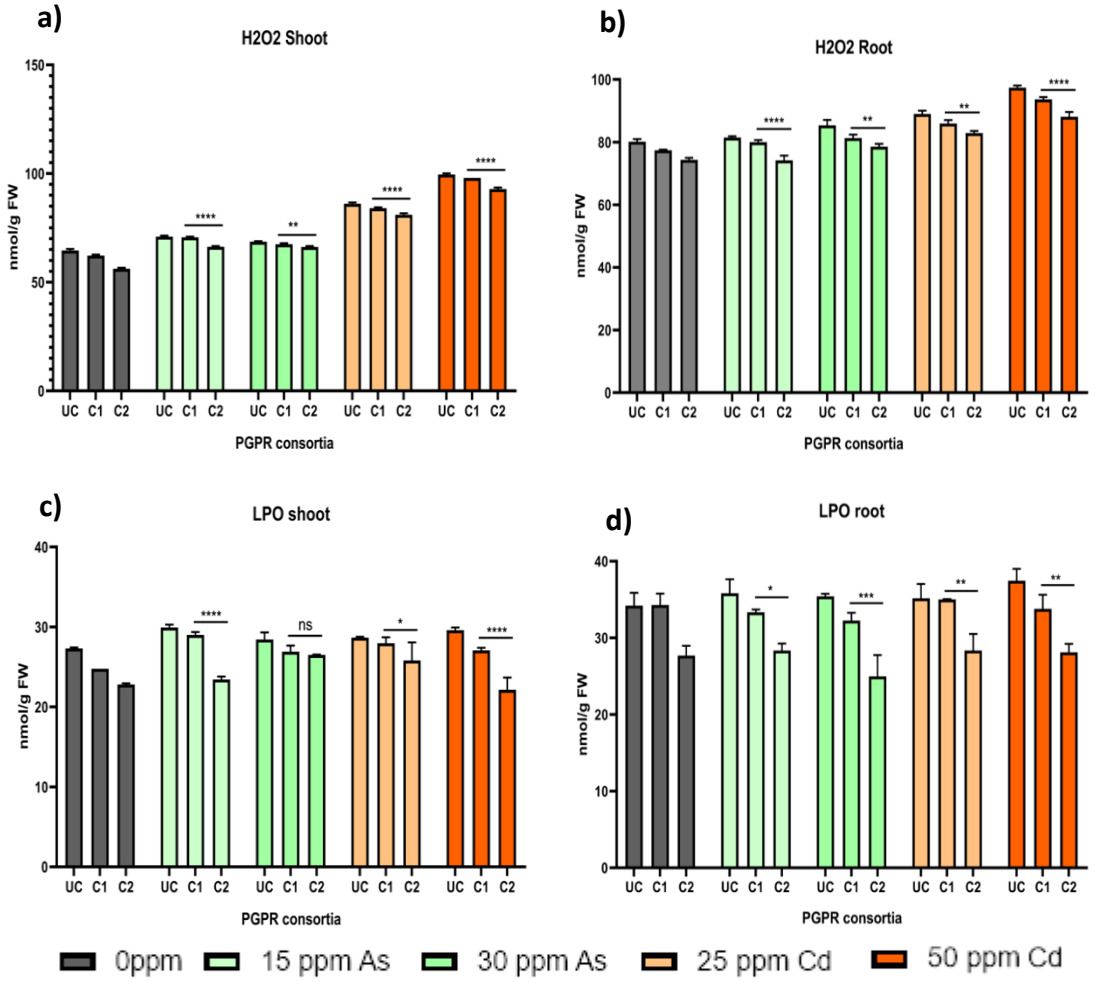


Figure 4.22 H2O2 content in a) Shoot and b) Root, MDA content in c) Shoot and d) Root of the seedlings treated with PGPR consortium growing in Arsenic stress (0,15 and 30 ppm) & Cadmium stress (0,25,50)

In shoots at 0 ppm, lowest GR levels were recorded in C2 treated seedlings, while at 15 ppm and 30 ppm arsenic stress the C2 treated seedlings had significantly higher GR in comparison to C1 treated seedlings and similar observations were recorded for 25 ppm and 50 ppm cadmium stress (Figure 4.23.c). While its opposite was observed in the roots. At 0 ppm the C2 had highest GR level. While at 15 ppm and 30 ppm arsenic stress, C2 treated seedlings had significantly lower GR compared to C1, still it was higher that untreated. At 25 ppm cadmium stress, similar observations were recorded. AT 50 ppm cadmium stress, C2 treated seedlings had heights levels of GR (Figure 4.23.d).

