# EFFECT OF SALINITY ON GROWTH AND NITROGEN YIELD OF INOCULATED AND N FERTILIZED CHICKPEA (*Cicer aritinum*)

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## ABSTRACT

Singly- and doubly-labelled antibiotic resistant mutants of streptomycin (250 µg/ml) and spectinomycin (250 µg/ml) were isolated from chickpea *Rhizobium* 2-ICAR-UNK-Ch-191 (Ch191). All mutants exhibited similar characteristics to the wild-parent type in their response to nodulation and tolerance of salinity and temperature. Salinity (1.0 ds/m) decreased root and shoot dry weight, total nodule number and nodule weight. Inoculated plants accumulated more N compared to N fertilized plants. The results indicate that the *Rhizobium* is more salt-tolerant than the cultivar and therefore, there is a clear need for greater salt-tolerance in chickpea. The results also indicate that *Rhizobium* strain Ch191 is effectively fixing N in saline and non-saline conditions.

## INTRODUCTION

Chickpea ranks fifteenth among the food crops, and third among the pulse crops in the world (Singh and Malhotra 1984). It is cultivated in arid areas because it is adapted to low water availability, however, the plant growth was reported to fail in arid areas under salt-stress (Lauter and Munns 1986). The limitation of production of this crop under sal-stress may be due to the salt sensitivity of the chickpea symbiosis (Lauter *et al.*, 1981).

Nitrogen assimilating chickpea plants are reported to be more tolerant to salinity than are $N_2$ fixing chickpea (*Cicer aritinum*) plants (Lauter *et al.*, 1981), soybean (*Glycine max*) (Abdel-Wahab and Zahran 1981; Wilson 1970), and faba bean (*Vicia faba*) (Yousef and Sprent 1983).

The aims of this work were to isolate antibiotic resistant mutants of *Rhizobium* strain Ch191 and to assess the effect of continuous salinity on growth and N yield of inoculated or N fertilized chickpea plants.

## Materials and Methods

*Rhizobium strains*

*Rhizobium sp. (Cicer)* strain 2-ICAR-UNK-Ch-191 (Ch191) was obtained from the

International Centre for Agricultural Research in the Dry Areas (ICARDA). The strain was maintained at 4°C on yeast extract mannitol (YEM) agar slopes incorporating 3.0 g $l^{-1}$ $CaCO_3$ (Vincent 1970). Chickpea cultivars ILC 482 and ILC 1919 were obtained from ICARDA.

***Isolation of antibiotic resistant mutants***

Stock solutions of streptomycin sulphate and spectinomycin dihydrochloride were prepared in distilled water, sterilized by passing through a 0.2 μm membrane filter and stored at 4°C. *Rhizobium* strain Ch191 ($10^8$-$10^9$ cfu/ml) was exposed to 250 μg/ml spectinomycin dihydrochloride or streptomycin sulphate in duplicate and incubated at 25°C for a period of 3 to 14 days in defined medium. Control medium without antibiotics was also used. After further incubation, the suspensions from the antibiotic-amended and from non-amended cultures were streaked onto YEMA plates with the same concentrations of antibiotics. The same procedure was followed to develop double resistance of the two antibiotics by using spectinomycin-resistant mutants in selecting for doubly labelled mutants which were also resistant to streptomycin.

***Salt-tolerance***

The isolated singly-labelled and doubly-labelled mutants and their wild-type parent (Ch191) were screened for salt-tolerance in defined medium containing a range of NaCl concentration from 0 to 0.51 M NaCl.

Chickpea rhizobia strains Ch191 and $Ch191^{strspc}$ were cultured in the defined glutamate- arabinose- glactose medium (Elsheikh and Wood, 1990) with or without 0.34 M NaCl. The medium (15 ml in 50 ml conical flasks) was inoculated to give an initial count of $10^2$-$10^3$ cfu $ml^{-1}$. The cultures were incubated at 90 rpm at 25°C. Two replicates were included per treatment. Growth was assessed by following viable counts at 2 days intervals for 10 days.

***Tolerance to high temperature***

*Rhizobium* strain Ch191 and the singly- and doubly-labelled rhizobia mutants isolated were grown in defined medium and then each strain was streaked on solid yeast extract mannitol agar (Vincent 1970) medium in petri-dishes in triplicate for each temperature. The plates were incubated at 25, 37 or 45°C and cheched daily for colony development for 14 days.

