

Effect of Heavy Metals on Growth of *Rhizobium*

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Abstract— *Rhizobium* isolates were obtained from sewage sludge of Mula-Mutha River. The isolated rhizobia were found to form root nodules on soybean. Cross-inoculation studies were carried out with soybean legume. Studies on the effect of five metals (Fe, Al, Mo, Co, Hg) were carried out on all strains of *Rhizobium*. The effects were assessed on bacterial growth in culture. Cobalt (Co) & Mercury (Hg) was found to have detrimental effect in vitro condition at all its concentrations. Iron (Fe), Molybdenum (Mo), Aluminum (Al) was found to support bacterial growth at all the concentrations in culture of *Rhizobium-1*, *Rhizobium-2*, *Rhizobium-3*, *Rhizobium-4*, and *Rhizobium-5* strains of bacteria. These *Rhizobial* isolates were screened for their ability of Siderophores production. One of the goals of present study is the selection of strains of rhizobia which are highly effective nitrogen fixers and biocontrol agents against most plant pathogens from their ability to produce siderophores.

Keywords— Heavy metals, nitrogen fixer, *Rhizobium*, Sewage sludge, Siderophores

1 INTRODUCTION

An example of an economically important symbiosis in nature is the nitrogen fixing association of leguminous plants with soil bacteria of the genus *Rhizobium*. This association leads to the formation of nitrogen fixing root nodules. Estimates of atmospheric nitrogen fixed by the *Rhizobium*/legume association vary depending on Environmental conditions, the effectiveness of the *Rhizobium*/legume association. Extensive use of chemicals to control plant diseases has disturbed the delicate ecological balance of the soil, leading to groundwater contamination, development of resistant races of pathogen and health risks to humans. However, considerable attention has been paid to plant growth-promoting rhizobacteria (PGPR), as the best alternative to chemicals, to facilitate eco-friendly biological control of soil and seed-borne pathogens. One of the goals of present study is the selection of strains of rhizobia which are highly effective nitrogen fixers and biocontrol agents against most plant pathogens from their ability to produce Siderophores.

Rhizobia encompass a range of bacterial genera, including *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Allorhizobium*, and *Azorhizobium*, which are able to establish a symbiosis with leguminous plants. Biological nitrogen fixation is an important component of sustainable agriculture, and rhizobial inoculants have been applied frequently as biofertilizers. It is unique because they live in a symbiotic relationship with legumes common crop and forage legumes are peas, beans, clover, and soybean. Among plant-microbe interactions, legume-*Rhizobium* interactions are unique because they supply 80-90% of total nitrogen requirement of legumes. Majorly *Rhizobium* is isolated from soil so it is the main source from which it can be isolated but recently, *Rhizobium* isolated from sewage sludge sample has been identified & characterized. It is also seen that sewage sludge is directly applied to soil so as to check whether the bacteria forms symbiotic association between plant & itself. *Rhizobium* culture was charac-

terized & identified by Biochemical testing of the isolates.

A Cross-inoculation study was carried out with different crops like Beans for *Rhizobium phaseoli*, *Rhizobium meliloti* for methi, *Rhizobium japonicum* for soybean etc. The principle of cross-inoculation grouping is based on the ability of an isolate of *Rhizobium* to form nodules in a limited number of species of legumes related to one another. Various seeds like beans, methi, Alfalfa, mung & soybeans were used to carry out cross-inoculation study in which soybeans showed good results.

Effect of heavy metals on the growth of *Rhizobium* species is checked. According to Giller *et al.* (1998), effect of heavy metals depends upon duration of exposure, dose and type of metal used. Kinkle *et al.* (1994) reported that *Sinorhizobium meliloti* strains have high tolerance ability to various heavy metals. In legume *Rhizobium* symbiosis, maximum yield is possible when there is suitable condition for both partners. Present investigation aims to analyze the effect of aluminum, iron, Mercury, cobalt and molybdenum on rhizobial growth.

Siderophores are low molecular weight (generally <10,000Da), iron-coordinating, organic compounds produced by most aerobic and facultative anaerobic microorganisms to combat low-iron stress. It was seen that *Rhizobium* also produces siderophores which was detected on CAS (Chrome Azurol S) Agar medium. Yellowish orange zone is seen around the organism.

