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Effects of zinc on cell viability and cell surface components of *Rhizobium* sp isolated from root nodules of *Trifolium alexandrinum*

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In this study, thirty three isolates of *Rhizobium* isolated from root nodules of *Trifolium alexandrinum* were selected for their cell viability in terms of log 10 cfu/ml under zinc stress (0-100 mg/l zinc) at different time intervals i.e. 24, 48 and 72 hours The results showed that rhizobial cell viability was maintained throughout the 72 hours incubation period at all concentration of zinc, Maximum growth in terms of log 10 cfu was recorded at 48 hours incubation period. Population count of nineteen isolates kept on increasing up to 50 mg/l zinc concentration and thereafter it declined with the increase in zinc concentration, on the basis of these results nineteen isolates were grouped as zinc tolerant strains and rest fourteen isolates were considered as zinc sensitive strains because population count of these isolates was increasing up to 25 mg/l zinc and thereafter it declines with the increase in zinc concentration The cell surface components of *Rhizobium* play an important role for maintaining nodulation in leguminous plants, The zinc tolerant and sensitive strains were also screened for cell surface component.The experimental results of our study reveal that under zinc stress, the tolerant organisms are better to adapted the stressful environmental conditions Therefore supplementation of soil with optimum amount of zinc in soils along with biological formulation will be beneficial so as to enhance the process of symbiotic nitrogen fixation.

Key words: cell surface components, cell viability, *Rhizobium, Trifolium alexandrinum*

Introduction

All over the world a very few agriculturalists have the luxury of crop production under ideal conditions. For the great majority and particularly in the third world, stresses of one form or another are an integral part of the crop cycle and may pose a severe yield constraint (Graham, 1992). In agricultural lands ,biological nitrogen fixation is the sole alternative for chemicals, many

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environmental conditions are limiting factors for growth and activity of both the nitrogen fixing plants and root nodulating bacteria.. Typical environmental stresses flood by the legume nodules and their symbionts may include photosynthate deprivation, water stress, salt stress, acidity, alkalinity, nutrient deficiency, soil nitrate, temperature, heavy metals and biocides etc (Zahran, 1999). Most of the rhizobia are very sensitive to soil environmental factors, which affect their dinitrogen fixation capacity and hence the productivity of legumes (Kulkarni and Nautiyal, 2000). Most of the cultivated legumes are exposed to agrochemicals such as plant protecting agrochemicals and fertilizers which not only contain essential nutrients but also comprise of contaminants such as heavy metals. Due to their toxic nature many of the chemicals cause a threat to symbiotic nitrogen fixation (Martensson, 1992). Elevated amounts of heavy metals could be a result of the use of contaminated inorganic or organic fertilizers or contamination by aerial deposition and are deleterious to both the soil micro organisms and plant beneficial microbial processes (Giller *et al.*1998).

Heavy metals in the environment arise from natural sources or directly and indirectly from human activities such as rapid industrialization, urbanization and anthropogenic sources, threatening the environment and human health. In United State alone, more than 50,000 metal contamined sites await remediation many of them superfunds sites. The study of regional variations and the anthrogenic contamination by metals of soil is very important for environmental planning and monitoring in urban areas. Governments worldwide are establishing research and demonstration programs to use this potential. Geographical Information System (GIS) can be used to identify soil contamination hot spot areas and to assess potential pollutant sources in an urban community (Li *et al*. 2004).

Some metals, such as cobalt, copper, nickel and zinc serve as micronutrients and are used for redox processes, to stabilize molecules through electrostatic interactions, as components of various enzymes and for regulation of osmotic pressure. Most of the metals are nonessential, have no nutritive value and are potentially toxic to micro organisms. Zinc, a divalent heavy metal with saturated d – orbital favour tetrahedral coordination for stable metalloenzyme complexes and there by regulate the various process of cell metabolism. It acts as micronutrient on one hand and environmental toxic factor on the other and is known to affect nodulation and dinitrogen fixation. (Munns, 1977; Smith, 1982). Successful development of nodules by rhizobial species on legume roots involve in surface interactions between the two interacting species at many different stages of development (Long, 1989; Brewin, 1991). Rhizobial surface component play an important role in deciding the host compatibility

and in bringing about the infection leading to nodulation and nitrogen fixation (Swamynathan and Singh, 1995). These surface components include acidic expolysaccharide, neutral beta glucans, lipopolysaccharides and to an extent the elements responsible for motility of rhizobial cells (Gray and Rolfe, 1990, Reuber *et al.* 1991) As the cell surface components of *Rhizobium* are signs for maintaining nodulation in leguminous plants, the isolates were screened for cell surface production under zinc stress. .

Material and methods

Bacteria and Media used

Thirty three(RGM 1, RGM 2, RGM 4, RGM 7, RGM 8, RGM 9, RGM 10, RGM 11, RGM 13, RGM 14, RGM 16, RGM 20, RGM 22, RGM 25, RGM 27, RGM 33, RGM 34, RGM 43, RGM 44, RGM 46, RGM 48, RGM 49, RGM 50, RGM 51, RGM 59, RGM 60, RGM 62, RGM 64, RGM 68, RGM 69, RGM 71, RGM 72, RGM 82) bacterial strains used in this study were selected from a collection of eighty five isolates of Barseem (*Trifolium alexandrinum*) rhizobia.. These strains were selected on the basis of their nodulation ,biochemical, physiological and antibiotic resistance patterns.

