STUDIES ON STRESS TOLERANCE OF *RHIZOBIUM* ISOLATED FROM *SESBANIA* WITH REFERENCE TO HEAVY METALS, FUNGICIDE, ANTIBIOTICS, BILE SALTS AND ANIONIC DETERGENTS.

[1]Vaishali Mane, [2]Deepak Thakare, [3] Shubhada Nayak
[1]Assistant Professor, [1]Microbiology Department, Karm**a**veer Bhaurao Patil College, Vashi, Navi Mumbai, India
[2]Former Head, [2]Microbiology Department, Karm**a**veer Bhaurao Patil College, Vashi, Navi Mumbai, India
[3]Head, [3]Microbiology Department, Karm**a**veer Bhaurao Patil College, Vashi, Navi Mumbai, India

Abstract: This work aimed to evaluate stress tolerance of nine *Rhizobium* isolates obtained from *Sesbania bispinosa* root samples collected from Navi Mumbai coastal region with reference to heavy metals, fungicide, antibiotics and bile salts. Effects of 100µg – 500µg concentrations of 13 heavy metal salts (Ag_2SO_4, KI, $Co_3(PO4)_2$, $MnCl_2$,$(NH_4)_2MoO_4$, LiCl, $ZnSO_4$, $CuSO_4$, $FeSO_4$, $K_2Cr_2O_7$, $CdCl_2$, $HgCl_2$, $Pb(CH_3COO)_2$) on the growth of 9 isolates of *Rhizobium*.The results clearly show different levels of intrinsic tolerance of *Rhizobium* strains to the applied concentrations of heavy metals in terms of growth. All the strains displayed the lowest intrinsic tolerance when growing on 100µg of $ZnSO_4$, $CuSO_4$, $K_2Cr_2O_7$, $CdCl_2$ & $HgCl_2$while most strains were found to show the highest intrinsic tolerance of 500µg of $Co_3(PO4)_2$, $MnCl_2$,$(NH_4)_2MoO_4$, LiCl, KI and $Pb(CH_3COO)_2$.The effect of the fungicide on the isolates of *Rhizobium* was variable, depending on the concentration of fungicide Bavistine and the isolate. All of the *Rhizobium* isolates showed resistance to the 10µg whereas 55% of isolates showed tolerance upto 30 µg/disc fungicide. One isolate could tolerate 40 µg/disc fungicide. Effect of different concentrations of 32 antibiotics on growth *of Sesbania Rhizobium spp* was studied. Nearly 90% isolates showed highest intrinsic resistance to Augmentine, Bacitracin, Cefuroxime, Cephotoxime, Ceptrixime, Erythromycin, Furozolidone, Gentamycin, Kanamycin, Lincomycin, Neomycin,Optochin, Penicillin G & Trimethoprim and around 55% isolates exhibited intrinsic resistance to some antibiotics like Chloromphenicol, Ciprofloxacin, Methicillin, Pipericillin, & Ofloxacine. Highest intrinsic tolerance to surfactant i.e. 10% sodium taurocholate used in this study was shown by 3 isolates. Highest tolerance to anionic detergent (sodium lauryl sulphate) upto 6% was exhibited by isolate IS-02. In future these stress tolerant *Rhizobium* spp. isolates can be successfully employed for biotechnological applications in terms of bioremediation and biomineralization.

Keywords: ***Rhizobium, Sesbania bispinosa*, Heavy metals, Fungicides, Antibiotics, surfactant, sodium taurocholate, stress tolerance**

1. INTRODUCTION

Rhizobia are genetically diverse and physiologically heterogeneous group of Gram negative, motile rod shaped bacteria, which belong to family *Rhizobiacae* (**Hirsch *et al.*, 2001**). It is first identified in root nodules of legumes in 1888 showing symbiotic nitrogen fixation and forming nodules on the roots or rarely on the stem of legume hosts, within which the bacteria fix atmospheric nitrogen into ammonia (**Quatrini *et al.*, 2002**). Effective survival ability of bacteria even under adverse environmental conditions is obligate requirement for a fully functional symbiosis. *Rhizobium* has nif genes which encodes for nitrogenase enzyme system provides a constant source of reduced nitrogen to the plant and this bacterial symbiotic association with leguminous plant reduces the dependence on nitrogenous chemical fertilizers for the growth of leguminous crops (**Dilworth and Parker, 1969**).

Sesbania is a genus of nearly 60 species of tropical legume out of which 40 have so far been reported to nodulation by *Rhizobium* spp (**Sprent, 2001**). Most of *Sesbania* species' natural occurrence or habitat is wet or flooded soils. It serves as a potential green manure

in wetland rice production as it can fix large quantities of nitrogen due to symbiosis with Rhizobium spp. (**James *et al.*, 2001**). Nodulation in *Sesbania* may be induced by a variety of *rhizobia*, including *Azorhizobium* spp.(**Dreyfus *et al.*, 1988; Gonçalves and Moreira, 2004**; Within the soil, *rhizobia* frequently encounter various stresses that affect their growth, their initial steps of symbiosis and the capability of nitrogen fixation (**Zahran, 1999**). Before understanding field, an application of promising inoculants, identification of certain markers (e.g., heavy metal, pH, antibiotic resistance) is necessary for ecological competitiveness (**Ausili*et al.*, 2002; Dourin*et al.*, 1 996; Evans *et al.*, 1980; Zahran, 1999**).

Heavy metals toxicity to nitrogen-fixing rhizobia and the nodulation process mediated by them has been the subject of intense research.Even though reports on effect of heavy metals on rhizobia are contradictory, several studies have validated that some of these metals are incompatible and can be toxic to both rhizobia (**Broos*et al.*, 2005**) and legumes (**Broos*et al.*, 2004**). Different heavy metals exhibit variable degree of deviations in rhizobial populations, in addition it can also cause multiple effects on legume plants & symbiosis between them. Heavy metals exhibit toxic effects on the plant metabolism. For instance, a higher concentration of metals may induce interaction with sulfhydryl groups, leading to the inactivation of plant protein (**Assche and Clijsters, 1990**). On the other hand, the growth and plant growth-promoting activities of microorganisms can be altered because of a high concentration of metals (**Broos*et al.*, 2004**).