2 Materials and Methods:

2.1. Bacterial strains & Growth conditions:

Sewage sludge sample was collected from Mula Mutha River in Pune. Physicochemical parameters of collected sew-

age sample were checked. From this sample 10 rhizobial isolates were obtained on YEMA medium. Their colony morphology & Gram staining was checked. The isolates were checked for their fast growing ability on yeast extract mannitol medium plates containing bromothymol blue (Trinick, 1980, Vincent, 1970). The isolates giving acid reaction on YEMA-BTB considered as fast growing & those which giving alkaline reaction considered as slow growing rhizobia. Cultures were also streaked on YEMA containing congo red to prevent any contamination of *Agro bacterium spp.* Cultures were grown aerobically at 28°C for 4 days.

2.2. Cross-inoculation studies:

The principle of cross-inoculation grouping is based on the ability of an isolate of *Rhizobium* to form nodules in a limited number of species of legumes related to one another. Cross inoculation studies were carried with soybean legume by coating seeds with rhizobial isolates. Surface sterilized seeds of soybean legume inoculated with log phase of rhizobial culture & sown in pots containing sterilized soil. Each pot was watered with sterile distilled water upto 45 days. After 45 days the plants were uprooted & the nodule formation was checked.

2.3. *In vitro* effects of metals on specific growth rate of *Rhizobium*-

Effect of five metals Al, Fe, Mo & Hg, Co was investigated on five *Rhizobium* strains isolated from sewage sludge nodulating soybean legume. Effects of these metals were studied on 0µM, 25µM, 50µM, 75µM & 100µM concentrations of their corresponding salts (AlCl₃.6H₂O, FeCl₃ & Na₂Mo₄.2H₂O, HgCl₂, CoCl₂). The effects were assessed on bacterial growth *in vitro*.

2.4. Bacterial culture:

A loop full of log phase culture of each strain was inoculated separately in flasks containing Yeast extract Mannitol broth medium amended with different salt concentrations of Al, Fe, Co, Hg and Mo. The inoculated flasks were then placed at 28 ±1 on shaker at speed 150 rpm. To obtain growth rate, the optical density of each flask was measured after every 4 hours up to 40 hours at wavelength 610 nm. Specific growth rate of five strains were calculated using the formula given by Stanier et al.(1985)

$$\text{Specific growth} = \frac{\text{Log OD}_1 - \text{Log OD}_0}{T_1 - T_0}$$

Where OD₁ = Log value of O.D. of culture at time t hour

Where OD₀ = Log value of O.D. of culture at time t₀ hour

T₁-T₀ = Difference between time interval or duration of incubation.

2.5. Siderophore Production-

Siderophore production was detected by Chrome-Azuroil S (CAS) assay (Schwyn and Neilands, 1987).

3. Results:

3.1. Isolation & identification of *Rhizobium* isolates

Rhizobium isolated from sewage sludge sample was first streaked on Jensen's medium to identify isolates then from that selected colonies of the isolates is streaked on Yeast Extract Mannitol Agar Medium (YEMA) then depending upon colony morphology, Gram character, cultural characteristics compared from Bergey's manual for classification the isolates were identified.

3.2. Cross inoculation studies:-

The principle of cross-inoculation grouping is based on the ability of an isolate of *Rhizobium* to form nodules in a limited number of species of legumes related to one another. All *rhizobia* that could form nodules on roots of certain legume types have been collectively taken as a species on the basis bacteria forming nodules to the specific legume the *rhizobial* isolates can be identified. This isolate shows nodulation with the soya beans seeds confirming that they may be *Rhizobium japonicum*. Out of 10 rhizobial isolates obtained only five was found to show nodule formation with soybean legume.

3.3. *In vitro* effect of different concentration of heavy metals on growth of *Rhizobium* isolates:

Different concentrations of heavy metals were used. & their effects on the growth of *Rhizobium* isolates were checked.

