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## Personnel involved in the research

The research was carried out by the principal investigator with the assistance of a part time research technician, Nancy Matchett, and several undergraduate research aides. These included Gary Schlosser, now employed at the Los Alamos Scientific Laboratory; Thomas Munson, a student in mechanical engineering; and Virginia Salas who is completing a degree in biology. These outstanding students were supported, in part, by the Crimson Scholar Program at NMSU. Karen Zahler also served as a research assistant. She has recently completed a M.S. degree in Botany at Ohio University. No graduate students were supported by the work.

Abstract

The physiology of the response of R. meliloti to salt stress was investigated. It was found that the growth of the bacterium is not inhibited simply as a consequence of the increased osmolarity of the medium caused by the addition of salts but by the effect of a particular salt. The bacteria were shown to accumulate glutamic acid in response to inhibitory concentrations of sodium chloride suggesting that this amino acid may act as an osmoticum. However, the addition of glutamic acid to the growth medium did not protect the bacterium from inhibition by sodium chloride even though the amino acid was transported and metabolized.

The effects of salts commonly found in irrigation water on growth of Rhizobium meliloti, the bacterium that establishes a symbiotic relationship with alfalfa (Medicago sativa) were investigated to determine how this might affect alfalfa production. The growth rate of this bacteria was not affected by sodium, potassium, magnesium, chloride, sulfate, bicarbonate or phosphate, except in concentrations much greater than can be tolerated by alfalfa cultivars currently in use. Growth of this bacteria was not affected by alkaline pH. These results indicate that saline and alkaline conditions do not limit alfalfa yields because of the effects of these conditions on growth of the symbiotic bacteria.

The work was expanded to include the rhizobia that establish a symbiotic relationship with peanuts (Arachis hypogaea). 19 strains were tested for the effect of saline and alkaline conditions on the growth rate of these bacteria. With one exception, the growth of all strains was inhibited by modest levels of sodium chloride when the pH was slightly alkaline. These preliminary results indicate that saline and alkaline conditions could limit peanut yields because of the effect on these condition on growth of the symbiotic bacteria.

Key Words: Rhizobium, Alfalfa (Medicago Sativa), Peanuts (Arachis hypogaea), salinity, alkaline, osmoregulation

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# OVERALL SUMMARY AND CONCLUSIONS

## Introduction

Bacteria of the genus Rhizobium are of great importance in agriculture because these bacteria can establish a symbiotic relationship with legume plants that results in biological nitrogen fixation. These bacteria reduce atmospheric nitrogen ($N_2$) to form ammonia ($NH_3$) which can be utilized by the plant for amino acid synthesis. The plant does not then require chemical fertilizers. Synthesis and distribution of chemical fertilizers is very energy intensive. In third world nations, the political, economic and educational infrastructure required for manufacture and distribution of chemical fertilizers limits their use.

## Objectives

The question of how non-halophillic bacteria, such as the rhizobia, respond to salt stress is a question of basic biological significance (Yancey et al., 1982). Bacteria could provide a useful model system for investigations of the phenomenon. R. meliloti has a sophisticated genetic system that can be exploited. Thus, quite apart from its agricultural importance, it provides a convenient system to study the fundamental biological phenomenon.

However, the question of how rhizobia respond to salt stress is also a question of immediate practical importance since these bacteria enter into a valuable symbiotic relationship with legume plants. This research project was initiated investigate the physiological response of R. meliloti to salt stress and to determine if biological nitrogen fixation by rhizobia could be adversely affected by the bacterium's ability to contend with

alkaline and saline conditions found in water used for irrigation. An examination of the literature indicated that most research with rhizobia has centered on the effects of acidic conditions and the effects of minerals such as aluminum that are mobilized by acidic conditions. Questions germane to arid regions have not been investigated extensively. The work was inititiated with Rhizobium meliloti, the bacterium that infects alfalfa (Medicago sativa). The basic physiological problem of how the bacterium deals with saline and alkaline conditions was addressed. Using the techniques and insight gained from these initial studies, it was possible to access the possible effects these conditions would have on the production of alfalfa. At the request of Dr. David Hsi (Middle Rio Grande Experiment Station), the research was expanded to include rhizobia that infect peanuts (Arachis hypogaea).

**Conclusions**

The response of non-halophillic bacteria to salts is not a familiar area. Our research represents the first time this question has been addressed in Rhizobium meliloti. Our results showed that the growth of R. meliloti is not inhibited by high osomolarity per se. Some salts, magnesium chloride for one, inhibited growth at fairly low concentrations while others, sodium chloride for example, inhibited growth at concentrations making the osmolarity of the medium much higher. When grown with high concentrations of sodium chloride, R. meliloti like many other non-halophillic bacteria, was found to accumulate glutamic acid in very large amounts. The role of this accumulation is uncertain. Glutamic acid, when added to the growth medium, did

not make the bacteria more resistant to growth inhibition by salt (Botsford, 1983a).

The effect of salts and alkaline pH on growth of R. meliloti with respect to alfalfa production was examined. Strains isolated from alfalfa fields in New Mexico and "classical" laboratory strains were tested and all grew best at slightly alkaline pH. Several strains isolated from alfalfa fields grew well at pH 9. The alkaline pH encountered in irrigation water should not limit the growth of R. meliloti. Of the various cations tested: sodium, potassium, magnesium, ammonium, none were found to inhibit growth except in concentrations much greater than are ever encountered in agricultural soils. For example, sodium chloride was found to inhibit growth rate by 50% at a concentration of 291 mM, about 17,000 ppm. Similarly, the anions tested, chloride, sulfate, bicarbonate, and phosphate inhibited growth only at very high concentrations. These results show that production of alfalfa in arid regions does not appear to be limited by the bacterial symbiot's ability to grow under saline and alkaline conditions (Botsford, submitted for publication).

In contrast, the work with the rhizobia that infect peanuts showed that growth of these bacteria is inhibited by alkaline pH (pH 8.0) and by quite modest concentrations of sodium chloride, particularly when the pH is alkaline. These results, although preliminary, suggest that the production of peanuts could very well be limited by the symbiotic bacterium's ability to deal with saline and alkaline conditions (Botsford, 1983b).

## Studies of the physiological response of *R. meliloti* to salt stress

**Introduction:** Traditionally, media for rhizobia contain much lower concentrations of minerals than do media designed for the more familiar enteric coliform bacteria (Vincent, 1975). There are some reports that most strains of *R. meliloti* are unable to grow in media intended for enteric coliform bacteria (Meade and Signer, 1977). These observations suggested that rhizobia could be unusually sensitive to the effects of minerals or conditions of high osmolarity. The initial goal of the research was to characterize the response of *R. meliloti* to inhibitory concentrations of salt.