Mostly crop diseases are controlled in legume fields using fungicides has enormous effect on increasing yield and amended quality (**Fox *et al.*, 2007**). Fungicides shows dual effect as it interfere with plant and has a detrimental effect on most of soil microflora (**Castro *et al.*, 1997; Malik and Tesfai, 1983; Lennox and Alexander, 1981**). Some research has been carried out on the effect of different fungicides on *Rhizobium* bacteria growth and nitrogen fixation capacity (**Rennie and Dubetz, 1984; Tu, 1982, 1981**). *Rhizobium* isolates have diverse wide resistance to fungicides (**Diğrak and Kirbağ, 1996; Martyniuk*et al.*, 1999a; 1999b**) and antibiotics (**Alexander *et al.*, 2006; Hungria *et al.*, 2001**).

Inoculation of stress tolerant strains of rhizobia may enhance the nodulation and nitrogen fixation ability of plants under stress conditions. The ability of legume hosts to grow and survive in saline conditions is improved when they are inoculated with stress tolerant strains of rhizobia (**Zou*et al.*, 1995; Hashem*et al* 1998; Shamseldin and Werner, 2005**). Rhizobial populations vary in their tolerance to major environmental factors (**Ulrich and Zaspel, 2000; Mahobia and Mahna, 2002; Sridhar *et al.*, 2005; Wei *et al.*, 2008; Biswas*et al.*, 2008**).

In mangrove ecosystem or in highly contaminated soils, these soil organisms are likely to bear systems to survive with toxic metals in their habitats (**C´anovas*et al.* 2003**).Due to their high stress tolerance capacity these microorganisms are very useful for biotechnological applications in terms of bioremediation and biominerilization. Therefore, there is a need to study the microorganisms of these soils. Navi Mumbai coastal region is one of the major mangrove forests in India where diversity has not been studied thoroughly.

Therefore, in the present study, an attempt has been made to study the stress tolerance of Rhizobial isolates obtained from *Sesbania bispinosa* growing in mangrove soils of Navi Mumbai coastal region to appreciate the functionalities of the stress tolerant rhizobia. Further, these strains were evaluated for their stress tolerance with reference to 13 heavy metal salts, Fungicide carbendazim, 32 antibiotics & Surfactant (sodium taurocholate).

2. MATERIAL AND METHODS

2.1 Bacteria and Media used:

Nine (IS 1, IS 2, IS 3, IS 6, IS 7, IS 10, IS 13, IS 15 & IS 16) rhizobial strains used in this study were selected from a collection of twenty isolates of *Sesbania bispinosa*. These strains were selected on the basis of their morphological, cultural, biochemical, physiological characteristics and nodulation patterns.

These strains formed pink-brown effective nodules on *Sesbania spp.* under controlled conditions. For routine growth of bacterial cultures, Yeast Extract Mannitol agar medium was used (**Vincent, 1970**).The purified cultures were maintained on Yeast Extract Mannitol agar slants. (**Subha Rao, 1988**).

2.2 Screening for heavy metal tolerance:

Rhizobium isolates of *Sesbania bispinosa* were evaluated for their resistance against thirteen different heavy metals using 5 levels of concentrations. The resistance of isolates to heavy metals was evaluated on solid YEMA medium. The plates were surface inoculated with 0.1 ml of culture with cell density of about 10^8 cells using sterile glass spreader. The stock solutions of following heavy metals (100 µg/disc –500µg/disc) were prepared using analytical grade heavy metal salts of Silver sulphate (Ag_2SO_4), Potasium iodide (KI), Cobalt phosphate($Co_3(PO4)_2$), Ammonium molybdate($(NH_4)_2MoO_4$), Lithium chloride (LiCl), Zinc sulphate ($ZnSO_4$), Copper sulphate ($CuSO_4$), Ferrous sulphate ($FeSO_4$), Potassium dichromate ($K_2Cr_2O_7$), Cadmium chloride ($CdCl_2.H_2O$), Mercuric chloride($HgCl_2$), Lead acetate ($Pb(CH_3COO)_2$.Control was maintained using sterile deionized water. Sterile whatman filter paper discs dipped in the respective heavy metal salt solution, excess solution removed and then placed over the swabbed YEMA plates and incubated at 30° C for 48-72 hours. Isolates were considered resistant when growth occurred around disc and sensitive when zone of inhibition around the discs was detected**(Abigarg *et al* 2013, Cigdem Kucuk *et al* 2012, Fazal Hadi *et al* 2010, S.P.Paudyal *et al* 2007).**.

2.3 Fungicide tolerance study:

Sterile YEMA plates were inoculated with 0.1 ml of culture with cell density of 10^8 cells/ml of *Rhizobium* using sterile glass spreader. Stock solutions of Fungicide concentration ranging from 0.1% to 0.4% were prepared using sterile distilled water. Sterile discs were dipped in the respective fungicide solution and then placed over the inoculated YEMA plate and then the plates were incubated at room temperature for 72 hours.The inhibition of *Rhizobium* isolates by fungicides was checked by the zone of inhibition around the discs (**Kale *et al*, 1989**).

2.4 Determination of Intrinsic Antibiotic Resistance:

The intrinsic antibiotic resistance of *Rhizobial* isolates to 32 standard antibiotics namely Amoxycillin, Ampicillin, Augmentine, Bacitracin, Cefuroxime, Cephotoxime, Ceptrixime,Chloromphenicol, Ciprofloxacin, Clindamycin, Doxycycline, Erythromycin, Furozolidone, Gentamycin, Kanamycin, Ketoconazole, Lincomycin, Methicillin, Neomycin, Nitrofurantoine, Ofloxacine, Optochin, Penicillin G, Pipericillin, Streptomycin, Sulphamethizole, Tobramycin, Trimethoprim & Vancomycin [Hi-Media] with variable µg/disc concentration were assessed according to the standard method. Antibiotic discs were placed on the surface of agar, YEM agar medium inoculated with 0.1 ml of liquid culture containing about 10^8 cells and then the plates incubated at room temperature for 5 days. The experiment was conducted in triplicates for each treatment. Isolates were (was) considered resistant to antibiotic when growth occurred around disc and sensitive when zone of inhibition around the discs was detected (**Mishra *et al*, 2009***)*.

