Nature Environment and Pollution Technology
An International Quarterly Scientific Journal

ISSN: 0972-6268	Vol. 13	No. 2	pp. 369-374	2014

Original Research Paper

Biodecolourisation of Azo Dye Reactive Red 22 by *Bacillus infantis* strain AAA isolated from Seawater and Toxicity Assessment of Degraded Metabolites

K. V. Bhaskara Rao and A. S. Arun Prasad*
Division of Environmental Biotechnology, School of Bio Sciences and Technology, VIT University, Vellore-632 014
*School of Biosciences and Technology, VIT University, Vellore-632 014, Tamil Nadu, India
Corresponding Author: K. V. Bhaskara Rao

Nat. Env. & Poll. Tech.
Website: www.neptjournal.com
Received: 31-10-2013
Accepted: 2-12-2013

Key Words:
Biodecolorization
Bacteria
Reactive Red 22
Toxicity assessment

ABSTRACT

Decolorization and degradation of Azo dye Reactive Red-22 (RR-22) by a bacterial strain isolated from marine seawater and identified as *Bacillus infantis* sp. AAA was assessed. Maximum decolorization (95%) of RR-22 (100 mg/L) was achieved at pH 9, 37°C and 3% salt concentration under static conditions in 6h. Various environmental characteristics such as pH, temperature, salt concentration and dye concentration were analysed to determine the optimum decolorization conditions. Decolorization analysis was carried out by UV-vis spectrophotometer. The FTIR spectra showed the absence of –N=N– in the degraded sample. The HPLC chromatogram showed the presence of multiple peaks at different retention times conforming degradation. The phytotoxicity results exhibited the toxic nature of RR-22 compared to the less toxicity of degraded metabolites on seeds of *Vigna mungo* and *Vigna radiata* showing 30% and 20% germination.

INTRODUCTION

Textile industries are presently one of the largest and most important industries in the global economy in terms of output, foreign exchange earnings and employment generation. The use of complex synthetic dyes has increased considerably in these industries (Asia et al. 2006, Mishra & Tripathy 1993). Dyes are natural or synthetic substances which are used to add or change the colour of a substance. Dyes have wide applications in many different industries like textiles, cosmetics, paper, pharmaceutical, leather and food because of their large variety of colour shades, high wet fastness profiles, ease of application and minimum energy consumption (Wang et al. 2009). At the same time these dyes are also harmful to the environment. Because of their synthetic origin and complex aromatic molecular structures they are difficult to degrade. So, dyeing effluent discharge from these industries into the water bodies and wastewater treatment systems is currently causing a major source of environmental pollution (Banat et al. 1996). The three most common groups of dyes are azo, anthraquinone and phthalocyanine dyes (Wang et al. 2009). Azo dyes are the leading group of dyes used in the textile industries. The presence of one or more azo groups (-N=N-) is responsible for the colour of these dyes (O'Neill et al. 2000). Because of their complex structure they cannot be easily degraded under natural conditions and thus are not typically removed from water by conventional wastewater treatment methods (Hildenbrand et al. 1999). They are also resistant to chemical and microbial attacks and can remain stable in light and washing conditions (Rajaguru et al. 2000).

Disposal of dyes into the water bodies affects the aesthetic merit, water transparency, gas solubility, photosynthetic activity of hydrophytes by reducing light penetration and also damages the food chain of most of the marine organisms (Aravindhan et al. 2007). Colour can be removed by a number of physical and chemical methods but with limitations such as high cost, formation of hazardous by-products and intensive energy requirements (Sarioglu et al. 2007). Biological methods have prevailed over these defects as they are cost effective and eco-friendly. Sea is an unexploited habitat of new and potentially useful biologically active microorganisms possessing great diversity. Dyeing effluents contain large amount of salts and the use of marine microorganisms is suitable owing to their resistance to salinity. The objective of the present study is to assess the dye decolourising potential of *Bacillus infantis* strain AAA isolated from marine seawater sample collected from Chennai beach, India. The strain AAA was evaluated for its ability to decolourise the azo dye Reactive Red 22 at various pH, temperature, salinity and dye concentration under static conditions. The degradation was analysed by FTIR and HPLC methods. Phytotoxicity study upon seeds of *Vigna mungo* and *Vigna radiata* showed the less toxic nature of degraded metabolites.

