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Effects of Different Salt Concentrations and pH on Growth of *Rhizobium* sp. and a Cowpea - *Rhizobium* Association

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Abstract: The root environment of a legume undergoes constant changes as a result of chemical reactions taking place within the soil and these exert fluctuating osmotic pressure on the rhizosphere and its associated rhizobacterium. *Rhizobium* produces root nodules and is subjected to changes in the soil environmental conditions including salt concentrations and soil acidity. An experiment was conducted to find out the effects of varying salt concentrations ranging from 0.005 M to 0.200 M NaCl and a pH range of pH 3-9 on growth of *Rhizobium* as well as the cowpea associated with the Rhizobium. *Rhizobium* species from cowpea were capable of osmoadaptation and were found to tolerate a relatively high salt concentration of up to 0.200 M NaCl. However, the population count was inversely proportional to the salt concentration with high growth (30.0-31.6 x 10^4 cfu/mL) at lower concentrations of 0.005-0.010 M and low growth (7.4-19.2 x 10^4cfu/mL) at higher salt concentrations of 0.050-0.200M. The optimal pH range for the growth of the *Rhizobium* sp was pH 6-7 while lower or higher pH values recorded lower population counts. The present study revealed a low yield for the cowpea at higher salinity and low pH. To improve the yield of cowpea in a saline soil with low pH, it is essential to reduce the soil pH to a range of 6-8 and desalinate to enhance the growth of the cowpea as well as the *Rhizobium* sp. associated with it.

Key words: Cowpea - *Rhizobium* association · salt concentrations · pH

INTRODUCTION

The rhizosphere is a complex ecosystem consisting of the soil immediately adjacent to the roots, the root surface and its overlaying slime coat and the endorhizosphere [1]. This sphere about the root is influenced by root activities such as the exudation of organic substances (amino acids, organic acids, sugars and carbohydrates) and several other chemicals including salts. *Rhizobium* species are soil bacteria, which display symbiotic interaction with specific legume hosts and most of these are sensitive to fluctuations in the quality and quantity of chemicals in the rhizosphere and hence affect the growth and productivity of the whole plant [2]. Most crops are sensitive to relatively high levels of salinity and acidity. In the case of legumes, there are additional problems because it is not only the plants but also the symbiotic R*hizobium* bacteria, which are sensitive both at the free-living stage as well as during the symbiotic relationship [3]. Mensah *et al.* [4] have reported that if the salt concentrations are raised above 1.5% NaCl in the root environment of some leguminous plants such as *Leuceana leucocephala*, the biomass of the plant reduces. Thus, according to Rafiq [5], at higher salt concentrations, the nodules associated with the *Leuceana* sp. are more numerous but reduced in size. The effects of varying pH levels on the growth of *Rhizobium* sp. in pure culture and *Rhizobium* inoculated plants have been recorded by some workers [6,7].

The present study aims at determining the effects of different salt concentrations and pH levels on the growth of the cowpea and *Rhizobium* species associated with the cowpea in the field.

MATERIALS AND METHODS

Sources of materials: Fresh and healthy root nodules from four weeks old cowpea plants were collected and thoroughly washed with sterile distilled water and then surface sterilized using 70% alcohol. The nodules were then crushed in a drop of sterile water on a sterile glass slide using a scapel. The suspension obtained was then streaked on petri-dishes containing Yeast Extract Mannitol Agar (YEMA) composed of the following

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dissolved in 1000 mL of water: NaCl 0.1 g, Mannitol 10.0 g, $MgSO_4.7H_2O$ 0.2 g, $CaCO_3$ 0.5 g, Yeast extract 0.5 g, Agar 15.0 g, $FeCl_2$ $6H_2O$, 0.02 M (4 drops).

The *Rhizobium* strain obtained during the isolation procedure was sub- cultured on a series of YEMA plates to obtain a pure culture. The stock culture of pure *Rhizobium* was inoculated on a YEMA plate for 48 h at room temperature (26±2°C) and then stored at 4-6°C for use in the various experiments of this study.

Varying Sodium Chloride and pH levels on growth of *Rhizobium* isolates: A bacterial suspension from the pure isolates of *Rhizobium* was prepared and inoculated into six replicates of broth tubes containing the same nutrients used in the isolation process (but no agar) at different salt concentrations to obtain 0.005, 0.010, 0.050, 0.1000 and 0.200 M of NaCl [5]. A seventh set with no NaCl other than the 0.1 g/10000 mL required by the microbe for healthy growth was prepared to serve as control. The bacterial population was adjusted to 10^3 cfu/mL of the broth culture and was incubated at room temperature (26±2°C) and allowed to grow for 10 days. After the 10^{th} day of incubation, the absorbance of the resultant growth of the bacteria in the broth tubes was measured using a spectrophotometer at a wavelength of 540 nm. The population count, of each broth tube was determined using the methods of Vincent [8]. To determine the effects of pH on the rate of growth of *Rhizobium*, the procedure above was repeated using nutrient media at different pH levels. This was achieved by adjusting the media to varying pH values from 3-9 using a pH meter and 0.5 M NaOH/HCl stock solutions. The initial bacterial count for each broth tube was adjusted to 10^3 cfu/mL of broth culture. At the end of the 10^{th} day, the population count of each broth tube was then determined.