2.5 Screening for tolerance to surfactant (Sodium taurocholate):

The screening of rhizobium tolerance to sodium taurocholate like surfactant was checked on sterile YEMA medium containing 0.5% -10% sodium taurocholate concentrations. Loop full liquid culture containing about 10^8 cells of rhizobia were spot inoculated & plates incubated at 30ºC for 5 days with 3replicates for each treatment.Isolates were considered resistant to sodium taurocholate when growth occurred on media and sensitive when growth was absent on medium containing sodium taurocholate. Control was maintained on sterile YEMA without sodium taurocholate. (**D.B. Thakare, 1999**).

2.6 Screening for tolerance to anionic detergent (Sodium lauryl sulphate):

The screening of rhizobium tolerance to anionic detergent like Sodium lauryl Sulphate (SLS) was checked on sterile YEM broth medium containing 1% -10% Sodium lauryl Sulphate concentrations. 0.1 ml culture containing about 10^8 cells of rhizobia were inoculated & broths incubated at 30ºC for 5 days with 3 replicates for each treatment. Isolates were considered resistant to sodium taurocholate when growth in the form of turbidity occurred in broth media and sensitive when growth and turbidity was absent in medium containing sodium taurocholate. Control was maintained on sterile YEM broth without Sodium lauryl Sulphate. (**D.B. Thakare, 1999**).

3. RESULTS & DISCUSSION:

3.1 Screening for heavy metal tolerance of *Sesbania Rhizobium spp.*

Sesbania Rhizobium spp. exhibited variable tolerance level to 13 different heavy metals under study [details of sensitivity & tolerance is given in figure. 1.a & 1.b Almost 90% *Rhizobia* spp. showed tolerance to 500µg/disc Cobalt phosphate, Manganese chloride, Lithium chloride, Potassium iodide, Lead acetate & Ammonium molybdate. In addition luxuriant growth & excess EPS production was observed as the concentration of Ammonium molybdate is increased. However 80 % *Rhizobia* spp. found to be sensitive to 100 µg of Zinc sulphate, Copper sulphate, Potassium dichromate, Cadmium chloride & Mercuric chloride. In case of Silver sulphate & Ferrous sulphate 45 % strains found to exhibit tolerance upto 500 µg concentration & remaining isolates exhibited variable sensitivity pattern towards it. Negative control did not show inhibition of isolates around discs.

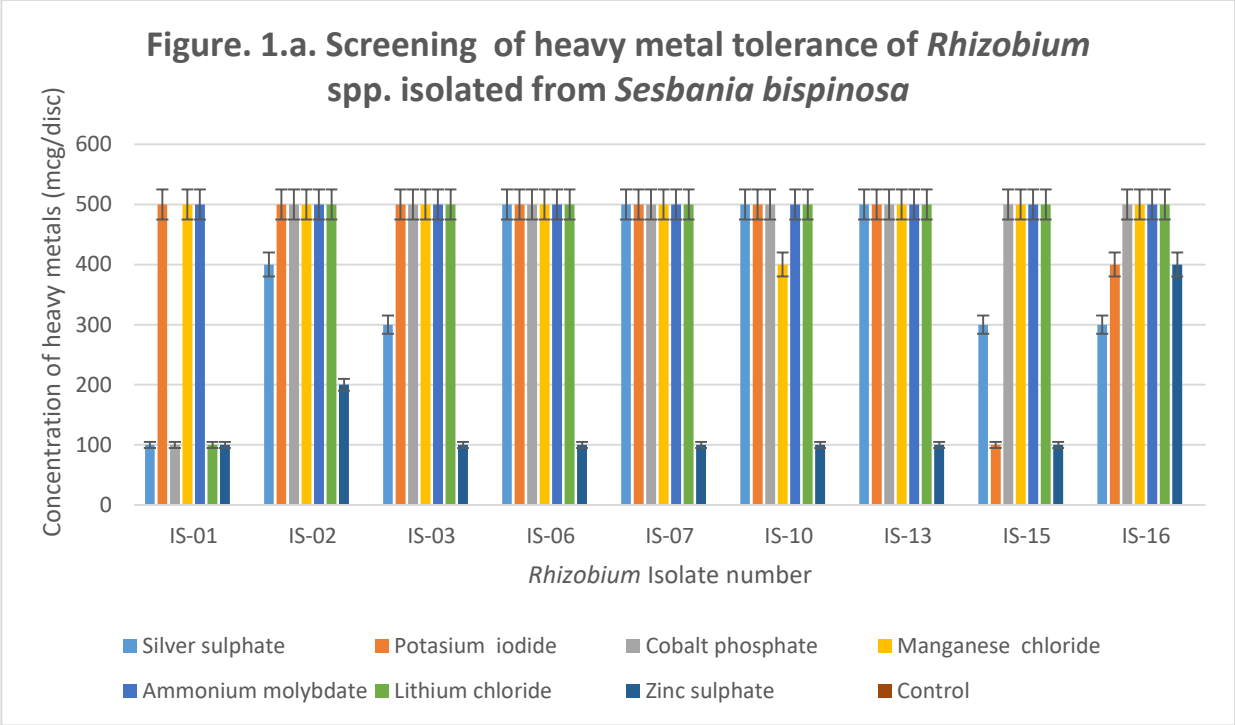


Figure. 1.a. Screening of heavy metal tolerance of *Rhizobium* spp. isolated from *Sesbania bispinosa*