***Nodulation of chickpea***

Two chickpea cultivars ILC482 and ILC1919 were grown in sterile N-free solution used for germination in the sterile boiling tubes (20×7.5 cm which were closed with cotton wool bungs) was as previously described (Cooper 1978). They were inoculated ($10^5$-$10^6$ cfu/ml) by the wild-type parent and antibiotic resistant mutants in triplicate. One pre-germinated, surface sterilized seed (0.2% $HgCl_2$ for 3 minutes followed by five rinses in distilled water) was grown in each tube.

Inoculation was carried out immediately after transplanting. The tubes were held in blackened boxes with shoots exposed to atmosphere in controlled environment growth room at 20-22°C and were illuminated at 300 $\mu Em^{-2}s^{-1}$ for a 16 hour day. Extra sterile N-free solution was added every three days. Three replicates were included per treatment. Plants were checked daily for the presence of nodules for 30 days.

***Survival of mutants in soil***

Antibiotic-resistant mutant strain $Ch191^{strspc}$ was added to non-sterile soils. The soil was air dried and passed through 2.0 mm sieve. A moisture release curve was developed by using a membrane pressure plate apparatus. The soil was also calibrated for the electrical conductivity (ECe) when increasing amounts of NaCl and $CaCl_2$ were added at a constant Ca/Na ratio of 0.25 on molar basis. Salinity levels used were (dS/m) 32.5 and 50.7 (equivalent to 0.34 and 0.51 M NaCl respectively). Samples of 10 g soils were prepared as -0.03 MPa (-0.3 bars) or -1.5 MPa (-15 bars) with one of the salinity levels in 100 ml glass bottles with screw caps. The inoculum was added from a freshly prepared culture to give an initial count of $10^5$-$10^6$ cfu/g of soil. The inoculated samples were incubated at 25°C and they were weighed every two days to maintain constant moisture content. Viable counts were made at 0, 10, 20 and 40 days after inoculation.

***Pot experiment***

Chickpea was either uninoculated, inoculated with *Rhizobium* strain $Ch191^{strspc}$ or supplied with N fertilizer. Two salinity ($NaCl+CaCl_2$) levels equivalent to 0 and 1.0 dS $m^{-1}$ at 25°C (8.6 and mM NaCl) were used for the irrigation of chickpea cultivar ILC 482 which was grown in soil in pots (1.0 Kg soil $pot^{-1}$). Inoculation was carried out immediately after transplanting, whereas the salt was added in the irrigation water which started 3-5 days after transplanting. Nitrogen was added as $NH_4NO_3$ to uninoculated plants to give an equivalent of 100 kg N $ha^{-1}$ (volume basis). Control plants were uninoculated and received no fertilizer. There were three replicates per treatment and the plants were maintained in a growth room at 20-22°C and illuminated at 300$\mu Em^{-2}s^{-1}$ for a 16 hour day.

The plants were harvested 4, 6 or 8 weeks after starting the salt treatment. Dry weights and N content of shoots (leaves and stems) and roots were determined, as well as nodule numbers, average nodule weight per plant and nodules sizes. N fixed was determined by the N difference method (I-U) where I is the total N content in the inoculated plants and U is the total N in uninoculated plants; similarly the N benefit due to the fertilizer was calculated as N-U where N is the total N in fertilized plants.

## Results and Discussion

*Salt-tolerance*

*Rhizobium sp. (Cicer)* strain Ch191 tolerated NaCl up to 0.43 M. Singly- and

doubly-labbled mutants showed tolerance to NaCl similar to that of their wild-type parent. The results indicate that the mutants retained their ability to tolerate salt.

Mutant strain Ch191$^{strspc}$ showed similar growth pattern to the wildtype parent (Ch191) (Fig. 1). Similar results were reported for doubly-labeled mutants of *Rhizobium* phaseoli and cowpea rhizobia (Msumali 1983).

***Tolerance to high temperature***

*Rhizobium sp. (Cicer)* strain Ch191 and all antibiotic resistant mutants developed colonies >2.0 mm in diameter on YEM agar plates within 3.5 days at 25°C and 3.5 days at 37°C. However, none of the strains could tolerate 45°C. Characters such as tolerance to high temperatures, high pH values, high salt levels and better infection and effectiveness can be developed by mutations (Rai and Parasd 1983; Schuller *et al.*, 1988). However, there were no differences between the wild-type parents and their antibiotic-resistant mutants in their response to temperature.

