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# *In vitro* study on the effect of heavy metals on *Rhizobial* and *Pseudomonas flourescence* isolates

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**ABSTRACT**
*This study investigates the impact of heavy metals on Rhizobial and Pseudomonas flourescence isolates. In the Present study, Fifteen Pseudomonas fluorescence and 15 Rhizobium strains were isolated from rhizospheric soils of Groundnut, Black gram, Green gram, Red gram, Soybean, Sunflower, Maize and Rice soils of student farm and college farm, College of Agriculture, Rajendranagar, PJTSAU, Hyderabad, Telangana state with a view to screen out heavy metal tolerant isolates., Selected isolates were biochemically characterized also evaluated for their plant growth promoting traits and tested for their tolerance to heavy metalsi.e As (Arsenic 1-100 ppm), Cd (Cadmium 1-100ppm) &Hg ( Mercury 0.5-50ppm) using respective broth under in vitro conditions. After screening for above conditions result showed that two Rhizobial isolates RR-1, GNR-1 isolates were tolerant to almost all conditions of heavy metals (except Hg 50ppm), It is also evident that thePseudomonas flourescence isolates showing all other positive capabilities such as GGR-1 & MP-1 both were sensitive to heavy metal Hg at concentration of 25 ppm & 1 ppm respectively.*
***Key words**: PGPR, Pseudomonas sp., Rhizobium sp Heavy metals tolerance, As, Cd & Hg*

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## INTRODUCTION

Soil is one of the most important natural resource different microorganisms thrive on abundantly present nutrients therein and through various interactions play a pivotal role in cycling of nutrients and pedogenesis (Ahemad and Kibret, 2013), among pollutant, abundant amounts of heavy metals (Cd, Cu, Pb Cr, which are known to be toxic in nature) contaminated soils through various natural and manmade activities (Liu et al. 2013), heavy metals found longer time in soil due to non- biodegradable in its nature and pose a risk to human health through come into the food chain (Lal et al.2013). Without bacteria soil would not be fertile and organic matter such as straw or leaves would accumulate within a short time. Heavy metal contamination is worldwide (Nigari, 1990) Since beginning of industrial revolution,pollution of biosphere by toxic metals has more widely increased and application of metal containing pesticides, fertilizers and sewage sludge soil become contaminated with metals (Gille*r et al*., 2001).The main environmental pollution is the heavy metal pollution of soil and its negative impact on agriculture which affects the animal as well as human health. Most common heavy metals contamination are Cd, Cr, Cu, Hg, Pb and Ni (Zhon and Qiu, 2005). The pollution of the ecosystem by heavy metal is a real threat to the environment because metal cannot be naturally degraded like organic pollutants and persist in the ecosystem having accumulated in different parts of the food chain so that metal toxicity may affect all forms of life in the world (Igwe et al., 2005) and the high level of heavy metals influencing both microbial population and metabolic processes (Ahamed et al., 2004).The ability of an organism to survive in an environment with high metal concentration reflects its capacity to tolerate metals (Azza et al., 2009).

Mallikarjuna *et al*.( 2015) reported that Pea plant (*Pisum sativum*) can grow in some heavy metal contaminated soils. Based on that, they studied the individual effects of several doses of Zn, Ni, Cu, Cr and Cd on the growth of live Pea plants using solid growth media. The doses used in the present study were 0, 20, 40, 60, 80ppm. The seed germination and plant growth were significantly affected by Cd and Cr at 60 ppm, as well as by Cu and Ni at 80 ppm and higher concentrations (P<1%). Zn did not affect seed germination.

