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Nodule formation, distribution and symbiotic efficacy of *Vigna unguiculata* L. under different soil salinity regimes

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Abstract: *Rhizobium*-legume symbiosis is one of the most well-established symbiotic nitrogen fixing system for agronomic studies. The current study aimed to test the hypothesis that screening for salt-tolerant rhizobial strains or salt-tolerant cultivar does not necessarily promise a salt-tolerant symbiotic system, as the symbiotic system is more sensitive to salt stress than the bacterium and/or the plant. In fact, the current study reveals that there is a decrease in salt tolerance of the symbiotic system by 1 dS/m , and also that there is a gradual shift in the spatial distribution of the nodules from the primary roots to the secondary roots under increased salt levels, and is time-dependant. Thus, the current study confirms that there is a need to screen for salt-tolerant symbiotic *Rhizobium*-legume system for producing efficient root nodules, thereby an efficient repository for nitrogen fixation.

Keywords: Cowpea, nodulation, rhizobium, root system, salt stress, symbiotic efficacy. **Abbreviation**: BNF –Biological Nitrogen Fixation; STI- Salt Tolerance Index; DAS- Days After Sowing; YEMA –Yeast Extract Mannitol Agar; YEMB – Yeast Extract Mannitol Broth

تشكيل وتوزيع الفعالية التكافلية للعقد البكتيرية لنبات *Vigna unguiculata* L. تحت أنظمة مختلفة لملوحة التربة

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ملخص: تعتبر ***Vigna unguiculata* L.** من النباتات البقولية واحدة من اهم وانجح العلاقات التكافلية لتثبيت عنصر النيتروجين فى معظم الدراسات الحقلية الزراعية، تهدف هذه الدراسة لاختبار فرضية عمليات الفرز والكشف عن سلالات ***Rhizobium*** المتحملة للملوحة او الأصناف المتحملة لها وليس بالضرورة فقط تقنية واعدة لتحديد نظم تحمل ال ***Rhizobium*** للملوحة ، وتعتبر الانظمة التكافلية اكثر حساسية لضغوطات التملح من البكتريا او من النبات نفسه ، وفى الواقع بينت الدراسة الحالية هناك انخفاض فى التحمل للملوحة فى الانظمة التكافلية بنسبة **1 dS/m** وايضا هناك تحول تدريجى للتوزيع المكافئ بالعقد من الجذور الأولية إلى الجذور الثانوية مع ارتفاع نسبة التملح مع تحييد عنصر الزمن ولهذا كله فان الدراسة الحالية أثبتت ان هناك حاجة للكشف تحت الأنظمة الملحية عن ***Rhizobium*** البقولية التكافلية لمدى كفأتها لإنتاج العقل الجذرية الفعالة لتثبيت عنصر النيتروجين .

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Introduction

Biological Nitrogen Fixation (BNF) is an effective alternative natural source of nitrogen made available to the soil. The total annual terrestrial inputs of nitrogen from BNF, ranges from 139 million to 175 million tonnes of nitrogen (Burns and Hardy, 1975; Paul, 1988), with the nitrogen fixing symbiotic association growing in the arable land accounting for 25 to 30% (35 million to 44 million tonnes of nitrogen). Even though the accuracy of these figures is debatable (Sprent and Sprent, 1990), they illustrate the relative importance of BNF in cropping systems and the magnitude of potential opportunities available to replace the 80 to 90 million tonnes of nitrogenous fertilizers applied annually to agricultural land (Peoples et al., 1995a; Peoples et al. 1995b). Among the various organisms involved in the fixation of nitrogen to the soil, the symbiotic association, especially that of the leguminous plants with the rhizobia has the greatest quantitative impact on the nitrogen cycle (Brockwell et al., 1995; Peoples et al., 1995a). However, several environmental conditions are limiting the efficient growth and activity of nitrogen-fixing plants. As the most serious threat faced by agriculture in arid and semi-arid regions is salinity (Rao and Sharma, 1995), selecting a salt tolerant rhizobial strain or a salt tolerant legume, the probability for the success of the partnership has been a failure. This is because legume-*Rhizobium* symbiosis and nodule formation on the legumes is more sensitive to salt or osmotic stress than the rhizobia or the plant (Zahran and Sprent, 1986; El-Shinnawi et al., 1989; Velagaleti et al., 1990; Zahran, 1991).