Towards this goal, a completely defined medium of low osmolarity was developed affording growth rates comparable to those observed with undefined media containing yeast extract. Using this medium, the effect of various salts on the growth rate of *R. meliloti* was determined. The effect of such potential osmotica as proline and glutamic acid on the inhibition of growth was also determined.

### Materials and Methods

*Cultures.* *Rhizobium meliloti* NSI was obtained from Dr. Harold Evans (Oregon State University, Corvallis, Oregon). *R. meliloti* 102f51 was obtained from Dr. Winston Brill (University of Wisconsin, Madison, Wisconsin). Cultures were maintained on VM agar slants (Vincent, 1975) and were transferred several times each year. Both strains were able to nodulate alfalfa. Several

times in the course of the experiments, these strains were retested for their ability to form nodules with axenic alfalfa plants (Vincent, 1975).

Media. The complex medium, (VM) contained per liter: 0.5 g $K_2HPO_4$; 0.4 g yeast extract; 0.1 g NaCl. Mannitol (1% w/v) was used as a carbon energy source. The final pH of this medium was found to be 7.5. 1.5% agar was added for solid medium (Vincent, 1975).

The completely defined medium (MDM) contained: MOPS (morpholinopropane sulfonic acid), 10 mM; Tricene (N-tris(hydroxymethyl)methyl-glycine), 1 mM; ammonium chloride, 10 mM; potassium phosphate, 1 mM; potassium sulfate, 0.270 mM; magnesium chloride, 0.6 mM; calcium chloride, 10 uM; ferric chloride, 1 uM; boric acid 64 nM; cobalt chloride, 6 pM; copper sulfate, 44 pM; manganese sulfate, 6 pM; molybdic acid, 3 nM; riboflavin, 5 uM; p-amino benzoic acid, 1.4 uM; nicotinic acid, 16 uM; biotin, 8 uM; pyridoxine HCl, 10 uM; thiamin HCl, 6 uM; inositol, 7 uM. Mannitol at a final concentration of 1% (w/v) was used as carbon energy source. The pH of the medium was adjusted to 7.5 with KOH. The medium has a calculated osmolarity (the sum of concentrations of all ions and solutes with the exception of $H^+$) of 73 mOs (milliosmolar). The pH of components found to alter the pH (e.g. glutamic acid) was adjusted to 7.5 before addition. The medium was usually made up in 2X concentration to permit the addition of the various solutes. Solutes were usually made up in 10X the final concentration. The medium was sterilized by filtration.

Growth Conditions. Inocula were grown in 10 ml VM or MDM for approximately 24 hours. If grown in VM, cells were washed three times with sterile twice distilled water. Enough cells were used to inoculate 10 ml cultures in MDM with the compounds to be tested to provide an initial turbidity of approximately 10 Klett Units. Comparable results were obtained when inocula were prepared in either VM or MDM.

Cells were grown in 50 ml Erlenmeyer flasks fitted with side arms permitting measurements of the turbidity of the culture. Flasks were filled to no more than 20% capacity. All growth rates were determined with cells growing at 30 C in an incubator shaker.

The turbidity of the culture was measured with a Klett Summerson Colorimeter fitted with the green filter. Measurements for growth were terminated at 150 KU. 150 KU was found to be equivalent to $1.4 \times 10^9$ cells $ml^{-1}$ and to an absorbancy at 660 nm of 0.44 measured with the Bausch and Lomb Spectronic 20 or 0.636 measured with the Pye Unicam 350 sepctrophotometer. Cell numbers were no longer linear with respect to absorbancy above this value. The pH of the medium was not changed when experiments were terminated at this absorbancy. This medium permitted the strains used to grow with generation times of approximately 2.8 $hr^{-1}$.

The growth rates of cultures in exponential phase were determined from linear regressions of $\log_{10}$ absorbancy (turbidity) vs. time (in h). Typically 10 to 12 measurements of the absorbancy were used. Data were not used if the $r^2$ for the

regression line was less than 0.95. The instantaneous growth rate, $\mu$, is defined as ln 10 x slope and has dimensions of $h^{-1}$. g, the generation time for the culture, is equal to $\ln 2/\mu$. Within experiments, the standard error of the mean was approximately 10%. Differences of less than 10% were considered to be significant only when the values reported represent means from several different experiments.

With the exception of the amino acid analysis, nearly all experiments were done with both strains NSI and 102f51. Results were comparable with both strains. The results with 102f51 are reported.

Amino acid analysis of cells. For amino acid analysis, 100 ml cultures were grown overnight in MDM medium with additions as indicated. Cells were harvested by centrifugation. After centrifugation, centrifuge tubes were drained and the inside of the tubes were wiped dry. 10 ml 50 mM sodium citrate buffer (pH 5) were added to each centrifuge tube. Tubes were then placed in boiling water bath for 2 minutes. Cell debris was removed by centrifugation at 25,000 x G for 15 min. The supernatant fraction was analyzed for free amino acids with a Durrum 400 amino acid analyzer using nor-leucine as the internal standard. The pellet fraction was resuspended in 10 ml 0.1 NaOH and analyzed for protein content by the method of Lowry et al. (1951) using bovine serum albumin as standard.

**Results**

Inhibition of growth of R. meliloti by salts: In Table 1 the results of many experiments to determine the effects of various

salts are summarized. Sodium chloride, in concentrations of 100

Table 1

**Inhibition of growth of *R. meliloti* 102f51 by various salts**

| Addition | Concentration | Relative Growth[a] | Concentration for 50% reduction[b] |
|---|---|---|---|
| NaCl | 100 mM | 96 | 291 mM |
| $Na_2SO_4$ | 50 mM | 94 | n.d. |
| $NaCH_3COO$ | 100 mM | n.g. | 58 mM |
| $NaHCO_3$ | 100 mM | 26 | 50 mM |
| $NaPO_4^{--}$ | 100 mM[c] | 36 | -- |
| KCl | 100 mM | 89 | 281 mM |
| $K_2SO_4$ | 50 mM | 108 | n.d. |
| $KPO_4^{--}$ | 100 mM | 58 | -- |
| $MgCl_2$ | 50 mM | n.g. | 26.9 mM |
| $NH_4Cl$ | 100 mM | 100 | -- |

[a]Relative Growth: Instantaneous growth rate $\mu$ for the sample divided by the instantaneous growth rate for the control(s) x 100. n.g., no growth.

[b]Concentration for 50% reduction of the instantaneous growth rate: The values were calculated for those solutes that inhibit growth in proportion to their concentration from a linear regression analysis of growth rate vs concentration using the expression $Y/2 = mx + b$ where x = that concentration of solute that reduces the maximum growth rate by half and b = the Y intercept for the regression line. The results from several experiments with each salt were included. At least 5 concentrations of each solute were used. If the regression coefficient ($r^2$) was less than .8 it was assumed that there was not a linear relationship between solute concentration and growth rate. These are indicated by --. n.d., not determined.

mM is only slightly inhibitory for *R. meliloti*. Potassium chloride and sodium chloride inhibited growth comparably (Table 1, lines 1 and 6) indicating that these two ions, potassium and sodium have similar effects.