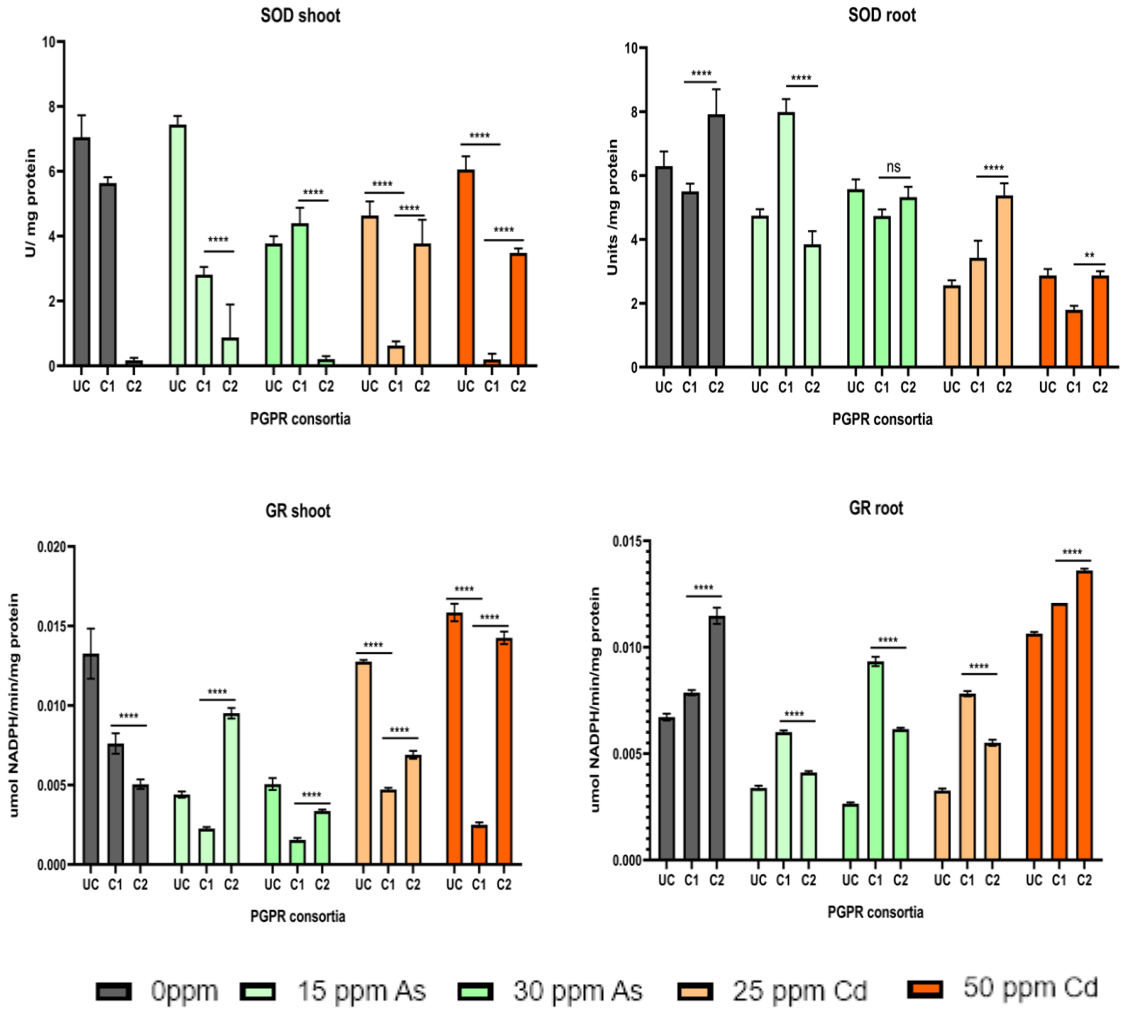


Figure 4.23 SOD levels in a) Shoot and b) Root, GR levels in c) Shoot and d) Root of the seedlings treated with PGPR consortium growing in Arsenic stress (0,15 and 30 ppm) & Cadmium stress (0,25,50)