***Nodulation of chickpea***

Chickpea cultivars varied in their response to inoculation. The wild-parent strain (Ch191) and all other antibiotic resistant mutant nodulated cultivar ILC482 after 9 days from inoculation. None of the strains nodulated ILC1919.

***Survival of mutants in soil***

The effects of salt concentration and water potential on growth and multiplication of *Rhizobium sp. (Cicer)* strain Ch191$^{strsp}$ were significant ($P < 0.0001$). This indicates the sensitivity of this strain of rhizobia (fast-growing) to water stress (Fig. 2). The strain Ch191$^{strspc}$ was more sensitive to 0.51 M salt than at -1.5 MPa than -0.03 MPa.

There is an inverse relationship between soil moisture tension and salinity in microenvironments. Bushby and Marshall (1977) have concluded that slow-growing rhizobia tolerate desiccated sandy soil better than do fast-growing species.

***Pot experiment***

*Shoot and root weights*

Salinity had very little effect on the dry weight of shoots, however, the root dry weights were significantly reduced ($P<0.0001$) for all treatments (Fig. 3). The inhibitory effect of salt on plants receiving no salt appeared after six weeks from treatment; no significant difference was observed in the fourth week. Roots appeared to be more sensitive to salinity than shoots even at the very low salinity level of 1.0 dS/m (8.6 mM NaCl).

Inoculated and N fertilized plants showed better root growth than the control (uninoculated and unfertilized) plants ($P<0.0001$) in both normal and saline conditions. Under salt stress, shoot growth of N fertilized plants was better than that of the inoculated plants (Fig. 3). The results show that this cultivar is sensitive to salinity.

In general, growth of N fertilized plants was better than inoculated plants in both saline and non-saline conditions, however, in some instances the differences were not significant. There was therefore no strong evidence that chickpea fixing $N_2$ were more sensitive to salinity than N fertilized plants. The increase in dry weight in the N fertilized plants may be due to the ability of such plants to utilize readily available N forms (ammonium nitrate may improve the ion balance in the N fertilized plants) rather than to fix it. The growth of N fertilized plants was found to be less affected by salts compared to the inoculated plants. This is true for chickpea (Lauter *et al.*, 1981), faba bean (Yousef and Sprent 1983). In the results reported here no major differences were observed between inoculated or N fertilized chickpea plants. This is may be due to the low salinity concentration used (1.0 dS/m) or the symbiosis process in this particular cultivar-*Rhizobium* interaction may be less sensitive to this salinity.

***Nodulation***

No nodules were found on all uninoculated plants in saline and non-saline treatments. Salinity significantly ($P<0.05$) reduced the total nodule number per plant (Fig. 4a). Salinity also significantly reduced ($P<0.0001$) the total number of large and medium nodule sizes but not the initiation of new nodules as shown by the presence of small nodules (Fig. 4a). In a treatment not reported which combined inoculation and N fertilizer nodulation was reduced, but not inhibited by fertilizer indicating that *Rhizobium sp. (Cicer)* strain $Ch191^{strspc}$ can form nodules in the presence of 100Kg N/ha.

The total nodule weight per plant was significantly decreased ($P<0.0001$) by salinity (Fig. 4b). Average nodule weight increased with increase in salinity level. The values were (mg per nodule) 3.6 and 3.6 after four weeks, 3.9 and 4.6 after six weeks and 5.7 and 5.8 after eight weeks for inoculated and inoculated and salt treated plants respectively.

In general, the total nodule number per plant increased with time in all treatments indicating the tolerance of this *Rhizobium* to salinity and nitrate (data for nitrate is not shown). The results in agreement with the results of Hafeez *et al.*, (1988) who reported that the nodulation of *Vigna radiata* was reduced to about half at salinity level of ECe 5.0 dS/m as compared to 1.4 dS/m; however, nodulation was completely depressed when salinity was reised to 10.0 dS/m regardless of the plant growth stage. The total nodule number, nodule weight and N content per plant of chickpea and mung bean decreased with increase in salinity treatment, which interfered with the initiation of nodules but not with their further development (Balasubramanian and Sinha 1976a; b).

Increase in average nodule weight under salt-stress reported here was also reported under stressed conditions in field bean at low temperature (Fyson and Sprent 1982), and under salt-stress in field bean (Yousef and Sprent 1983), chickpea (Lauter *et al.*, 1981; Balasubramanian and Sinha 1976). The increase in average nodule weight under stressed conditions may partly compensated for reduce specific activity (Yousef and Sprent 1983).