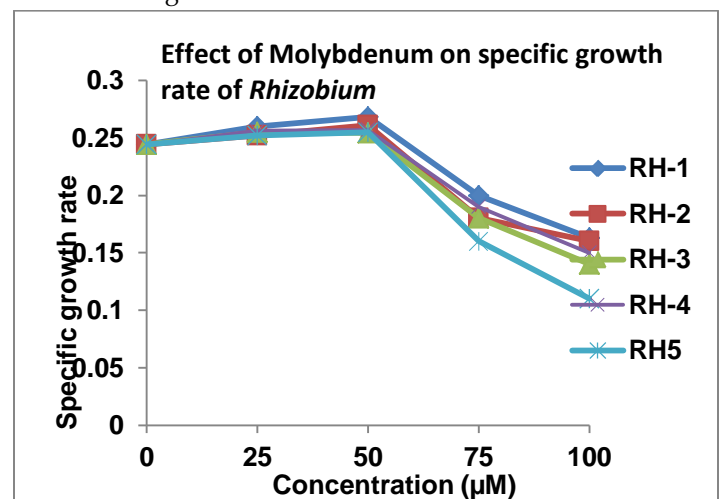


Fig 1: Effect of Molybdenum on specific growth rate of *Rhizobium*

can also have ability to bind other metals such as actinides & heavy metals. It was found that on the CAS medium *Rhizobium* isolates shows yellowish orange zone around the colony. Azurool S (CAS) assay of Schwyn and Neilands [16]. This assay results in an unknown structure of a Fe-CAS-hexadecyltrimethylammonium (HDTMA) complex, at pH 5.6 that changes from a blue to an orange-yellow color as the Fe is removed from the complex.(Fig.5)