Magnesium ion was found to inhibit growth significantly at much lower concentrations than sodium or potassium (Table 1, line 8). Ammonium ion was found to inhibit growth completely at concentrations greater than 100 mM but to have no effect on growth at a concentration of 100 mM (Table 1, line 9).

Growth of the bacteria was affected by the anion present. While 100 mM sodium chloride and 50 mM sodium sulfate had little effect on growth, 100 mM sodium acetate inhibited growth of the bacterium completely (Table 1, line 3). Sodium propionate at the same concentration had little effect on growth indicating that the effect is specific for the acetate ion. Similarly 100 mM sodium bicarbonate inhibited growth significantly indicating that the bicarbonate ion is inhibitory (Table 1, line 4).

The effect of phosphate was anomalous. When phosphate was present at a concentration of 25 mM or greater, growth was inhibited. This inhibition of growth did not appear to be due to the potassium or sodium present as a counter ion. 100 mM sodium (or potassium) phosphate buffer is 166 mEq with respect to sodium ion when poised at pH 7.5. This concentration of sodium or potassium phosphate inhibits growth no more than 25% (calculated from the data showing the concentration of sodium and potassium required for 50% inhibition of growth). 100 mM potassium phosphate inhibits growth much more than 60% (Table 1, line 5) indicating that the phosphate per se is inhibitory. The relationship between inhibition of growth and the concentration of phosphate was not linear. When phosphate was present at 100 mM or greater, the results were variable. In some experiments the lag phase of the culture was protracted, sometimes as much as 24

hours. After this lag, cells grew at a reduced rate. Cells could be taken from cultures which had emerged from lag phase, washed and inoculated into fresh medium with 100 mM phosphate. Cells would grow without a lag phase but at the characteristic low rate. In other experiments, the lag phase was not protracted but the growth rate of the cells was comparable to that of cultures after the protracted lag phase.

Neither sodium chloride nor sodium sulfate was found to inhibit growth at a concentration of 50 mM (Table 1, lines 2 and 7). The inhibition of growth by magnesium chloride and magnesium sulfate was comparable. These results indicate that chloride and sulfate anions have comparable effects on growth.

The effect of most of the solutes at increasing concentrations was tested. For those solutes that inhibited growth in direct proportion to their concentration, the concentration inhibiting growth by 50% was calculated (Table 1, right column) using the slope of the linear regression fitted to the data. The concentration of solutes required for 50% inhibition of growth show that the growth rate of cells is not influenced simply by increased osmolarity but to the particular ion. For example, 100 mM sodium chloride, sodium bicarbonate, and sodium acetate all increase the osmolarity of the medium by 200 mOs yet these three salts do not inhibit growth comparably.

The addition of 400 mM glycerol, increasing the osmolarity of the medium by 400 mOs, did not affect growth of R. meliloti again showing that increasing the osmolarity of the growth medium is not inhibitory (data not presented).

**Effect of Added Osmotica:** Solutes which cells accumulate in response to increased osmolarity are termed "osmotica" (Greenway and Munns, 1980). Proline appears to serve as an osmoticum for Salmonella typhimurium (Csonka, 1980) and for Staphylococcus aureus (Koujima et al., 1978). Other non-halophilic bacteria accumulate glutamate or gamma amino butyric acid (Measures, 1975). The effect of added glutamate and proline on inhibition of growth by sodium chloride, sodium acetate, potassium phosphate and magnesium chloride was tested (Table 2). With one exception, experiment 3, the addition of glutamate or proline to the growth medium had little effect on the growth rate. With added sodium chloride, neither glutamate nor proline had resulted in a significant stimulation of growth. Glutamate appeared to have a slightly stimulatory effect with respect to the inhibition of growth caused by sodium acetate and magnesium chloride. Glutamate did not have a consistent stimulatory effect with respect to the addition of phosphate. But as pointed out in the previous section, the effect of phosphate was variable.

Betaine is accumulated by some plants in response to salt stress (Greenway and Munns, 1980). The addition of betaine, like the addition of proline or glutamate had little effect on the growth rate of R. meliloti in the presence of any of the four salts tested (data not presented). Glutamate, proline, and betaine could serve nearly as well as ammonium as sole sources of nitrogen indicating they are transported and metabolized by R. meliloti.

Table 2

**Effect of added glutamate and proline**

| | | Relative Growth Rate[a] | | |
|---|---|---|---|---|
| | addition | none | glutamate[b] | proline |
| Expt. 1 | none | 100 | 96 | 114 |
| | NaCl, 400 mM | 28 | 39 | 43 |
| Expt. 2 | none | 100 | 112 | 112 |
| | NaCl, 250 mM | 50 | 46 | 63 |
| Expt. 3 | none | 100 | 134 | -- |
| | NaCl, 250 mM | 51 | 84 | -- |
| | NaCl, 400 mM | 29 | 30 | -- |
| Expt. 4 | none | 100 | 102 | 91 |
| | $NaCH_3COO$, 50 mM | 33 | 65 | 40 |
| | $KPO_4^{--}$, 100 mM | 69 | 79 | 73 |
| | $MgCl_2$, 20 mM | 53 | 69 | 57 |

[a]Relative growth rate as in Table 1.

[b]Concentration of glutamate and proline added was 1 mM in experiment 1 and 10 mM in the other experiments.

**Accumulation of Glutamate:** The results of experiments to determine if glutamate or proline is accumulated by cells growing in inhibitory concentrations of sodium chloride are shown in Table 3. These experiments show that the accumulation of glutamate in the cellular fraction is at least 5 times greater when cells are grown with 250 mM NaCl than in the controls. It should be pointed out that cells were not washed and no correction was made for amino acids trapped in the intercellular space. Therefore, these values show only relative increases in

the glutamate present and can not be used to calculate the intracellular concentration of the amino acid. These data, nevertheless, show that R. meliloti accumulates glutamate in response to the addition of 250 mM NaCl when growing in minimal medium. Proline was detected in small amounts but its concentration did not appear to be affected by the presence of inhibiting concentrations of sodium chloride. Gamma amino butyric acid was not detected in either experiment.

