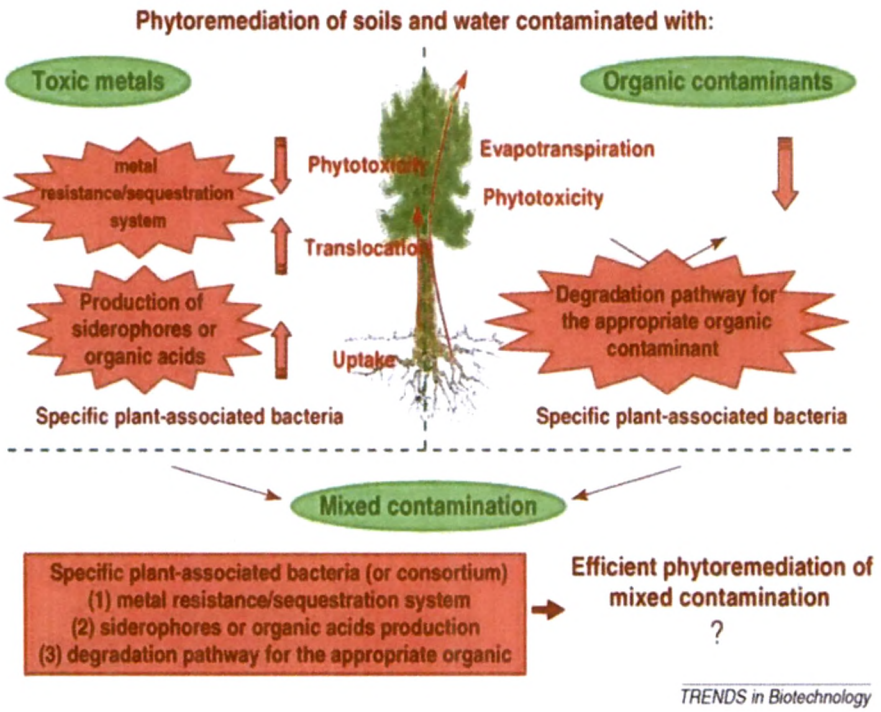
Chapter 6



Determining the alleviation of Cd toxicity and biocontrol abilities of *H. seropedicae* Z67 harboring *pqq* gene clusters in rice plants.

6.1: Introduction

Microorganisms colonize at rhizosphere and rhizoplane and use the nutrients secreted by plants (Hiltner, 1904). Some rhizobacteria promote plant growth and control plant pathogens. These are known as plant growth promoting rhizobacteria (PGPR). Atmospheric nitrogen can also be fixed by rhizobacteria, which is consequently used by the plants for growth and development. Rhizobacteria also show synergistic inhibition of plant pathogens (Schippers, 1992). PGPR include *Azotobacter, Azospirillum, Pseudomonas, Acetobacter, Burkholderia, Bacillus, Paenibacillus*, and some are members of the *Enterobacteriaceae*. There are various mechanisms involved in growth promotion such as nitrogen fixation, production of auxins, gibberellins, cytokinins, ethylene, solubilization of phosphorous, antibiotics production, lytic enzymes which helps to increases in root permeability, for the colonization which reduces competition for the available nutrients and root sites, which could enhance the uptake of essential plant nutrients etc. (Weyens et al., 2009). Plant diseases caused by plant pathogens such as fungi, bacteria, viruses, nematodes etc are controlled by PGPR (Saharan and Nehra, 2011). Blast and leaf blight diseases in rice (Oryza sativa) are caused by *Xanthomonas oryzae and Magnaporthe oryzae*, which are most destructive diseases (Haruhiko et al., 2013, Liu et al., 2013). *Rhizoctonia solani* causes sheath blight disease in rice and results in low crop yield (Sundravadana et al., 2007). Chemicals used to control this disease have not been very effective (Noda et al., 1999).

Heavy metal toxicity in soil poses a major challenge for sustainability of crop growth and yield (Gill et al., 2011). Cadmium (Cd) is one of the heavy metal which causes extremely high toxicity to plants (Mattioni et al., 1976; Adriano, 1986). Cd toxicity causes chlorosis and reduces plant growth (Choudhury and Panda, 2004). Cd toxicity to plants is mediated by generating reactive oxygen species (ROS) causing cell death (Davies and Cury, 1991; Molassiotis *et al.*, 2006). Salinity and Cd toxicity is known to significantly reduce plant productivity of wheat, barley and rice (Balestrasse et al., 2004). Drought, salinity and cadmium stresses involve reactive oxygen species.