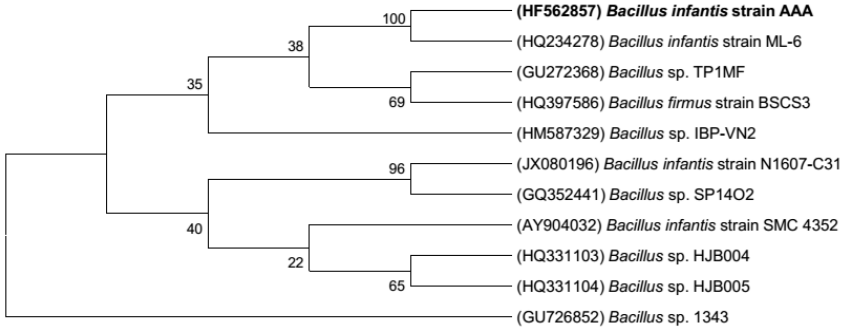

Fig. 1: Phylogenetic tree of *Bacillus infantis* sp. AAA.

MATERIALS AND METHODS

Chemicals: Azo dye Reactive Red 22 (RR-22) was a generous gift from local textile mill in Coimbatore. HPLC grade methanol was purchased from Sigma. Dye Stock Solution was made by dissolving 1000 mg/L of RR- 22 in 1L of distilled water.

Isolation, screening and identification of bacteria from marine sea sample: Collected marine sample was serially diluted from 10^{-1} to 10^{-7} and plated on Marine Zobell agar by spread plate technique and was incubated at 37°C for 24h. After incubation period, distinct colonies were separated and streaked on Luria Bertani Agar plates containing 3% salt (NaCl). Preliminary screening for azo dye (RR-22) decolorization was carried out in 50mL LB broth containing casein enzymic hydrolysate 10 g/L, Yeast extract 5 g/L, NaCl 10 g/L with 5 mg/L of RR-22. To the overnight grown isolates 5 mg/L of RR-22 was added and incubated under static conditions for decolorization. The marine bacterial strain, which showed highest potential for decolorization, was selected and identified at molecular level by amplifying the 16S rRNA gene as described earlier (Attschul et al. 1997). The obtained 16S rRNA gene sequence was aligned with closely related sequences from EMBL using CLUSTAL W (Thompson et al. 1997). A phylogenetic tree was constructed using the software package MEGA version 5 (Tamura et al. 2011).

Decolorization at various environmental parameters: Decolorization experiments were carried out at various pH (7, 8, 9, and 10), temperature (32, 37, 40 and 45°C), salt concentrations (1, 2, 3, 4, 5%) and increasing dye concentration (l00-500 mg/L). Decolorization experiments were carried out in 250 mL conical flasks containing 100mL of LB broth. 1% inoculum was added to the flask and kept for shaking at 130 rpm for overnight. 100 mg/L of RR-22 was added to the overnight grown culture and incubated under static conditions for decolorization. At regular intervals (0h, 2h, 4h and 6h) 4 mL sample was withdrawn and centrifuged at 10,000 rpm for 15min. Supernatant obtained was analysed by UV visible spectroscopy at 555 nm (λ_{max} of RR-22). All the experiments were carried out in triplicate. Decolorization rate was calculated and expressed as decolorization percentage:

$$\text{Decolorization (\%)} = (A_0 - A)/A_0 \times 100$$

Where A_0 is the initial absorbance and A is the absorbance of medium after decolorization at the λ_{max} (nm) of dye.

Degradation analysis by FTIR and HPLC: After decolorization, 100 mL of sample was withdrawn and centrifuged at 10,000 rpm for 15min. The metabolites were extracted from supernatant using equal volume of ethyl acetate. The extract were dried over anhydrous Na_2SO_4 and evaporated to dryness in a rotary evaporator. The analysis was carried out in the mid IR region ranging from 400-4000 cm^{-1} with 16 scan speed. The samples were mixed with spectroscopically pure KBr in the ratio of 1:9 and analysis was carried out. The sample was evaporated in rotary vacuum and was dissolved in methanol. HPLC analysis was carried out on C18 column. The mobile phase used was methanol : water (50:50)