Salt concentrations and pH on agronomic charactes of *V. unguiculata*: A thick suspension of agar cultures of the *Rhizobium* in 10% sucrose solution was made. The suspension was made to a concentration of 10^3 cells/seed following the methods outlined in an earlier report by Vincent [8]. The cowpea seeds were sterilized by washing them 1% HgCl solution followed by copious rinsing with sterile water. The suspension was then used to surface inoculate the sterilized cowpea seeds. Surface inoculation of the seeds were carried out by placing some seeds in a beaker and pouring the suspension of inocula into it and stirred thoroughly before planting in sterile loamy sand soil.

Table 1: Effects of different salt concentrations on the growth of *Rhizobium* in broth tubes for 10 days

Conc. of salt (NaCl) M	Absorbance at 540 nm	Population count cfu/mL x10^4
0.000	0.83	29.3
0.005	0.85	30.0
0.010	0.90	31.6
0.020	0.80	22.5
0.050	0.62	19.2
0.100	0.41	12.5
0.200	0.24	7.4
LSD (p=0.05)	0.13	4.5

The soils for the planting were maintained at different pH values of 3, 4, 5, 6, 7, 8 and 9 by irrigating the soil with a buffer solution of appropriate pH. The salt concentrations were maintained at 0.0, 0.005, 0.010, 0.20, 0.05, 0.100 and 0.200 M of NaCl solutions throughout the growth period to obtain the desired salinity pH and salinity checks were carried out on weekly bases and the levels of either of them adjusted using the appropriate solutions. The growth rates of the plants in the treated soils were assessed by determining the survival percentage at 21 days after planting, plant height, shoot and root dry weight and seed yield/plant. The number and sizes of the nodules (measured as weight/10 nodules developed/plant) were also recorded at the onset of flowering (42 days after planting).

RESULTS

There were slight increases in the optical densities of the broth between 0.005 and 0.010 M (NaCl; Table 1) and then decrease in optical density (as indicated by mean absorbance) with increasing concentration of salt. Significant differences ($p \leq 0.05$) were recorded between absorbance readings of the *Rhizobium* growing in low salt concentration and those in high salt concentrations. There were changes in the population count of the bacterial in the growth broth. The growth indicates that the bacteria preferred lower salt concentrations and grew heavily with a higher count whereas at higher salt concentrations the growth was scanty and recorded only sight increases in population over the initial density of 10^3cfu/mL of broth culture. The observed differences in growth (population count) were significant from each other ($p \leq 0.05$). The field studies indicated decreases in survival percentages, shoot and dry weights; plant height and seed yield with increasing salt concentrations (Table 2). The number of nodules per plant generally

Table 2: Effects of different salt concentrations on nodulation and some Agronomic characters of Cowpea Inoculation with *Rhizobium*

Concentration (m)	Survival (%)	No. of Nodules/ Plant	Mean wt. of 10 nodules (g)	Shoot dry wt. (g)	Root dry wt. (g)
0.000	86.6	52.7	2.1	10.8	4.4
0.005	88.2	56.5	2.4	11.5	4.5
0.010	75.4	53.3	2.0	12.1	4.5
0.020	68.1	57.4	1.8	10.4	3.9
0.050	51.6	55.3	1.9	10.6	3.4
0.100	45.8	38.6	1.6	8.5	2.8
0.200	22.8	30.7	1.1	7.6	2.9
LSD (p=0.05)	15.4	14.2	0.5	3.5	0.7

Table 3: Effects of different pH values on the growth of *Rhizobium* in broth tubes for 10 days

pH value	Absorbance at 540 nm	Population count cfu/mL x 10^4
3	0.20	0.52
4	0.33	11.30
5	0.45	15.40
6	0.80	22.40
7	0.84	29.20
8	0.65	20.70
9	0.38	13.00
LSD (p=0.05)	0.12	3.50

Table 4: ffects of different pH levels on nodulation and some agronomic characters of cowpea plants inoculated with *Rhizobium*

pH level	Survival (%)	No. of Nodules/ Plant	Mean wt. of 10 nodules (g)	Shoot dry wt. (g)	Root dry wt. (g)
3	20.2	11.7	1.1	3.7	3.8
4	55.3	36.5	1.6	8.4	4.2
5	54.7	44.5	1.9	9.8	4.9
6	79.6	57.3	1.9	11.2	5.7
7	88.9	60.8	2.1	10.8	5.8
8	68.7	58.9	2.1	9.5	4.6
9	40.3	45.1	1.8	8.0	4.7
LSD (p=0.05)	10.9	19.2	0.4	2.7	0.9

increased with increasing salt concentration between 0.005-0.050 NaCl but nodule weight was maintained at near control levels. However at higher concentrations of 0.100-0.200 M, there were simultaneous reductions in number of nodules and size (measured as wt. of 10 nodules).