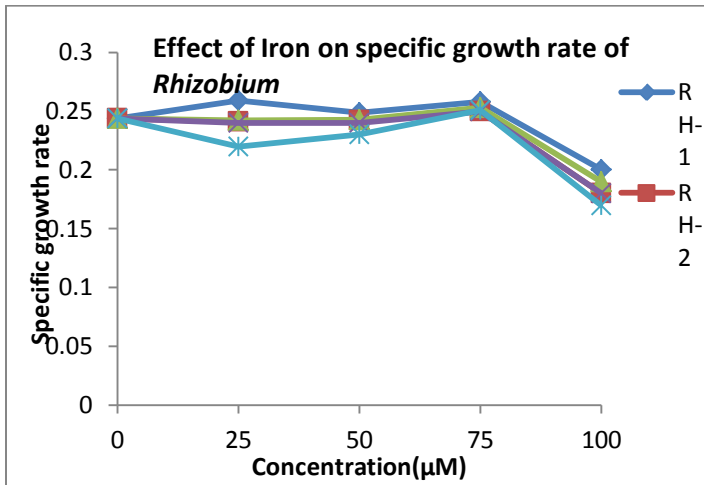


Fig 2: Effect of Iron on specific growth rate of Rhizobium

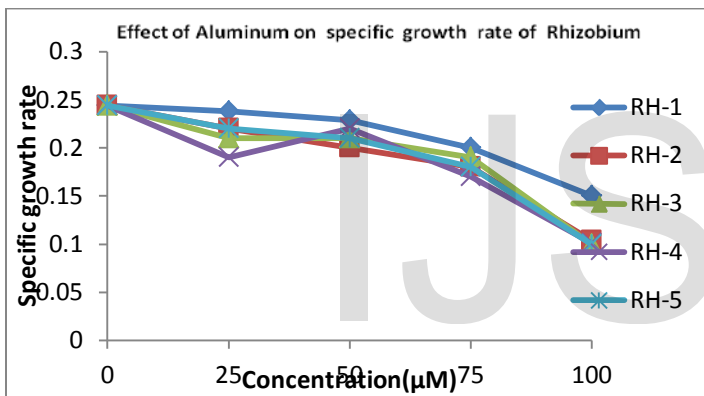


Fig 3: Effect of Aluminium on specific growth rate of Rhizobium

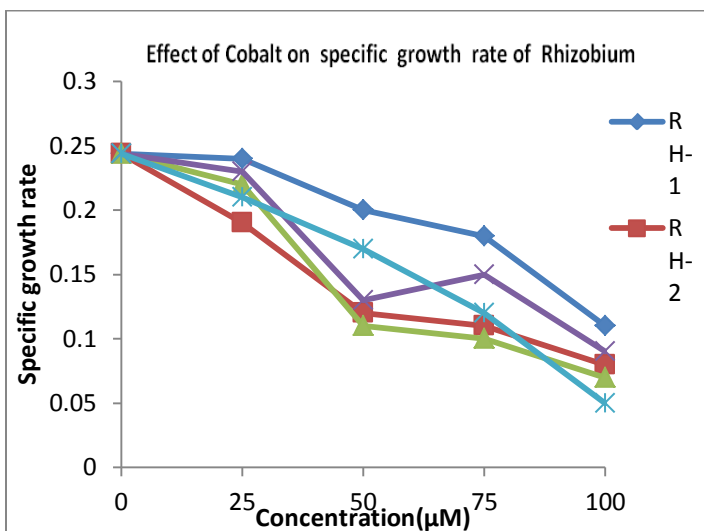


Fig 4: Effect of Cobalt on specific growth rate of Rhizobium

3.4. Identification of Siderophores production in Rhizobium isolates:

Siderophores have high specificity for iron besides this they

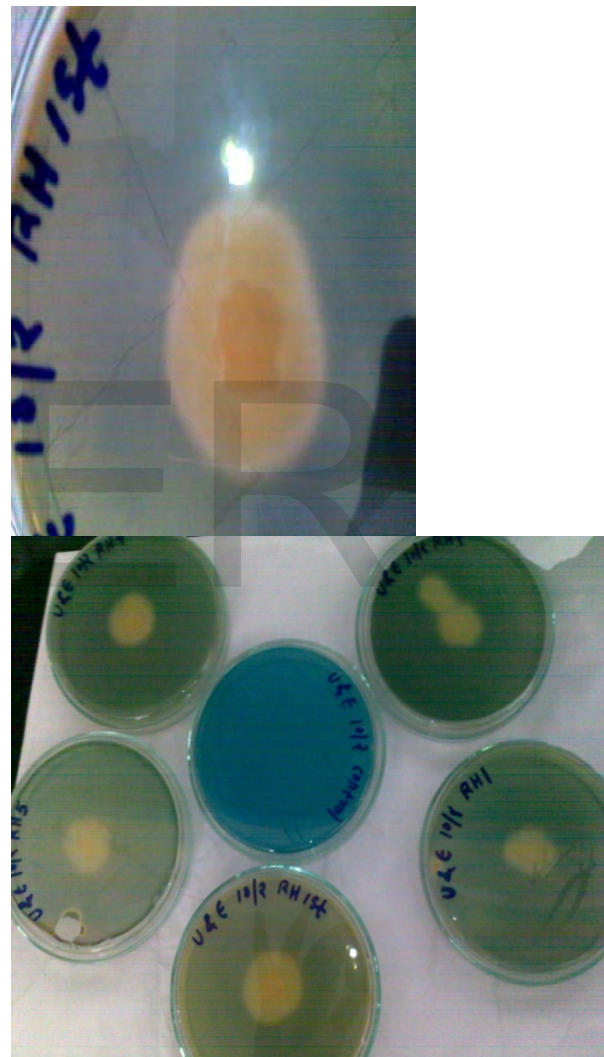


Fig5: Siderophore detection by five strains of Rhizobium

Discussion:

In the present paper we have isolated ten rhizobial strains from the sewage sample. Gram staining & motility of these ten strains were checked. They were found Gram negative & motile bacteria. After performing cross inoculation studies by coating the seeds of soybean legume with rhizobial cultures, the obtained results revealed that only five strains were able to form root nodules on the soybean leg-

ume. Therefore we continued our studies with these five rhizobial isolates. There are two groups of rhizobia, one is *Bradyrhizobium* and another is *Rhizobium*. Rhizobia coming under *Bradyrhizobium* show alkaline reaction on yeast extract mannitol agar containing bromothymol blue, & these are slow growing rhizobia. Whereas rhizobia belong to the *Rhizobium* shows acid reaction on yeast extract mannitol agar medium containing BTB, these are fast growing rhizobia. Therefore to know under which group isolated rhizobia belongs we streaked cultures of rhizobia on YEMA containing BTB & incubated at 28°C for 4 days. Results obtained revealed that these were fast growing rhizobia showing acid reaction on YEMA-BTB medium. Thus it indicates that rhizobia isolated from sewage sludge sample are belongs to the *Rhizobium* group. At Pune the domestic sewage and the other wastes is drained in the Mula-Mutha River. The physicochemical characteristics of sewage was studied considering parameters like pH, alkalinity, BOD, COD, chlorides, total nitrogen, total phosphorus, potassium, calcium, nitrates, nitrites, oil and grease, organic carbon. Results of these physicochemical characteristics of sewage revealed that the quality of sewage water was favorable as far as its agriculture use is concerned. These values of different physicochemical parameters noted in this study showed below the tolerance limits for irrigation purpose given by ISI 1986.