Durve *et al.* (2012) isolated *Pseudomonas aeruginosa* and *Brevibacillus choshinensis*have the properties to resist and accumulate high levels of heavy metals Cadmium (3000 ppm), Lead (600 ppm), Arsenic (1500 ppm) and Mercury (500 ppm) and can resist various antibiotics and pesticides and showed multi-drug resistance.The residual heavy metals (Cadmium, Lead, Arsenic and Mercury) after bioaccumulation were analyzed using ICP-AES technique and the isolates showed 70-80% bioaccumulation of heavy metals.Figueira *et al*. (2005) reported that heavy metal tolerance of *Rhizobium* isolates was screened by plating in YEM media supplemented with metal (Zn, Pb, Co, Cd, Ni and Cr) of increasing concentrations: 0, 0.065, 0.125, 0.165, 0.210, 0.250, 0.500, 0.750, 1.000, 2.000 and 3.000 mM for *Rhizobium leguminosarum*biovar *viciae.*

Raja *et al.*(2006) stated that growth rate of the sewage isolates in the presence of heavy metal (Cd, Ni, As and Pb) were consistently slower than that of the control, In this study, *Pseudomonas* sps were resistance to cadmium 7mM in TY agar plate.

Carrascoa (2005) isolated 100 *Rhizobium* strains, 41 of them being resistant to high concentrations of As(300 mg lK1), Cu (100 mg lK1) and Pb (500 mg/ lK). Their phenotypes and bioaccumulation potentials have been characterised by their growth rates in media supplemented with As and heavy metals. Several *Rhizobium* were symbiotically effective in the contaminated soils. Nodule establishment is more affected by heavy metals than $N_2$-fixation.

The present study is aimed to determine how much the isolates of *Rhizobium* and *Pseudomonas flourescence*strains are tolerant and sensitive to increasing concentrations of Arsenic, cadmium and mercury. This study may extend to open new area of research like heavy metals in soil at high levels, which may affect growth and activity of the selected strains.

## MATERIALS AND METHODS

### 1. ISOLATION OF *RHIZOBIAL* AND *PSEUDOMONAS FLUORESCENCE* ISOLATES FROM DIFFERENT RHIZOSPHERE SOILS.

Efficient plant growth promoting isolates are collected from different rhizosphere soils of different crops and these isolates were tested for their purity and preservation in Dept.of Agricultural Microbiology & Bioenergy, College of Agriculture, PJTSAU, Rajendranagar, Hyderabad

Pure isolates were isolated by streaking isolates on respective media plates and screened for following Plant growth promoting properties like phosphate solubilization, ammonia production,Nitrogen fixation efficiency by Acetylene reduction assay(ARA) and Indole Acetic Acid Production

### 2. EFFECT OF HEAVY METALS ON *RHIZOBIAL AND PSEUDOMONAS FLORESCENCE* ISOLATES

Isolates were screened for their ability to tolerate different and heavy metals (As, Cd and Hg) using respective broth. YEMA broth used for *Rhizobial* isolates and Kings B broth used for *Pseudomonas flourescence.* Test tubes of respective broth were amended with different concentrations of arsenic, cadmium and mercury. Each isolate was inoculated into the different concentrations of arsenic, cadmium ranging from 1, 5, 10, 50 and 100 ppm and mercury 0.5, 1, 10, 25 and 50 ppm. Inoculated tubes were incubated at $30^0$C for 24 h after which growth of each isolate was determined spectrophotometrically by measuring the optical density (O.D) at 600 nm. Growth was quantified relative to growth of control containing un inoculated medium used as a blank. After this, the isolates that exhibited greater amount of growth at 50 and 100 ppm and above were selected for further study.

## RESULTS AND DISCUSSION

### *3.* SCREENING OF *Rhizobial* AND *Pseudomonas fluorescence* ISOLATES FOR HEAVY METAL TOLERANCE

#### 3.1 Effect of heavy metals on *Rhizobial* isolates:

Heavy metal concentration in polluted soils tend to influence growth of PGPR and in tern crop yields. In the present study different heavy metal concentration in the normal soils (permissible limit 1-5 ppm) and in polluted soils (25 - 50 ppm) were taken screening the tolerance of isolated PGPR. (Fig 3.1)