These strains formed pinkish and effective nodules on *Trifolium alexandrinum* under controlled conditions.For routine growth of bacterial cultures, YEM agar medium was used (Vincent, 1970).

Zinc Tolerance Test for Bacterial Strains

Cell suspensions were prepared by inoculating 50ml of YEM broth with approximately 10^6 rhizobial cells, and then incubating in a shaker at 80 oscillations per minute for a time period of 48 hours. Cultures were then transferred to 150ml centrifuge bottles and centrifuged at 3000xg for 10 min. The supernatants were than discarded and the cells resuspended in 30ml of sterile deionized water. This washing procedure was repeated twice before the washed cells were finally resuspended in 30ml of sterile deionized water.

Stock Solution of the zinc was made using zinc chloride. The test solutions were then made by diluting the stock solutions as required for concentrations of 0 – 100 mg/l Zinc chloride. The pH of each solution was adjusted to between 6.5 and 7.0 by the addition of every dilute HCl (aq) or NaOH (aq). The test solutions were filter sterilized, using a disposable filter with 0.2 micrometer pore size.

10 ml of each of the test solutions was placed in a 50ml flask and then inoculated with 30 micro liters of washed cells. The flasks were than placed in

a shaker at 80 oscillations per minute at 30 °C for 72 hours. Cell viability was assessed after 24, 48 and 72 hours using a technique for counting viable cells. Control solutions which were not inoculated with rhizobia, were also incorporated into the experiment in order to detect any possible contamination.

Six Single colonies from agar plates with different concentration of Zn which individual strain could tolerate and six single colonies from the control plates with no Zn, were subcultured in defined liquid growth medium at 28°C until turbidity developed then a loop full of culture was transferred onto YEMA slopes and stored at 4°C. Surface Properties (Nakanishi *et al*.1976).

Test for Production of β-Glucan

Rhizobium strains were streaked on YEM agar having aniline blue at a rate of 1mg/ml. They were given different Zinc stress (0-100mg/l) as $ZnCl_2$. Plates were incubated at 28°C for 2 days. Those shows blue coloured colony were considered to be β Glucan positive and white colored colony were negative.

Test for Production of Lipopolysaccharide

Rhizobium strains were streaked on TY medium having Sodium deoxycholate (SDC) at a rate of 1mg/ml and incubated at 28°C for 2 days. LPS producing strains of *Rhizobium* shows growth.

Test for Production of Succinylated Exopolysaccharides (EPSI)

Bacterial strains to be tested were streaked on YEMA plates having Calcofluor at a rate of 0.02%. They are given different Zinc stress (0-100mg/l) as $ZnCl_2$ and incubated at 28°C for 48 hours. Bacteria producing succinoglycan exhibit a blue - green fluorescence under ultra violet light if grown on media containing Calcofluor.

Test for Motility (Swamynathan and Singh, 1995)

Rhizobium strains were spotted on swarm plates having tryptone yeast Extract medium with 0.3% agar. They are given different Zinc stress (0-100mg/ml) as ZnCl2 and incubated at 28°C for 48 hours. The motility of bacterial strains was determined by spreading the colonies in the swarm plates. The diameter of the motility/Swarming behavior was measured in centimeter units

Results

Rhizobial Cell Viability at Different Concentration of Zinc

Rhizobium strains were exposed to various concentrations of zinc (0-100 mg/l) in terms of cfu (log 10 cfu/ml) at different time intervals i.e. 24, 48 and 72 hours. Rhizobial cell viability was maintained throughout the 72 hours growth period in both zinc treated and no treated medium. Maximum cfu was obtained at 48 hours incubation period. Population count was increased in 19 strains (RGM 1, RGM 2, RGM 7, RGM 8, RGM 10, RGM 11, RGM 13, RGM 14, RGM 25, RGM 27, RGM 33, RGM 43, RGM 44, RGM 51, RGM 59, RGM 60, RGM 62, RGM 71 and RGM 82) up to 50mg/l of zinc concentration and there after it decreases and reached to minimum at 100 mg/l Zinc. Fourteen strains (RGM 4, RGM 9, RGM 16, RGM 20, RGM 22, RGM 34, RGM 46, RGM 48, RGM 49, RGM 50, RGM 64, RGM 68, RGM 69 and RGM 72) showed increase in log 10 cfu up to 25 mg/l of zinc and after that growth was decreased. Based on the above results, isolates showed maximum growth upto 50 mg/l of zinc concentration, were grouped as zinc tolerant strains and others were zinc sensitive.