In further studies effects of heavy metals such as Fe, Al, Mo & Co, Hg were assessed for bacterial growth in culture (invitro). In these studies effects of all these metals at different concentrations such as 25µM, 50µM, 75µM, and 100µM were observed on the specific growth rate of rhizobia. The results obtained revealed that aluminum has detrimental effect on growth rate of rhizobial under *invitro* conditions. Wood & Cooper (1988) reported inhibition of multiplication of rhizobial strains at 50µM Al concentration.

Al toxicity is of great problem under acidic medium as solubility of free Al ion (Al⁺⁺⁺) increases rapidly under acidic conditions (McLean, 1976) As rhizobial strains studied here are fast growing acid producing bacteria it promotes Al to show its full toxicity. This study indicated that Al was inhibiting the bacterial growth (fig 3). Other metal such as Fe & Al was found to show stimulatory effect on the growth of rhizobial up to certain concentration (25µM, 50µM, 75µM) (fig1 &2). Both iron and molybdenum being the key components of enzyme nitrogenase & nitrate reductase. Apart from being & important cofactor of enzyme complex iron is also an inevitable component of various other cellular components & products like leghaemoglobin (Bergersen 1978, Dudeja et al 1997). Molybdenum is also an important component of nitrogenase enzyme complex (Solaiman, 1999) Fu & Tabatabai (1989) observed the inhibitive effects of Fe & Mo beyond a certain limit. Soil deficient in Mo produces poor & ineffectively nodulated legumes. Among these five metals, mercury & cobalt was showing detrimental effect on the growth of rhizobial beyond 25 µM concentration (fig3&4).

Siderophores production was detected by all these five strains of rhizobia (fig 5). It is well known that iron is specifically required for N₂ fixing system. It is involved in the synthesis of the nitrogenase, leghaemoglobin, ferredoxin, and

hydrogenase & cytochrome.

Consequently iron deficiency may affect symbiotic fixation by impairing, *Rhizobium* survival, establishment of functional nodules or host photosynthesis & energy transfer to the bacteroids (Johnson & Barton, 1993). Although extensive research has been directed to correct chlorosis (iron deficiency) by the application of available iron compounds to the soil & by selective plant breeding to produce Fe chlorosis resistant cultivars, only during the last decades the possible implications of Siderophores production by rhizobial strains has been considered as a potential way to improve nodulation & N₂ fixation in iron deficiency conditions. The beneficial effect of using Siderophores producing strains of *Bradyrhizobium spp.* & *Rhizobium meliloti* were reported by Gill et al (1991) & Arora et al (2001) respectively. Moreover Siderophores producing ability might favour the persistence of rhizobia in iron deficient soils (Lesueur et al 1995). Recently more attention is given to the Siderophores producing *Rhizobium spp.* Because of their ability to exhibit antagonistic effect against plant pathogens such as *Fusarium*, *Macrophomina phaseolina*.

Since the source of this *Rhizobium* is sewage sludge, it means that these strains have resistant to many factors present in the sludge. Therefore this can be used as efficient biofertilizers & are superior to *Rhizobium* present in the soil because of its resistance.

Conclusion:

Significantly increased biomasses of plant indicate that rhizobia isolated from sewage sludge have naturally potential ability to promote the growth of soybean legume but the ability of performance depends on the proper association between rhizobial strains and legume plant species. The concentration level of rhizobial inoculation is another important factor for seed germination and plant growth. The results confirm earlier studies indicating that strains of rhizobia isolated from sewage sludge can promote growth of legume plant, possibly through mechanisms that involve changes in growth physiology and root morphology. Also they have ability to resist heavy metals. More rhizobial strains should be screened from sewage sludge of different sites and places through laboratory and field experiments to exploit their potential as PGPR for sustainable plant production.

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