The effect of pH on the *Rhizobium* strain under investigations is given in Table 3. At pH 3, the bacterial count was least ($0.52x10^4$cfu/mL) while the highest bacterial count $29.2x10^4$ cfu/mL was recorded at pH 7. Similarly, the absorbance readings of the broth under different pH levels revealed that pH 3 recorded the lowest absorbance while pH 7 recorded the highest. The difference in absorbance values between pH 3 and pH 7 was significant ($p \leq 0.05$).

There were changes in survival percentage, number of nodules/plant, nodule size shoot and root dry wt and seed yield due to pH. In all parameters, a pH range of 5-8 recorded optimal values (Table 4). The lowest values in terms of survival percentages and seed yield were record at pH 3.

DISCUSSION

The results of the present studies revealed that salt concentrations in the growth medium ($p \leq 0.05$) had significant effect on the growth of *Rhizobium*. Table 1 indicates an increase in the rhizobial growth between 0.005-0.010 M NaCl concentrations and then decreases at higher concentrations of 0.020-0.200M. The results also provide an explanation with respect to which samples are identical in terms of the response to the salt concentrations and which samples are significantly different from each other. The results recorded significant differences in the mean values of growth in terms of cfu/mL and absorbance due to the salt treatment as determined by the Least Significant Difference (LSD) at 5% probability.

Rhizobium strains capable of growing at NaCl concentration of up to 0.500 M have been isolated from a melilotus plant [3]. However from this study, the *Rhizobium* strain isolated from cowpea grew lightly in terms of colony formation and population density at 0.200 M NaCl salt concentrations, but the growth was heavy at lower salt concentrations (0-0.005 M NaCl). Previous studies reported by Talibart *et al.* [9], have shown that changes in osmotic potential exerted by salt concentration alter the structure of lipopolysaccharides of bacteria in response to salt stress and that rhizobia accumulate several solutes to overcome the osmotic stress induced by salt when growing in association with the host plant. It has been reported by Diejomaoh [10] that morphological changes including reduced plant height and yield are mainly due to the effect of the salt on the plant rather than changes due to the symbiotic relationship with the *Rhizobium*. Furthermore, Diejomaoh [10] stated that the reductions in plant height and dry weight biomass are as a result of decreases in the size of the individual cells/ structures which enable the plant to accumulate solutes to balance the high osmotic pressure in the rhizosphere medium.

The result of the effects of different pH values on the growth of *Rhizobium* species in culture shows that the *Rhizobium* strain grew well at pH values of between 6-8 with optimal growth density at pH 7 in accordance with Selmen [11], who recorded that the optimum pH of the growth of *Rhizobium* is between 6 and 7. In the present study, pH 7 also recorded maximum absorbance for the broth experiment as well as exhibited heavy growth as measured by the population count on the 10^{th} day after incubation. At a pH range of 6-8, there were maximum values in plant height, dry weights and yield and this is similar to earlier observations [12]. The reduced plant height and yield recorded at lower pH values of 3 and 4 and at higher pH value of 9 are as a result of the effects of the pH on both the *Rhizobium* strain and the host plant. The sensitivity for red clover rhizobia to soil acidity in pure culture and as symbionts with the host plant has previously been reported by Lindstrom and Myllyniemi [13]. The successful germination and establishment of *Vigna* species under acid soil conditions of pH 4-6 recorded in the present report indicated that it may be tolerant to acid soils. However, acidity *per se* does not appear to affect the growth of most plants and that the effects of pH are secondary- increasing the solubility of Mn and other heavy metals as well as Al under acidic conditions.

Franco and Munns [14] have demonstrated the importance of acidity and aluminum toxicity in limiting nodulation and nitrogen fixation on the growth of *Phaseolus vulgaris.* Aluminum toxicity restricts root growth and phosphorus uptake and hence affects productivity. Acidity has direct effect on the survival and growth of *Rhizobium* bacteria, which fixes nitrogen in association with legumes as observed in the present study. Since very scanty growth was recorded for the *Rhizobium* at pH 3 on a few of the plants those plants that survived at that treatment level relied more on different sources of nitrogen for their growth activities, other than what is fixed by *Rhizobium.* Thus, the cumulative effects of the increased concentration of Al and Mn in the soil as a result of soil acidity as well as effects of acidity on the *Rhizobium,* resulted in the observed decreases in the shoot/root dry weight and seed yield/plant recorded in this report.

It could be suggested that cowpea performs poorly in terms of biomass accumulation and seed yield in acidic soils of low pH (<4) and high salinity (>0.050 M NaCl) as a result of effects of these factors on the plant and its associated symbionts. Both acidity and high salinity reduced the growth rate and survival of the *Rhizobium* sp. and hence the productivity of the host plant.

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