Organic acids produced by rhizobacteria can efficiently bind with heavy metals and release P from mineral phosphates for plant growth (Gyansehwar et al, 2002; Khan et al., 2009; Archana et al., 2012). Organic acids have metal chelating ability which leads to decrease oxidative stress against cadmium (Kavita et al., 2008). Plant growth promoting bacteria inoculation to crop seeds alleviated salt stress (Dodd and Perez-Alfocea, 2012). Rhizobacteria possess several traits that can alter heavy metals stress through the release of chelating substances, acidification and changes the redox potential (Leong, 1986; Smith and Read, 1997; McGrath et al., 2001; Whiting et al., 2001; Lasat, 2002). Bacterial glucose dehydrogenases (GDH) require pyrroloquinoline quinone (PQQ) as a redox cofactor for activity which determines phosphate solubilization (Goosen et al., 1989; Meulenberg et al., 1992; Goldstien 1985). PQQ is known to promote plant growth and reduce oxidative stress which could be attributed to its very high catalytic redoxing cycling efficiency of ~ 20,000 cycles (Choi et al., 2008; Rucker et al., 2009). Additionally, PQQ is involved in production of antimicrobial compounds (Schnider et al., 1995; Han et al., 2008; de Werra et al., 2009; Guo et al., 2009).

H. seropedicae Z67 is an endophytic nitrogen-fixing bacterium which colonizes roots of economically important crops such as rice, sugarcane, and wheat (Pedrosa et al., 2001; da Silva et al., 2003; Monteiro et al., 2003). *Herbaspirillum* sp. has been isolated from the interior of roots, stem, and leaves of many Graminaceous plants (Baldani et al., 1992; DSbereiner et al., 1994; Olivares et al., 1996). *Herbaspirillum* spp. has the pectinolytic and cellulolytic enzymes that are essential for the cell wall degradation of plant during infection (Elbeltagy et al., 2001; Gyaneshwar et al., 2002). *H. seropedicae* Z67 increased biomass and N content of rice plants equivalent to the treatment with 40 kg of N/ha (Pereira and Baldani 1995). Thus the present study determines the potential of *H. seropedicae* transformants secreting PQQ in protecting the rice seedlings against the Cd^{+2} exposure.

6.2: EXPERIMENTAL DESIGN

6.2.1: Bacterial strains used in this study

Table 6.1: Bacterial strains used in this study

Plasmid/Strains	Characteristics	Source or Reference
Hs (pOK53)	35Kb plasmid contains 13.4-kb of *pqq* gene cluster of *P. fluorescens* B16 Tet^r (40μg/ml)	This study
Hs (pSS2)	25Kb plasmid contains 5.1-kb of *pqq* gene cluster of *A. calcoaceticus* Tet^r (40μg/ml)	This study
E. coli DH10B	Host strain for routine DNA manipulation experiments and plasmid maintenance. $Strp^r$ (50μg/ml)	Invitrogen,USA
E. coli DH10B	pUC18*gfp* Amp^r (100 μg/ml)	This study
pJNK2	pBBR1MCS2, Km^r (50 μg/ml) with 13.4 Kb *pqq* gene cluster of *Pseudomonas fluorescens* B16.	This study
pJNK5	pUCPM18, Gm^r (20μg/ml) with 5.1 Kb *pqq* gene cluster of *Acinetobacter calcoaceticus*.	This study
pJNK6	pJNK5, Gm^r (20μg/ml) with *gad* operon 3.8 Kb of *Pseudomonas putida* KT 2440	This study
Hs	Nitrogen fixing rice endophyte , Nal^r(10μg/ml)	Baldani et al., 1986
Hs (pUCPM18)Gm^r	*H. seropedicae* Z67 Nal^r (10μg/ml) with (pUCPM18)Gm^r (20μg/ml)	This study
Hs (pJNK1)	*H. seropedicae* Z67 Nal^r(10μg/ml) with pBBR1MCS2, Km^r (50μg/ml)	This study
Hs (pJNK5)	*H. seropedicae* Z67 Nal^r(10μg/ml) with pJNK5Gm^r (20μg/ml)	This study
Hs (pJNK6)	*H. seropedicae* Z67 Nal^r(20μg/ml) with pJNK6Gm^r (20μg/ml)	This study
Enterobacter asburiae PSI3		Gyaneshwar et al., 1998

6.2.2: Bacterial strains, plasmids, Media, and culture conditions.