The salt injury on the symbiotic interaction not only inhibits the formation of the nodules, but also thereby leads to the reduction of the growth of the host plant. Other effects of salinity on the nodulation, includes formation of non-functional nodules with abnormal structure, and degradation of peribacteroid membrane (Bolanos et al., 2003). Bacterial chemotaxis, colonization, root hair curling (Zahran, 1986; Zahran and Sprent, 1986; Tu, 1981) and deformation (Singleton et al., 1982), reduction in nodular respiration (Delgado et al. 1994; Walsh, 1995), leghemoglobin content (Delgado et al., 1993) and impaired N-fixing activity (Bordeleau and Prevost, 1994) have also been observed as adverse effects of salt. Hence, screening for a salt tolerant symbiotic legume-rhizobium system is more important.

Among the dietary legumes of the world, cowpea, *Vigna unguiculata* L., stands sixth in consumption. Cowpea has been categorized as a salt tolerant plant with EC_e of 5.0 dS/m (California Fertilizer Association, 1980), (Ayers and Westcot, 1985) and, so it does not escape from salt injuries altogether. A decrease in the shoot dry matter, shoot/root ratio, nitrate uptake, leaf-nitrate reductase (NR) activity and shoot-nitrate content of the plant (de Sileveria et al., 1999; Maas and Poss, 1989) under salt stress, though rhizobial strains isolated from *Vigna unguiculata* L. nodules are known to be tolerant upto 450 mM NaCl (Mpepereki et al., 1997). However, very little is known on the effect of salinity on the changes in the pattern and distribution of nodules in the different regions of the root system, and on their efficacy to establish a symbiotic association, in other words, the ability of the *Rhizobium*-legume to develop a partnership for forming nodules, a repository for fixing nitrogen that can be used by the legume and energy resources for the bacterium. Results of the work carried out in these perspectives are discussed in this paper.

Materials and Methods

Study sites and species

Rhizobial strains were isolated from groundnut (*Arachis hypogaea* L.), a cross-inoculating legume variety for cowpea, and a widely cultivated legume in the district, were collected from different fields in and around Dindigul, Tamil Nadu, India. Cowpea seeds for pot culture were also collected from authenticated resources.

Screening of salt tolerant rhizobial strains

Rhizobial strains were isolated from *Arachis hypogaea* L. nodules, a cross inoculating group of cowpea. The isolated strains were characterized and authenticated using standard microbiological and biological methods. Pure culture of the isolated strains, GRI I, GRI II, CP I, CP II and CP III, were maintained till the end in YEMA slants.

Strains were classified as slow-growers and fast-growers using a biochemical method, by growing them on Bromothymol Blue (BTB) medium, yeast extract mannitol agar supplemented with 0.5% alcoholic solution of bromothymol blue.

Halotolerance of the strains were determined based on the exponential growth and relative salt tolerance of the strains using turbidometric growth technique (Singleton et al., 1982). Isolated strains were inoculated in YEMB in 9:1 ratio and incubated at 22°C for 48 hours. The diluted culture was then transferred to 250 ml Erlenmeyer flasks until the initial optical density of the YEMB read 0.08 nm, and this was considered zero time. Inoculated flasks were then incubated at 26°C with constant shaking (70 rpm) using an Orbital-shaking incubator. All readings were recorded at an interval of one hour for 8 hours, at an optical density of 600 nm.

Comparative readings of the growth of the strains under different salt treatments were taken by supplementing YEMB with different salt (NaCl : $CaCl_2$ in 7:3 ratio) concentrations ranging from 0 mM, 100 mM, 200 mM, 300 mM, 400 mM and 500 mM. Turbidometric readings thus obtained were used for calculating the exponential growth rate (μ) and the doubling time (t_d) of the individual strains using equations given by Schlegel et al. (2002), and a sodium response curve was derived using these values.

Salt tolerance index of the plant

Cowpea plants were grown in *in vitro* using Knop's solution (Rachel and Ravindran, 2006) supplemented with nitrate in Erlenmayer's flask, with differing salt concentrations adjusted to 0, 1, 2, 3 and 4 dS/m, harvested on the 15th day after sowing, and salt tolerance index of the genotype was calculated using the formula adopted by Garg and Singla (2004).