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Table 3

**Accumulation of glutamate in R. meliloti**

| NaCl | Supplemental Amino acid | Accumulated amino acid[a] glutamate | proline |
|---|---|---|---|
| Experiment 1 | | | |
| 0 | -- | 26.5 | ud[b] |
| 0 | 10 mM glutamate | 59.0 | ud |
| 250 mM | -- | 132.3 | ud |
| 250 mM | 10 mM glutamate | 320.9 | ud |
| Experiment 2 | | | |
| 0 | -- | 0.9 | 0.7 |
| 0 | 10 mM proline | 12.6 | 1.7 |
| 250 mM | -- | 125.9 | 0.8 |
| 250 mM | 10 mM proline | 120.3 | 8.7 |

a Concentration of amino acids reported as u moles/mg protein

b ud, undetectable, less than 60 nmoles/mg protein

**Effects of alkaline pH and salts commonly found in irrigation water on growth of R. meliloti: Limitation of alfalfa production.**

The productivity of nearly all commercially useful legume crops is reduced by saline conditions (Maas and Hoffman, 1976). The sensitivity of the symbiotic rhizobia to these conditions could affect the productivity of these crops (Epstein et al., 1980). The symbiosis between R. meliloti and its host plant, alfalfa, (Medicago sativa) offers a convenient system to study the effects of salts on the legume-rhizobium relationship.

Studies of the effect of salts on the growth of R. meliloti provide an initial attempt to determine if establishment of the legume-rhizobium relationship could be limited by the bacterium's ability to grow independent of the host plant. Studies of the effects of salts on the growth of Rhizobia are limited. Vincent (Vincent, 1962) determined the effects of calcium and magnesium on the growth rate and final numbers of R. trifolii. Several groups have offered data suggesting that sodium chloride can reduce the final numbers of various rhizobia including R. meliloti growing in complex media containing yeast extract (Bhardwaj, 1975; Singleton et al., 1982; Subba Rao et al., 1972; Yader and Vyass, 1971). However, results obtained with media containing yeast extract can be ambiguous because yeast extract has been shown to affect the growth of rhizobia (Skinner et al., 1977). Furthermore, the growth rate of a culture rather than the final numbers is a better indication of the ability of the bacteria to adapt to the situation. We have found that

comparable final numbers whenever cells regardless of the growth rate afforded by the experimental conditions.

The effect of alkaline pH on growth of R. meliloti was addressed because it is generally thought that rhizobia favor neutral or slightly acidic conditions (Nutman, 1975) and irrigation water is usually alkaline because of the formation of bicarbonate salts (Kamphorst and Bott, 1972).

The effect of salt on the survival of R. meliloti was examined because in many arid regions, the soil is dry between irrigation seasons. We wanted to see if the indigenous R. meliloti could be expected to persist in fields not planted with alfalfa.

**Materials and Methods**

Strains: R. meliloti NSI was obtained from Dr. Harold Evans (Oregon State University). Strain 102f51 was obtained from Dr. Winston Brill (University of Wisconsin). Five additional laboratory strains were obtained from various sources including the American Type Culture Collection. However, preliminary results with these strains were comparable to those obtained with strains NSI and 102f51. The other laboratory strains were not examined further. "Field strains" were isolated from alfalfa fields in different regions of New Mexico.

Isolation of field strains: Rhizobia were isolated from alfalfa fields by suspending 10 g soil taken from near the root in 90 ml sterile water. This soil suspension was serially diluted. One ml of each dilution was used to inoculate alfalfa plants growing axenically in test tube culture. Rhizobia were then isolated

from the root nodules of the plant receiving the highest dilution of the bacterial suspension and cloned several times by streaking on VM plates. The isolates were grown in liquid culture and were used to inoculate axenic plants a second time. The bacteria were isolated from the nodules a second time and stock cultures prepared. Strains were isolated from about 80% of the soil samples with this technique.

All strains of rhizoboia used were tested for their ability to establish effective nodules in alfalfa growing axenically. The techniques used to grow alfalfa plants axenically, to infect the plants with rhizobia, to sterilize the root nodules and to isolate the rhizobia were essentially those described by Vincent (1975).

Other techniques and media used were described in the previous section of this report and have been published (Botsford, 1983a).

**Results**

Effect of pH on growth of R. meliloti: The effect of alkaline pH on the growth of R. meliloti was examined (Table 4). These data show that with the exception of strain NSI, a laboratory strain, all strains grew well at alkaline pH. Cells were grown in MDM medium adjusted to the desired pH. Experiments were terminated at 150 Klett Units. When cells had grown to this absorbance, the pH was not changed from the initial pH. Strains MV1, MV2 and MV3 are field strains isolated from alfalfa fields receiving water from the Rio Grande (river) near Las Cruces, New Mexico. The pH of the Rio Grande at Las Cruces is about 8.0. These data indi-

cate that the alkaline pH encountered in irrigation water does not limit growth of R. meliloti.

Effect of sodium and potassium on growth: The results of numerous experiments to determine the effects of sodium and potassium on growth of R. meliloti 1025f1 are summarized in Figure 1. The MDM medium does not contain any added sodium.

Table 4

**Effect of pH on Growth of R meliloti**

| | Growth Rate[a] | | | | |
|---|---|---|---|---|---|
| pH | 102f51 | NSI | MV1 | MV2 | MV3 |
| 6.5 | 0.06 | 0.03 | 0.13 | 0.06 | 0.04 |
| 7.0 | 0.12 | 0.12 | 0.20 | 0.09 | 0.11 |
| 7.5 | 0.20 | 0.17 | 0.19 | 0.09 | 0.14 |
| 8.0 | 0.23 | 0.19 | 0.21 | 0.15 | 0.17 |
| 8.5 | 0.24 | n.g. | 0.26 | 0.16 | 0.20 |
| 9.0 | 0.21 | n.g. | 0.09 | 0.18 | 0.20 |

[a]Growth rate reported as the instantaneous growth rate, $\mu$ in units of $hr_{-1}$. n.g. = no growth. Strains 10f51 and NSI are laboratory strains, strains MV1, MV2, and MV3 were isolated from alfalfa fields in New Mexico.

These data show that sodium, at least in more than trace amounts, is not required for growth of R. meliloti. Reagent grade chemicals and twice distilled water were used in all experiments so only very small amounts of sodium were present as contaminants. Significant inhibition of growth was observed when the concentration of either NaCl or KCl is greater than 100 mM.