Bacterial strains and plasmids used for this work have been listed in Table **1.** DNA manipulation and molecular biology experiments were performed using *E. coli* DH10B as the host strain using standard protocols (Sambrook and Russell, 2001). Plasmid bearing derivatives of *E. coli* DH10B were routinely grown at 37^0C on Luria Bertani broth (L.B) and maintained on Luria Bertani Agar (L.A) plate with 40 μg ml^{-1} streptomycin. *H. seropedicae* Z67 was routinely grown at 30^0C on Luria Bertani (L.B) and maintained on semisolid JNFb medium pH 5.8 plates containing 10 μg ml^{-1}Nalidixic acid at 30°C (Baldani et al., 1986). For nitrogen fixation, *H. seropedicae* Z67 JNFb medium was devoid of a nitrogen source. Further, plasmids pUCPM18Gm^r as vector control, pJNK1, pSS2, pOK53, pJNK5 and pJNK6 were transformed by electroporation in *H. seropedicae* Z67 as described by Unge et al. (1998). Fungal and bacterial pathogens were maintained on potato dextrose agar (PDA) plate.

6.2.3: Antibacterial and antifungal activity of *Hs* transformants

Antibacterial and antifungal agar spot assay test was carried out by using method described by Murugalatha and Mohankumar (2011). Fresh culture of *Hs* Z67 transformants and obtained by growing a single colony inoculated in 3ml LB under shake conditions at 30°C was dispensed in 1.5ml sterile centrifuge tubes, pelleted at 9, 200x *g* and washed thrice with sterile normal saline and used to determine antibacterial and antifungal activity against pathogenic bacteria and fungus, pathogens used in this study are (i) Pathogenic bacteria – *Serratia, Shingella, Salmonela typhimurium* and *Xhanthomonas oryzae* (rice plant pathogen), and (ii) Pathogenic fungi - *Fusorium oxosporium, Botrytis cinerea, Rhizoctonia solani* (rice plant pathogen) and *Magnaporthe gresia* (rice plant pathogen). The fungal pathogens *Fusorium oxosporium* and *Botrytis* were generous gift from Dr Arun Arya, Department of Botany, M. S. Univerity of Baroda, Baroda while *Magnaporthe gresia* and *Xhanthomonas oryzae* were obtained from Indian Type Culture Collection, New Delhi. All pathogenic bacteria were placed on potato dextrose agar (PDA) plate for 5 min to get dry, *Hs* transformants saline washed

culture was spotted on in uniform concentration on plates, and plates were kept for drying and placed at 30° C for 24 h. In case of antifungal activity, fungus was placed at center of PDA plate and allowed to grow on plate for 36 h, saline washed *Hs* transformant culture was spotted on plates, plates were kept for drying and placed at 30° C for 3-4 days.

6.3: Results:

6.3.1: PQQ secretion by *H. seropedicae* Z67 tranformants

The pOK53 plasmid containing *pqq* gene cluster of *P. fluorescens* B16, pSS2 plasmid containing *pqq* gene cluster of *A. calcoaceticus* and *pqq E* gene of *E. herbicola* were transformed in *H. seropedicae* by electroporation. *H. seropedicae* Z67 tranformants *Hs* (pOK53) and *Hs* (pSS2) had shown fluorescence property which was equivalent to *E. coli* containing pUC18*gfp* (**Fig 6.1**). Fluorescence was seen on normal Luria agar plate as well as on JNFb medium agar plate. *H. seropedicace* Z67 and pBBR1MCS2Km^r transformant did not show any fluorescence. On the other hand, fluorescence was also not seen in *pqqE* transformant.