***Total N***

Inoculation and N fertilization significantly increased ($P<0.0001$) the total plant N in both shoots and roots of chickpea (Table 1). However, salinity significantly decreased ($P<0.0001$) the total N in both shoots and roots. Inoculated and N fertilized plants accumulated more total N per plant in their tissues than control plants in normal and saline conditions. The total N in the shoots and the roots of the inoculated or N fertilized chickpea plants were increased by 3-7 folds compared to that of uninoculated and non-fertilized (control) plants.

***Nitrogen fixation***

Total nodule number and nodule weight nodule weight per chickpea plant were decreased by increasing salinity, however, average nodule weight and $N_2$ fixation were increased. Wilson (1970) reported that during salinity treatment the development of new nodules, and $N_2$ fixation by the existing soybean nodules, were greatly inhibited, with a resulting marked decline in plant N concentration. At 100 mM NaCl nodulation and $N_2$ fixation were not completely inhibited in faba bean however they were significantly reduced (Yousef and Sprent 1983). Salts reduced total nodule number thereby depressing total nodule activity in spite of an increase in specific nodule activity.

The proportion of $N_2$ fixed (I-U/U) or assimilated (N-U/U) per plant (Shoot + root) reported here were 3.28 and 2.90 under non-saline conditions, and 5.11 and 4.49 under saline (1.0 dS/m) for inoculated and N fertilized plants respectively. This indicates neither symbiosis nor N assimilation were inhibited by salinity. $N_2$ fixing plants differ from the N fertilized plants in that (i) they must form root nodules, (ii) they have a biochemically different mechanism for uptake and transport of N, and (iii) often they must withstand a lag in growth resulting from N-deficiency prior to optimum rates of $N_2$ fixation (Lauter *et al.*, 1981). Sensitivity of the inoculated plants to salinity may be due to the accumulation of $Na^+$ and $Cl^-$ in their tissues.

Overall, the results show that salinity reduced shoot and root dry *weights, nodule number and nodule weight in chickpea plants.* Neither $N_2$ fixation by inoculated plants nor N benefit by N fertilized plants was inhibited by salinity. This may indicate that this chickpea-*Rhizobium* symbiosis is tolerant to sailnity. The results also indicate *Rhizobium* strain Ch191 is more salt-tolerant than chickpea cultivar ILC 482 and therefore, there is a clear need for greater salt-tolerance in chickpea. Similar results were reported by Lauter *et al.*, (1981) and Singleton *et al.*, (1982). This strain needed to be tested fully under the field conditions before any recommendation can be made concerning its suitability for inoculant production in normal and saline conditions.

## References


Abdelwahab A. M. and Zahran H. H. (1981) Effects of salt-stress on nitrogenase activity and growth of four legumes. Biol. Plant. 23: 16-23.

Balasubramanian V. and Sinha S. K. (1976a) Nodulation and nitrogen fixation in chickpea (Cicer arietinum L.) under salt stress. J. Agric. Sci., Cambridge 87: 465-466.

Balasubramanian V. and Sinha S. K. (1976b) Effects of salt stress on growth, nodulation and nitrogen fixation in cowpea and mung beans. Physiol. Plant. 36: 197-200.

Bushby H. V.A. and Marshall K.C. (1977) Some factors affecting the survival of root-nodule bacteria on desiccation. Soil. Biol. Biochem. 9: 143-147.

Cooper J.E. (1978) A method for testing *Rhizobium* effectiveness at low pH. Soil. Biol. Biochem. 10: 18-83.

Elsheikh E.A.E. and Wood M. (1990) Salt effects on survival and multiplication of chickpea and soybean rhizobia. Soil. Biol. Biochem. 22:343-347.

Fyson A. and Sprent J. I. (1982) The development of primary root nodules on *Vicia faba* L. grown at two temperatures. Annal. Bot. 50:681-692.

Hafeez F.Y., Aslam Z. and Malik K. A. (1988) Effect of salinity and inoculation on growth, nitrogen fixation and nutrient uptake of *Vigna radiata* (L.) Wilczek. Plant Soil 1060 3-8.