Population count of zinc tolerant isolates at 24 hours incubation period under different concentrations of zinc is shown in Fig. 1. Maximum cfu under treatments with 0, 25, 50, 75 and 100 mg/l of zinc were 9.41, 9.45, 9.46, 9.37 and 9.32 log 10cfu/ml respectively for RGM 59 which is followed by RGM 7 i.e. 9.38, 9.43, 9.45, 9.35 and 9.30 log 10 cfu at above concentration, after that cfu for RGM 71, RGM 25, RGM 43 and RGM 10 were recorded. Zinc sensitive isolate RGM 49 shows minimum cfu under different treatments, were 8.25, 8.27, 7.95, 7.92 and 7.47 log 10 cfu/ml for 0, 25, 50, 75 and 100 mg/l of zinc respectively at 24 hour incubation period (Fig. 4). Population count of zinc tolerant isolates at 48 hours incubation period under different concentrations of zinc is shown in Fig 2.0. Maximum cfu under treatments with 0, 25, 50, 75 and 100 mg/l of zinc were 9.42, 9.47, 9.51, 9.4 and 9.36 log 10 cfu/ml respectively for RGM 59. Zinc sensitive isolate RGM 49 shows minimum cfu under different treatments, were 8.58, 8.7, 8.32, 8.3 and 7.1 log 10 cfu/ml for 0, 25, 50, 75 and 100 mg/l of zinc respectively at 48 hour incubation period (Fig. 5).
Population count of zinc tolerant isolates at 72 hours incubation period under different concentrations of zinc is shown in Fig. 3. Maximum cfu under treatments with 0, 25, 50, 75 and 100 mg/l of zinc were 9.38, 9.4, 9.43, 9.35 and 9.3 log 10 cfu/ml respectively for RGM 59. Zinc sensitive isolate RGM 49 shows minimum cfu under different treatments, were 8.34, 8.48, 8.25, 8.08 and 7.0 log 10 cfu/ml for 0, 25, 50, 75 and 100 mg/l of zinc respectively at 72 hour incubation period (Fig. 6).

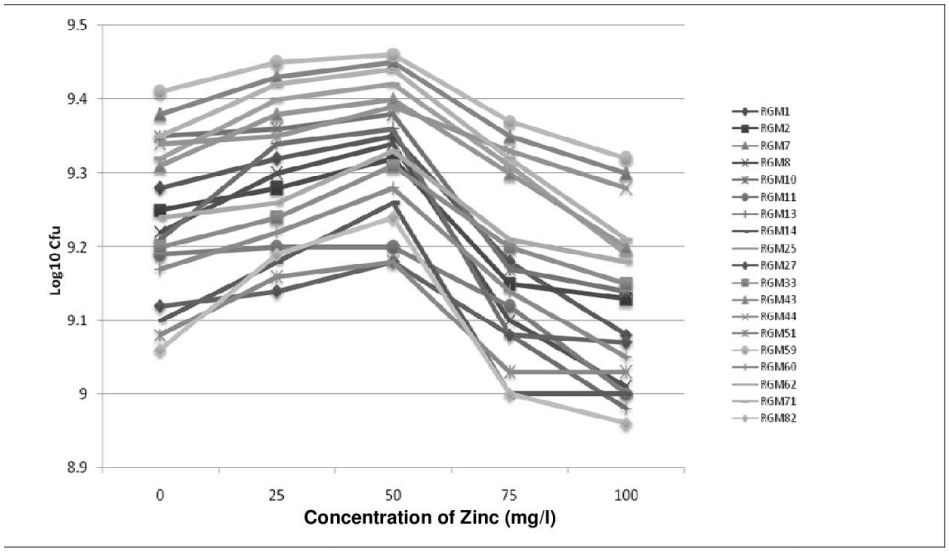

Fig. 1: Rhizobial cell viability of Zinc tolerant strains under Zinc stress in terms of log10 Cfu/ml at 24 hours incubation period

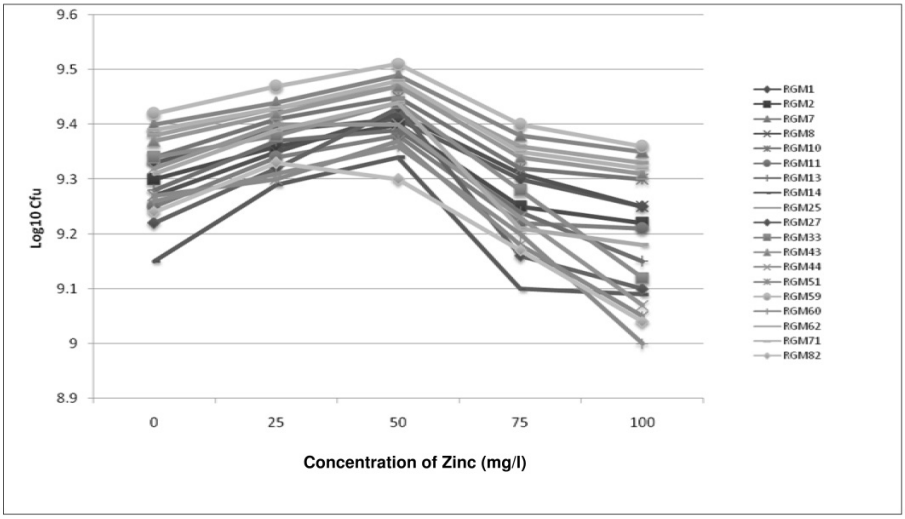

Fig. 2: Rhizobial cell viability of Zinc tolerant strains under Zinc stress in terms of log10 Cfu/ml at 48 hours incubation period