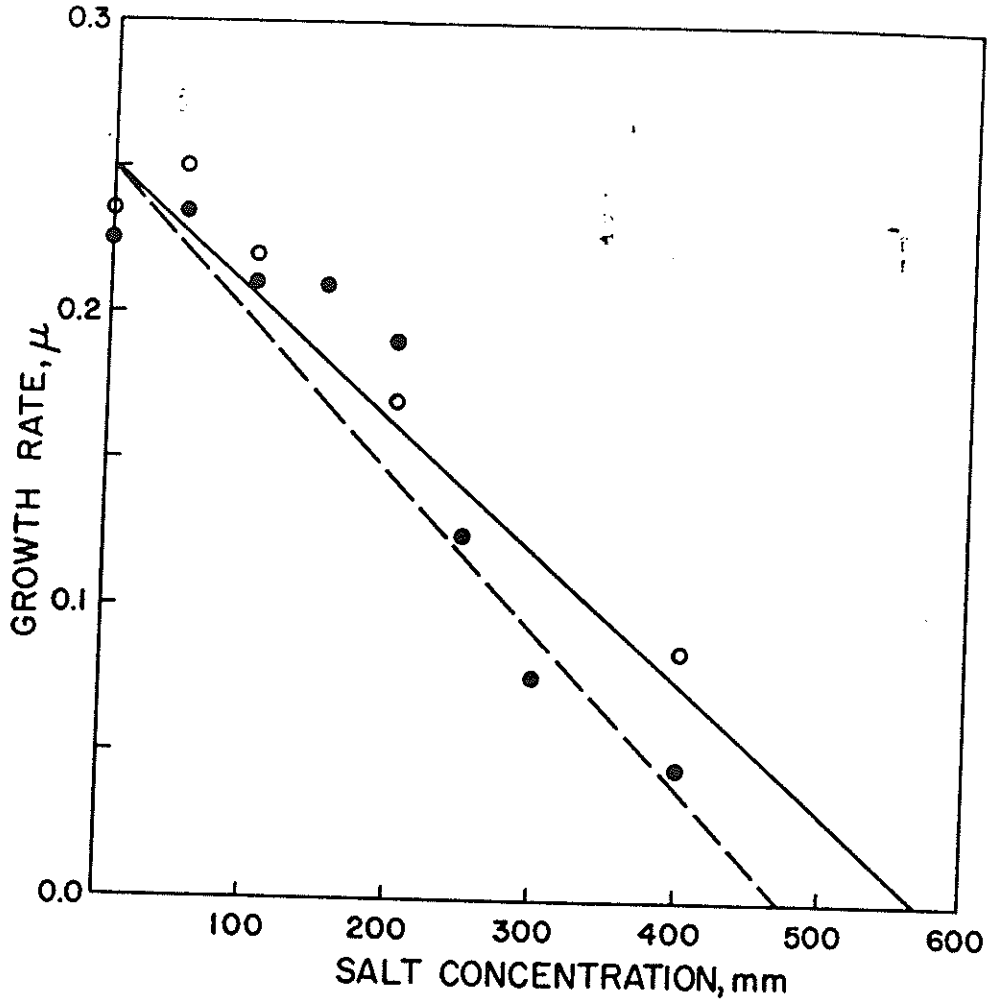


Figure 1

**Effect of sodium and potassium chloride on growth rate of *R. meliloti* strain 102f51.**

Cells were grown with MDM medium. The values presented represent the means from at least six separate experiments specifically intended to test the effects of NaCl and KCl on growth. The open circles represent the results with NaCl, the closed circles represent results with KCl. The lines are the linear regressions fitted to the points. Growth rate is reported as in Table 1. The concentration of NaCl resulting in a 50% reduction in the growth rate was determined to be 291 mM while the concentration of KCl resulting in 50% reduction of the growth rate was determined to be 251 mM. These values were calculated from the slope of the least squares fit regression line of growth rate vs. concentration of the salt with the relationship $Y/2 = mx + b$ where $Y/2$ = 50% of the maximum growth, b is the Y intercept for the regression line, and x is the concentration of salt required to reduce growth by half.

NaCl and KCl inhibited the growth rate of strain 102f51 by half at concentrations calculated (as described in the figure legend) to be 291 (equivalent to 6693 ppm $Na_+$) and 251 mM (equivalent to 9814 ppm $K_+$) respectively.

Both sodium chloride and potassium chloride at a concentration of 50 mM appeared to stimulate growth of the bacterium slightly. The stimulation of growth of strain 102f51 by 50 mM NaCl was examined in more detail. The addition of a 10 fold excess of the trace elements mixture did not stimulate growth of this strain indicating the the medium is not limiting for one of these elements. Thus stimulation of growth by the sodium ion was probably not due to contaminating trace elements in the salt. The increase in osmolarity of the growth medium by the addition of 50 mM NaCl is not responsible for this stimulation because the osmolarity of the medium could be increased by addition of 100 mM MOPS or even 400 mM glycerol without affecting the growth rate (results not presented). Similar results were obtained with strain NSI and other strains indicating the effect is not peculiar to strain 102f51.

The effects of increasing concentrations of $Na_2SO_4$ and $K_2SO_4$ were comparable to the effects of NaCl and KCl indicating that chloride and sulfate anions have comparable effects (data not presented).

In Table 5, the results of experiments to test the effect of sodium and potassium ions on growth of some of the strains isolated from irrigated alfalfa fields in New Mexico are presented. These data show differences among the various strains were not dramatic. With the exception of strain CHA, a field

isolate, the growth of none of the strains was inhibited more than 10% by the addition of 100 mM NaCl. And with the exception of strain BES, growth of all the strains was inhibited slightly more by potassium ion than by sodium ion.

Strain MV3, when first isolated, required NaCl at a concentration of at least 10 mM for growth. Potassium chloride would not replace the requirement for sodium. After several

Table 5

**Effect of salts on growth rate of field strains of *R. meliloti***

| | Relative Growth Rate[a] | | | |
|---|---|---|---|---|
| | Salt Added[b] | | | |
| Strain | NaCl | KCl | $Na_2SO_4$ | $K_2SO_4$ |
| CHA | 80 | 62 | 73 | 64 |
| A110 | 96 | 97 | 98 | 102 |
| A111 | 90 | 92 | 97 | 98 |
| H550 | 101 | 77 | 96 | 90 |
| BES | 128 | 73 | 131 | 71 |
| MV1 | 94 | 117 | 125 | -- |
| MV2 | 116 | 113 | 109 | -- |
| MV3 | 106 | -- | -- | -- |

[a]Relative Growth Rate (as in Table 4) of culture growing in the presence of the salt/growth rate of culture growing in the absence of the salt x 100.

[b]Sodium and potassium chloride present in 100 mM concentration. Sodium and potassium sulfate present in 50 mM concentration.

transfers over a six month period, this strain no longer exhibited the requirement. This strain was still capable of nodulating alfalfa and reducing acetylene after losing the

requirement for sodium. This observation suggests that the response of R. meliloti to salt can be altered after subculturing in laboratory media.

Interaction between sodium chloride and pH. Since both acidic pH and sodium chloride in high concentrations inhibit growth of R. meliloti, it was desired to determine if these two parameters interact with respect to inhibition of growth. The data presented in Figure 2 are representative of a series of experiments with various strains. These data show that NaCl inhibits growth more severely when the medium is slightly acidic than when the medium is neutral or slightly alkaline. This indicates interaction between acidic pH and salinity. Similar results were obtained when the concentration of NaCl was 100 mM, inhibition of growth was significant when the medium was poised at an acidic pH but was only minimal at neutral or alkaline pH. These data show that the alkaline pH of irrigation water does not exacerbate the inhibition of growth by salinity.