Lauter D. J. and Munns D. N. (1986) Salt resistance of chickpea genotypes in solutions salinized with NaCl or $Na_2So_4$. Plant Soil 95: 271-279

Lauter D. J., Munns D. N. and Clarkin K.L. (1981) Salt response of chickpeas influenced by N supply. Agron. J. 73:961-966.

Rai R. and Prasad V. (1983) Salinity tolerance of *Rhizobium* mutants: growth and relative efficiency of symbiotic nitrogenn fixation. Soil Biol. Biochem. 15: 217-219.

Singh K. B. and Malhtra R. S. (1984) Collection and evaluation of chickpea genetic resources. In Genetic resources and their explotitation-chickpea, faba beans and lentils. (J.R. Witcombe and W. Erskine eds. Martinus Nijhoff. Netherlands.

Singleton P.W., Elswaify S. A. and Bohlool B. B. (1982) Effect of salinity on *Rhizobium* growth and survival. Appl. Env. Microbiol. 44: 884-890.

Vincent J.M. (1970) A manual for the Practical Study of Root Nodule Bacteria. Blackwell Scientific Publications, Oxford.

Wilson J.R. (1970) Response to salinity in glycine: VI Some effects of a range of short term salt-stresses on the growth, nodulation and nitrogen fixation of *Glycine whightii* (Formerly javanica). Aust. J. Agric. Res. 21:571-582.

Yousef A. N. and Sprent J. I. (1988) Effects of NaCl on growth, nitrogen incorporation and chemical composition of inoculated and $NH_4NO_3$ fertilized *Vicia faba* (L.) plants. J. Exp. Bot. 34: 941-950.

Table 1 The effect of salinity (NaCl + $CaCl_2$) on total N (mg/plant) in shoots and roots of chickpea cultivar ILC 482 either N fertilized (N), inoculated (I) with *Rhizobium sp. (Cicer)* strain Ch191 or control (U). Three replicates per treatment.
(a) Shoots SE ± 13.02

| Treatment | Harvest 4 weeks | 6 weeks | 8 weeks |
|---|---|---|---|
| No Salt | | | |
| Control (U) | 24.3 | 30.87 | 40.00 |
| Inoculated (I) | 67.53 | 157.13 | 184.93 |
| N fertilized (N) | 59.70 | 127.80 | 163.37 |
| I-U | ND | ND | 144.9 |
| N-U | ND | ND | 123.4 |
| 1.0 DS/m (8.6 mM NaCl) | | | |
| Control (U) | 19.4 | 19.70 | 21.23 |
| Inoculated (I) | 48.33 | 84.37 | 148.76 |
| N fertilized (N) | 48.47 | 67.47 | 131.53 |
| I-U | ND | ND | 127.5 |
| N-U | ND | ND | 110.3 |

**(b) Roots N SE ±**

| Treatment | Harvest 4 weeks | 6 weeks | 8 weeks |
|---|---|---|---|
| No salt | | | |
| Control (U) | 12.03 | 13.93 | 18.20 |
| Inoculated (I) | 37.33 | 56.17 | 64.10 |
| N fertilized (N) | 32.70 | 53.20 | 63.40 |
| I-U | ND | ND | 45.9 |
| N-U | ND | ND | 45.2 |
| 1.0 dS/m (8.6 mM NaCl) | | | |
| Control (U) | 9.18 | 12.43 | 10.93 |
| Inoculated (I) | 27.47 | 30.17 | 47.60 |
| N fertilized (N) | 28.80 | 31.47 | 42.73 |
| I-U | ND | ND | 36.7 |
| N-U | ND | ND | 31.8 |