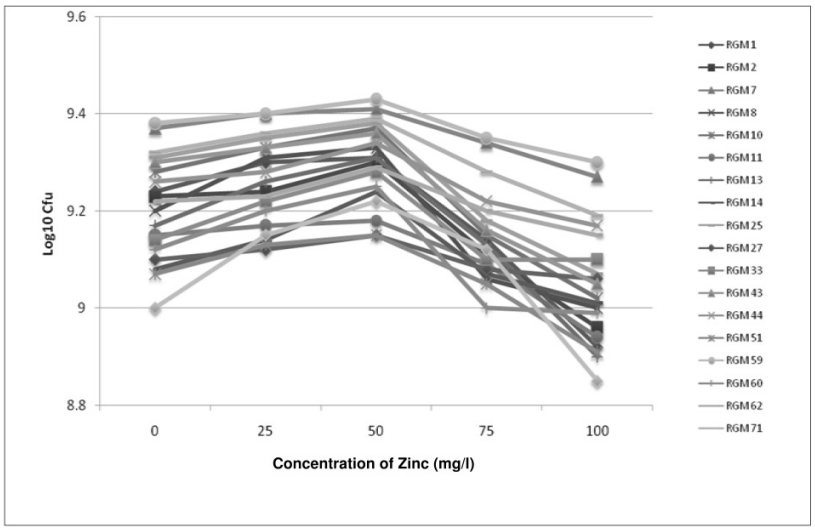

Fig. 3: Rhizobial cell viability of Zinc tolerant strains under Zinc stress in terms of log10 Cfu/ml at 72 hours incubation period

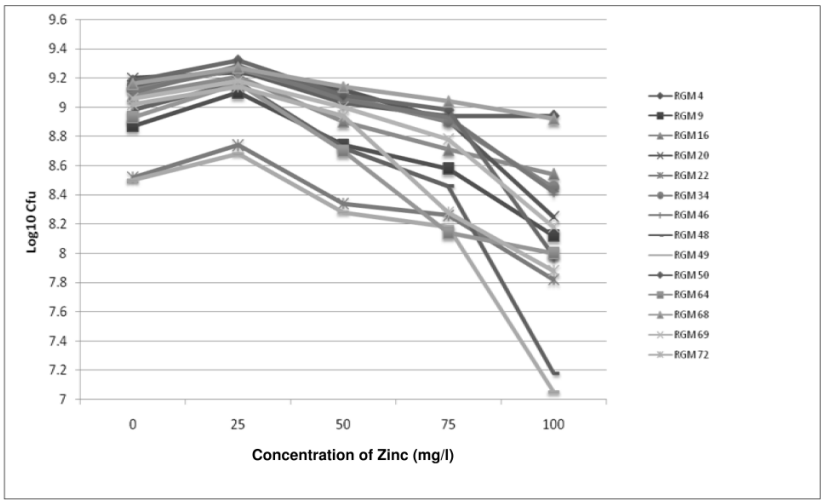

Fig. 4: Rhizobial cell viability of Zinc sensitive strains under Zinc stress in terms of log10 Cfu/ml at 24 hours incubation period

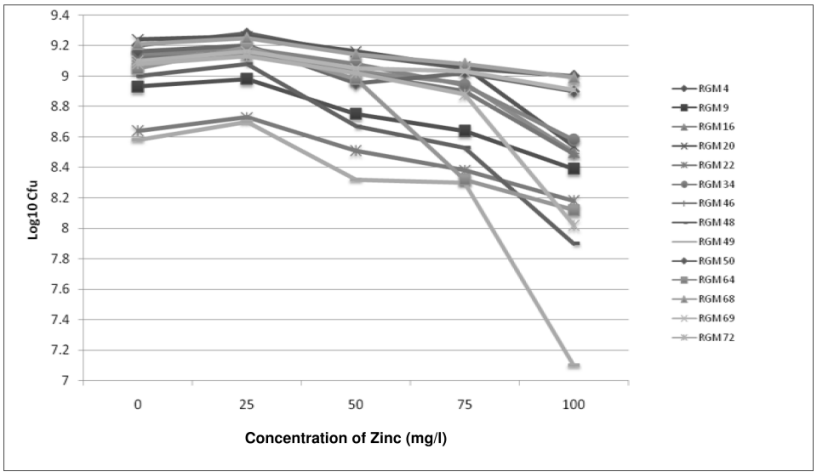

Fig. 5: Rhizobial cell viability of Zinc sensitive strains under Zinc stress in terms of log10 Cfu/ml at 48 hours incubation period

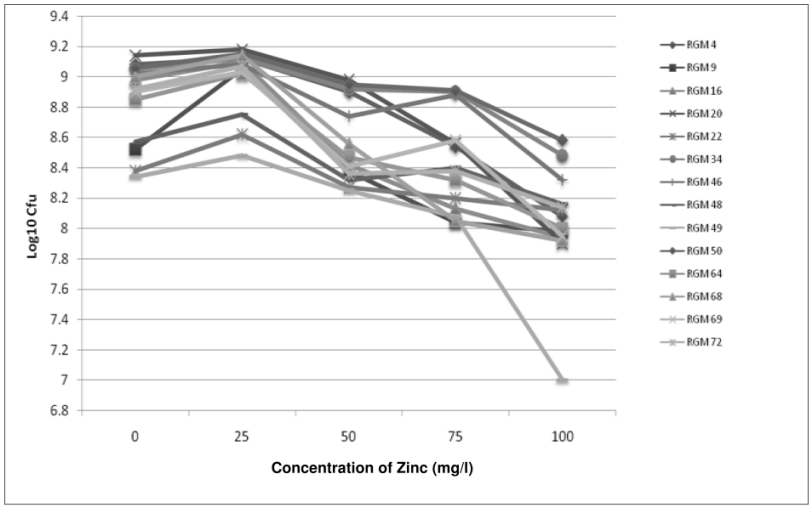

Fig. 6: Rhizobial cell viability of Zinc sensitive strains under Zinc stress in terms of log10 Cfu/ml at 72 hours incubation period

Cell Surface Properties of Rhizobium Under Zinc Stress

Test for the Production of ß-Glucans

The different *Rhizobium* isolates were tested for the presence of Cyclic ß-Glucans on the basis of their staining with aniline blue at different zinc concentrations. ß-Glucan production was decreased with an increase in the concentrations of zinc in the medium in both zinc tolerant and sensitive strains.