Inhibition of growth by additional solutes. The effect of the other salts commonly found in irrigation water including phosphate, magnesium, bicarbonate, and ammonium, were tested. The results of representative experiments with strain 102f51 are presented in Table 6. These concentrations are all much greater than are normally encountered in water used for irrigation (Goldman and Wetzel, 1966).

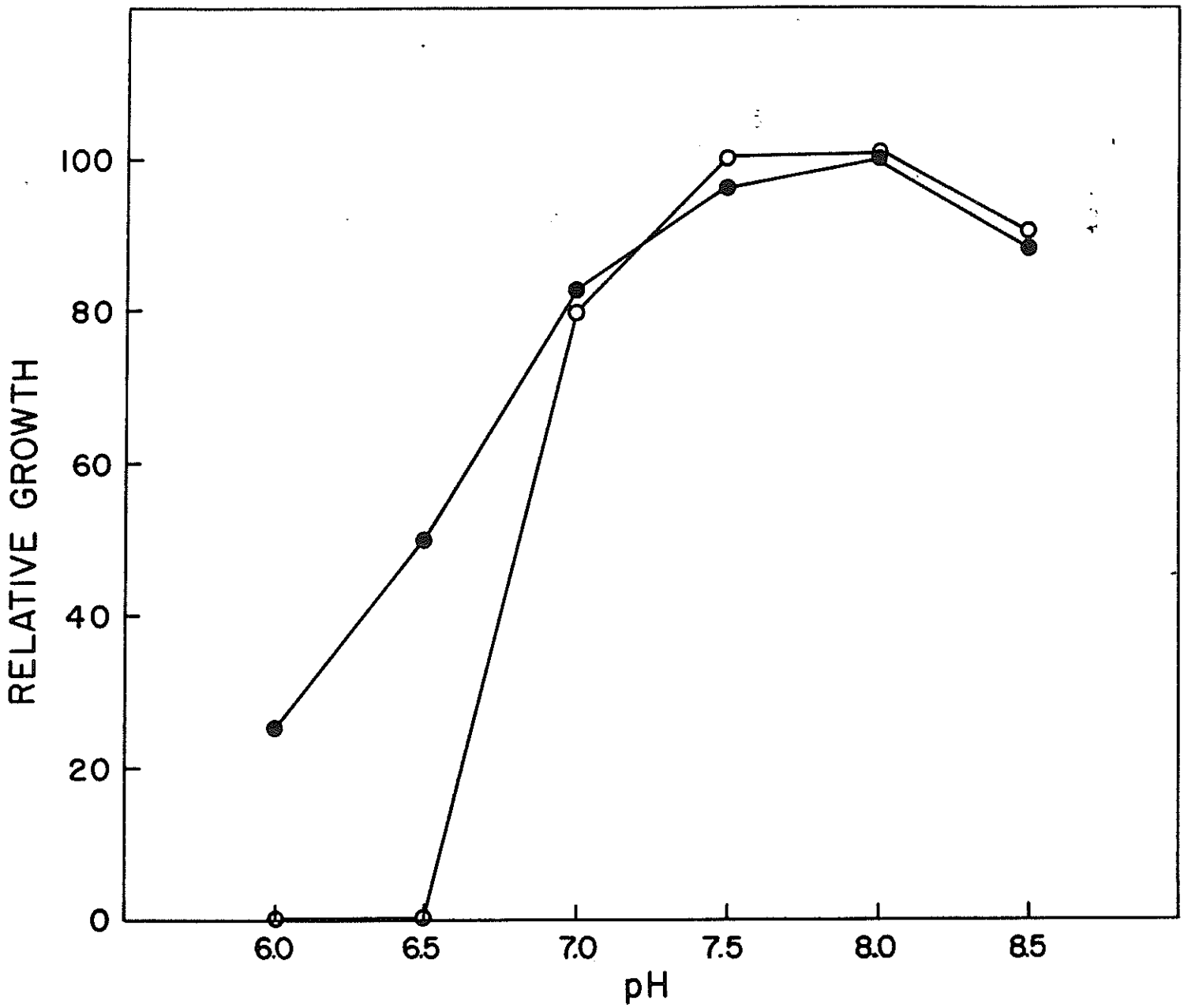


Figure 2

**Interaction between pH and inhibition of growth by NaCl**

Relative Growth reported as in Table 4 with the growth rate normalized to the maximum growth rate for cells growing with or without the added NaCl at the optimum pH. The closed circles represent values for the control. The open circles represent values for the cultures receiving 250 mM NaCl.

Effect of NaCl on survival of R. meliloti: The effect of NaCl on survival of R. meliloti was tested in two ways. First spontaneous mutants of strain NSI resistant to 50 ug/ml streptomycin were isolated. There cells were used to inoculate local agricultural soil in flower pots. The pots were then watered to saturation (a little water drained from the pots) with water containing 100 mM or 250 mM NaCl. The pots were held in the green house. Soil samples were taken at weekly intervals for 12 weeks and viable numbers of total cells were determined by plate counts with plate count agar. Numbers of R. meliloti were determined from plate counts using VM agar with streptomycin. As the pots dried both total counts and streptomycin resistant counts decreased approximately 2 orders of magnitude. Once the soil had dried, the numbers remained essentially constant. There was no difference between controls and pots watered with solutions of NaCl.

The effect of NaCl on survival of R. meliloti was also tested by suspending cells in water containing 100 mM or 250 mM NaCl. The cells were held in the $30_o$ incubator. Samples were taken at weekly intervals and numbers determined by plate counts. Again there was no significant difference between cell numbers in the controls and cells suspended in water containing NaCl. These data indicate that the survival of R. meliloti is not affected by NaCl.

**Discussion**

The data presented demonstrate that growth of R. meliloti is inhibited by the addition of minerals commonly found in soil and in irrigation water. However, the concentrations required are

several times greater than are commonly found in soil (Reisenauer, 1966) and in water (Goldman and Wetzel, 1966). The concentration of NaCl required to inhibit growth of R. meliloti is much greater than the concentration of NaCl that can be tolerated by alfalfa cultivars currently available. Yields of alfalfa are reduced 50% in soils with a conductance of 8.85 (reported as E. C. in units of m mho/cm in water saturated soil samples) (Brown and Hayward, 1956, Maas and Hoffman, 1976). Using the approximation, E. C. = 1.0 = 640 ppm dissolved salts (Kamphorst and Bott, 1972) and assuming all the dissolved salts to be NaCl, a conductance of 8.85 is equivalent to 5,491 ppm or 95 mM NaCl. This concentration of NaCl has little effect on the growth of all but one of the strains tested (Table 6).

