WORLD JOURNAL OF PHARMACY AND PHARMACEUTICAL SCIENCES

SJIF Impact Factor 5.210

Volume 5, Issue 1, 1468-1479 Research Article ISSN 2278 – 4357

UTILIZATION OF ^{15}N DILUTION ANALYSIS FOR MEASURING EFFICIENCY OF BIOLOGICAL NITROGEN FIXATION UNDER SOIL SALINITY STRESS

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Article Received on 20 Nov 2015,

Revised on 11 Dec 2015,
Accepted on 31 Dec 2015

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ABSTRACT

The current work was conducted to evaluate the effect of salts content of soil on the efficiency of Biological nitrogen fixation by local rhizobium strains using the ^{15}N dilution technique. Local strains obtained from Iraqi provinces, from Basra down south to Sulymania up the north, were isolated from cowpea rhizospher. They were purified, identified, Authenticated, characterized and propagated. Salt content, equivalent to 3, 6, and 9 dSm^{-1}, effect on BNF%, nodules number, plant dry weight and total N content of cowpea were determined. Isolates showed different efficiency of BNF under different salinity levels. Generally, BNF of all isolates were the least at the highest level of soil salinity, which is 9.0 dSm^{-1}. Local isolates respond differently to different soil salinity levels. The third level of soil salinity, which is equivalent to 112 mM, is the critical salinity level of BNF for all isolates. However, nodules number was significantly decrease with the increase of soil salt content. Plant dry weight under all isolates decrease linearly with the increase of soil salinity.

KEYWORDS: Rhizobium, local Iraqi strains, Cowpea, Total N, Nodules number, dry weight.

INTRODUCTION

Increases in the salinity of soils or water decrease crop productivity and may lead to marked changes in the growth pattern of plants (Cordovilla *et al*., 1994). Tate 1995 reported that increasing of salt concentrations may have a detrimental effect on soil microbial population as a result of direct toxicity as well as through osmotic stress. The introduction of plants capable of surviving under these conditions (salt-tolerant plants) is worth investigating (Delgado *et al*., 1994). Delgado *et al*. (1994) concluded that salinity decreases plant growth and yield, depending upon the plant species, salinity levels, and ionic composition of the salts. Cordovilla *et al*. (1995a) and Cordovilla *et al*. (1995b) concluded that the salinity response of legumes varies greatly and depends on such factors as climatic conditions, soil properties, and the stage of growth. Plant dry weight under all isolates decrease linearly with the increase of soil salinity.

It has been reported by Velagaleti *et al*. (1990); Zahran (1991a); Zahran and Sprent (1986) that the legume-Rhizobium symbioses and nodule formation on legumes are more sensitive to salt or osmotic stress than are the rhizobia. Tu (1981) found that salt stress inhibits the initial steps of Rhizobium-legume symbioses. Soybean root hairs showed little curling or deformation when inoculated with *Bradyrhizobium japonicum* in the presence of 170 mM NaCl, and nodulation was completely suppressed by 210 mM NaCl. Zahran (1986); Zahran and Sprent (1986) observe that bacterial colonization and root hair curling of Vicia faba were reduced in the presence of 50 to 100 mM NaCl or 100 to 200 mM polyethylene glycol as osmoticum. The proportion of root hairs containing infection threads was reduced by 30 and 52% in the presence of NaCl and polyethylene glycol, respectively.

Delgado *et al*. (1994); Ikeda *et al*. (1992); Walsh (1995) attributed the reduction of N_2-fixing activity by salt stress to the reduction in respiration of the nodules and a reduction in cytosolic protein production, specifically leghemoglobin, by nodules. Cordovilla *et al*. (1995a) on the other hand, concluded that the depressive effect of salt stress on N_2 fixation by legumes was directly related to the salt-induced decline in dry weight and N content in the shoot. Other scientists (Sprent and Zahran (1988) and Zahran and Abu-Gharbia (1995) stated that the salt-induced distortions in nodule structure could also be reasons for the decline in the N_2 fixation rate by legumes subject to salt stress. Furthermore, Georgiev and Atkias (1993) observed that reduction in photosynthetic activity affect N_2 fixation by legumes under salt stress. Although the root nodule-colonizing bacteria of the genera Rhizobium and

Bradyrhizobium are more salt tolerant than their legume hosts, they show marked variation in salt tolerance. Growth of a number of rhizobia was inhibited by 100 mM NaCl (Yelton *et al*., 1983), while some rhizobia, e.g., Rhizobium meliloti, were tolerant to 300 to 700 mM NaCl (Helemish *et al*., 1991; Mohammad *et al*., 1991; Embalomatis *et al*., 1994).

Many species of bacteria adapted to saline conditions by the intracellular accumulation of low-molecular-weight organic solutes called osmolytes (Csonka and Hanson 1991). The accumulation of osmolytes is thought to counteract the dehydration effect of low water activity in the medium but not to interfere with macromolecular structure or function (Smith *et al*., 1994).