ND = not determined

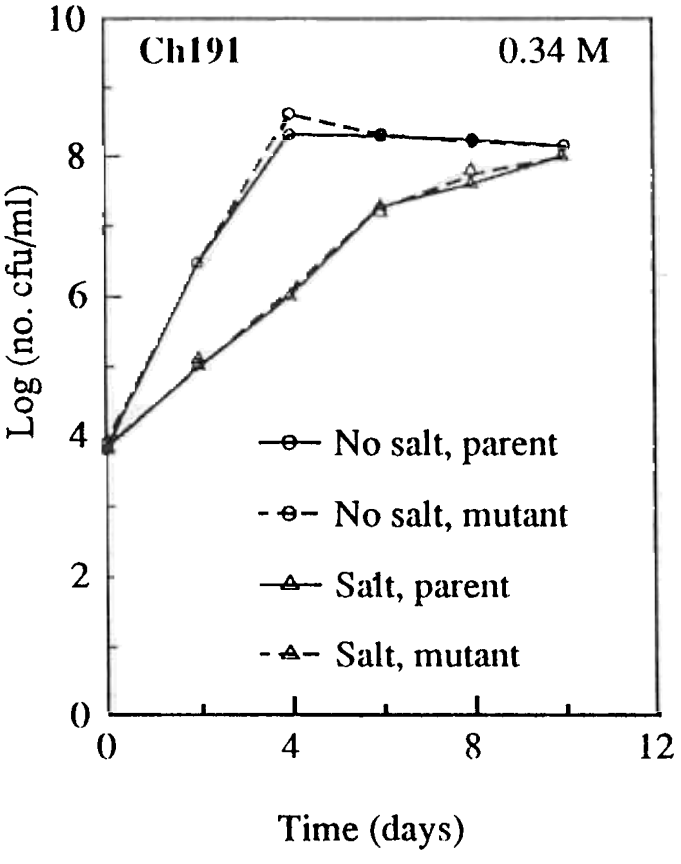


Fig. 1 Growth of wild-type parent and antibiotic-resistant mutant of chickpea Rhizobium strain Ch191 in normal and salt-stressed defined media. Two replicates per treatment.

