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Ajayi OO
1. Department of Microbiology, Faculty of Science, University of Ibadan, Ibadan, Nigeria
2. Soil microbiology unit, International Institute of Tropical Agriculture, Ibadan, Nigeria

Amao TO
Department of Microbiology, Faculty of Science, University of Ibadan, Ibadan, Nigeria

Bitire T
Northwest University South Africa, South Africa

Dianda M
1. Soil microbiology unit, International Institute of Tropical Agriculture, Ibadan, Nigeria
2. Laboratoire de Microbiologie forestière (INERA/DEF) BP 7047 Ouagadougou 03, Burkina Faso

Fagade OE
Department of Microbiology, Faculty of Science, University of Ibadan, Ibadan, Nigeria

Correspondence
Ajayi OO
3. Department of Microbiology, Faculty of Science, University of Ibadan, Ibadan, Nigeria
4. Soil microbiology unit, International Institute of Tropical Agriculture, Ibadan, Nigeria

Impactive role of reactive chlorine in chlorinated water and the survival of selected rhizobia strains commonly used as inoculant

Ajayi OO, Amao TO, Bitire T, Dianda M and Fagade OE

Abstract
Chlorination is a commonly used method for the disinfection of water in many homes and farms. The use of chlorinated water is believed to kill or affect rhizobia negatively, this study was carried out to determine the effect of different concentrations of NaCl and chlorine powder (100 mg/L- 300 mg/L) on the growth of selected Rhizobia strains including USDA110, USDA136, 532C, RACA6 and FA3 which are *Bradyrhizobium japonicum* strains commonly used for soybean. They were inoculated on chlorine induced Congo-red medium and incubated for 10days and growth was studied from the 3rd day. The growth of the strains were unaffected by NaCl, FA3 and RACA6 showed no growth at 150mg/L on the 3rd day but after 7 days RACA6 showed signs of growth at 150 mg/L. the growth of USDA110 and 532C was affected at 250 mg/L and 300 mg/L respectively from the 3rd day till the 10th day. USDA136 was unaffected even at 300 mg/L from the 3rd day through the 10th day. Chlorination had effect on the growth of the strains particularly strain FA3 and RACA6, therefore it is highly important to monitor the amount of chlorine in tap water that will be used in watering soybean plants particularly those inoculated with these strains.

Keywords: Growth, *Bradhyrhizobium*, chlorination, water

1. Introduction

Water is highly essential for the growth of plants including legumes especially soybean. Rhizobia are also essential for the growth of legumes where they form symbiotic relationships with legumes and fix nitrogen in their root. This has led to exploring the potential of these beneficial rhizobia as inoculant in maximizing the productivity and increasing the nitrogen input in farming systems. According to Hartley *et al*. (2012) [1], the quality of water used in the process of inoculating seeds is often overlooked as a potential cause of early rhizobia death. They carried out a test to determine the effect of water quality in the on the survival of rhizobia in cultures prepared with distilled water and unfiltered tap water. It was observed that the survival of rhizobia in distilled water was higher than those in the other water sources (Hartley *et al*., 2012) [1]. In essence the quality of water affects rhizobia growth. Studies also carried out by Novozymes a leading bio-inoculant industry stated that the use of chlorinated water out of a tap can be an issue as chlorinated water will kill the rhizobia (Treloar, 2013) [5]. This maybe as a result of chlorination (which is known to be an antagonism mechanism in some bacteria) occurring as a result of the production of reactive chlorine (HOCl, hypohalom acid) as suggested by Oberg (2002) [4]; Van pee and Unversucht (2003) [3]; Bengtson *et al*. (2009) [2] this reactive chlorine is usually used to sterilize or disinfect water. Chlorinated water is also believed to cause leaching (Tsobedi *et al*., 2022) [23].

Some legumes like *Prosopis*, *Acacia* (Zhang *et al*., 1991) [22], and *Medicago sativa* (Abdel-Wahab and Zahran; 1983) [6], are able to tolerate salt, although their leguminous hosts may have less tolerance to salt than are their rhizobia symbiont. Studies have also shown that the symbiotic legume-Rhizobium association and the formation of nodules may have high sensitive to salt or osmotic stress than are their rhizobia (El-Shinnawi *et al*., 1989, Velagaleti *et al*., 1990, Zahran *et al*., 1986, Zahran *et al*., 1991b) [12, 18, 21, 20]. It has also been shown that salt stress may be an inhibitor for the initial steps of *Rhizobium*-legume symbioses. The root hair of Soybean plants showed little curling and deformation when inoculation with *Bradyrhizobium japonicum* was done at concentrations of 170 mM NaCl, and nodule formation was completely inhibited at concentrations of 210 mM NaCl.