Pot culture experiments

The effect of varying concentration of salinity on the symbiotic association was evaluated in pot culture experiments. Circular plastic pots were filled with a mixture of thoroughly sifted and sterilized soil, sand and farmyard manure in proportion of 2:2:1 by volume. Seeds were surface sterilized with 0.1% mercuric chloride for 2 minutes, then washed in sterile water thrice and germinated in pots. The pots were treated with saline solutions (prepared from a mixture of NaCl, $CaCl_2$ and Na_2SO_4 in the ratio of 7:2:1 (w/v) of varying electrical conductivity, 0, 1, 2, 3 and 4 dS/m, incrementally. The pots were treated with the salt solution three days prior to sowing until the desired level of the salt has been attained and these levels were maintained throughout the growing period by fortificating the soil with saline solutions at weekly intervals. The electrical conductivity of the pot soil was measured using a Systronic conductivity meter (Type 302) adopting the methodology of Ryan et al. (2001), originally done by Richard (1954). The controls were irrigated using tap water alone. Surface sterilized seed were sown with 1 ml aliquot of 24 hours old culture solution. They were then treated with 1 ml of the aliquot solution 24-hour culture solution consecutively for three days. Initially five plants were sown, on the 15th day, two of the five plants were removed, and three plants of uniform size were maintained in each pot. Plants were sampled and analysed on the 15th and the 30th day after sowing, and the pots were randomly shuffled on a daily basis.

Measurements

Triplicates of each treatment were maintained and the following parameters were analysed:

1. Salt tolerance index of the plant (Garg and Singla, 2004).
2. Mean root length, number of secondary roots, nodule fresh weight, total number of nodules formed, number of nodules formed on the primary and the secondary roots and shape of the root nodules.
3. The symbiotic efficacy (efficiency to nodulate) on the primary or the taproot and the secondary roots of the plant was calculated using the derived formula:

$$\text{Symbiotic Efficacy of Primary Roots} = \frac{\text{Number of Nodules in the Primary Roots}}{\text{Total Number of Nodules}} \times \text{Mean Root Length} \quad \text{---- Eqn. 1}$$

$$\text{Symbiotic Efficacy Of Secondary Roots} = \frac{\text{Number of Nodules in the Secondary Roots}}{\text{Total Number of Nodules}} \times \text{Total Number of Secondary Roots} \quad \text{---- Eqn. 2}$$

4. F-test was performed to study the distance of variances in the symbiotic efficacy on the secondary and primary roots of the plant by the tested rhizobial strains. Statistical Analyses were performed using MS-Excel 2007.

Results and Discussion

Screening for halo tolerant strains

Five rhizobial strains, namely GRI I, GRI II, CP I, CP II and CP III were isolated from peanut plant nodules. All strains were fast growers expect for CP II, which is a slow grower. Based on the results obtained from the halo tolerance experiments, CP II was found to be the halo tolerant strain and was used for further studies. The performance of the other strains when subjected to salt stress were in the following order - GRI I and GRI II, CP I and CP III as evident in the sodium response curve for exponential growth rate during logarithmic phase (Fig. I.). The exponential trend line shows that CP II shows an upward trend with increasing salt concentrations unlike GRI I and GRI II that has more or less similar growth rate in all concentrations of salt supplemented to the medium; and CP I and CP III, contrarily showed a downward trend.