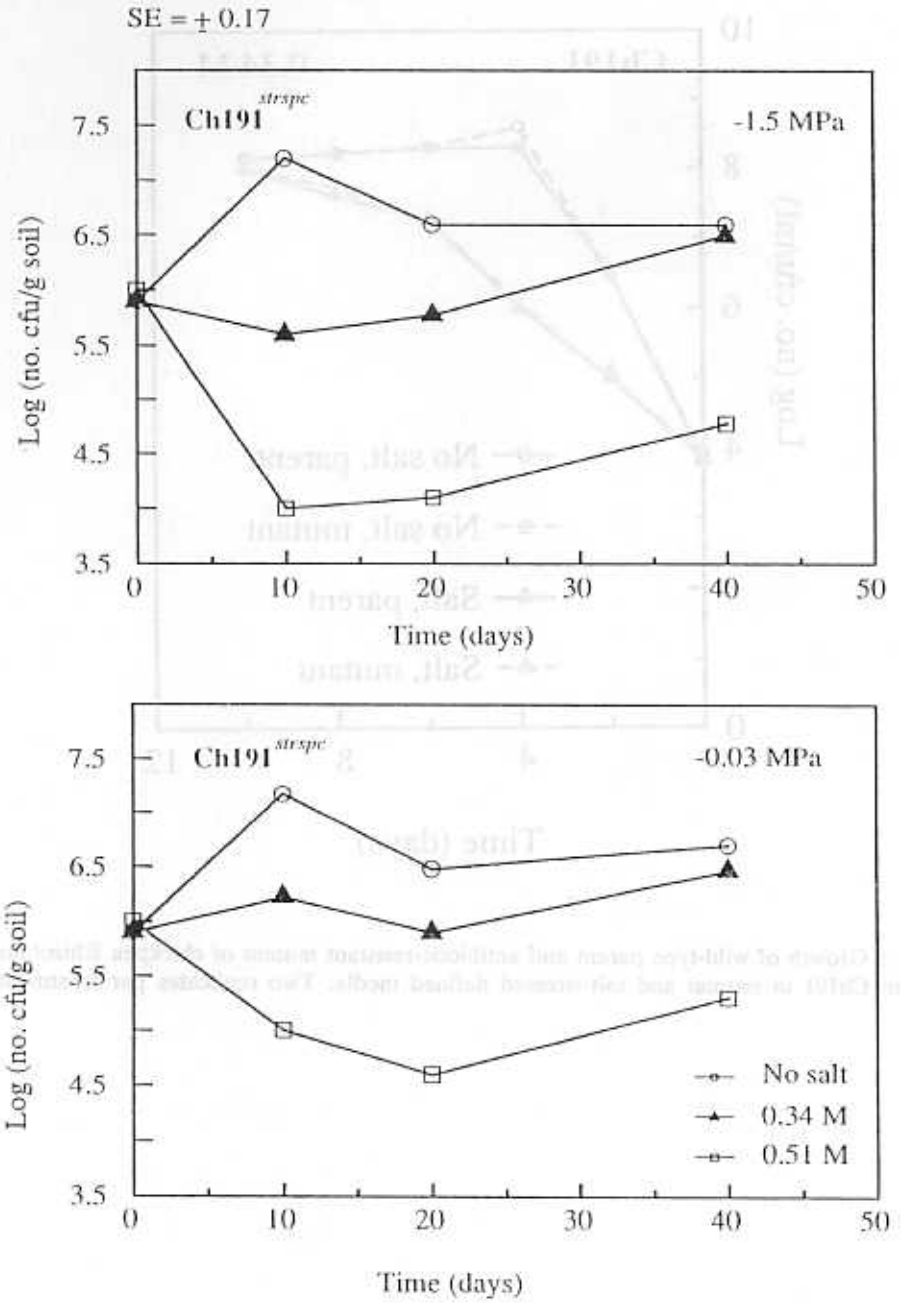


Fig.2 Recovery of antibiotic-resistant mutant of chickpea Rhizobium strain Ch191 from non-sterile soil under different salt and moisture levels. Three replicates per treatment.

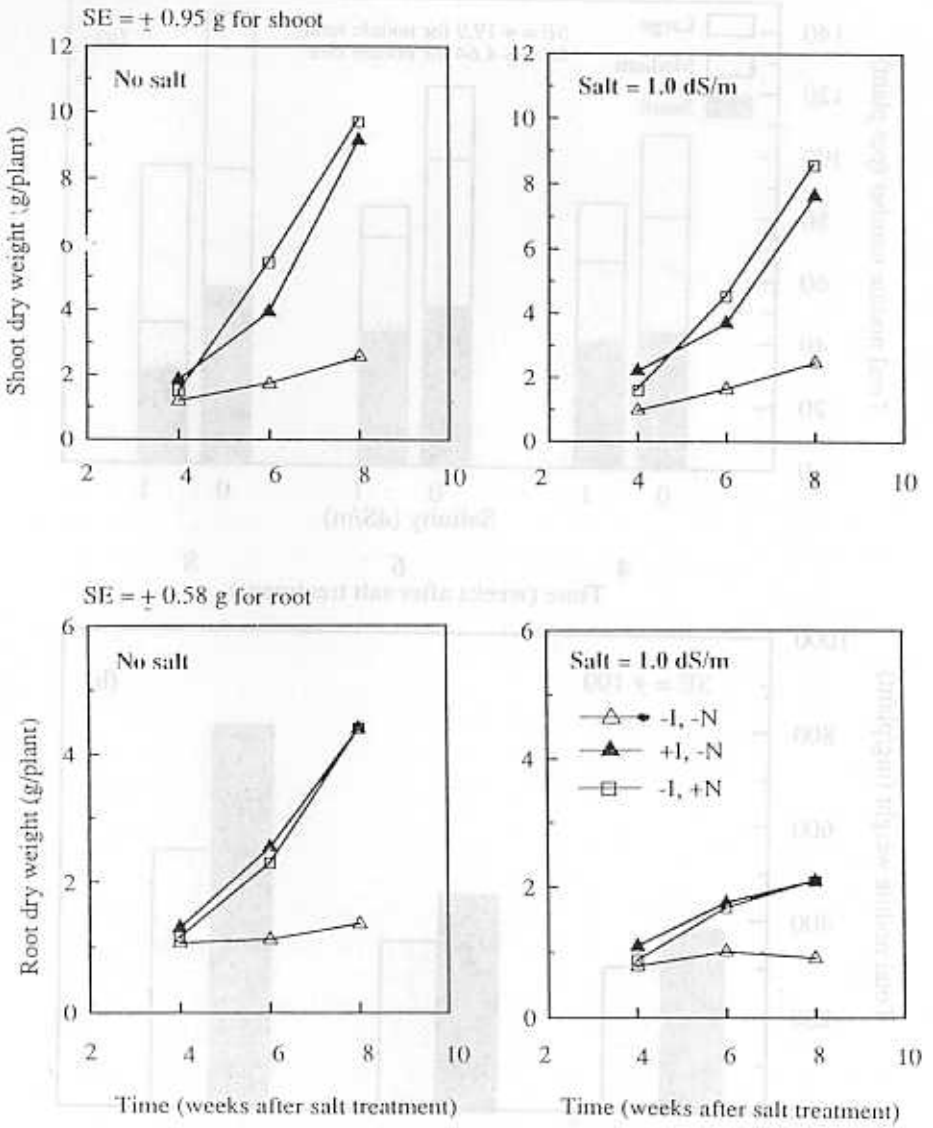


Fig.3 The effect of salinity (NaCl +$CaCl_2$) on shoot and root dry wieght of chickpea cultivar ILC either uninoculated and not N fertilized (−I, −N), inoculated with *Rhizobium* strain $Ch191_{strspe}$ (+I, −N) or uninoculated and N fertilized (−I, +N). Three replicates per treatment.