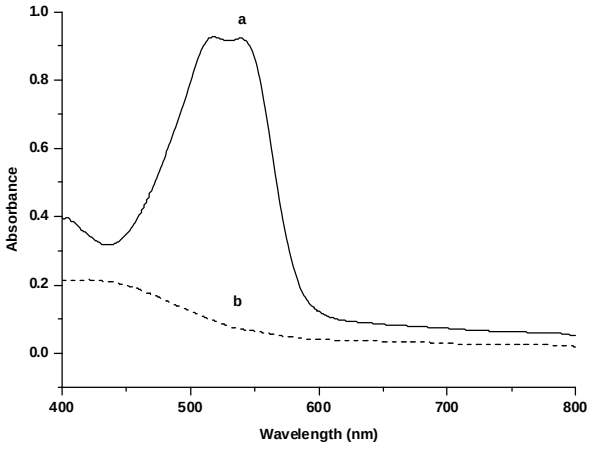

Fig. 2: UV Scan of RR-22 decolorization by *B. infantis* sp. AAA. Sample withdrawn at a) 0 h, b) 6 h.

ratio with flow rate of 1mL per min and UV detector at 555nm.

Phytotoxicity assay: Phytotoxicity assay of the dye and the degraded sample was carried out on *Vigna mungo* and *Vigna radiata*. The seeds were surface sterilized using 0.1% $HgCl_2$. Seeds were interacted separately in conical flask with dye (RR-22) and degraded metabolites at 100 ppm concentration. Seeds in distilled water were used as control. The conical flasks were kept at 100 rpm for 2 h for interaction. After interaction, the seeds were placed in sterile Petri plates and incubated in dark until germination. The sprouted seeds were planted in pots for growth. After a period of 7 days, the growth was assessed by measuring the root and shoot length.

RESULTS AND DISCUSSION

Isolation, screening and identification of bacteria from marine sea sample: A total of 10 different bacterial isolates were obtained from marine seawater. Based on the results of preliminary decolorization of RR-22, only 1 strain showed good decolourising activity in LB broth. The strain AAA possessed the ability to decolourise the azo dye RR-22 (100 mg/L) with 95% rate at pH 9, 37°C with 3% NaCl in a time span of 6 h under static conditions. The potential strain was identified as *Bacillus infantis* sp. AAA based on 16S rRNA gene sequence analysis (1471 bp). BLAST analysis of the 16S rRNA sequence comparison showed that strain AAA had the closest phylogenetic affiliation to the genus *Bacillus*. The phylogenetic tree indicates that the strain AAA had 100% similarity with *Bacillus infantis* strain ML-6. Phylogenetic relationship between *Bacillus infantis* sp. AAA and other related microorganisms is shown in Fig. 1.

Decolorization analysis by UV-Vis spectrophotometer: RR-22 decolorization by *Bacillus infantis* sp. AAA was monitored spectrophotometrically at 555 nm (λ_{max} of RR-22). *B. infantis* sp. AAA decolourised 100 mg/L of RR-22 at pH 9, 37°C with 3% NaCl in LB broth under static conditions. The dye peak at 0 h was found to be decreased with time by the end of 6 h (Fig. 2).

Decolorization at various environmental parameters: The process of decolorization was found to be affected by a number of physico-chemical characteristics such as pH, temperature, salinity and dye concentration (Pearcea et al. 2003). Decolorization rate of RR-22 increased with rise in pH. Decolorization rate of about 57% and 64% was observed at pH 7 and pH 8. At pH 9 optimal decolorization rate of 95% was noticed in 6 h (Fig. 3a). Lowest rate of decolorization (27%) was seen at pH 10. Similar result was shown by *Planococcus* sp. MC01 in decolourising Orange