The percentage of ß-Glucan production were 100%, 100%, 92%, 74% and 52.7% at 0, 25, 50, 75 and 100mg/l of zinc respectively (Fig. 7). Production of ß-Glucan at 25 mg/l was same as control. Nine isolates i.e. RGM 1, RGM 7, RGM 10, RGM 25, RGM 43, RGM 51, RGM 59, RGM 71 and RGM 82 shows blue colour colony at 100 mg/l of zinc. ß-Glucan activity of zinc sensitive isolates are shown in Fig. 8. At different concentrations of Zinc, percentage of ß-Glucan production of zinc sensitive strains are 92%, 74%, 52.7%, 28.5% and 28.5% respectively. ß-Glucan production at 75 mg/l and 100 mg/l zinc was same and only four out of fourteen isolates shows blue colour colony at these concentrations.

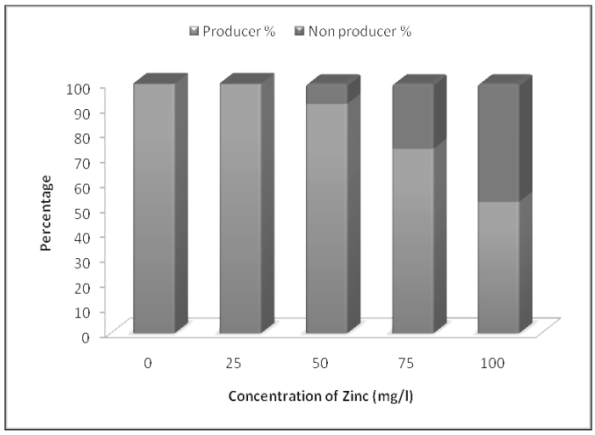

Fig.: 7 Production of ß-Glucan of zinc tolerant isolates

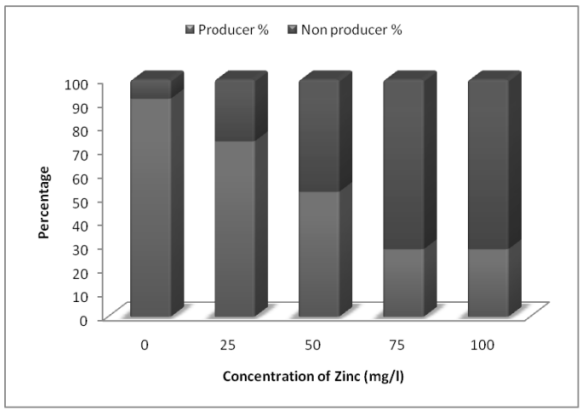

Fig. 8 Production of ß-Glucan of zinc sensitive isolates

Test for the Production of Lipopolysaccharide

Rhizobium isolates were streaked on TY plates containing 1mg/ml sodiumdeoxycholate (SDC) to test the presence of lipopolysaccharide at different concentrations of zinc. Zinc tolerant isolates have a little effect of lipopolysaccharide production under various zinc concentrations. The percentage of lipopolysaccharide production in zinc tolerant isolates were 78.9%, 68.4%, 84.2%, 89.4% and 63.1% at 0, 25, 50, 75 and 100 mg/l zinc (Fig. 9). Highest lipopolysaccharide production was seen at 75 mg/l zinc i.e. only two isolates (RGM 1 and RGM 14) do not shows growth at 75 mg/l zinc. Lipopolysaccharide production in zinc sensitive isolates increases upto 50 mg/l zinc, beyond this concentration i.e. at 75 and 100 mg/l, a decline was recorded in lipopolysaccharide production. Lipopolysaccharide production percentage in case of zinc sensitive isolates were 78.5%, 78.5%, 85.7%, 57.1% and 35.7% at 0, 25, 50, 75 and 100 mg/l zinc (Fig. 10). Lowest lipopolysaccharide production was recorded at 100 mg/l of zinc.