#### (A) Arsenic tolerance of *Rhizobium*:

Out of 15 *Rhizobial* isolates four isolates (27.00%) (GGR-1>GNR-2>SFR-2>BGR-1) could tolerant to 1 ppm Arsenic concentration. Maximum O.D was recorded for GGR-1 (0.55 OD) & four isolates (27.00%) (GGR-1>GNR-2>SFR-2>BGR-1) able to survive 5ppm arsenic concentration. Maximum OD value for GGR-1. Only four isolates (27.00%) (GGR-1>GNR-2> BGR-1>SFR-2) could tolerant to 10 ppm arsenic concentration. Maximum O.D value 0.32 was recorded for GGR-1**.** At 50 ppm arsenic concentration only three isolates (20.00%) GGR-1>GNR-2>BGR-1) able to survive maximum OD value was recorded for GGR-1. Increasing the concentration of arsenic up to 100 ppm three isolates GGR-1>GNR-2>>BGR-1) (20.00%) able to grow maximum OD 0.13 was recorded for GGR-1

#### (B) Cadmium tolerance of *Rhizobium*

Out of 15 *Rhizobial* isolates six isolates (40.00%) in the order of RR-1>MR-3>RGR-1>GNR-2>GGR-2>GNR-1 could tolerant to 1 ppm cadmium concentration. At 5 ppm six isolates (40.00%) (RR-1, RR-2, MR-3,GNR-1,GNR-2,GGR-2)showing the value of O.D 0.35-0.58. In this decreased growth of *Rhizobial* isolates with increasing metal concentration was observed. At 10 ppm nine isolates (RR-2,MR-3,MR-4,GNR-1,GNR-2,GGR-1,GGR-2,SFR-1,RGR-1) were able to survive as OD value recorded for them was >0.2. Maximum value (0.36) of OD at 10 ppm was observed for MR-3. At 50 ppm ten isolates (80.00%) (RR-1, RR-2, MR-3, MR-4, GNR-1, GNR-2, GGR-1, GGR-2, SFR-1) were able to survive as OD value recorded for them was >0.13. Maximum value (0.24) of OD at 50 ppm was observed for GNR-2. Increasing the concentration of cadmium level up to 100 ppm six isolates (40.00%) (RR-1, RR-2, MR-3, MR-4, GNR-1, GNR-2) were able to survive as OD value recorded for them was 0.085-0.16. Maximum value (0.16) of OD at 100 ppm was observed for RR-1&MR-3.

**(C) Hg tolerance of *Rhiobium***

Out of 15 *Rhizobial* isolates four isolates (27.00%) (RR-1,GNR-1,GNR-2, SYR-1) able to survive in the range of 0.5 ppm Hg concentration and showing the value of O.D 0.67 -0.77. Maximum value (0.77) of OD at 0.5 ppm was observed for RR-1.Increasing the concentration of Hg to 1 ppm 10 isolates (67.00%) (RR-1, RR-2, MR-1, MR-2, MR-3, BGR-1, GNR-1, GNR-2, SFR-1, SFR-2) able to survive but O.D value is low in the range of 0.44-0.50. Maximum O.D value was observed for RR-1. At 10 ppm Hg concentration 11 isolates (73.00%) (RR-1, MR-1, MR-2, MR-3, BGR-1, GNR-1, GNR-2, GGR-1, SFR-1, SFR-2) able survive to O.D in the range of 0.31-0.38. Highest O.D was observed for MR-1. At 25 ppm Hg concentration 9 isolates (63.00%) (RR-1, MR-1, MR-3, BGR-1, GNR-1, GNR-2, GGR-1, SFR-2, RGR-1,SYR-1) and could tolerant O.D value is in the range of 0.20-0.25. Maximum O.D was observed for MR-1.Only three isolates (20.00%) (GGR-1, GGR-2,SYR-1) could be tolerant to 50 ppm Hg concentration O.D was recorded for them was 0.12-0.16 . Maximum OD was observed for SYR-1.