Zahran (1991b) illustrated that successful Rhizobium-legume symbioses under salt stress require the selection of salt-tolerant rhizobia from those indigenous to saline soils.

El-Mokadem *et al*. (1991) showed that Inoculation of legumes by salt-tolerant strains of Rhizobium *leguminosarum* bv trifolii and *Rhizobium meliloti* enhanced nodulation and N content under salt stress up to 1% NaCl. Under saline conditions, the salt-tolerant strains of *Rhizobium sp*. formed more effective N_2-fixing symbiosis with soybean than did the salt-sensitive strains (El-Sheikh and Wood, 1995). It was concluded from these results that salt-tolerant strains of Rhizobium can nodulate legumes and form effective N_2-fixing symbioses in soils with moderate salinity. Therefore, inoculation of various legumes with salt-tolerant strains of rhizobia will improve N_2 fixation in saline environments (Zou *et al*., 1995). However Craig *et al*. (1991) concluded that tolerance of the legume host to salt is the most important factor in determining the success of compatible Rhizobium strains to form successful symbiosis under conditions of high soil salinity. Evidence presented in the literature suggests a need to select plant genotypes that are tolerant to salt stress and then match them with the salt-tolerant and effective strain of rhizobia (Velagaleti *et al*., 1989 and Cordovilla *et al*., 1995b).

Reviewing the literature indicates the importance of obtaining salt tolerant rhizobium species to be used as inoculant of legumes to increase crop productivity under salinity stress. However, there is contrasting evidence concerning salt tolerance of rhizobium. Therefore, the current study was conducted to evaluate effect of salt content of soils on local rhizobium strains isolated from various locations along the climatic change of Iraqi provinces using ^{15}N dilution technique.

MATERIAL AND METHODS

Collection of nodules

Cowpea root nodules were collected from 20 locations from different field sites in 10 governorates in Iraq, belonging to the most important cowpea production area in Iraq (Basrah, Dhi-Qar, Misan, Wasit, Babil, Al Anbar, Baghdad, Salahudein, Suleimanyah, and Ninevah).

Effective and healthy cowpea root nodules were collected from young, healthy and green cowpea plants of 45 to 60 days old. Cowpea plants in the sites were simply excavated from cultivated field and adhering soil particles were carefully removed. The roots were found to have nodules of various size and stage of development of which reddish to pinkish healthy nodules of 0.3 to 0.6 mm diameter were excised from the root along with 0.5-1.0 mm root part on the both sides of nodule attachment. Excised nodules were washed in tap water; shade dried for 1-2 hours and collected in nodule collection vials. The vials were labeled and were stored at $4\pm1\,^{0}$C for short-term storage.

Bacterial Isolates: Isolation of cowpea rhizobium colonies and maintenance of pure culture and authentication of isolates by infection Tests were all done as it found in unpublished data Alsaedi 2015. Furthermore, Growth on Yeast Manitol Agar-Bromothymol Blue (YMA-BTB) reaction was also done after unpublished data Alsaedi 2015.

Effect of soil salinity levels on Efficiency of BNF of the isolates.

Under this experiment efficiency of six isolates and controls affected by three levels of salinity was evaluated in a greenhouse experiments, using ^{15}N technique, with three replicates in a plastic pots of 24 cm diameter and 20 cm height. Pots were cleaned and swabbed with absolute ethanol and kept for 10 minutes. Therefore, 21 pots were assigned for each level. Samples of five kg of each sterilized soil were transferred in the cleaned pots. The pots were transferred to the greenhouse.

Efficiency of BNF of the six isolates and control (no isolates added) were evaluated in sandy loam soil (medium texture soil) with three levels of salinity. Levels of salinity were added to irrigation water. These levels are.

S1: first level at which salinity of irrigation water is 3 dSm^{-1} (37 mM NaCl)

S2: Medium salinity level which salinity of irrigation water is 6 dSm^{-1} (74 mM NaCl)

S3: High salinity level at which salinity of irrigation water is 9 dSm^{-1} (112 mM NaCl)

It is important to mention here that initial salts content of irrigation water were determined and salinity level was brought up to the selected level. Nitrogen, P and K were added to the soil at the same rate of the first experiment. Taking in consideration that amount of Nitrogen added at all rates of N application was labeled with ^{15}N of 10 a.a. (access atoms).

RESULTS AND DISCUSSION

Effect of soil salinity level on Biological Nitrogen Fixation (BNF)

Figure (1) shows BNF % of the six isolates as affected by salinity levels of the soil. As stated in material and methods section, medium texture soil with three salinity levels was used for this investigation.