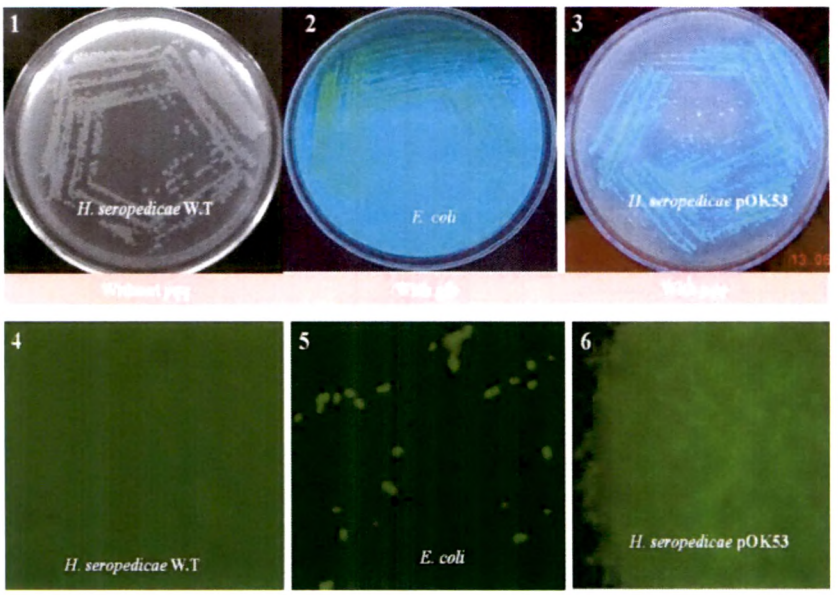


Fig. 6.1: PQQ fluorescence of *E. coli* and *H. seropedicae* Z67 transformants containing *pqq* genes. 1, 2 and 3- culture plates under UV light, 4, 5 and 6 cells under fluorescence microscopy. 1, 4- *Hs* native strain. 2, 5- *E. coli* with pUC18*gfp*. 3, 6- *Hs* (pOK53).

6.3.2: Antibacterial activity of *H. seropedicae* transformants secreting PQQ.

H. seropedicae Z67 harboring *pqq* gene clusters were used for antimicrobial activity against pathogenic bacteria. *H. seropedicae Z67* containing *pqq* gene clusters transformants have shown antimicrobial activity against *Serratia, Shingella, S. typhimurium* and *X. oryzae,* while *H. seropedicae* containing *pqqE* has not shown zone of inhibition against any pathogenic bacteria (**Fig. 6.2**). *E. asburiae* PSI3 has shown antibacterial activity against *X. oryzae*.

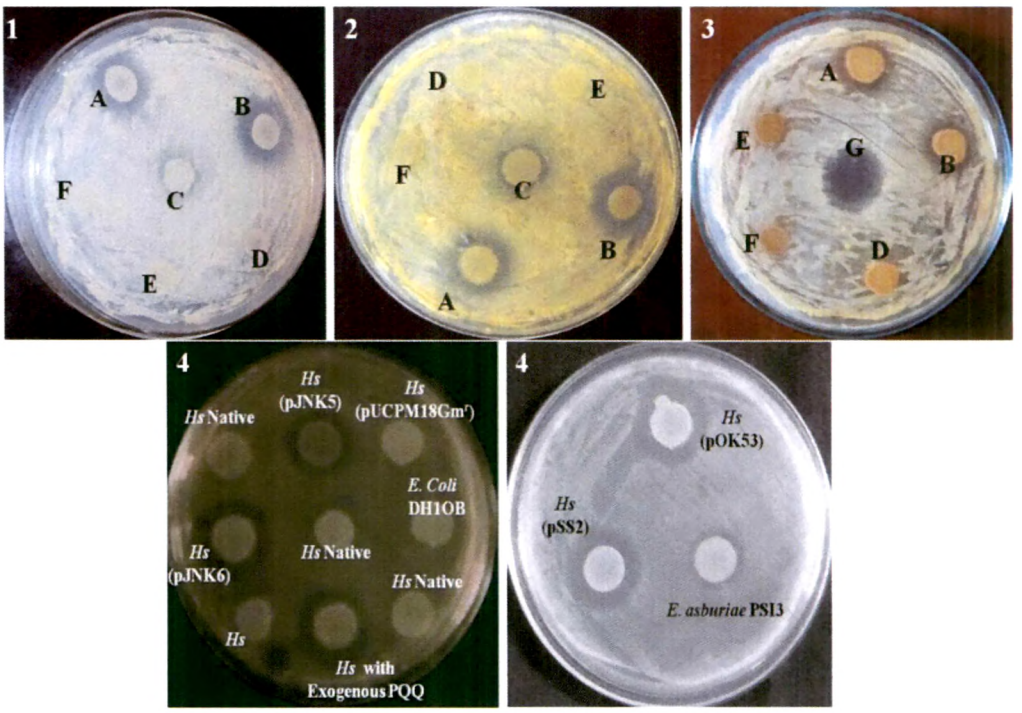


Fig. 6.2: Antibacterial activity of *H. seropedicae* Z67 containing *pqq* genes. 1- *Serratia*; 2- *Shingella* ; 3- *Salmonela typhimurium* and 4 - *Xhanthomonas oryzae*. A- *Hs* (pOK53), B- *Hs* (pSS2), C- supernatant of culture A, D- *Hs* native, E- *Hs* (pJNK1), F- *Hs* (pBBR1MCS2Km^r), G- Concentrated extract from culture A.

6.3.3: Antifungal activity

H. seropedicae Z67 containing *pqq* gene clusters showed antifungal activity against *F. oxosporium, B. cinerea, R. solani* and *M. gresia* (**Fig. 6.3**). In contrast to antibacterial activity, *pqqE* has shown zone of inhibition against pathogenic fungi. *H. seropedicae Z67* native culture was unable to show antifungal activity.