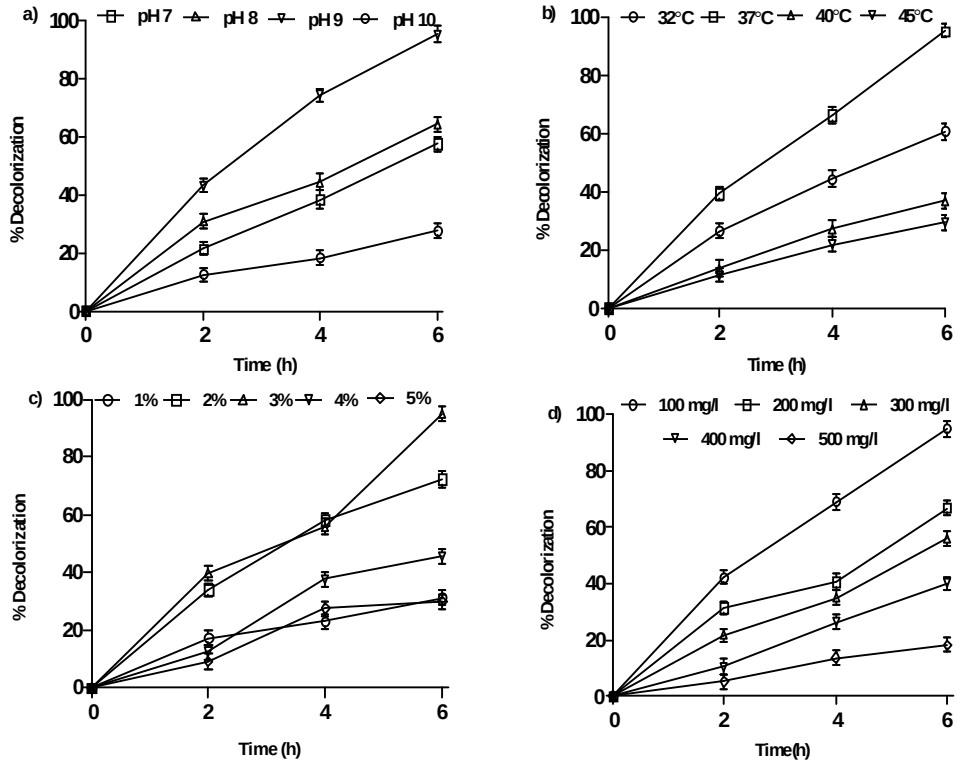

Fig. 3: Effect of a) pH, b) temperature, c) salinity, d) dye concentration on RR-2 decolorization by *Bacillus infantis* sp. AAA.

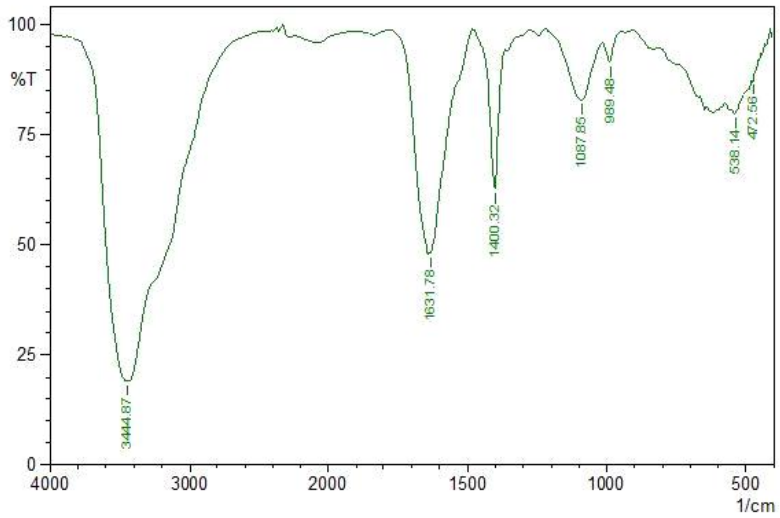

Fig. 4: FTIR spectrum of dye Reactive Red-22.

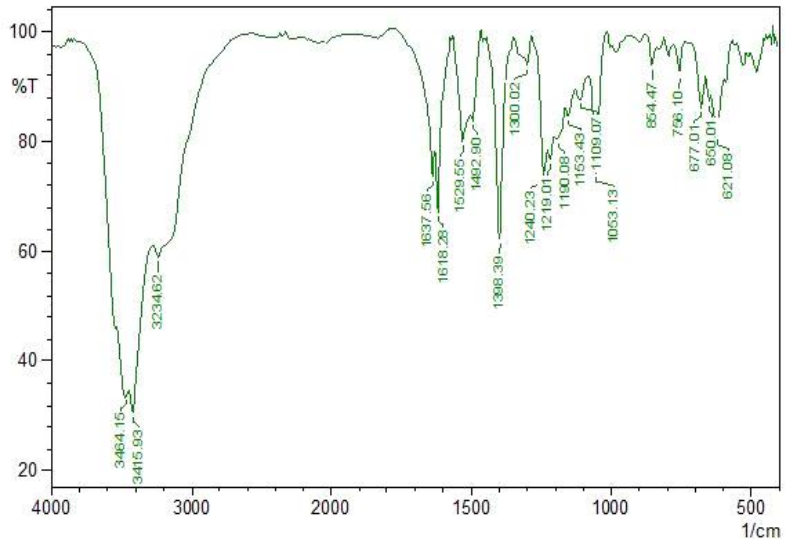

Fig. 5: FTIR of RR-22 degraded metabolite.