In the previous experiments (Section 4.3.4 and 4.3.5), GMO PGPR treated seedlings/ coated seeds would have received more glutathione compared to the wild type treated seedlings in heavy metal stress conditions (Arsenic and Cadmium). Similarly, in consortium experiment the GMO consortium treated seedlings (C2) would have received more glutathione compared to the wildtype consortium treated seedlings (C1), as the GMO bacteria secretes more glutathione compared to wildtype bacteria (Results, chapter 3 and 5). In the current experiment seeds were coated with multiple (3) PGPR and sown in the soil. As the soil is unautoclaved, presence of other PGPR microorganisms could not be denied.[55] There is a high possibility that the cross talk of PGPR and plant will differ in both type of experiments (Single PGPR and Consortium PGPR). In single bacteria experiments generally, there will be no competition while in multiple bacteria experiment there will be competition between the bacterial species.

[56] In our single PGPR experiment since we coated only one type of bacteria to the seeds and also autoclaved the soil, so it sure that whatever the effect we see on plants is due to those bacteria only. While in consortium experiment, multiple bacteria would have interacted and they might have competed with one another or might have lived in a commensal relation with one another, just like in a natural condition whenever any farm soil is treated with PGPR.

Glutathione released by the PGPR used in this study will be taken up by plants as the PGPR are known to improve the nutrient uptake capacity in plants.[57,58] Also, the GSH released by our PGPR will be absorbed by other microbes (mutual cross feeding)[59,60] native to the rhizosphere. GSH absorbed by the plants takes part in neutralization of ROS and chelation of the metal ions inside the plants. After this interaction mostly GSH would have converted to GSSG, which could cause an increase in GSSG levels in the plant tissues (roots or shoots) and the source of this GSSG is external (i.e., absorbed from rhizosphere). An increment in GSSG levels upregulates glutathione reductase (GR) enzyme[15,16] in GMO treated seedlings compared to the wild type treated seedlings. The lowered ROS levels will eventually lower the antioxidant enzymes levels, except GR enzyme.[15,16] Therefore, in single PGPR experiments (Section 4.3.4 and 4.3.5), the antioxidant enzyme levels are lowered on application of GMO PGPR compared to wild type PGPR. GSH released by GMO consortium in the rhizosphere will be utilized by other microorganisms present in the soil. The release of GSH in rhizosphere full of microorganisms can highly improve the transport activity in PGPB and rhizobacteria[61] and enhance the cross talk between the microbes and between plants and microbes which can establish a healthy bacterial ecology in rhizosphere.[62] Besides this nutrient starvation and heavy metal concentration can also greatly affect the GSH production in rhizobacteria.[63] In case of wild type consortium treated seedlings, less free glutathione is secreted in rhizosphere by wild type bacteria compared to GMO bacteria. Comparatively less glutathione will be available for the plants and PGPR, therefore less GSH will be absorbed by the seedlings treated with wild type consortium. But the stress levels are same for both GMO and wildtype consortia treated seedlings. As the GMO rhizobia of the GMO consortium (C2) are capable of releasing more glutathione, the GMO consortia (C2) will be healthier compared to the wildtype consortium (C1) in heavy metal stress conditions. Also, it is known that the consortium of bacteria can trap and neutralize heavy metals more efficiently compared to monoculture,[64] so it can also be said that GMO consortia can trap heavy metals more efficiently than wildtype consortium. Wildtype consortium treated seedlings (C1) and untreated seedlings (UC) would have absorbed more

heavy metals compared to the GMO consortium treated seedlings, which would have increased the heavy metal content in the plant tissues and in turn ROS increased (Figure 4.22).