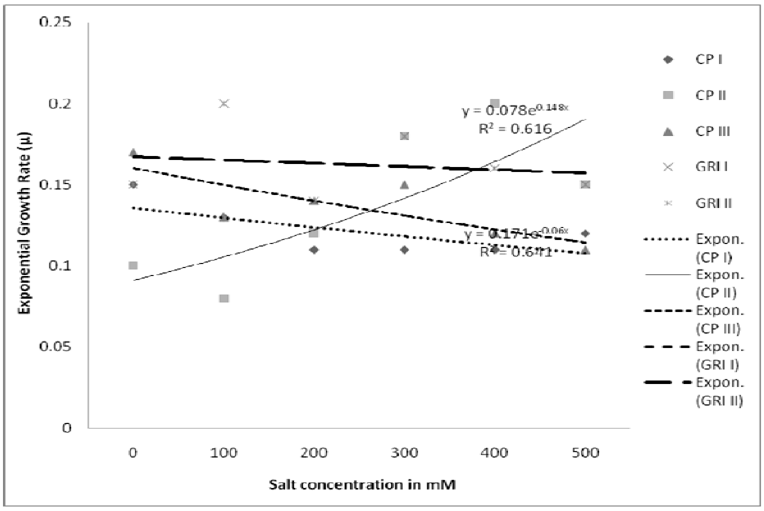

Figure 1. Exponential growth rate under varying salt stress: Trend line showing the exponential variation in the growth rate of isolated rhizobial strains under different salt concentration. CP II shows an upward trend with increasing salt concentrations unlike GRI I and II that has more or less similar growth rate and CP I and CP III shows a downward trend.

Even though the salt tolerance of the rhizobial strains are usually calculated based on their doubling time, in this case, for the convenience of forecasting how successfully a symbiotic association can be established, the influence of salt stress on the exponential growth rate is calculated. The rhizobial strains has to be in their exponential phase of growth for a longer period of time, so that they can establish themselves, in the legume rhizosphere (Schlegel et al., 2002); while, a higher doubling time is favourable to overcome the effects of salinity. This is because in culture solutions the fast-growing rhizobia isolated from peanut cultivars, has been more tolerant to salt than the slow-growing rhizobia. Hence, a strain that can strike a balance of these two features, was found to be observed in CP II, and was thus selected for further evaluation.

Pot Culture Experiments

Salt tolerance index of the plant as evaluated on the 15th and 30th day after sowing, showed that the genotype used in this experiment had a tolerance to salt up to 3 dS/m (Table 1). However, plants were still subjected to a higher concentration of salt of 4 dS/m, in order to test the performance of the symbiotic system.

Table 1. Salt Tolerance Index of *Vigna unguiculata – Rhizobium* sp.

Salt Treatment	Salt Tolerance Index (STI)	
	15 DAS	30 DAS
0	100	100
1	146.11	57.33
2	141.45	55.75
3	94.19	50.12
4	19.92	40.08

Results of the pot culture experiments showed that the symbiotic system tolerated salt up to 2 dS/m on the 15th day after sowing, but the level of tolerance started to decrease from 3 dS/m. In contrast to the controls, the symbiotic system succumbed to salt stress by the 30th day after sowing at 1 dS/m.

The penetration of the tap root system into the soil also seemed to reduce under saline conditions, with a considerable decrease in the primary root length when the salt concentration was increased to 4 dS/m on the 15th day after sowing, and a further decline on the 30th day after sowing probably owing to death of the tissue. On the contrary, an increase in salt concentrations resulted in an increase in the number of secondary roots, though does not seemed to be pronounced as the number of the secondary roots on the 15th and the 30th day after sowing did not increase with increasing salt concentrations (Table 2).

In spite of the effect of salt injury on the plant seemed to be marked on the 30th day after sowing, the effect of salinity on the nodules formed does not seem to correlate with the salt tolerance of the plant on the 15th or 30th day after sowing, as there was no variation in the size, shape and fresh weight of the nodules. However, there was marked salinity imposed shift in the spatial distribution of nodules that thereby influencing the efficiency of nodules formed, that is, the symbiotic efficacy (Table 3). The efficiency for nodule formation on the taproot of the plant is on a declining trend with increasing salt concentration (2 dS/m) on 30th day after sowing; and also, there was a difference in the colour of the nodules formed in the primary and secondary roots (Table 2). Nevertheless, there was a reversal trend in the symbiotic efficacy on secondary roots of the plants on the 30th day after sowing, which also seemed to fall in line with the salt tolerance level of the symbiotic system, a decline in the efficacy beginning from an increase in salt concentration from 2 dS/m to 3 dS/m.

Table 2. Morphometric variations in *Rhizobium* sp. (CP II) *Vigna unguiculata* symbiotic relationship under salt stress.