Salinity in the soil can limit symbiosis they affect the survival growth rate of Rhizobium spp. in the soil and rhizosphere, inhibit the process of infection, affect the functioning of the root nodule and reduce the growth of the plant, process of photosynthesis, and nitrogen demand.

Salinity poses a threat to agriculture in many regions of the world (Rao *et al*., 1991a) [27], and roughly 40% of the world's land surface have been categorized as having salinity issues (Cordovilla *et al*., 1994) [7] especially in the tropical and Mediterranean regions. The increased salinity of soils and use of chlorinated water supplies for irrigation have resulted in decreasing production in many crop plants and drastic change in the pattern of growth in several plants (Cordovilla *et al*., 1994) [7]. Also, when there is increased salt concentration detrimental effects may be seen in the soil microbial populations because of the toxicity and osmotic stress (Delgado *et al*., 1994; Tate; 1995, Glenn *et al*., 1999) [11, 16, 14]. Salinity is known to decrease the plant growth and its yield, depending upon the plant species, salinity levels, and ionic composition of the salts (Delgado *et al*., 1994) [11]. As has been discovered in most cultivated crops, the response of legumes to salinity varies largely depending on factors like climatic conditions, soil properties, and the stage of growth (Cordovilla *et al*., 1995a; 1995b; 1995c) [8-10]. Also, reports have been made on the variability in tolerance to salty conditions among crop legumes (Zahran *et al*., 1991a, Zahran *et al*., 1991b) [19-20]. Some legumes, e.g., *Vicia faba*, *Phaseolus vulgaris*, and *Glycine max*, are more salt tolerant than others, e.g., *Pisum sativum*.

Since soil salinity may directly affect either symbiont or affect their interaction (Tsobedi *et al*., 2022) [23], it is essential to study the sensitive of rhizobia strains to salinity. This will ensure that proper efforts are directed to help improve the tolerance of more sensitive symbiont or processes in symbiosis. In essence the quality of water may be a major factor affecting the adaptability and growth of rhizobia when used to inoculate soybean on the field. Very little work has been done to study the effects of salinity and chlorinating agent (for treating water) on Rhizobium growth (Tsobedi *et al*., 2022) [23]. This study was carried out to observe the effect on varying concentrations of reactive chlorine which is commonly being used in the treatment of water sources especially well water etc. it was done with the aim of determining how harmful chlorine in water can be for the survival of rhizobia as this is worth investigating.

2 Methodologies

2.1 Rhizobia strain

Cultures used for this study were USDA136, USDA110, FA3, RACA6 and 532C which are *Bradyrhizobium japonicum* strains specific for soybean were obtained from the collection held at soil microbiology laboratory International Institute for Tropical Agriculture, Ibadan Nigeria.

2.2 Purchase of Chlorine containing agent

Chlorine reagent (Ca (Clo) 2nH_2O) for treating water was obtained at the Ogunpa market in Ibadan, Oyo State Nigeria the type purchased was the hth Granular calcium hypochlorite (68% chlorine) made in USA

2.3 Preparation of media

Congo red media (Mannitol 10g, K_2HPO_4 0.5g, $MgSO_4$. 7H_2O, NaCl 0.1g, Yeast extract 0.5g, distilled water 1L, Congo red 10ml (Stock solution 0.25gCongo red dye/100 ml i.e. 25ppm) was prepared. Calcium hypochorite and NaCl salt (SigmaAldrich) was incorporated into the CR media in concentrations of 100 mg/L, 150 mg/L, 200 mg/L, 250 mg/L, 300 mg/L separately. The media was sterilized at 121 °C and 1 atm for 15 mins allowed to cool to 45°C, poured in petri dishes and then allowed to set. Plates were then used for point inoculation.

2.4 Culturing of Rhizobia strains

Rhizobia strains were used inoculated into broth which was confirmed to be at least 10^9 cfu/ml. 1µl of the broth was then inoculated (using the point inoculation method) in Congo-red medium incorporated with increasing concentration (100-300 mg/L) of chlorine and NaCl aseptically using three replicates for each concentration. The plates were incubated at 28 °c for 10days during which there growth pattern was monitored. Growth pattern were checked on the 3rd, 5th, 7th and 10th day and recorded.

2.5 Statistical analysis

The collected data were analyzed for correlation (Pearson's) using SPSS version 20 and Fisher's least significance was used to compare means at $p \leq 0.05$ and $p \leq 0.01$.