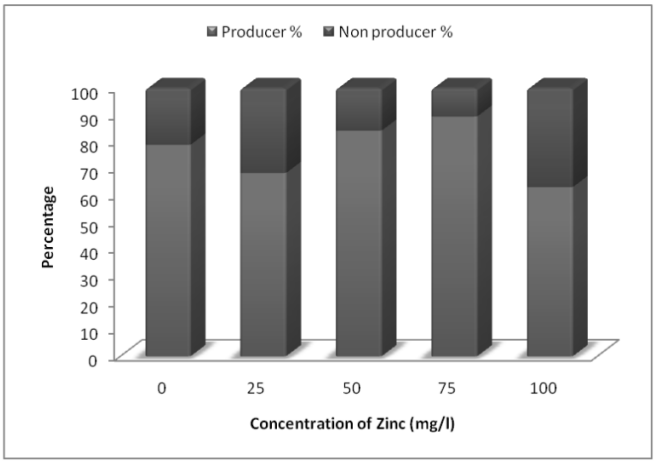

Fig. 9 Production of Lipopolysaccharide of zinc tolerant isolates

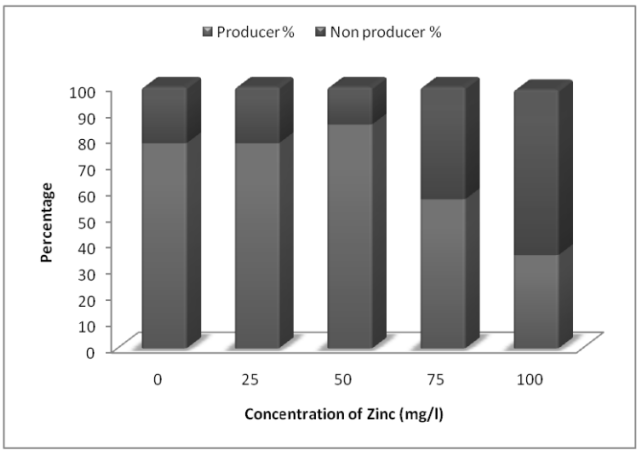

Fig. 10 Production of Lipopolysaccharide of zinc sensitive isolates

Test for the production of Exopolysaccharide I (EPSI)

Rhizobium strains were tested for the production of EPSI on the basis of calcofluor staining at various concentrations of zinc. The calcofluor phenotype of different isolates varied from No or Dim or Bright fluorescence to very Bright fluorescence with mucoid type. All zinc tolerant isolates showed EPSI production at all concentrations of zinc, but very bright fluorescence with mucoid type observed at 50 mg/l zinc (Table 1). Therefore 50 mg/l zinc was optimum concentration for EPSI production by zinc tolerant strains. There was a decrease in EPSI production with an increase in zinc concentration of zinc sensitive isolates. At 50 and 75 mg/l, most of the zinc sensitive isolates shows dim fluorescence. Five out of fourteen isolates showed fluorescence at 100 mg/l zinc (Table 2).

Table 1. Test for the production of Succinylated exopolysaccharide by Zinc tolerant strains under Zinc stress

Strains	0 mg/l	25 mg/l	50 mg/l	75 mg/l	100 mg/l
RGM 1	++	++	+++	++	++
RGM 2	++	++	+++	++	+
RGM 7	++	++	+++	++	+
RGM 8	++	++	+++	++	+
RGM10	++	++	+++	++	++
RGM11	++	++	+++	++	++
RGM13	++	+	+++	++	+
RGM14	++	++	+++	++	+

RGM25	++	+	+++	++	+
RGM27	++	++	+++	++	+
RGM23	++	++	+++	++	+
RGM43	++	+	+++	++	+
RGM44	++	+	+++	++	++
RGM51	++	++	+++	++	++
RGM59	++	++	+++	++	++
RGM60	+	+	+++	++	+
RGM62	++	++	+++	++	+
RGM71	+	+	+++	++	++
RGM82	++	++	+++	++	+

- No fluorescence ++ Bright fluorescence
+ Dim fluorescence +++ Very bright fluorescence with slightly mucoid phenotype

Table 2. Test for the production of Succinylated exopolysaccharide by Zinc Sensitive strains under Zinc stress

Strains	**0 mg/l**	**25 mg/l**	**50 mg/l**	**75 mg/l**	**100 mg/l**
RGM 4	++	++	+	+	+
RGM 9	++	++	+	+	-
RGM16	++	++	+	+	-
RGM20	++	++	+	+	+
RGM22	++	++	+	+	-
RGM34	++	++	+	+	-
RGM46	++	++	+	+	+
RGM48	++	++	+	+	-
RGM49	++	++	+	+	-
RGM50	++	++	+	+	+
RGM64	++	++	+	+	-
RGM68	++	++	+	+	-
RGM69	++	++	+	+	-
RGM72	++	++	+	+	+

- No fluorescence ++ Bright fluorescence
+ Dim fluorescence +++ Very bright fluorescence with slightly mucoid p

Swarming Activity of Rhizobium Strains Under Zinc Stress

Swarming activity of zinc tolerant and zinc sensitive strains under different zinc stress conditions are shown in the Fig. 11 and Fig. 12. Swarming activity at 25 mg/l, was higher in zinc tolerant isolates than that of control, with the increase in concentration of zinc in the medium, there takes place a drastic decrease in motality. Zinc tolerant strains maintained the swarming behavior up to 100mg/l of zinc with a reduction of the diameter from 3.6 cm to 1.5 cm. Most of the zinc sensitive strains were even failed to grow above 75 mg/l zinc. Percentage of motile zinc sensitive strains are 85.7%, 70.14% and 57.14% at

50, 75, 100 mg/l zinc concentration respectively. Maximum diameter of zinc sensitive strain was 2.3 cm at 25 mg/l zinc. Swarming behaviour of RGM 59 was maximum in zinc tolerant strains. Diameter of RGM 59 was increased from 3.4 cm in control to 3.6 cm in 25 mg/l of $Zncl^2$ and beyond 25 mg/l it declines, reached 3.0 cm at 100 mg/l of the same. Similar trend was observed in zinc sensitive isolates. RGM 59 was highly motile followed by RGM 7, RGM 71, RGM 25, RGM 43 and RGM 10.