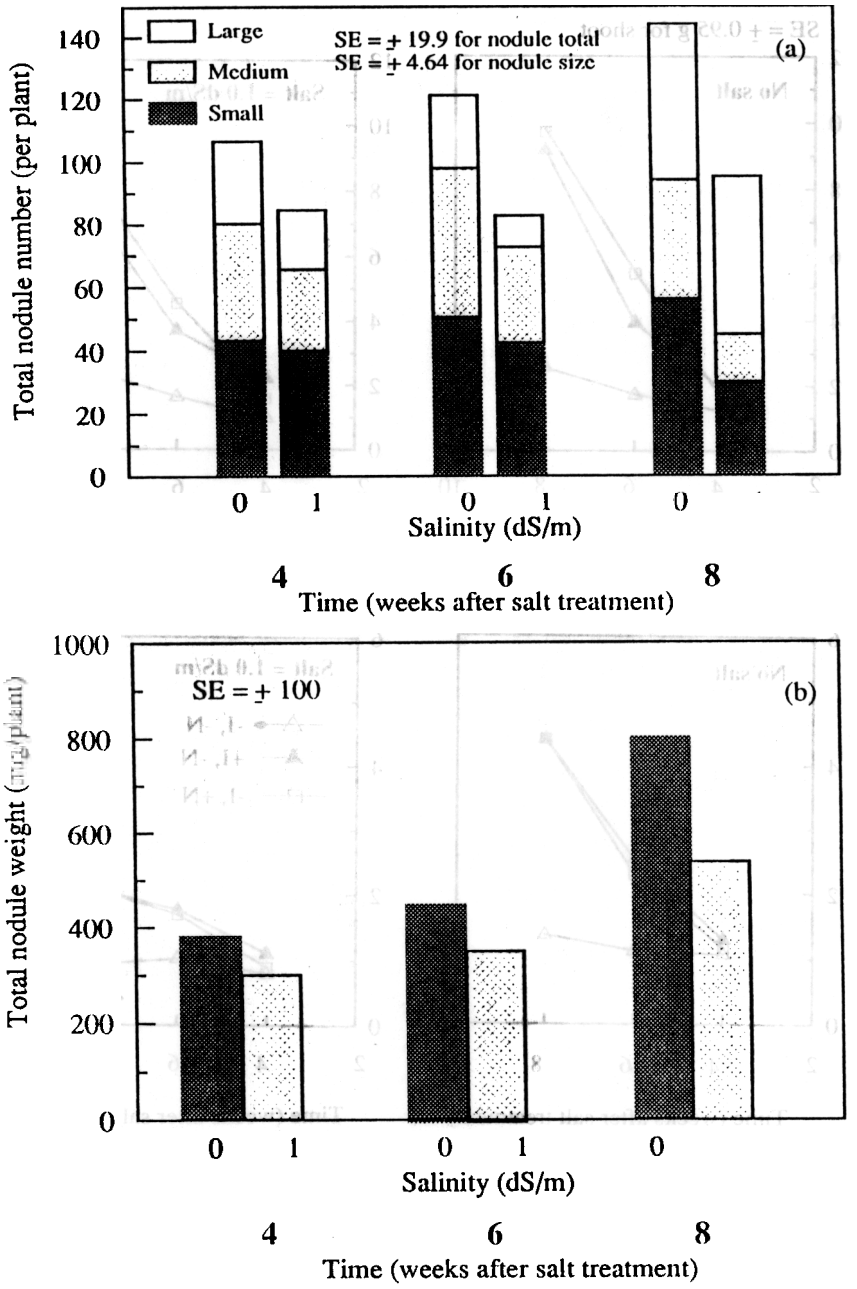


Fig. 4 The effect of salinity (NaCl + $CaCl_2$) on (a) total nodule number per plant and nodule size (b) nodule weight for chickpea cultivar ILC 482 inoculated with *Rhizobium* strain $CH191_{strsps}$. Three replicates per treatment.