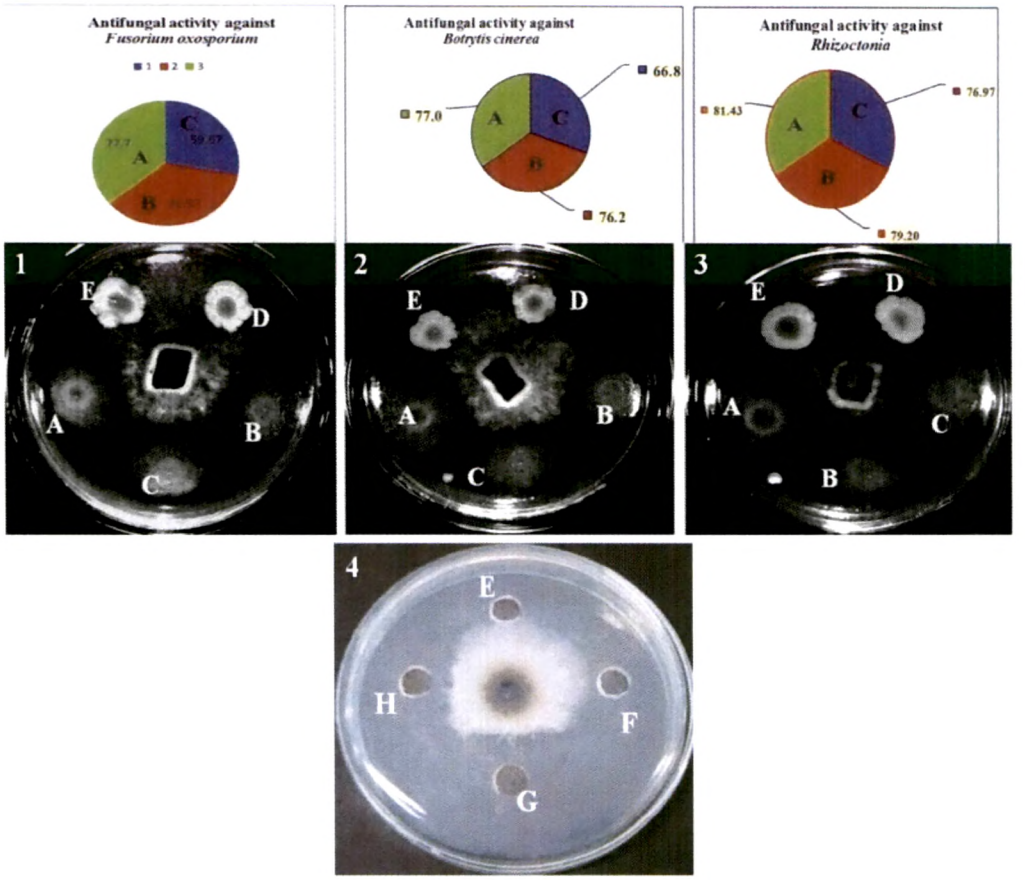


Fig. 6.3: Antifungal activity of *H. seropedicae* Z67 containing *pqq* gene cluster. 1- *Fusorium oxosporium*, 2- *Botrytis cenerea*, 3- *Rhizoctonia solani*, 4- *Magnaporthe gresia* A- *Hs* (pOK53), B- *Hs* (pSS2), C- *Hs* (pJNK1), D- *Hs* (pBBR1MCS2Kan^r), E- *Hs* native , F- *Hs* (pUVPM18Gm^r), G- *Hs* (pJNK5) and H- *Hs* (pJNK6).

6.3.4: Effect of *H. seropedicae* Z67 transformants *Hs* (pJNK5) and *Hs* (pJNK6) on cadmium tolerance.

Hs native and *Hs* (pUCPM18) did not secrete PQQ while *Hs* (pJNK5) and *Hs* (pJNK6) secreted ~1.15 µM and ~1.17 µM PQQ, respectively, in M9 minimal medium. Since PQQ is known to confer tolerance to Cd, *Hs* transformants were monitored for minimum inhibitory and tolerance levels. *Hs* native and *Hs* (pUCPM18) were able to

tolerate 40 µM of $CdCl_2$ minimum inhibitory concentration (MIC). However, *Hs* (pJNK5) and *Hs* (pJNK6) was able to grow and tolerate up to 100 µM $CdCl_2$.

6.3.5: *H. seropedicae* Z67 *pqq* transformants (pJNK5) and (pJNK6) effect on growth of rice seedling against cadmium stress.