Table 6

Inhibition of growth of strain 102f51 by various salts[a]

| Salt | Concentration | Relative Growth rate[b] |
|---|---|---|
| NaCl | 200 mM | 50 |
| $Na_2SO_4$ | 50 mM | 94 |
| $NaHPO_4$- | 100 mM | 36 |
| $NaHCO_3$ | 50 mM | 59 |
| KCl | 200 mM | 72 |
| $K_2SO_4$ | 50 mM | 88 |
| $KHPO_4$- | 100 mM | 58 |
| $MgCl_2$ | 20 mM | 53 |
| $NH_4Cl$ | 100 mM | 101 |

[a]The values represent the means of at least two experiments.
[b]Relative growth as in Table 4.

There is mounting interest in developing more salt tolerant plant varieties (Espstein et al., 1980). A cultured cell line of alfalfa capable of growing (in cell culture) with 1% NaCl has been selected (Croughan et al., 1978). 1% NaCl is equivalent to 172 mM. This concentration of NaCl inhibits growth of R. meliloti less than 50% (Figure 1).

The concentration of dissolved solutes soil will increase as soil dries. The relationship between moisture content and the osmotic potential of soil is linear. However, the water available to plants and microorganisms is determined not simply by the osmotic potential. Rather, the water available to plants and microorganisms is a function of the water potential in the soil. The water potential is the sum of the osmotic potential, determined by the concentration of dissolved solutes in the soil and the matrix potential resulting from physical factors such as size and shape of soil particles. Typically, mesic plants such as alfalfa stop growing when the water potential reaches -1.5 to -2.6 MPa. Gram negative bacteria stop growing when the water potential reaches -0.1 to -2 MPa. In clay or loam soil, water potentials in this range can be achieved when the moisture content decreases as little as 10% (Griffin, 1972; Griffin, 1981).

Growth of rhizobia in the soil is the first step in the establishment of the symbiotic relationship. However, salinity could inhibit the establishment of an effective symbiotic relationship apart from any effects it might have on the growth rate of rhizobia growing independent of the host (Bauer, 1981). Salinity could affect motility of the bacteria, the ability of

the bacteria to adsorb to the root surface, or the ability of the bacteria to penetrate the root. The plants' response to salinity (7) could affect the ability of the bacteria to establish an effective relationship. The data presented here show only that the saline and alkaline conditions found in irrigated soils do not affect the growth rate of the bacteria living independent of the host plant. Thus the initial step in establishment of an effective relationship is not limited by these conditions.

**Effect of alkaline pH and salinity on growth of rhizobia infecting peanuts.**

It has been proposed to select strains of peanuts more resistant to saline conditions for cultivation with irrigation in arid regions (Dr. David Hsi, Middle Rio Grande Experiment Station, personal communication). The experiments presented here were undertaken to determine if alkaline and saline conditions could slow the growth rate of rhizobia that grow symbiotically with peanuts. While this is not a critical problem in the United States where most peanuts are grown in the Southeast in acidic soils with adequate rainfall, it is a critical question in many developing tropical countries where peanuts make an important contribution to nutrition.

The question of the effect of sodium chloride on the slow growing rhizobia (Bradyrhizobia) has been addressed. With chick peas, it has been shown that the sensitivity of the cultivar to salinity is affected by the source of nitrogen. When chick peas are grown with rhizobia providing nitrogen to the plant, growth is inhibited by lower concentrations of sodium chloride than when ammonium nitrate provides the nitrogen source (Lauter et al., 1981). As these authors point out, any program to select more salt tolerant legume cultivars must consider the source of nitrogen. The growth of legumes with nitrogen provided by rhizobia could be limited by the ability of the bacterial symbiot to tolerate saline and alkaline conditions.

The effect of acid conditions particularly with regard to aluminum toxicity has been addressed (for example see Keyser and

Munns, 1979). The effect of NaCl on rhizobia under acidic conditions has also been investigated (Steinborn and Roughly, 1975). The effect of salinity on growth and survival of rhizobia from the tropics has been studied (Singleton et al., 1982). There are several reports of the effects of saline and alkaline conditions on final numbers of rhizobia (Yader and Vyass, 1971; Bhardwaj, 1979) but the growth rate rather than final numbers is more indicative of the bacterium's ability to cope with to the situation. Strains of R. japonicum have been selected for greater resistance to NaCl (Upchurch and Elkan, 1977). The question of the effect of saline and alkaline conditions on the rhizobia that infect peanuts (Arachis hypogea) has not been addressed to the best of my knowledge.

**Methods and Materials**

Bacterial Strains: Rhizobia are usually classified with respect to their host plant. Peanuts are infected by a variety of rhizobia and the rhizobia infecting peanuts have not been given species names. These bacteria will be referred to simply as "peanut rhizobia." Cultures were obtained from the Nitragin Corporation (Milwaukee, Wisconsin) and from Dr. William Lindemann, Department of Crop and Soil Science, New Mexico State University. The strains used are listed in Table 7.

Stock cultures were maintained on yeast extract galactose agar slants (Vincent, 1975) and transferred regularly. Stock cultures were routinely streaked on yeast extract galactose agar Media: The experiments required a medium of low osmolarity that would support the growth of all strains of peanut rhizobia.

Several completely defined media designed for growth of R. japonicum were tested but not all the strains of peanut rhizobia would grow with these. Cells were grown in Vincent's galactose medium consisting of: (g/l) $K_2HPO_4$, 0.5; $MgSO_4$ $7H_20$, 0.2; NaCl 0.1; Yeast extract, 0.4; galactose, 10. The final pH of the medium was adjusted with KOH or HCl (Vincent, 1975). For solid media, agar was added to 1.5%. The addition of 10 mM glutamate to this medium did not affect the growth rate of the three strains tested. The concentration of yeast extract could be doubled without affecting growth. However, increasing the yeast extract to 2 g/l inhibited growth of all strains tested.

Growth Experiments: Cells were grown in 50 ml Erlenmeyer flasks fitted with side arms permitting optical measurements. Flasks were never filled to more than 20% capacity. Cells were grown in an incubator shaker at 200 revolutions $min_{-1}$ at $30_o$. Flasks were fitted with cotton stoppers with loosely fitting aluminum foil caps to minimize evaporation.

Inocula were prepared from cells grown to stationary phase in the medium without supplemental NaCl poised at pH 6.5. Cells were washed 3 times in sterile tap water by centrifugation. Enough cells were used to make the initial absorbancy of the culture equal to approximately 10 Klett units.