**3.2. Effect of heavy metals on *Pseudomonas fluorescence* isolates**

**(A) Arsenic tolerance of *Pseudomonas fluorescence***

Out of 15 *Pseudomonas fluorescence* isolates 4 isolates (27.00%) (MP-1, MP-3, SFP-1, GNP-1) could tolerant to 1 ppm arsenic concentration. Out of 4 SFP-1 showed maximum tolerance. Only 4 isolates (27.00%) (MP-1, MP-3, SFP-1,GGP-1) could tolerant to 5 ppm arsenic concentration. Only 4 isolates (27.00%) (MP-1,MP-3,SFP-1, GGP-1) and could tolerant to 10 ppm Arsenic concentration. only 3 (20.00%) (MP-1, MP-3, SFP-1) tolerant to 50&100ppm arsenic could concentration

Out of 15 *Pseudomonas fluorescence* isolates MP-1, MP-3, SFP-1 showed tolerance in all concentrations and SFP-1 showed tolerance to As, Cd& Hg metals up to 100 ppm concentration.

**(B) Cadmium tolerance of *Pseudomonas fluorescence***

Out of 15 *Pseudomonas fluorescence* isolates six isolates (40.00%) (RGP-1, MP-1,SFP-1,SFP-2,GNP-1,BGP-1) could be tolerant to 1 ppm cadmium concentration. At 5 ppm cadmium 5 isolates (33.00%) (MP-1, RP-4, SFP-1, GNP-1, BGP-1). Increasing the concentration of Arsenic 10 ppm five isolates (33.00%) (MP-1, SFP-1, SFP-2, GNP-1, BGP-1). At 50 ppmisolates only three isolates (20.00%) (SFP-1,GNP-1, BGP-1) able to survive. Only three isolates (20.00%) (SFP-1, GNP-1, BGP-1) and could tolerant to 100 ppm cadmium concentration. Out of 15 isolates SFP-1, GNP-1, BGP-1 isolates was tolerance in all concentrations.

**(C) Hg tolerance of *Pseudomonas fluorescence***

Out of 15*Pseudomonas fluorescence* isolates nine isolates (60.00%) (RGP-1, RGP-2, MP-1, MP-3, SFP-1, SFP-2, GNP-1, BGP-1, SYP-1) could tolerant to 0.5 ppm Hg concentration. Maximum growth was recorded for GNP-1. Only 6 isolates (40.00%) (RGP-2, MP-3, SFP-1, SFP-2, GNP-1, BGP-1) could tolerace to 1 ppm Hg concentration. Maximum OD value recorded for GNP-1. Only four isolates (27.00%) (SFP-2, GNP-1, RGP-2, BGP-1) could be tolerant to 10 ppm Hg concentration GNP-1 was recorded for maximum OD. At 25&50 ppm only two isolates (13.00%) (SFP-2, GNP-1) able to survive, increasing the concentration of Hg decreasing the growth was observed.

Out of 15 *Pseudomonas fluorescence* only two isolates SFP-1 &GNP-1 showed tolerance in all concentrations.


**Tolerance of *Rhizobium* to HgTolerance of *Pseudomonas flourescence* to cadmium**
**3.1 Heavy metal tolerance of *Rhizobial* and *Pseudomonas fluorescence* isolates**

## DISCUSSION

After screening for above conditions, it was found that the two *Rhizobial* isolates RR-1, GNR-1 isolates were tolerant to almost all conditions of heavy metals (except Hg 50ppm), abiotic stresses (temperature $45^0$C).

This indicates that these two isolates had good tolerance to above tested adverse conditions in addition to having multiple beneficial activity such as nitrogen fixation, phosphate solubilisation, growth promoters through IAA production

Future study with these isolates using them in pot cultures and followed by field experiments will help in establishing their potential to be used as biofertilizers.

The isolates GGP-1 also showed tolerance to abiotic stresses such as temperature, drought, salinity and pH at different levels. However, these isolates did not show any tolerance to heavy metals. Interestingly, the isolate MP-1 which was exhibiting antagonism also showed tolerance to heavy metals but without phosphate solubilization and IAA production. It is also evident that the isolates showing all other positive capabilities such as GGR-1 & MP-1 both were sensitive to heavy metal Hg at concentration of 25 ppm & 1 ppm respectively.

The data obtained in the present study suggest that *Pseudomonas flourescence* isolates GGP-1, MP-1 would be ideal organisms for further study in pot culture and field experiments to exploit their PGPR potential for a good biofertilizers production.

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