Under hydroponic condition, rice seedling treated with 10 µM cadmium showed growth inhibition on root and shoot length up to ~80% and ~70% by respectively **(Fig. 6.4)**. *Hs* native and *Hs* (pUCPM18Gm^r) reduced inhibition ~35% when compared with independent Cd treated seedlings, while *Hs* (pJNK5) and *Hs* (pJNK6) showed growth promotion of rice seedlings by reducing stress up to ~55% and ~65%, respectively. On the other hand, PQQ treated seedlings with 10 µM cadmium had root and shoot length of rice similar to that of untreated seedlings **(Fig. 6.5).**

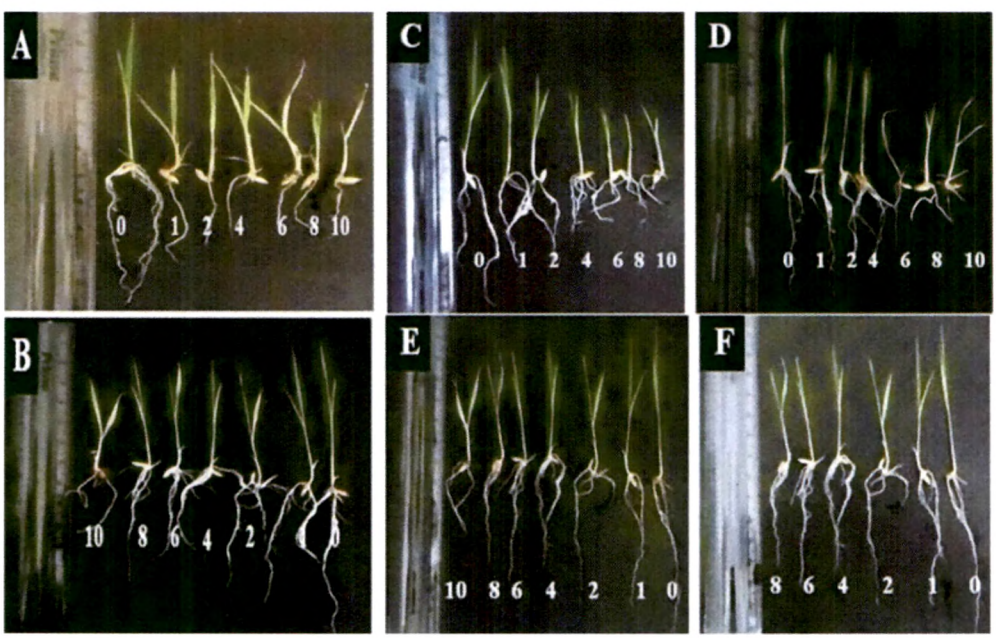


Fig. 6.4: Effect of *Hs* transformants on rice seedling treated with cadmium. A-$CdCl_2$, B- $CdCl_2$ with PQQ, C- *Hs* with $CdCl_2$, D- *Hs* (pUCPM18Gm^r) with $CdCl_2$, E- *Hs* (pJNK5) with $CdCl_2$, F- *Hs* (pJNK6) with $CdCl_2$; Concentration used ($CdCl_2$- 10 µM, PQQ- 100 nM).

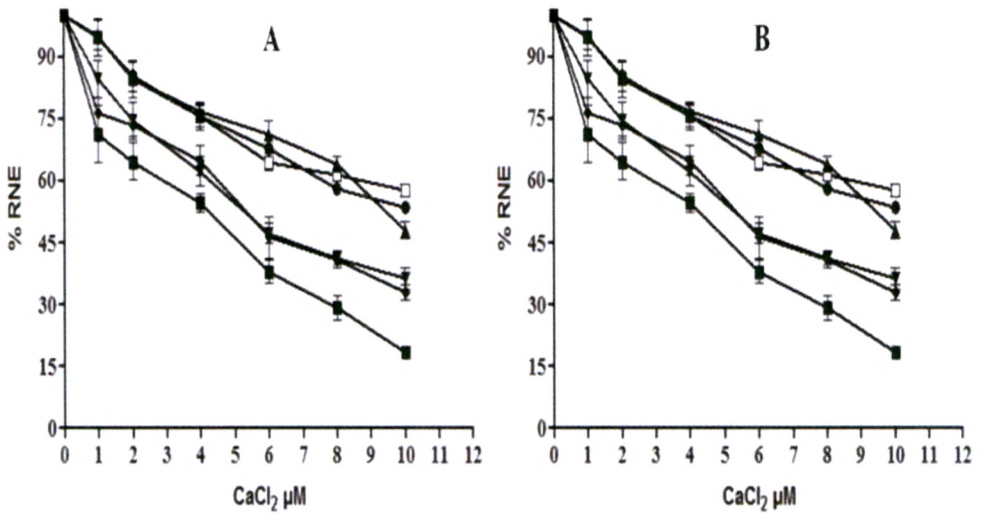


Fig.6.5: Relative net elongation (%) of root and shoot of rice seedling (RNE). (A, B- $CaCl_2$ treated, A-% RNE of root; B-% RNE of shoot C- % RNE of root and shoot, NaCl treated). **(A, B)** ■- $CaCl_2$; ▲- Exogenous PQQ; ▼- *Hs* (wild type); ◆- *Hs* (pUCPM18Gm^r); ●- *Hs* (pJNK5) ; □- *Hs* (pJNK6).