3. Results

3.1 Day three

By the third day, active growth was observed in all the strains in NaCl at all concentrations used while no growth was observed at 300 mg/L in USDA110, RACA6 and 532C and at 250 mg/L no growth was seen in USDA110, RACA6 while growth was seen in 532C (Table 3.1). No growth was observed in RACA 6 at all in all the concentrations used and no growth was seen at concentrations of 150 mg/L and above for FA3.

Table 1: Growth of *Bradyrhizobium japonicum* strains on the 3rd day

Strain	100 mg/L		150 mg/L		200 mg/L		250 mg/L		300 mg/L	
	NaCl	RC	NaCl	RC	NaCl	RC	NaCl	RC	NaCl	RC
USDA110	G	G	G	G	G	G	G	NG	G	NG
RACA6	G	G	G	NG	G	NG	G	NG	G	NG
USDA136	G	G	G	G	G	G	G	G	G	G
532C	G	G	G	G	G	G	G	G	G	NG
FA3	G	G	G	NG	G	NG	G	NG	G	NG

NaCl: Sodium Chloride, RC: Calcium hypochlorite, NG: no growth G: growth

3.2 Day five

On the 5th day, all strains that showed no growth at 150 mg/L showed signs of growth while at 200 mg/L no growth was observed in only RACA6. FA3 showed growth at all concentrations by the 5th day but the growth pattern of USDA110, RACA 6 and 532C remained the same at 250 mg/L and 300 mg/L (Table 3.2). Active growth was observed in all the strains in NaCl at all concentrations used.

Table 2: Growth of *Bradyrhizobium japonicum* strains on the 5th day

Strain	100 mg/L		150 mg/L		200 mg/L		250 mg/L		300 mg/L	
	NaCl	RC	NaCl	RC	NaCl	RC	NaCl	RC	NaCl	RC
USDA110	G	G	G	G	G	G	G	NG	G	NG
RACA6	G	G	G	G	G	NG	G	NG	G	NG
USDA136	G	G	G	G	G	G	G	G	G	G
532C	G	G	G	G	G	G	G	G	G	NG
FA3	G	G	G	G	G	G	G	G	G	G

NaCl: Sodium Chloride, RC: Calcium hypochlorite, NG: no growth G: growth

3.3 Day Seven
At 200 mg/L of Calcium hypochlorite all strains showed signs of growth. RACA 6 and 532C showed signs of growth at 250 mg/L but still showed no signs of growth at 300 mg/L while USDA110 showed no growth at 250 mg/L and 300 mg/L and active growth was observed in all the strains in NaCl at all concentrations used (Table 3.3).

Table 3: Growth of *Bradyrhizobium japonicum* strains on the 7th day

Strain	100 mg/L		150 mg/L		200 mg/L		250 mg/L		300 mg/L	
	NaCl	RC	NaCl	RC	NaCl	RC	NaCl	RC	NaCl	RC
USDA110	G	G	G	G	G	G	G	NG	G	NG
RACA6	G	G	G	G	G	G	G	G	G	G
USDA136	G	G	G	G	G	G	G	G	G	G
532C	G	G	G	G	G	G	G	G	G	NG
FA3	G	G	G	G	G	G	G	G	G	G

NaCl: Sodium Chloride, RC: Calcium hypochorite, NG: no growth G: growth

3.4 Day Ten
The growth pattern remained the same as was observed on the 7th day with no signs of growth at 250 mg/L and at 300 mg/L in USDA110 and 532C. Active growth was observed in all the strains in NaCl at all concentrations used (Table 3.4).

Table 4: Growth of *Bradyrhizobium japonicum* strains on the 10th day

Strain	100 mg/L		150 mg/L		200 mg/L		250 mg/L		300 mg/L	
	NaCl	RC	NaCl	RC	NaCl	RC	NaCl	RC	NaCl	RC
USDA110	G	G	G	G	G	G	G	NG	G	NG
RACA6	G	G	G	G	G	G	G	G	G	NG
USDA136	G	G	G	G	G	G	G	G	G	G
532C	G	G	G	G	G	G	G	G	G	NG
FA3	G	G	G	G	G	G	G	G	G	G

NaCl: Sodium Chloride, RC: Calcium hypochorite, NG: no growth G: growth

3.5 Effect of treatment, concentrations and number of days on growth of the strains
Treatment, number of days and concentration had significant effect on the growth of the strains. The combined effects of treatment, strain, day and concentration had significant effects on the growth of the strains. The combined effects of treatment and concentration had no significant effects on the growth of the strains, also the combined effects of day and concentration had no significant effects on the growth of the strains. Likewise, the combined effects of strain and day had no significant effects on the growth of the strains. Finally the combined effects of strain and concentration had significant effects on the growth of the strains (table 3.5).