During arsenic stress, in consortium experiment the SOD levels in shoot and roots decrease in C2 treated seedlings compared to C1 treated seedlings, which was consistent with previous findings.[15] Increase in GR levels in shoots of C2 treated seedlings compared to C1 treated seedlings is also consistent with previous findings.[15] But the reverse is observed in roots, which could be due to less absorption of arsenic by C2 treated seedlings compared to C1 treated seedlings. In shoots C2 treated seedlings GR level is high due to more absorption of GSH from rhizosphere, which is released by GMO consortium, while in roots C1 treated seedlings GR level is higher compared to C2. Increase in GR enzymes in plant tissue in not only due to more absorption of external GSH, but it can also be the function of stress. Many studies have proved that overexpression GSH and GR in heavy metal stress conditions has a very important role in plant protection.[65] Plants under stress produce GSH in their tissues which is converted to GSSG on encounter with ROS or translocated heavy metals. In the case of consortium experiment, we know that since GMO bacteria used in C2 are capable of producing more glutathione than C1, and they would have decreased the translocation of arsenic in the case of C2 treated seedlings compared to C1 treated seedlings. Since more arsenic would have reached tissues of C1 treated seedlings, therefore in response to these heavy metals in their tissue GR in the roots might have increase. This is reflected in the low biomass of C1 in comparison to C2 (Figure 4.18.c and 4.20).

In cadmium stress the SOD levels in shoots of untreated group was higher compared to the treated group, which is also consistent with previous findings,[16] as the coated seeds will receive more protective biomolecules compared to untreated. Comparing only C1 and C2, the SOD levels in C2 were higher compared to C1 which could be due to significantly low fresh weight of C1 treated seedlings (Figure 4.18) as C1 treated seedlings might have absorbed more cadmium. In roots also the SOD levels were highest in C2 group seedlings compared to C1 and untreated group. Toxicity of cadmium metal might have killed most of the native PGPR of untreated group and coated wild type PGPR of C1 consortium, while the C2 consortium bacteria which release more glutathione might have absorbed more cadmium inside them. Many studies have shown the effectiveness of GSH on bacterial survival as GSH have an excellent antioxidant property and reports are there which shows that it reduces the bioavailability of heavy metals. The plant which absorbs more heavy metals have lower biomass and shows reduced the growth characteristics (Figure 4.18, 19), thus SOD levels

would have decrease in the seedlings treated with C1 compared to C2. First point of contact for heavy metals is roots so we can observe that UC group roots have very less biomass and less SOD. It is also important to note that SOD levels in shoot of UC group are significantly higher than the SOD levels in roots of UC group. This proves that more cadmium is translocated into shoots compared to roots, damaging roots more (figure 4.18.b) and getting stored in shoots (Vacuoles of cells). Increase in the GR levels in shoots of C2 treated seedlings compared to C1 treated seedlings is also consistent with previous findings.[16] But the reverse is observed in roots, which could be due to less absorption of cadmium by C2 treated seedlings compared to C1 treated seedlings. Explanation of this part goes hand in hand with the above explanation made for arsenic stress.

4.4 Conclusion

During individual PGPR experiments we observed that the GMO PGPR were able to reduce heavy metal induced ROS in fenugreek seedlings growing in heavy metal stress (arsenic and cadmium) by lowering the antioxidant enzyme levels, except GR enzyme. Chlorophyll and Carotenoids amount, biomass and growth parameters of the GMO PGPR treated seedlings also increased significantly compared to other wild type PGPR treated seedlings. So, we conclude that the genetically modified rhizobia are more effective for fenugreek growing in arsenic and cadmium contaminated soils compared to other PGPRs used in this study.

In the case of consortia there is more complex interplay between Plant-Coated PGPR-Other native PGPR of the soil. Such interplay can have slightly or totally different mechanism of protection against heavy metals which could have slightly different effects of the plants. More understanding is required to crack the complex cross talk between the PGPRs in rhizosphere and understand how their mechanism of protection could have slightly different effect on plants compared to the individual PGRP. But one thing is clear that the PGPR consortia was able to reduce heavy metal induced ROS in fenugreek seedlings and enhance the Chlorophyll and Carotenoids amount. Biomass and growth parameters of the GMO consortium treated seedlings also improved significantly compared to other wild type consortium treated seedlings. So, we conclude that the GMO consortia is more effective for fenugreek growing in arsenic and cadmium contaminated soils compared to wild type consortium.

4.5 References

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