6.3.6: Effect *H. seropedicae* transformants (pJNK5) and (pJNK6) on the antioxidant enzyme activity of rice seedlings exposed to cadmium.

Cadmium treated rice seedlings exhibited a significant change in the activities of CAT and SOD. CAT activity of control seedlings was 18 U and ~17 U while seedling treated with 10 µM cadmium showed ~3.4 folds increase in activity when compared with control plants without cadmium. Hs native and Hs (pUCPM18Gm^r) showed similar ~44 U of CAT and SOD activity. On the other hand Hs (pJNK5) and Hs (pJNK6) significantly reduced CAT and SOD activities by ~3 folds **(Fig. 6. 6)**. Similarly, PQQ treated plants showed significant decrease in CAT and SOD activity by ~ 3 folds in presence of 10 µM cadmium.

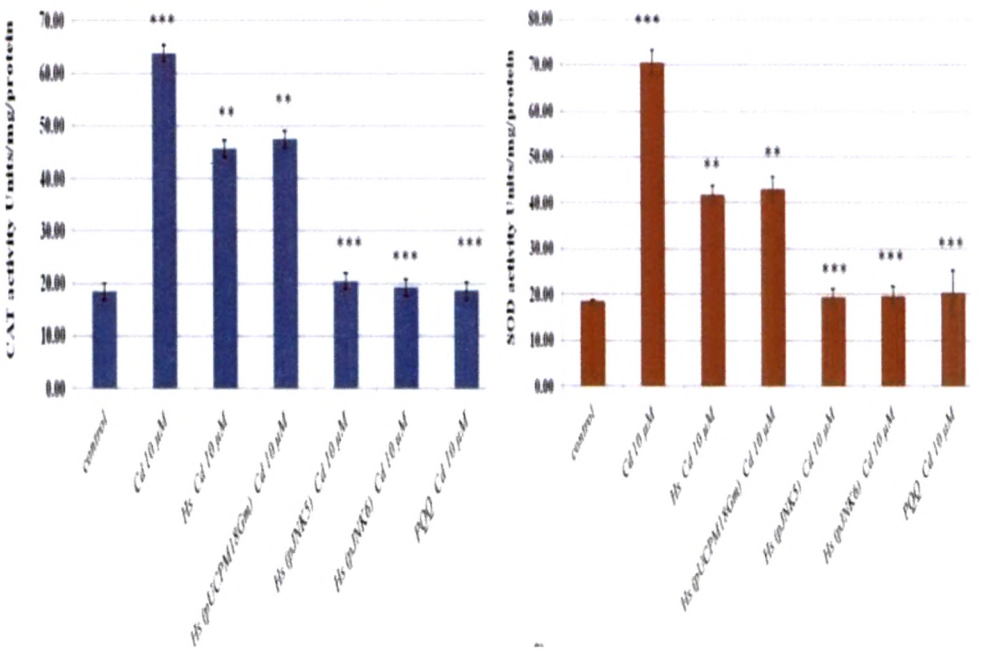


Fig. 6.6: CAT and SOD enzyme activities of rice seedlings inoculated with *H. seropedicae* Z67 transformants.

6.4: Discussion

Mineral phosphate solubilization by microorganisms is mediated by organic acids (Archana et al., 2012). Phosphate solubilising bacteria produce PQQ as cofactor for GDH which catalyzes gluconic acid production. In present study our results demonstrated the overexpression of *pqq* gene cluster and *gad* operon in *H. seropedicae* Z67 enhanced phosphate solubilization and secreted PQQ in medium. Plant inoculation studies showed that the transformants transported PQQ to plant tissues (**Chapter 5**). Present study demonstrated that *H. seropedicae* Z67 containing *pqq* gene cluster has shown fluorescence property equivalent to fluorescence of *E. coli* containing *egfp* gene on pUC18 which is a high copy plasmid. PQQ is known to show good fluorescence (Duine and Olsthoorn., 1998). Hence, overexpression of *pqq* genes in PGPR could also help in monitoring the presence of the bacteria in plant tissues and in the rhizosphere.