I at alkaline pH (Chen et al. 2013). Reactive Red-22 was decolourised with maximum rate of 95% by *B. infantis* sp. AAA at a temperature of 37°C (Fig. 3b). About 60% reduction in colour was noticed at 32°C. Decrease in decolorization rate was observed as temperature increased with rates of about 36% and 30% at 40°C and 45°C respectively. Similar phenomenon was also noticed with *Shewanella* sp. during decolorization of azo dyes (Khalid et al. 2008). Salt concentration of 3% exhibited optimal decolorization of 95% by *B. infantis* sp. AAA (Fig. 3c) Decolorization rate of about 31% and 72% was noticed with 1% and 2% NaCl concentration. As the salt concentration increased to 4% and 5% the rate of RR-22 decolorization decreased gradually with rates of about 45% and 30% respectively. This decrease in decolorization at higher salt concentrations could be due to the high osmotic pressure in *Bacillus infantis* sp. AAA inhibiting bacterial growth and causing cell death. Similar effect was noticed on bacterial decolorization of azo dyes in earlier studies (Wang et al. 2013, Kolekar et al. 2008). Effect of initial dye concentration on decolorization of RR-22 by *B. infantis* sp. AAA is shown in Fig. 3d. Strain AAA decolourised an initial dye concentration of 100 mg/L with 95% rate in 6 h under static conditions. As dye concentration increased the efficiency of strain AAA to decolourise RR-22 decreased with rates of decolorization of about 66%, 56% and 40% at 200, 300 and 400 mg/L dye respectively. Drastic decrease in decolorization rate (18%) was observed at an increased RR-22 concentration of 500 mg/L. This could

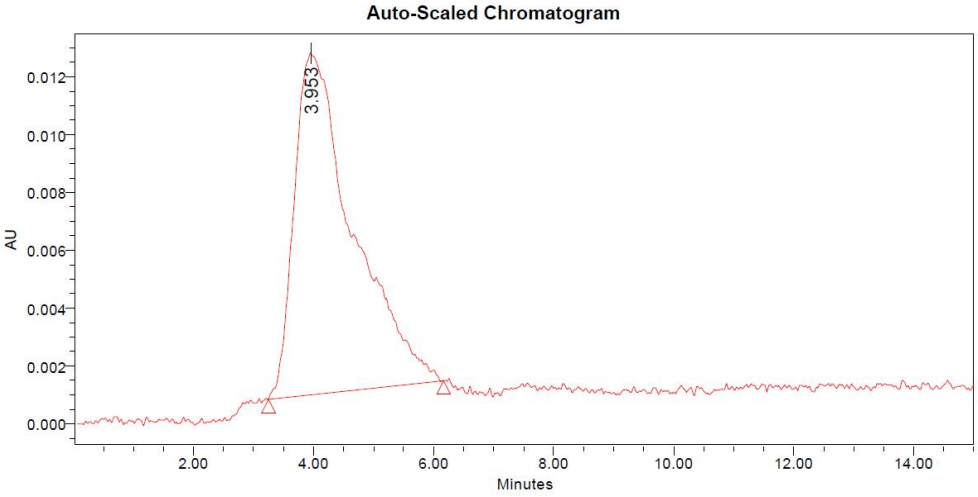

Fig. 6: HPLC profile of Reactive Red-22.