Growth was measured using the Klett Summerson Colorimeter as described previously (Botsford, 1983). The absorbancy of the cultures was checked at approximately 4 hour intervals for three days. These bacteria grow with generation times of 12 to 15 hours.

## Results and Discussion

Effect of saline and alkaline conditions on growth rate: The 19 strains collected from various sources were tested for the effect of NaCl, alkaline pH, and NaCl and alkaline pH in combination. The results of these experiments are presented in Table 8. When the pH of the growth medium was poised at pH 6.5, the growth of 7 of the 19 strains was inhibited by the addition of 50 mM NaCl (a difference of 20% or more in the growth rates was considered significant). Thus most strains of peanut rhizobia can tolerate 50 mM NaCl when the pH is slightly acidic.

It is generally assumed that rhizobia prefer slightly acidic conditions (Nutman, 1975). To determine how alkaline conditions affect growth of peanut rhizobia, the 19 strains were grown with the medium poised at pH 8.0. Growth of 9 of the 19 strains was reduced by alkaline conditions (Table 8).

To determine if saline and alkaline conditions might interact to reduce growth of peanut rhizobia, the effect of these two conditions in combination was tested. With one exception, growth of all the strains tested was inhibited by 50 mM NaCl when the pH of the growth medium was poised at 8.0 (Table 8). These results indicate that saline and alkaline conditions in combination inhibit the proliferation of peanut rhizobia.

50 mM NaCl is equivalent to 1450 ppm sodium and a electrical conductance of 4.5 mho/cm (Kamphorst and Bott, 1972). The yield of most legume crops, including peanuts is inhibited by salinity in this concentration (Maas and Hoffman, 1976).

The inhibition of growth by NaCl at alkaline pH was investigated in more detail in strains P-7, P-8, and P-10.

Growth of the three strains was inhibited more than 50% by 25 mM

Table 7

Strains used in these experiments

| Strain designation | Source | Comments |
|---|---|---|
| NM-212 | W. Lindemann | From New Mexico peanut fields |
| NM-214 | " | " |
| LP201 | " | Originally a commercial strain |
| LP203 | " | From high pH soil |
| LP204 | " | " |
| LP205 | " | " |
| LP206 | " | Originally a commercial strain |
| LP207 | " | " |
| LP208 | " | " |
| LP209 | " | Cowpea inoculum |
| LP210 | " | Originally from NifTAL collection |
| P-1 | Nitragin | Nitragin 8A11[a] |
| P-2 | " | Nitragin 8A50[a] |
| P-3 | " | Nitragin 8A54[a] |
| P-4 | " | Nitragin 25B7[b] |
| P-5 | " | Nitragin 150B1[c] |
| P-6 | " | Nitragin 41K2[d] |
| P-7 | " | Nitragin 8A44[a] |
| P-8 | " | Nitragin 8A57[a] |
| P-9 | " | Nitragin 8A62[a] |
| P-10 | " | Nitragin 8A63[a] |

[a] *Arachis hypogaea* is host plant
[b] *Centrosema pubescens* is host plant
[c] *Stylosanthes sundaicia* is host plant
[d] *Desmodium triflorum* is host plant
medium to check for purity.

NaCl with the medium poised at pH 8.5. At pH 6.5, inhibition was observed only when the NaCl concentration was greater than 100 mM (results not presented). These results again show how alkaline conditions exacerbate inhibition of growth by NaCl.

Effect of saline and alkaline conditions on survival: The effect of saline and alkaline conditions of the survival of peanut rhizobia was tested. Strain P-10 was chosen because of its sensitivity to alkaline pH and NaCl. Spontaneous mutants resistant to streptomycin (50 ug/ml) or to spectinomycin (100 ug/ml) were isolated using conventional techniques. These mutations did not affect the growth rate. Cells were grown in Vincent's Galactose medium, washed 3 times in double distilled water and were suspended in sterile water adjusted to pH 6.5 or 8.5 and supplemented with 0, 50 or 100 mM NaCl. Samples were plated at weekly intervals for 14 weeks. The viable cell numbers were determined by plate counts. None of the treatments resulted in a decrease of more than 2 logs in the numbers of survivors relative to the controls. No pattern could be discerned in the survival of the two strains, i.e. neither salinity nor pH appeared to affect the survival of the rhizobia. Originally, it had been planned to test the survival of peanut rhizobia in soil samples using the antibiotic resistant markers to select for the survivors. However, since this preliminary experiment indicated that these conditions do not affect survival, this experiment was not performed.

In conclusion, the results presented here suggest that the growth but not the survival of rhizobia commonly used to

Table 8

Effect of alkaline pH and 50 mM NaCl on growth of peanut rhizobia

| | Growth Rate $\mu_a$ | | | |
|---|---|---|---|---|
| | pH 6.5 | | pH 8.5 | |
| Strain | control | 50 mM NaCl | control | 50 mM NaCl |
| NM-212 | .035 | .064 | .046 | .062 |
| NM-214 | .023 | .002 | .014 | .009 |
| LP201 | .054 | .033 | .052 | .019 |
| LP203 | .056 | .036 | .057 | .043 |
| LP204 | .072 | .051 | .076 | .043 |
| LP205 | .027 | .018 | .014 | .011 |
| LP206 | .053 | .067 | .081 | - |
| LP208 | .039 | .035 | .039 | .011 |
| LP209 | .052 | .054 | .052 | .016 |
| P-1 | .058 | .054 | .046 | .044 |
| P-2 | .043 | .048 | .027 | .025 |
| P-3 | .084 | .067 | .055 | n.g. |
| P-4 | .076 | .065 | .059 | - |
| P-5 | .053 | .053 | .048 | .030 |
| P-6 | .016 | .015 | n.g. | n.g. |
| P-7 | .044 | .052 | .031 | .005 |
| P-8 | .047 | .054 | .015 | n.g. |
| P-9 | .050 | .047 | .038 | n.g. |
| P-10 | .054 | .056 | .021 | .013 |

[a]$\mu$ instantaneous growth rate as determined from the slope of KU vs. time as described in the Methods section. n.g., no detectable growth. -, not included.

inoculate peanuts is adversely affected by saline and alkaline conditions.

## FUTURE DIRECTIONS FOR RESEARCH

The work presents several interesting problems in basic bacterial physiology. The response of non-halophilic bacteria to salt stress is not a well studied area of research. Our report represents the second report of the accumulation of glutamate by rhizobia in response to NaCl, the first in a fast growing rhizobium. The question of the role of accumulated glutamate is currently being investigated using a genetic approach.

Since the growth of rhizobia infecting peanuts is inhibited by saline and alkaline conditions, particularly in combination, it would be valuable to compare and contrast the response of these slow growing rhizobia to these conditions with fast growing rhizobia such as R. meliloti. This observation points out yet one more difference between the fast growing rhizobia and slow growers.

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