Synopsis of the Ph.D. thesis on

Genetic modification strategies in Rhizobia to combat abiotic stress in legumes

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Introduction

Plants face various abiotic stresses such as salinity, drought, temperature and heavy metal contamination in soil. Abiotic stress generates ROS in plants which decreases the rate of photosynthesis and causes growth retardation.[1] ROS imbalance due to any stress results in oxidative damage in cells which can lead to cell death. Apart from its destructive activities, ROS are also known for their role as the secondary messenger in many cellular processes. An imbalance between ROS generation and antioxidant enzyme system can cause harmful effects in plants[2]. Uncontrolled anthropogenic activities have polluted the agricultural soils in many ways one of which is accumulation of heavy metals like Arsenic and Cadmium[3]. Both Arsenic and Cadmium from soil are taken up by plants and have been reported to generate oxidative stress. Heavy metals reduce overall growth of plants, causes structural alterations, gets accumulated in edible parts of plant and can ultimately cause death of plant [4,3]. The growth and protection of plants not only depends on the defense mechanism of plants but, it also depends on the plant growth promoting bacteria (PGPB) / Plant growth promoting rhizobacteria (PGPR) which lives in harmony within the root-rhizosphere plane. Some of them can even infect plant roots through cracks or through root hairs (e.g. Rhizobium) and establish a commensal relationship with plants. Rhizosphere is colonized by different soil bacteria species [5].PGPR/PGPB bring about growth promotion in plants, shows antimicrobial effects and protects plants from stresses like heavy metals/hydrocarbons/salinity/drought [6]. PGPR can combat heavy metals in varieties of ways as they possesses a mechanisms such as efflux pump [7], Siderophore production for heavy metal chelation [8], exopolosachrides production [9,10]. PGPR also produces glutathione which is one of most important antioxidant in all the living systems. It helps in chelating the heavy metals and also directly neutralizes the ROS produced by the oxidative actions of these heavy metals. It also helps in maintaining Ascorbate-Glutathione cycle, Redoxin cycle, helps in reducing the oxidized protein thiols and is a building block of Phytochelatin which chelates heavy metal ions[11]. Rhizobium interacts with legumes and forms root nodules in legume corps where it fix the atmospheric nitrogen and makes it available to the plants. Heavy metals like Arsenic has already been reported by delayed nodule formation and reduction in nitrogenase activity in legumes [12], similarly cadmium stress in soil also greatly inhibited nodulation in legumes[13]. Glutathione produced by PGPR can chelate this heavy metals like cadmium and later conjugates it with sulphide to deposit CdS intracellularly[14]. By this method it can decrease the availability of heavy metals for plants and reduce the deleterious effects. Glutathione is one of the most important antioxidant in cells. It is synthesized by 2 enzymes 1) y-glutamyl cysteine ligase and 2) glutathione synthase. Two genes, gshA and gshB genes encodes this enzymes respectively in most of the bacteria. The enzyme y-glutamate cysteine ligase is inhibited by glutathione (GSH) as a feedback mechanism. Gamma glutamate cysteine ligase is very important and mutations in gshA gene in some bacteria is responsible for inhibition of glutathione and biofilm production[15]. Also gshA mutation in rhizobium bacteria are linked with its inability to synthesize glutathione[16]. Glutathione also increases Siderophore production by bacteria which also acts as the metal chelators[17]. Also it has been seen that rhizobium lacking gshA and gshB genes (genes synthesizing enzymes responsible for glutathione biosynthesis) were unable to survive in minimal medium [16], which can indicate its inability to grow properly in soil without proper glutathione supplementation as the condition/ nutrition in root-rhizosphere plane is minimal. Plant glutathione cannot replace rhizobial glutathione as it is very important for preventing early nodule senescence, maintaining the symbiosome and protecting the bacteroids from ROS generated inside the symbiosome[18].