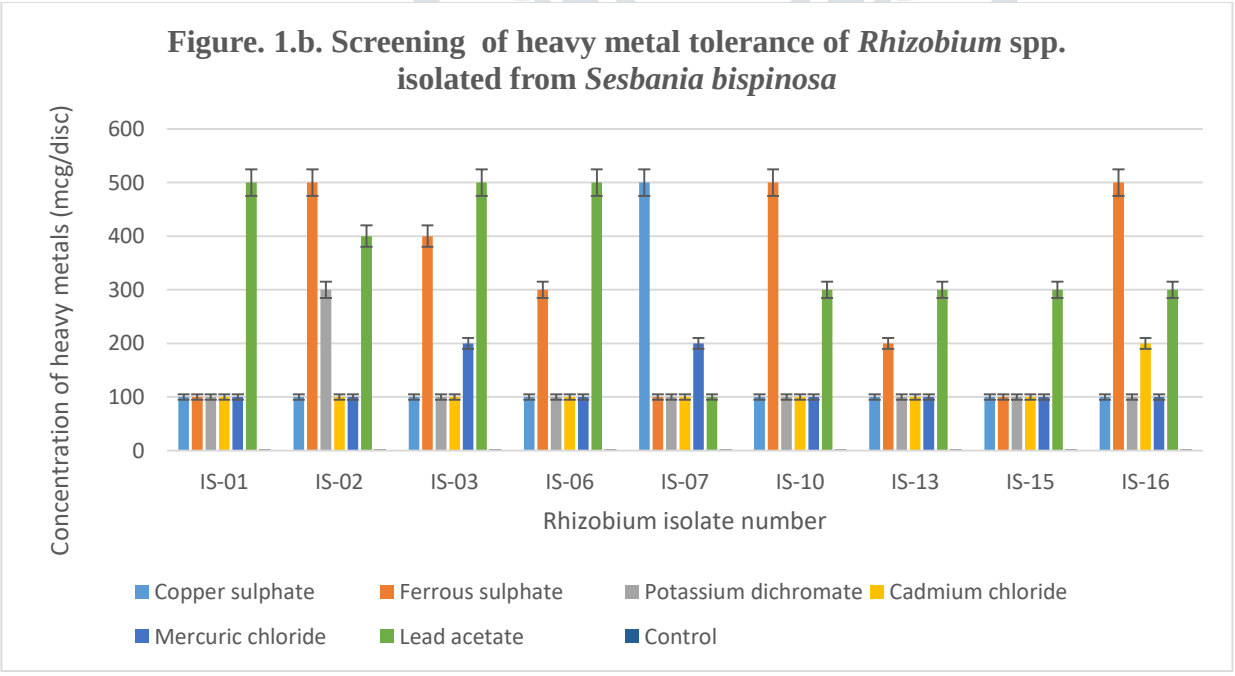
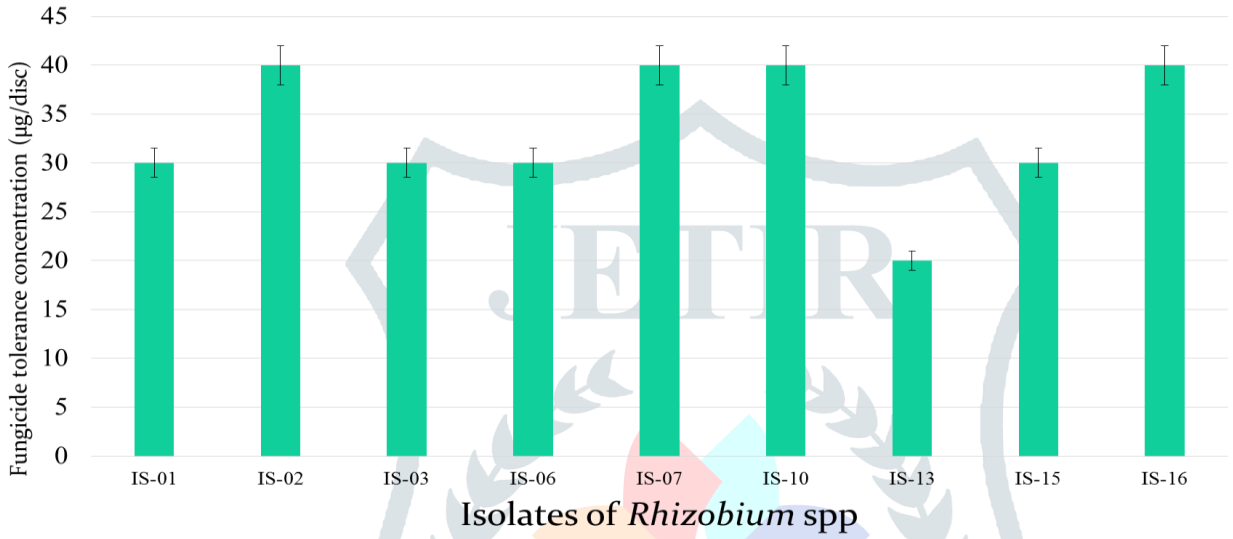


Figure. 1.b. Screening of heavy metal tolerance of *Rhizobium* spp. isolated from *Sesbania bispinosa*

3.2 Fungicide tolerance of *Sesbania Rhizobium* spp. towards standard Bavistin (Carbendazim 50%WP)

In this study increasing concentrations of fungicide decreased the growth of isolates. Figure2 shows that all isolates were resistant to 10µg Carbendazim, isolate no. IS-07 & IS -13 showed sensitivity to 20µg/disc Carbendazim. However IS-01, IS-02, IS-06, IS -10 & IS- 16 displayed tolerance to 30µg/disc Carbendazim, on the other hand Isolate IS-10 showed highest tolerance to fungicide upto 40µg/disc Carbendazim. These fungicide tolerant *Sesbania Rhizobium* isolates can be easily employed in the stressed agricultural regions where fungicides are used in excess concentration.