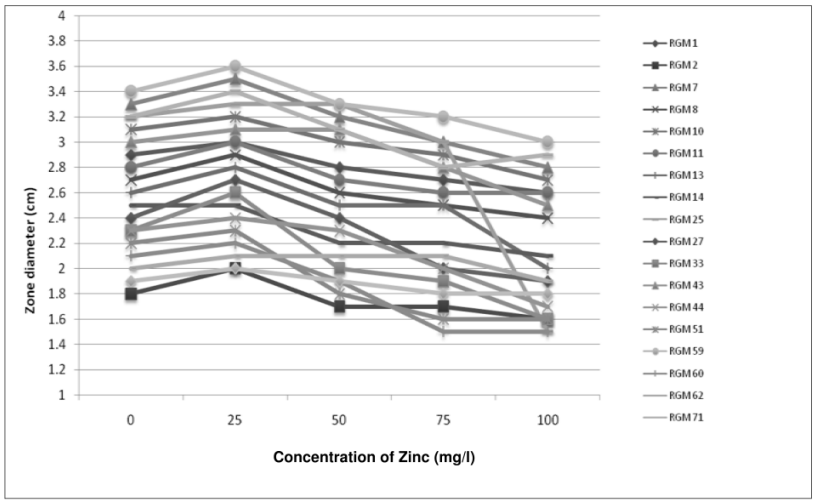

Fig. 11 Swarming activity of zinc tolerant *Rhizobium* isolates under zinc stress

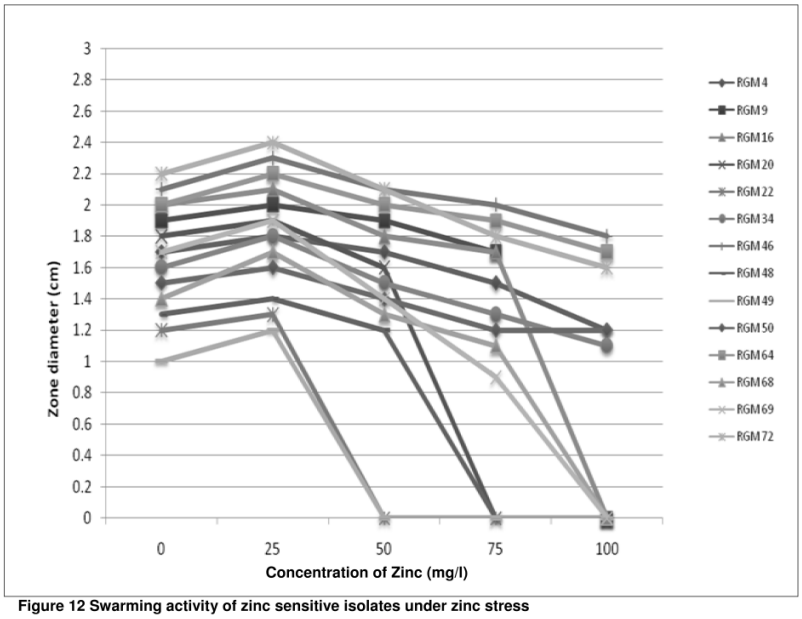

Figure 12 Swarming activity of zinc sensitive isolates under zinc stress

Discussions

Based on the antibiotic resistance, thirty three *Rhizobium* strains were selected for the study of their cell viability under zinc stress because according to Spain and Alm (2003) bacterial heavy metal tolerance evolved is due to the use of antibiotics. Cfu count of all *Rhizobium* isolates were recorded under normal and stress conditions at 24, 48 and 72 hours stages from the time of inoculation. The results of our study revealed that cell viability was maintained throughout the growth period. It appears that *Rhizobium* isolates tested to demonstrate an elevated tolerance may be important for the survival in the metal contaminated conditions.

Microorganisms tolerant to metal contaminants are known to have two mechanisms by which they protect themselves from the toxic effects of heavy metals. Firstly, Exclusion, as mediated by the extra polysaccharide capsule around the cell, has been shown to prevent cellular uptake of metals (Bitton and Freihofer, 1974; Mitra *et al*. 1975). The polysaccharide capsule around most species of rhizobia is quite thick and therefore should provide adequate protection against most metals. Secondly, several genera of soil microbes are known to produce phytochelatins, which may internally bind metals and thus effectively lower activity with in the cell. Combining this chelating ability with location in relatively uncontaminated microsites where metal availability is naturally reduced (i.e. areas high in humic compounds which are known for

their metal binding capabilities) rhizobia may well be able to thrive in what would normally be considered an inhospitable environment. Metal tolerance may be one mechanism which aids rhizobial survival in metal contaminated soil. It is important in this study that the maximum cfu count of ninteen strains was observed at 50mg/l Zn and fourteen isolates showed maximum Cfu at 25mg/l Zn, beyond these concentrations, there is a decline in Cfu values. Accroding to our results nineteen strains are grouped as zinc tolerant and rest fourteen were grouped as zinc sensitive isolates. Cfu count of zinc sensitive isolates was decreased up to 2 log cycles at 100 mg/l zinc. Lakzain *et al.*(2002) observed that zinc tolerant strains S_9 grew at all metal concentrations where as zinc sensitive strain F_6 did not grow at higher concentrations of zinc.