Table 5: Significance of the effect of interaction between treatment, concentration and number of days on the growth of the strains

Source	DF	Mean Square	F	Sig.
treatment	1	1.805	40.111	.008
concentration	4	1.143	7.659	.003
Strain	4	.280	2.333	.115
Day	3	.712	15.815	.510
treatment * concentration	4	.243	1.626	.231
treatment * Strain	4	.280	2.333	.115
treatment * Day	3	.045	.178	.910
concentration * Strain	16	.155	9.789	.000
concentration * Day	12	.149	1.000	.500
Strain * Day	12	.120	1.000	.500
treatment * concentration * Strain	16	.155	9.789	.000
treatment * concentration * Day	12	.149	9.421	.000
treatment * Strain * Day	12	.120	7.579	.000
concentration * Strain * Day	48	.016	1.000	.500
treatment * concentration * Strain * Day	48	.016	.	.

There was positive significant correlation between the growth and the number of days and the treatment applied showing that as the number of days increased the growth response in the strains increased, while there was a negative significant correlation between the growth and the concentration of the reactive chlorine and NaCl, i.e. as the concentration increased the growth reduced in the strains (Table 3.6).

Table 6: Correlation between parameters and their effects on the growth of the strains

	Strain	Day	Concentration	Treatment	Growth
Strain	1	.000	.000	.000	-.120
Day	.000	1	.000	.000	.232**
Concentration	.000	.000	1	.000	-.392**
Treatment	.000	.000	.000	1	.270**
Growth	-.120	.232**	-.392**	.270**	1

******. Correlation is significant at the 0.01 level (2-tailed).

4. Discussion
Rhizobia spp. are able to tolerate high levels of NaCl and can therefore grow and adapt in saline /salty soil conditions. These results differed from that obtained by Pillai and Sen; 1973, who showed that the rate of growth Rhizobium spp. increased with 1% NaCl added to broth media (EC-18.0 mS cm-1), but similar to the findings of Singleton *et al*. 1982 [26] and Steinborne and Roughley; 1975 [25] where it was shown that the growth of both R. trifolii and R. meliloti was slowed down by the adding of salt. The reactive chlorine used in treatment of water on the other hand affected the growth of the rhizobia strains at concentrations between 150 mg/L-200 mg/L at the 3rd day of growth, but bacteria appeared able to recover over longer growth periods by later showing signs of growth. This was similar to the results obtained in the studies carried out by Singleton *et al*. 1982 [26], where all of the four strains chosen from the experiment carried out for growth rate (17E and 21A which were used for salt-toleranance growth and Hawaii 5-0 and USDA 110 selected for salt-sensitive growth), and were able to survive the first exposures (5 h) to solutions containing EC of 43 mS cm-1. Significant reduction in growth after 5 days of exposure of USDA 110 to EC solutions at 43 mS cm-1, and Hawaii 5-0

after 27 days.

At concentrations of 250 mg/L - 300 mg/L strains growth were most affected particularly USDA110, RACA6 and 532C as they showed no signs of growth even till the 10th day this was similar to the report of Hartley *et al.* 2012 [1] and Treloar; 2013 [5], although RACA 6 started showing signs of growth on the 7th day. This was also similar to the findings obtained in the work of Singleton *et al*. 1982 [26s], where the survival of strains USDA 110 and 21A strain during soil tests were affected, especially at the most extreme treatment combination of 12 mS cm-1 and -15 bars of moisture tension, while two strains 17E and Hawaii 5-0 were not affected. It also showed that chlorinated water may affect the activity of nodulation in the plant by the Rhizobia spp. as the first two weeks are usually the period during which the rhizobia interacts with the root of the plant

5. Conclusion

NaCl showed no significant harmful effects on the growth of the Rhizobia cells at 150mg/L and above, while Reactive chlorine on the other hand affected the recovery and growth of the strains at concentrations of 250mg/L and above, and some of the strains were negatively affected permanently with no growth. This shows that the use of chlorinated water as water source for inoculated legumes maybe harmful to the Rhizobia spp. especially when freshly treated as concentrations of the chlorinating agent is usually still very high in the water.

6. Conflict of Interest

Not available

7. Financial Support

Not available

8. References

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