Figure. 2 Fungicide tolerance of Sesbania *Rhizobium* spp. towards standard Bavistin (Carbendazim 50%WP)



3.3 Determination of Intrinsic Antibiotic Resistance of *Rizhobium spp.*

Intrinsic antibiotic resistance of *Sesbania rhizobium spp*. studies as showm in Table 3.3 showed that all isolates exhibited resistance to some antibiotics namely Augmentin, Bacitracin (except IS-06 & IS-07), Cefuroxime, Cefotoxime, Ceptrixime (except IS-06), Erythromycin, Furozolidone, Kanamycin, Lincomycin, Optochin, Penicillin G & Trimethoprim. On the other hand with Amoxycillin, Doxycyclin, Streptomycin, sulphamethizole, Vancomycin; the isolates were found sensitive (except IS-02). Sensitivity towards Ampicillin, Ketoconzole and Tobramycin was shown by 33% of isolates. With Chloramphenicol, Methicillin and Pipericillin sensitivity was exhibited by almost 45% of isolates. 50% of isolates showed sensitivity towards Ciprofloxacine and Ofloxacine. Almost 80% isolates were sensitive to Gentamycin and Neomycin antibiotics. 65% isolates were found to be sensitive towards Nitrofurantoin and Tetracycline antibiotics.

Table 3.3. Intrinsic Antibiotic Resistance of *Rizhobium spp*

Antibiotibcs used	Concentration used	IS-01	IS - 02	IS - 03	IS - 06	IS - 07	IS -10	IS - 13	IS -15	IS - 16
Amoxycillin	**30 µg/disc**	30	08	36	18	50	30	28	36	31
Ampicillin	**10 µg/disc**	R	18	R	12	R	R	R	24	R
Augmentine	**30 µg/disc**	R	R	R	R	R	R	R	R	R
Bacitracin	**8 units/disc**	R	R	R	08	08	R	R	R	R
Cefuroxime	**30 µg/disc**	R	R	R	R	R	R	R	R	R
Cephotoxime	**30 µg/disc**	R	R	R	R	R	R	R	R	R
Ceptrixime	**30 µg/disc**	R	R	R	14	R	R	R	R	R

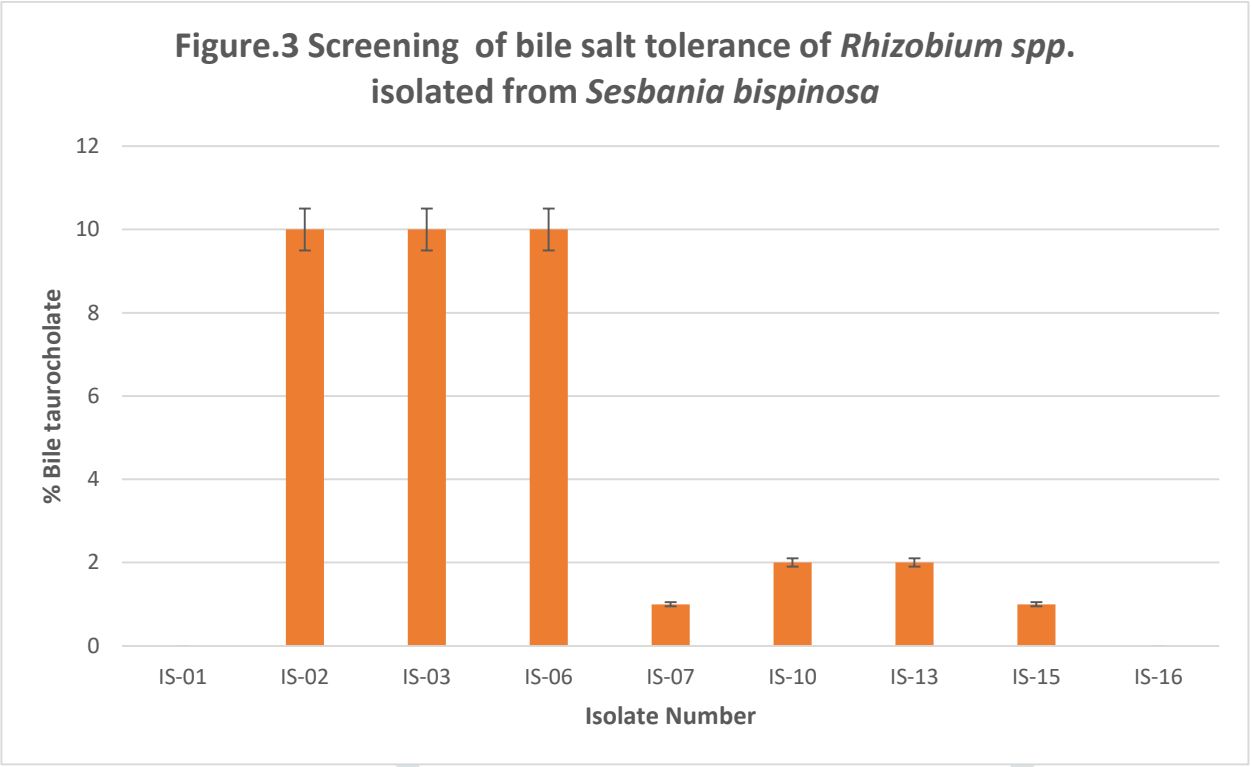
Chloromphenicol	**10 µg/disc**	R	17	R	29	R	28	32	R	R
Ciprofloxacin	**5 µg/disc**	26	25	R	30	R	40	42	R	R
Clindamycin	**2 µg/disc**	R	R	08	30	R	R	R	36	R
Doxycycline	**30 µg/disc**	40	19	40	23	60	52	52	62	44
Erythromycin	**15 µg/disc**	R	R	R	R	R	R	R	R	R
Furozolidone	**50 µg/disc**	R	R	R	R	R	R	R	R	R
Gentamycin	**10 µg/disc**	07	07	12	12	14	R	R	R	10
Kanamycin	**5 µg/disc**	R	R	R	R	R	R	R	R	R
Ketoconazole	**50 µg/disc**	R	R	08	20	10	R	R	R	R
Lincomycin	**2 µg/disc**	R	R	R	R	R	R	R	R	R
Methicillin	**5 µg/disc**	R	07	08	11	R	R	R	34	R
Neomycin	**30 µg/disc**	R	R	16	10	21	15	12	22	14
Nitrofurantoine	**300 µg/disc**	R	12	14	15	24	18	11	R	R
Ofloxacine	**5 µg/disc**	20	20	R	28	R	45	44	R	24
Optochin	**10 units/disc**	R	R	R	R	R	R	R	R	R
Penicillin G	**10 units/disc**	R	R	R	R	R	R	R	R	R
Pipericillin	**100 µg/disc**	R	21	08	17	R	R	R	32	R
Streptomycin	**25 µg/disc**	12	16	36	18	50	13	07	50	15
Sulphamethizole	**300 µg/disc**	26	25	56	30	52	29	22	25	40
Tetracycline	**30 µg/disc**	40	22	60	R	46	56	25	44	R
Tobramycin	**10 µg/disc**	R	R	R	26	12	R	R	R	44
Trimethoprim	**5 µg/disc**	R	R	R	R	R	R	R	R	R
Vancomycin	**30 µg/disc**	20	R	32	08	40	10	16	32	24