Salt Treatment in dS/m	Mean Root Length (Primary Root, MRL) in cm		Number of Nodules in the Primary Root		Colour of the Nodules in the Primary Root		Number of Secondary Roots		Number of Nodules in the Secondary Roots		Colour of the Nodules in the Secondary Roots		Total Number of Nodules	
	15 DAS	**30 DAS**	**15 DAS**	**30 DAS**	**15 DAS**	**30 DAS**	**15 DAS**	**30 DAS**	**15 DAS**	**30 DAS**	**15 DAS**	**30 DAS**	**15 DAS**	**30 DAS**
0	11.3	10.08	4	5	Pink	Pink	16	15	3	7	White to Pink	White to Pink	7	12
1	11.2	9.7	1	2	Pink	Pink	12	10	4	9	White to Pink	White to Pink	5	11
2	9.7	7	3	3	Pink	Pink	13	8	9	10	White to Pink	White to Pink	10	10
3	9.3	4	4	3	Pink	Pink	10	7	7	9	White to Pink	White to Pink	10	9
4	6.2	3	2	1	Pink	Pink	7	5	3	5	White to Pink	White to Pink	4	5

Table 3. Symbiotic efficacy of the selected rhizobial strain under varying concentrations of salt.

Salt Concentration dS/m	SE IN SR		SE IN PR		F - Test	
	15 DAS	30 DAS	15 DAS	30 DAS	15 DAS	30 DAS
0	6.86	8.75	6.46	4.5	0.41	0.999
1	9.6	8.18	2.24	1.76		
2	11.7	8	2.91	2.1		
3	7	7	3.72	1.33		
4	5.25	5	3.1	0.6		

Symbiotic efficacy was calculated as the ability to form nodules in plants grown in differing salt concentrations varying from ~0 dS/m to 4 dS/m.

Moreover, the distance of variances in the symbiotic efficacy on the 15th DAS and 30th DAS has increased nearly twice, from 0.4 to 0.9, as observed from the F-test calculations, thereby showing a time-dependant increase in the shift of trend towards nodule formation on the secondary roots.

The salt tolerance of the plant and the halo tolerance of the symbiotic system seemed to be differing from 3 dS/m to 2 dS/m that is a variation of 1 dS/m. However, for evaluating the efficiency of the symbiotic system the initial observation is on the ability of the symbiotic systems that can form efficient nodules under saline conditions. Yet another interesting feature that has been observed is the increase in the number of nodules formed in the secondary roots than in the primary roots under increasing salt concentrations. But this trend does not persist when the salt tolerance of the symbiotic system decreases, as it is obvious from the results that the symbiotic efficacy on secondary roots decreases at 3 dS/m while the salt tolerance index of the plant is up to 3 dS/m.

Nodule formation and its distributional pattern on the different regions of the roots have been observed and various conclusions and generalizations have been made under non-saline conditions. The formation of nodules, *per se*, has significant effect on the reduction in the growth of the primary root, and induces the formation of laterals or nodules. In fact, the formation of nodules on the taproot is considered as a desirable feature than on the secondary roots, as the nodules formed on the primary roots are efficient. At this juncture, it is also important to take into consideration that the underlying mechanisms in lateral root initiation and nodule formation are more or less similar (Lim, 1963; Dart and Pate, 1959; Nutman, 1959, 1948).

However, the formation of nodules on the lateral roots has also been observed in soybean plants during pod filling stages; and, Lim (1963) observed a similar effect when the number of bacteria in the legume rhizosphere decreased. Hence, the plausible explanations for this shift of nodule formation from primary roots to lateral roots could be due to a reduction in the number of bacteria over time owing its failure to establish in the legume rhizosphere due to decreased slime forming capability (Nilson, 1957), or reduced surface area for initiation of infection. This is because of the increase in the number of secondary roots, a “cross tolerance" mechanism (Malash and Khatab, 2008) to the osmotic stress or drought stress, thereby reducing the surface area exposed for the initiation of infection and colonisation by the *Rhizobium* sp. However, the actual mechanism involved can only be better explained only when more insights and knowledge is gained on the physiological and the biological mechanisms on the establishment of the *Rhizobium* in the legume rhizosphere, initiation, formation, and establishment, and efficiency of the nodules thus formed are understood.

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