Hs containing *pqq* gene clusters has shown antibacterial and antifungal activity against plant pathogens including rice pathogens *X. oryzae* and M. *gresia*. However, PQQ alone does not show any antibacterial and antifungal activity. *Hs* (pJNK6) showed lesser antibacterial and antifungal activity as compared with *Hs* (pJNK5). This is contrast to earlier studies which showed mutation in *pqq* genes increased the biosynthesis of biocontrol compound (pyoluteorin) (Schnider et al., 1995). On the other hand, over expression of *pqq* genes of *S. marcescens* in *Escherichia coli* has inhibited fungal growth of *M. gresia* (Kim et al., 2006). In *Rahnella aquatilis,* mutation in *gdh* or *pqq* resulted in loss of antibacterial compound formation (Guo et al., 2009). An antibacterial compound ABS has also been purified from this bacteria (Chen et al., 2009). Mutation in *pqqA* and *pqqB* genes of *Enterobacter intermedium* has lost antifungal activity against *M. gresia* (Han et al., 2008). PQQ–GDH are involved in production of antipathogen compounds (James and Gutterson 1986; Schnider et al. 1995; Han et al. 2008; de Werra et al. 2009; Guo et al. 2009; and Ahmed and Shahab, 2010). Gluconic acid is also known to directly inhibit pathogenic fungi (Kaur et al., 2006). Antibacterial and antifungal activities of *H. seropedicae* Z67 transformants could be mediated by compounds which require gluconic

acid as ABS compound of *R. aquatilis* contains uncharacterized sugar moiety (Chen et al., 2009). This also supported by the fact that *Hs* (pJNK6) showed lesser antibacterial activity as compared to *Hs* (pJNK5) which secrete 3mM and 22mM gluconic acid, respectively.

Cd significantly inhibits the growth of roots and earlier studies showed that chelation of Cd by organic acids including gluconic acid decrease the oxidative damage (Kavita et al., 2008). *Enterobacter asburiae* PSI3 secreting high levels of gluconic acid, decreased oxidative damage and improved the root growth of mung bean seedlings in presence of Cd. Cd caused oxidative damage of rice seedlings by altering the activities of CAT and SOD leading to increased generation of ROS as reported in many other studies (Mittler et al., 2004; Fariduddin et al., 2009; Verma et al., 2011). Our result showed that overexpression of *pqq* gene cluster and gad operon in *H. seropedicae* Z67 has reduced stress of rice seedlings and could tolerate 100 μM Cd while uninoculated rice plants could only tolerate up to 10 μM Cd. *H. seropedicae* Z67 transformants (pJNK5) and (pJNK6) showed similar extent of Cd tolerance but secrete gluconic and and 2-ketogluconic acids at different levels suggest that the tolerance could be mediated by PQQ. This is supported by the fact PQQ is very efficient antioxidant with a redox cycling of 20,000 (Rucker, 2009). PQQ as antioxidant plays an important role in DNA repair and reduces oxidative stress (Misra et al., 2004; Misra et al., 2010; Choi et al., 2008). PQQ has role in protection of cells against γ radiation, which protects cells from oxidative stress (Khairnar et al., 2003). In mammals, PQQ has shown to protect mitochondria from oxidative stress (Nukada et al., 2003). PQQ neutralizes reactive species by scavenging reactive oxygen species (ROS) (Klinman and Mu, 1994; McIntire, 1998; Morris et al., 1994; Misra et al., 2004). Additionally, PQQ supplementation to mung bean seedlings resulted in growth promotion (Shahab and Ahmed, 2011). Thus, *H. seropedicae* Z67 transformants secreting strong 2-ketogluconic acid along with PQQ can confer multiple benefits to rice plants by improving phosphate solubilization, growth promotion of PQQ, tolerance to Cd exposure and protection against bacterial as well as fungal pathogens.

ROS scavenging resulted in decrease of CAT and SOD activity whereas Cd treated rice seedlings showed increase in SOD and CAT which are important antioxidant enzymes for ROS scavenging (Miller et al., 2008). Similarly, increase in SOD and CAT activity during stress condition have been reported (Scebba et al., 2006; Mobin and Khan, 2007; Zhang et al., 2009). Our results demonstrated that PQQ has normalized CAT and SOD activity and protected rice seedling from Cd when compared with control plants, it indicates the PQQ showed antioxidant property against Cd toxicity. Thus, *H. seropedicae* Z67 secreting PQQ and 2-ketogluconic acid could benefit rice plants in multiple ways by improving the P status, N status and Cd tolerance which in turn significantly enhance the efficacy of the bacteria in field conditions.