Key : R - Resistant

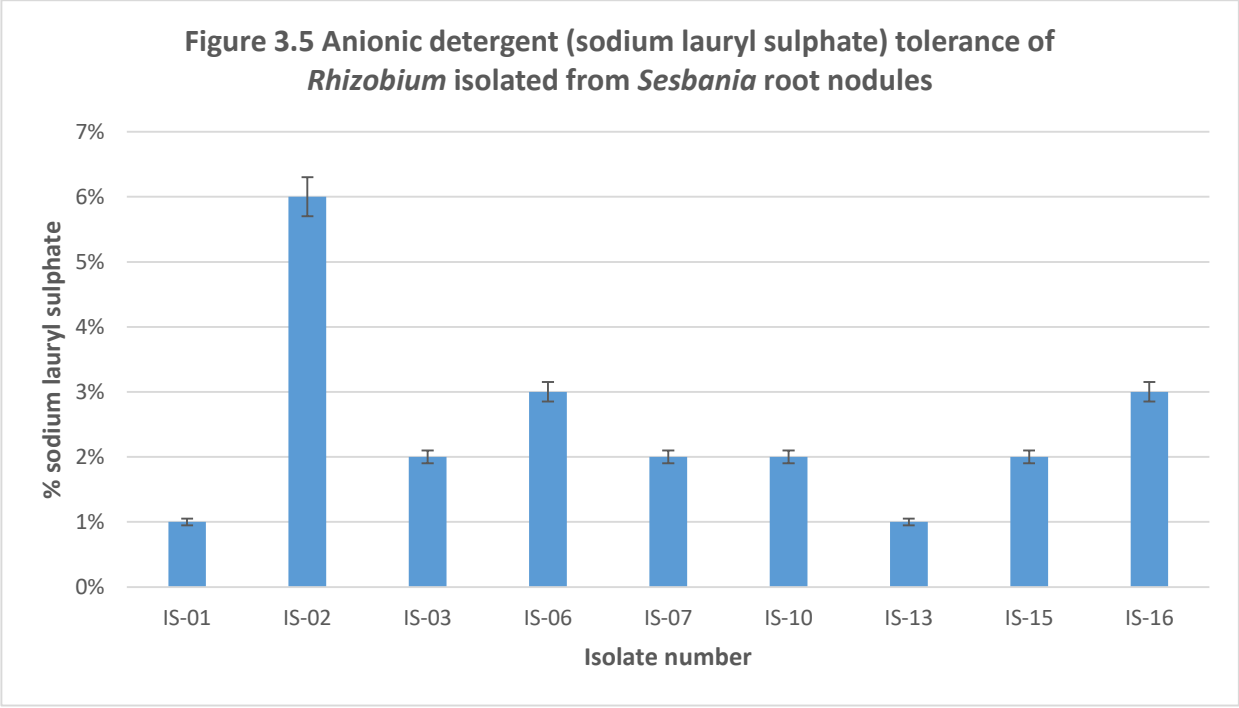
Zone of inhibition in mm

3.4 Surfactant (sodium taurocholate) tolerance of *Rhizobium spp*. isolated from *Sesbania* root nodules.

Highest tolerance to surfactant (sodium taurocholate) was shown by isolate IS-02, IS-03 & IS-06. Highest sensitivity to sodium taurocholate was observed in IS-01 & IS-16. Isolate number IS-07 & IS-15 just able to tolerate surfactant only up to 1% sodium taurocholate. Tolerance up to 2% was exhibited by IS-10 & IS-13. Isolates tolerating upto 10% taurocholate can be employed in sewage contaminated soils where both surfactant & bile salts addition is found to inhibit its microflora.



3.5 Anionic detergent (sodium lauryl sulphate) tolerance of *Rhizobium spp*. isolated from *Sesbania* root nodules.



As shown in figure 3.5 highest tolerance to anionic detergent (sodium lauryl sulphate) upto 6% was exhibited by isolate IS-02. Highest sensitivity to sodium lauryl sulphate was observed at 1% in IS-01 & IS-13. Isolate number IS-06 & IS-16 were able to tolerate anionic detergent only up to 3% sodium taurocholate. Tolerance up to 2% was exhibited by IS-13 & IS-15. Isolates tolerating anionic detergent (sodium lauryl sulphate) upto 6% can be employed in sewage contaminated soils where both surfactant and detergents addition is found to inhibit its microflora.

4. CONCLUSION:

The results of this investigation clearly indicate different levels of intrinsic tolerance of the *Sesbania rhizobia* strains in terms of their growth under stress conditions induced by various heavy metals, fungicide, antibiotics & bile salts concentrations (surfactants). Few rhizobial strains tested were found to display the lowest intrinsic tolerance whereas most strains showed the highest intrinsic tolerance to heavy metals, fungicide, antibiotics & bile salts used in this study. Differences in growth at various concentrations of test compounds were recorded for isolates obtained from *Sesbania bispinosia* root nodules is indicative of differences in their genetic structure originating from their indigenous biodiversity. Stress tolerant isolates are potential candidates which can be explored further in development of biofertilizers showing effectiveness in stressed soils contaminated with the heavy metals, fungicide, antibiotics & bile salts (surfactants).