In our study, a maximum log 10 Cfu of zinc tolerant strains was observed at 50 mg/l zinc. In this contest, Geeta *et al.* (2008) conducted experiments to study the effect of different concentrations of micronutrients, on the growth of *Bradyrhizobium japonicum* strains in liquid culture conditions. The supplementation of zinc at 75 ppm has recorded maximum log viable cell counts. The present study trend was that, with an increase in zinc concentrations up to certain limit Cfu count also increases and after that at higher concentrations it goes on decreasing. Similar trend was reported by Angle and Chaney (1991) in a study of metal contaminated soil, they found that the lowest *Rhizobium meliloti* population density was in the soil with the highest level of zinc and the highest populations of *R. meliloti* were in soils which were moderately contaminated. Rhizobial cell surface is a complex conglomerate of various polysaccharides that are known to play a major role in the development of symbiotic nodules. These surface components include cyclic β-glucans, lipopolysaccharides, exopolysaccharides and the elements responsible for motility of cells. We tested, if there are any changes on the rhizobial cell surface when associated with zinc stress. We used various dyes that interact specifically with these molecules. Production of cell surface molecules was determined by growing the strains on normal and stress medium containing aniline blue for β (1-3) glucans, sodium deoxycholate for lipopolysaccharides, calcofluor for exopolysaccharide I. Motility of the strains was determined in normal and stress media by using swarm plates.

Aniline blue is known to specifically bind the curdlan type of polysaccharides, of which β-glucan is a major component (Nakanishi *et al*. 1976). In the present study results showed that β-glucan production in both zinc tolerant and zinc sensitive strains was going on decreasing with the increase in zinc concentrations. Production of β-glucan at higher concentrations of zinc was absent in several strains. Absence of β-glucans has been linked to a

defective flagellum, that results in the absence of chemotactic response which in turns leads to the formation of ineffective nodules (Geremia *et al*. 1987).

Lipopolysaccharide is a major component of the bacterial outer membrane and for Rhizobial species, having a critical role in the establishment of an effective nitrogen fixing symbiosis with a legume host (Carlson *et al*. 1995). Our results showed that LPS production of zinc tolerant strains was going on increasing up to 75 mg/l zinc and thereafter it declines and in zinc sensitive strains LPS production was higher at 50 mg/l zinc. This indicates that LPS production in both zinc tolerant and zinc sensitive isolates was enhanced up to certain limit of zinc. Kannenberg and Brewin (1989) reported that alteration in LPS occurred in response to environmental changes. Further, LPS confirms, rhizobia resistance to sodium deoxycholate. According to Noel *et al.,* (1989) LPS is needed for normal nodule development. Exopolysaccharide synthesis appears to be a common feature associated with numerous microorganisms. Results from a previous studies indicated that rhizobial EPS production is necessary for successful nodulation of alfa-alfa, clovers and peas as well as other leguminous plants (Diebold and Noel, 1989). Some of the Rhizobia with mutation in exo, muc or gum genes on chromosomes or plasmids does not appear to able to nodulate host plants (Reed *et al*.1991); Anna *et al*., (2006) suggests that exopolysaccharides may be involved in invasion and nodule development, bacterial release from infection threads, bacterial development, suppression of plant defense response and protection against plant antimicrobial compounds.

In our study, strains grown on YEMA plates containing calcofluor and having different concentrations of zinc showed that zinc sensitive strains produce less exopolysaccharide than tolerant strains. According to Reuber and Walker (1993) exopolysaccharide give advantages by improving nutrient acquisition or providing protection from environmental stress and host defences. Dragana *et al.* (2006) also reported that exopolysaccharide producing mutant strains of *Rhizobium leguminosarum* bv *trifolii* tolerated very high concentrations of heavy metals than non mutant strains.

Swarming behaviour is the marker for the motility and chemotaxis. Chemotaxis and motility might have provided enhanced chemical or physical contact with the root, enhanced occupation of sites potentially suitable for infection and rapid or efficient infection development (Gulash *et al*. 1984). Our results showed that swarming activity of both zinc tolerant and zinc sensitive strains goes on increasing up to 25 mg/l zinc and thereafter with an increase in zinc concentration in the medium it decreases.The strains showing higher swarming behaviour were supposed to form better nodules. Ames and Bergman (1981) reported that the motile, chemotactic wild type strain was capable of

forming between 65% and 98% of the nodules when competing against equal numbers of non-flagelleted or nonmotile mutant isolates. In this contest Mellor *et al.* (1987) also found that motile strains of *Rhizobium trifolii* formed approximately five times more nodule than non motile strains and suggesting that motility is a factor in competition for nodule formation. of zinc on nodulation may perhaps be as much due to inhibition of motility as to direct toxicity especially at low concentrations. The present study concludes that under zinc stress conditions, tolerant strains successfully overcome the stressful environmental conditions by maintaining the factors, essential for nodulation like β-glucan, lipopolysaccharide, exopolysaccharide and motolity in an ideal system In legume *Rhizobium* symbiosis zinc is essential as a micronutrient on the one hand and become toxic, when agricultural fields are get polluted due to industrialization. Results of this study indicated that metal tolerant strains can be preferred for plantation of *Trifolium alexandrinum* in metal contamination areas.

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