The aim of our study is to check the protective effect of different rhizobia on fenugreek (*Trigonella foenum-graecum* L) growing in arsenic and cadmium contaminated soils. Fenugreek is an important legume with numerous health benefits[19] and consumed by people across Asia , central Asia and Europe. Fenugreek growing in metal contaminated soils has more oxidative stress which effects normal cellular processes and ultimately reduces the nutritional and medicinal value of crop[20]. Our interest is to genetically modify rhizobia in such a way that it can protect fenugreek from the oxidative

stress generated by Arsenic and Cadmium. This could ultimately improve its nutritional and medicinal values. Also fenugreek shows very good phytoextraction ability, which can accumulate metal ions in different parts of plants[21]. Such accumulation leads to bio magnification and ultimately effects an entire ecosystem. Rhizobia and other soil bacteria are capable of precipitation, immobilization, chelation and accumulation of metal ions and this property of PGPR decreases the bioavailability of harmful metal ions which are already present in rhizosphere of contaminated soils. This ultimately decreases the oxidative damage caused by heavy metals[22].

Hypothesis

So we hypothesize that expressing YbdK (a glutamate cysteine ligase) [EC 6.3.2.2] in rhizobium will help it produce enhanced glutathione and alleviate oxidative stress in fenugreek growing in heavy metal (Arsenic and Cadmium) contaminated soil. YbdK has not been expressed in any rhizobium for alleviation of heavy metal stress in fenugreek. Also in this study we are incorporating a very important broad host range rhizobium that is *S.fredii* NGR 234. It has an extensively well-developed secretion system and can colonize many species of legumes.

Objectives

1. **Cloning and expression of *Escherichia coli* DH10B y-glutamate cysteine ligase (YbdK) in rhizobia.**
   a. Cloning *Escherichia coli* DH10B ybdk gene in pBBR1MCS2 vector and transformation of *Sinorhizobium fredii* NGR 234 & *Sinorhizobium meliloti* (NAIMCC-B-00863)
   b. Comparing the production of glutathione by wild type and genetically modified rhizobium. [*Sinorhizobium fredii* NGR 234 & *Sinorhizobium meliloti* (NAIMCC-B-00863)]

2. **Determining the effect of rhizobia expressing y-glutamate cysteine ligase (YbdK) on abiotic stress mediated damage in fenugreek seedling**
   a. Effects of genetically modified *S. fredii* NGR 234 & *S.meliloti* on fenugreek seedling growth in soil leached with (As) Arsenic metal salt.
   b. Effects of genetically modified *S. fredii* NGR 234 & *S.meliloti* on fenugreek seedling growth in soil leached with (Cd) Cadmium metal salt.
   c. Effect of rhizobial consortium on fenugreek seedlings growing in As & Cd metal stress.

3. **Exploring the ability of genetically modified rhizobia for *invitro* production of heavy metal nanoparticles for combating metal stress.**
   a. *Invitro* synthesis of nanoparticles by PGPRs used in objective 1.
   b. Characterization and comparison of cadmium sulphide nanoparticles produced by wild type (WT) and genetically modified (GMO) *S. fredii* NGR 234.
   c. Characterization and comparison of cadmium sulphide nanoparticles produced by wild type and genetically modified *S. meliloti.*

Result and Discussion

Objective1

a. 1.1kb ybdk gene, encoding YbdK enzyme [EC 6.3.2.2] from *E.coli* Dh10B was cloned in a low copy number plasmid pBBR1MCS2 (5.1kb) having a derepressed *lac* promoter. This gave rise to a recombinant plasmid pPAT (6.2 kb). The nomenclature of the pPAT plasmid is as follows [plasmid- Pushpa Akash Tanvi]. Plasmid was confirmed by restriction digestion, PCR amplification of ybdk gene and sequencing which yielded proper results. Plasmid pPAT was transformed in two rhizobium strains 1. *Sinorhizobium fredii* NGR 234 2. *Sinorhizobium meliloti* (NAIMCC-B-00863). Proper transformation was confirmed by plasmid isolation form them and PCR amplification of ybdk gene form that plasmids which confirmed a proper transformation of rhizobia.