5. ACKNOWLEDGEMENT:

We the authors of this research paper express heartfelt gratitude to Dr. V. S. Shivankar, Principal, K. B. P. College &Research Promotion & Ethics Committee for providing financial support for this research work. We are also thankful to all the teaching faculty & non-teaching staff members of Department of Microbiology for their constant support and guidance.

6. REFERENCES:

1. Abhi Garg, Manoj Sharma, March 2013, Study of Stress Tolerant Forms of *Rhizobia i*solated from Trigonella Foenumgraecum in Semi Arid Region of Rajasthan. *International journal of scientific research,* Vol.2 no: 3, pp.336-339.
2. Alexander A, Laranjo M, Oliveira S (2006). Natural populations of chickpea rhizobia evaluated by antibiotic resistance profiles and molecular methods. Microbial Ecol. 51: 128-136.
3. Alm E (2003). Implication of microbial heavy metal tolerance in environment. Rev. Undergraduate Res. 2: 1-6.
4. Assche F. Van, Clijsters H. (1990) Effects of metals on enzyme activity in plants, Plant Cell Environ. 13, 195–206.
5. Ausili P, Borisov A, Lindblad P, Martensson A (2002). Cadmium affect the interaction between peas and root nodule bacteria. Acta Agric. Scand., Sect. B, Soil Plant Sci., 52: 8-17.
6. Biswas S., Das R.H. and Sharma G.L. 2008. Isolation and characterization of a novel cross-infective rhizobial from Bispinosaiaaculeata (Dhaincha). Current Microbiology. 56: 48-54.
7. Brockwell J., Bottomley P. J. and Thies J.E. 1995. Manipulation of rhizobia microflora for improving legume productivity and soil fertility: a critical assessment. Plant and Soil. 174: 143-180.
8. Broos K., Beyens H., Smolders E. (2005) Survival of rhizobia in soil is sensitive to elevated zinc in the absence of the host plant, Soil Biol. Biochem. 37, 573–579.
9. Broos K., Uyttebroek M., Mertens J., Smolders E. (2004) A survey of symbiotic nitrogen fixation by white clover grown on metal contaminated soils, Soil Biol. Biochem. 36, 633–640.
10. C´anovas D, Cases L, de Lorenzo V. 2003. Heavy metal tolerance and metal homeostasis in *Pseudomonas putida*as revealed by complete genome analysis. Environ Microbiol 5(12):1242–1256.
11. Castro IV, Ferreira EM, McGrath SP (1997). Effectiveness and genetic diversity of *Rhizobium leguminosarum*bv. *Trifolii* isolates in Portuguese soils polluted by industrial effluents. Soil Biology and Biochemistry 29:1209-1213.
12. CigdemKucuk, CenapCevheri12 March, 2012, Tolerance of *rhizobia* isolated from *Trifolium* species in Southeast region, Sanliurfa, Turkey. *African Journal of Agricultural Research,* Vol. 7 no: 10, pp. 1462-1467.
13. Deepak Thakare, 1999, Studies on *Rhizobia* with particular reference to *Aeschynomene*.
14. Dığrak M, Kırbağ S (1996). The effects of the some pesticides on soil microorganisms. Trend J. Agric. For. 20: 165-173.
15. Dilworth MJ, Parker CA (1969). Devolopment of the nitrogen fixing system in legumes. J. Theor. Biol. 25: 208-218 6.
16. Dourin P, Prevost D, Antoun H (1996). Classification of bacteria nodulating *Lathyrus japonicas* and *Lathyrus pratensis* in northern Quebec as strains of *Rhizobium leguminosarum*biovarviciae. Int. J. Syst. Bacteriol., 46: 1016–1024.