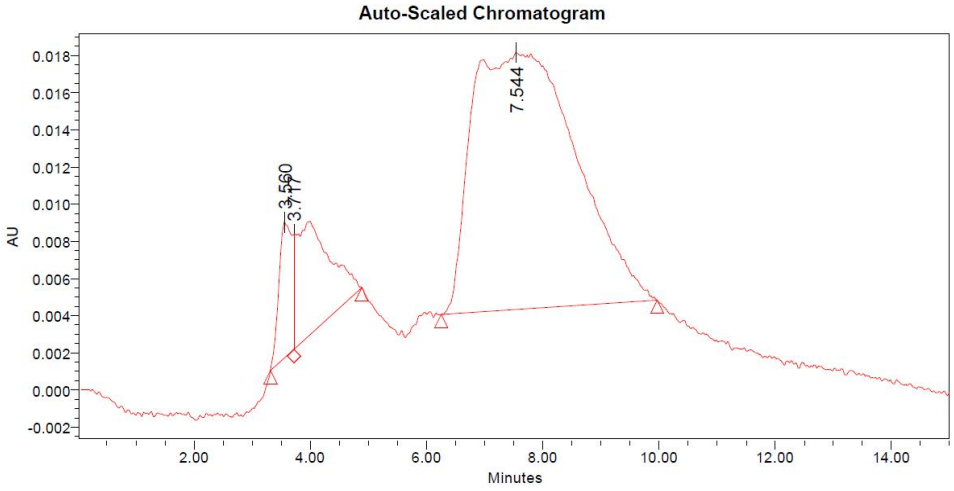

Fig. 7: HPLC chromatogram of degraded metabolites of RR-22.

be due to toxicity of dye at high concentrations affecting the growth and metabolic activity of the microorganism (Jadhav et al. 2008). Similar result was exhibited by *Shewanella oneidensis* MR-1 and *Deinococcus radiodurans* in the decolorization of Sudan I dye and Malachite green (Lv et al. 2013, Ji et al. 2012).

Degradation analysis by FT-IR and HPLC: The FT-IR analysis showed significant changes in peaks before and after dye degradation. In untreated dye there was an interaction between OH and NH_3 groups which resulted in the formation of a single broad peak at 3444.87cm^{-1}, N=N stretch was found at 1631.78 cm^{-1} and C-C=C asymmetric stretch was found at 1400.32cm^{-1} (Fig. 4). However, in the FTIR of degraded sample the bond between the OH and the NH_3 groups was broken and two peaks were formed at 3464.15 cm^{-1} and 3415.93cm^{-1}. N=N stretch was absent in the degraded sample and C=C asymmetric stretch was observed at 1398.39 cm^{-1} (Fig. 5). The major change observed was the absence of -N=N- stretch in the degraded dye product which indicated the degradation of RR-22 by *B. infantis* sp. AAA. The HPLC analysis of the dye sample withdrawn at 0 h showed a single peak with a retention time of 3.95 minutes (Fig. 6). As the degradation proceeded (6 h), the HPLC chromatogram of the degraded sample showed three peaks with retention time of 3.56, 3.71 and 7.54 minutes (Fig. 7).

Phytotoxicity assay: The result of phytotoxicity assay carried out on the seeds of *Vigna mungo* and *Vigna radiata* is depicted in Table 1. The seeds of *V. mungo* and *V. radiata* treated with degraded metabolites of RR-22 (100 ppm) and distilled water showed 100% germination rate compared to

Table 1: Phytotoxicity comparison of RR-22 and its extracted metabolites.

Parameters	*Vigna mungo*			*Vigna radiata*		
	Water	RR-22 (100 ppm)	Extracted metabolites (100 ppm)	Water	RR-22 (100 ppm)	Extracted metabolites (100 ppm)
Germination (%)	100	30	100	100	20	100
Shoot (cm)	7.15±0.21	2.87±0.1**	4.37±0.23*	8.11±0.13	2.13±0.51**	5.01±0.24
Root (cm)	2.79±0.21	00.43±0.11**	1.50±0.12	3.13±0.12	0.33±0.07**	1.32±0.11*

the very low germination rate in *V. mungo* (30%) and *V. radiata* (20%) seeds treated with dye (100 ppm). The mean of the shoot and root length of *V. mungo* was 7.15±0.21 cm and 2.79±0.21 cm, respectively, and in *V. radiata* the lengths were (8.11±0.13 cm, 3.13±0.12 cm) respectively with distilled water. The seeds treated with degraded metabolites of RR-22 showed good growth pattern in their shoot and root lengths as that of control (distilled water) with average values for *V. mungo* (4.37±0.23 and 1.50±0.12 cm) and *V. radiata* (5.01±0.24 and 0.33±0.12 cm) respectively. The root and shoot lengths of seeds treated with RR-22 (100 ppm) were affected drastically with mean values, *V. mungo* (2.87±0.1 and 0.43±0.11 cm) and *V.radiata* (2.13±0.51 and 0.33±0.07 cm) respectively.

Values were mean of three experiments ± SEM, significantly different from the control (seeds germinated in water) at $^{*}P< 0.05$, $^{**}P< 0.01$, by one-way analysis of variance (ANOVA) with Tukey-Kramer comparison test.

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