b. Expression of YbdK was checked by measuring the reduced glutathione produced by rhizobium. GSH/GSSG ration significantly increased in transformed rhizobia compare to their wild type counterparts. As glutathione is responsible to reduce the oxidative stress, we measured the fluorescence intensity of GMO and WT by fluorometer and fluorescence microscopy. We found out that GMO cells gave significantly lower fluorescence compared to WT, which indicated a proper expression of YbdK and enhanced glutathione production by GMO rhizobium, which could be related to the fact that fluorescence intensity is directly proportional to ROS. SDS-PAGE of the total protein from cell lysates of GMO and WT was compared (from both rhizobia) which showed a darker band in GMO in comparison to WT, which indicates a proper expression of YbdK in rhizobia. Also a spot assay was also performed to check the effect of Arsenic and Cadmium on growth of GMO and WT rhizobia, it was noted that GMO showed increased tolerance to metal ions at higher dilutions compared to the WT bacteria, which indirectly indicates that GMO is producing more glutathione compared to WT and is efficiently fighting the metal induced ROS generation inside them.

Objective2

a. Effect of Arsenic on growth and antioxidant profile of fenugreek was examined. Fenugreek seeds were coated with control, GMO and WT PGPR and sowed in the soil spiked with Arsenic. It was observed that root length, shoot length and total length of fenugreek treated with GMO bacteria increased significantly in comparison to WT bacteria. Similar pattern of results were obtained for fresh weight, dry weight and Glutathione reductase activity. Chlorophyll a, b, total and carotenoids increased significantly in fenugreek treated with GMO bacteria compared to the WT bacteria. While the parameters like H2O2, MDA, SOD and CATALASE significantly decreased for fenugreek treated with GMO bacteria compared to WT bacteria, which implies that Ybdk expression in rhizobia can reduce the arsenic induced oxidative damage in fenugreek seedlings.

b. Effect of Cadmium on growth and antioxidant profile of fenugreek was examined. Fenugreek seeds were coated with control, GMO and WT PGPR and sowed in the soil spiked with Cadmium. It was observed that root length, shoot length and total length of fenugreek treated with GMO bacteria increased significantly in comparison to WT bacteria. Similar pattern of results were obtained for fresh weight, dry weight and Glutathione reductase activity. Chlorophyll a, b, total and carotenoids increased significantly in fenugreek treated with GMO bacteria compared to the WT bacteria. While the parameters like H2O2, MDA, APX, SOD and CATALASE significantly decreased for fenugreek treated with GMO bacteria compared

to WT bacteria, which implies that Ybdk expression in rhizobia can reduce the cadmium induced oxidative damage in fenugreek seedlings.

c. As both GMO were effective on Cadmium as well as Arsenic, we made two consortium 1) Comprising **both WT rhizobia + control** (*Pseudomonas fluorescence* (NAIMCC-B-00342) [+ ve control for PGPR], Sinorhizobium fredii NGR 234 [WT] & Sinorhizobium meliloti (NAIMCC-B-00863) [WT] **(C1)) 2) Comprising both GMO bacteria + control** (*Pseudomonas fluorescence* (NAIMCC-B-00342) [+ ve control for PGPR], Sinorhizobium fredii NGR 234 [GMO] & Sinorhizobium meliloti (NAIMCC-B-00863) [GMO] **(C2))**. Fenugreek seedlings treated with C2 consortium and grown in Arsenic spiked soil showed a significant increase in chlorophyll and carotenoid content at higher stress, compared to the fenugreek seedling treated with C1 consortium. Total length of seedlings treated with C2 consortium increased significantly in comparison to the seedlings treated with C1 consortium. Other parameters like H2O2, glutathione estimation, MDA (data analysis remaining) CAT, APX etc. are yet to be done which could give a clear cut picture of effectiveness of C2 and C1 consortium. Fenugreek seedlings treated with C2 consortium and grown in Cadmium spiked soil showed a significant increase in chlorophyll and carotenoid content at lower and higher stress, compared to the fenugreek seedling treated with C1 consortium. Total length of seedlings treated with C2 consortium increased significantly in comparison to the seedlings treated with C1 consortium. Other parameters like H2O2, glutathione estimation, MDA (data analysis remaining) CAT, APX etc. are yet to be done which could give a clear cut picture of effectiveness of C2 and C1 consortium.