17. Dreyfus, B., Garcia, J.L., and Gillis, M. (1988) Characterization of *Azorhizobiumcaulinodans*gen. nov. sp.., a stem-nodulating nitrogen-fixing bacterium isolated from *Bispinosaia rostrata*. *Int J SystBacteriol*38: 89–98.
18. Evans L, Lewin KF, Vella FA (1980). Effect of nutrient medium pH on symbiotic nitrogen fixation by *Rhizobium leguminosarum*and *Pisumsativum.* Plant Soil, 56: 71-80.
19. Fazal Hadil, Asghari Banol 2010 Effect of diazotrophs (*Rhizobium and azatebactor*)on growth of maize (*zea mays* l.) and accumulationof lead (pb) in different plant parts, *P. J. Bot.*,Vol. 42 no. 6 pp. 4363-4370.
20. Fox JE, Gulledge J, Engelhaupt E, Burow ME, McLachlan JA (2007). Pesticides reduce symbiotic efficiency of nitrogen-fixing rhizobia and host plants. Process Nat. Acad. Sci. USA, 104: 10282-10287.
21. Gonçalves, M., and Moreira, F.M.S. (2004) Specificity of the legume *Sesbania virgate* (Caz.) pers. & its nodule isolates *Azorhizobium johannae* with other legume hosts and rhizobia. *Symbiosis* 36: 57–68.
22. Hashem F.M., Swelim D.M., Kuykendall L.D., Mohamed A.I., Abdel-Wahab S.M. and Hegazi N.I. 1998. Identification and characterization of salt- and thermo-tolerant Leucaena-nodulating Rhizobium strains. Biology and Fertility of Soils. 27: 335-341.
23. Heaton A.C.P., Rugh C.L., Wang N.J. and Meagher R.B., Phytoremediation of mercury- and methylmercury- polluted soils using genetically engineered plants, *J Soil Contamination*, 7, 497-509 (1998)
24. Hirsch, A. M., Lum, M. R. and Downie, J. A. (2001). What makes the rhizobia-legume Symbiosis so special? Plant physiol.127:1484-1492. 15. Jordan, D.C. (1984) Family III Rhizobiaceae, p 234-256.
25. Hungria M, de O'Cheuerie LM, Coca RG, Megias M (2001). Preliminary characterization of fast growing rhizobial isolated from soyabean nodules in Brazil. Soil Biol. Biochem. 33: 1349-1361.
26. James, E.K., Iannetta, P.P.M., Nixon, P.J., Whiston, A.J., Peat, L., Crawford, R.M.M., *et al.* (1996) Photosystem II and oxygen regulation in *Bispinosaia rostrate* stem nodules. *Plant Cell Environ* 19: 895–910.
27. James, E.K., Loureiro, M.F., Pott, A., Pott, V.J., Martins, C.M., Franco, A.A., and Sprent, J.I. (2001) Flooding tolerant legume symbioses from the Brazilian Pantanal. *New Phytol*150: 723–738.
28. Kale S. P, Murthy NBK, Raghu, K (1989). Effect of carbofuran, carbaryl and their metabolites on the growth of *Rhizobium* sp. and *Azotobacter chrooccum*. Bull. Environ. Contamin. Toxicol. 42: 769- 772.
29. Lennox LB, Alexander M (1981). Fungicide enhacement of nitrogen fixation and colonization of *Phaseolus vulgaris* by *Rhizobium phaseoli*. Appl. Environ. Microbiol. 41: 404-411.
30. MA, Tesfai K (1983). Compatibility of *Rhizobium japonicum* with commercial pesticides in vitro. Bull Environ. Contam. Toxicol. 31(4): 432-7.
31. Mahobia V. and Mahna S.K. 2002. Characterization of rhizobia isolated from Prosopis cineraria in Jodhpur region, Rajasthan, India. NFT News. 5: 3-5.
32. Martyniuk S, Oron J, Martyniuk M (1999a). Interaction between chemical seed dressings and *Bradyrhizobium*inoculant on lupine seeds. Bot. Lithuan. 3: 95-98.
33. Martyniuk S, Wozniakowska A, Martyniuk M, Oron J (1999b).Interaction between chemical dressings and *Rhizobium* inoculant on pea seeds. Prog. Plant Prot. 39(1): 120-125.
34. Mishra R. R.1, Dangar T. K.2, Rath B1 and Thatoi H. (2009). Characterization and evaluation of stress and heavy metal tolerance of some predominant Gram negative halotolerant bacteria from mangrove soils of Bhitarkanika, Orissa, India. African Journal of Biotechnology Vol. 8 (10), pp. 2224-2231.
35. Quatrini, P., Scaglion, G., Cardinale, M., Caradonna, F. and Puglia, A.M (2002). Bradyrhizobium sp. nodulatig the mediterranean shrub, Spanis broom (Spartium junceum L.).J .Appl.Microbiol.92:13-21.
36. Rennie RJ, Dubetz S (1984). Effect of fungicides and herbicides on nodulation and N2 fixation in soybean fields lacking indigenous *Rhizobium japonicum*. Argon. J. 76(3): 451-454.

37. Requena N., Perez-Solis E., Azcon-Aguilar C., Jeffries P. and Barea J.M., 2001. Management of indigenous plant–microbe symbioses aids restoration of desertified ecosystems. Applied and Environmental Microbiology. 67: 495-498.
38. S.P. Paudyal, Rishi R. Aryal, S.V.S. Chauhan and D.K. Maheshwari June 2007 Effect Of Heavy Metals On Growth Of *Rhizobium* Strains And Symbiotic Efficiency Of Two Species OfTropical Legumes, *Scientific World*, Vol. 5 no. 5, pp.27-32.
39. Shamseldin, A. & Werner, D. 2005. High salt and high pH tolerance of new isolated Rhizobium etli strains from Egyptian soils. Current Microbiology. 50: 11-16.
40. Sprent, J.I. (2001) *Nodulation in Legumes*. London, UK: Royal Botanic Gardens, Kew.
41. Sridhar K.R., Arun A.B., Narula N., Deubel A. and Merbech W. 2005. Patterns of sole-carbon-utilazation by fast growing coastal and dune rhizobia of the Southwest coast of India. Engineering in Life Sciences. 5: 425-430.
42. Thies J.E., Woomer P.L. and Singleton P.W. 1995. Enrichment of Bradyrhizobium spp. population in soil due to copping of the homologous host legume. Soil Biology and Biochemistry. 27: 633-636.
43. Tu CM (1981). Effect of fungicides on growth of *Rhizobium japonicum*and symbiotically grown soybean in soil under laboratory conditions. Prot. Ecol. 3: 41-46.
44. Ulrich A. and Zaspel I. 2000. Phylogenic diversity of rhizobial strains nodulating Robinia psudoacacia L. Microbiology. 146: 2997-3005.
45. Ulrich A. and Zaspel I. 2000. Phylogenic diversity of rhizobial strains nodulatingRobiniapsudoacacia L. Microbiology. 146: 2997-3005.
46. Vincent J.M., A manual for the practical study of root nodule bacteria. Blackwell Scientific Publications, Oxford (1970).
47. Wei G.H, Yang X.Y., Zhang Z.X., Yang Y.Z. and Lindstrom K. 2008. Strain Mesorhizobium sp. CCNWGX035; A stress tolerant isolate from Glycyrrhizaglabra displaying a wide host range of nodulation. Pedosphere. 18(1): 102-112.
48. Zahran H.H. 1999. Rhizobium-Legume Symbiosis and Nitrogen Fixation under Severe Conditions and in an Arid Climate. Microbiology and Molecular Biology Reviews. 63: 968-989.
49. Zou N, Dart PJ, Marcar NE (1995). Interaction of salinity and rhizobial strain on growth and nitrogen fixation by *Acacia ampliceps*. Soil Biol. Biochem. 24: 409-419.
50. Zou N., Dart P.J. and Marcar N.E. 1995. Interaction of salinity and rhizobial strain on growth and N_2-fixation by Acacia ampliceps. Soil Biology and Biochemistry. 27: 409-413.