Objective 3

a. Orange colored cadmium sulphide nanoparticles were synthesized by all the bacteria used in the study, which are ***Pseudomonas fluorescence*** **(NAIMCC-B-00342),** ***Sinorhizobium fredii*** **NGR 234,** ***Sinorhizobium fredii*** **NGR 234 (pPAT),** ***Sinorhizobium meliloti*** **(NAIMCC-B-00863),** ***Sinorhizobium meliloti*** **(NAIMCC-B-00863) (pPAT). (pPAT)** at the end of bacterial name refers to transformed bacteria. Cell free lysate (supernatant) of the growth media was used in biosynthesis of Cadmium sulphide nanoparticles. Late stationary phase supernatant was use for synthesis of nanoparticles.

b. SEM analysis revealed that GMO bacteria produced significantly smaller nanoparticles compared to WT bacteria, which indirectly implies that glutathione has been significantly overproduced by GMO and secreted outside the cells compared to the WT bacteria. FTIR analysis showed a decrease in transmittance of the peak at 1629 cm-1 for GMO bacteria compared to WT bacteria, indicates an augmented production of thiolated compound and XRD analysis showed that the lattice plane of CdS nanoparticles showed d spacing of 3.2 Å to the strongest peak (111) for the nanoparticles produced by both GMO and WT which indicates a face centered crystalline nanoparticle structure.

c. Analysis of FTIR, SEM, XRD results of the biosynthesized nanoparticles produced by *S.meliloti and P.fluoroscence* is pending.

Publications

1. **Dave A**, Khanna T, Robin P*. Exploiting Rhizobium for Cadmium Sulphide Nanoparticle Synthesis: Heterologous Expression of an *Escherichia coli DH10B* Enzyme, YbdK [EC: 6.3.2.2] in *Sinorhizobium fredii* NGR234. *J Pure Appl Microbiol.* Published online February 25, 2022. doi: 10.22207/JPAM.16.1.59

2. Tanvi Khanna, **Akash Dave**, Sejal Purani, Jagath Vedamurthy, Dhaval Jivani and Pushpa Robin*. Bauhinia variegata Bark Extract: Assessment of its Anti-proliferative and Apoptotic Activities on A549 and H460 Lung Cancer Cell Lines. J. Nat. Remedies; April 2022; 22(2): 175 – 195. doi: 10.18311/jnr/2022/28740.

3. Tanvi Khanna, **Akash Dave**, Pushpa Robin. Phytochemical evaluation & Chemical characterization of Bauhinia variegata L. bark extract by TGA/DSC, FT-IR and GC-MS analytical techniques: Pharmaceutical Aspects. Res J Chem Environ; 2022. (In Press)

Manuscript in preparation (Probable title)

1. **Akash Dave**, Tanvi Khanna, Pushpa Robin*. Alleviation of Arsenic induced stress in fenugreek seedlings by different rhizobium species expressing YbdK [EC 6.3.2.2].(Original research Article)

2. **Akash Dave**, Tanvi Khanna, Pushpa Robin*. Alleviation of Cadmium induced stress in fenugreek seedlings by different rhizobium species expressing YbdK [EC 6.3.2.2].(Original research Article)

Conferences

1. International Symposium on "Proteins and Biology" at The M.S. University of Baroda, 28th Feb-1st March 2022, Vadodara.
2. "International Conference on Emerging Trends in Biological Sciences" (ICETBS) at P.D. Patel institute of Applied Sciences, CHARUSAT, 9-11th January 2022, Anand.
3. Symposium on "Trends in Biochemistry and Inauguration of Prof. L.J. Parekh memorial series" at The M.S. University of Baroda, 27th & 28th September 2019, Vadodara. (Chief guest- Noble laureate Prof. Ada Yonath)
4. Workshop on "BIOLOGICAL APPLICATIONS OF MAGNETIC NANOPARTICLES" at The M.S. University of Baroda, 27th March 2019, Vadodara.
5. National Symposium on "Omics... to Structural Basis of Diseases" at The M.S. University of Baroda, Sep30-Oct1- 2016, Vadodara.
6. National seminar on "Molecular basis of diseases" at The M.S. University of Baroda ,1st &2nd August 2014, Vadodara.
7. Seminar on "Research to rupees" conducted by GSBTM at The M.S. University of Baroda, 6th June 2013, Vadodara.
8. National symposium, workshop & Lecture series on "Current trend in biological sciences" at M&N Virani Science College, 5th&6th March 2012, Rajkot.

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