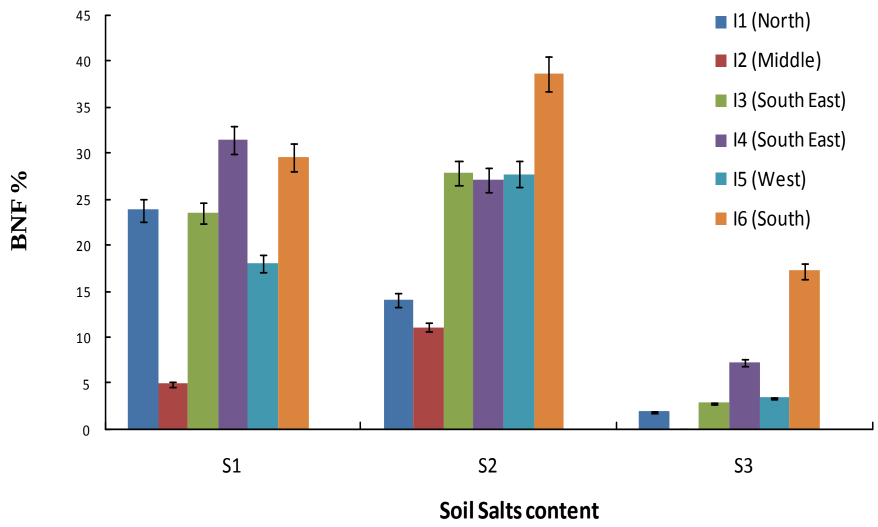

Figure (1.): Efficiency of local Bradyrhizobim isolates on BNF percent under soil salinity level specified.

Where : S1 = 3.0 dSm^{-1} S2 = 6.0 dSm^{-1} S3 = 9.0 dSm^{-1}

Obviously, isolates showed different efficiency of BNF under different salinity level. Generally BNF of all isolates were the least under the highest level of soil salinity, which is 9.0 dSm^{-1}. This result is in agreement with (Tate 1995), who found that soil salts reduce the BNF through its direct effect on bacterial growth and on plant growth as well. The result may be explained by reports of Delgado *et al*. (1994); Ikeda *et al*. (1992); Walsh (1995) who attributed the reduction of N_2-fixing activity by salt stress to the reduction in respiration of the nodules and a reduction in cytosolic protein production, specifically leghemoglobin, by nodules.

However, BNF of isolates 2, 3 and 5 under soil salinity level 2 (6 dSm^{-1}) is higher than under salinity level 1 (3 dSm^{-1}). This may be attributed to the fact that these 3 isolates which were obtained from mid, south and western part of the country, adapted to high salinity level and somewhat are drought resistance. There is evidence in the literature however, that isolates extracted from rizosphere of a plant grown in salt affected soil are of higher tolerant to salts and drought of those grown in soils of less salinity level. This may be supported by the finding of (Tate 1995) and Delgado *et al*. (1994) who concluded that salinity decreases plant growth and yield, depending upon the plant species, salinity level, and ionic composition of the salts.

Isolate number 2 is the least salt tolerance compared to the other 5 isolated under the three salinity levels. Isolates number 6, on the other hand, is the highest salt tolerant as indicated by the highest BNF% under the three-salinity level. Results also showed that BNF of all isolates is markedly reduced at salinity level of 9 dSm^{-1}. Apparently, the third level of soil salinity, which is equivalent to 112 mM, is the critical salinity level of BNF. This result is in agreement with (Yelton *et al*., 1983), who found that 100 mM soil salinity is the critical level. However, it is in contrast with the finding of Tu (1981) who found that 178 mM is the critical salinity level. These results may suggest that there is a serious need in Iraq to increase salt tolerance of N fixing microorganisms. That is due to the fact that most Iraqi soils contain relatively high salt contents. Furthermore, the quality of irrigation water is rather severely deteriorated over the past two decades.

Statistical analysis data showed that there is a significant variation ($P < 0.01$) in BNF as a percent under different salinity levels. Isolates are significantly varied in their ability to fix N under different salinity level. BNF% significantly decreases all over strains with the increase of salinity levels. In conclusion these results firmly suggest that other work for obtaining salt and drought tolerant strains in Iraq are very much needed.

Effect of soil salt content on nodules number

Number of nodules of the six isolates as affected by salinity levels of the soil generally decreases with the increase of the salinity levels of the soil (Fig. 2). This reduction in nodule may be attributed to the reduction in respiration of the nodules and a reduction in cytosolic protein production, specifically leghemoglobin, by nodules (Delgado *et al*. (1994); Ikeda *et al*. (1992); Walsh (1995).

Comparing the BNF % of the six isolates to the nodule number under the three salinity levels clearly shows that BNF % is more affected by salinity than the nodules number. Obviously, under the first salinity level isolate number 1 showed the highest nodules number while strain number 5 showed the least nodules number.

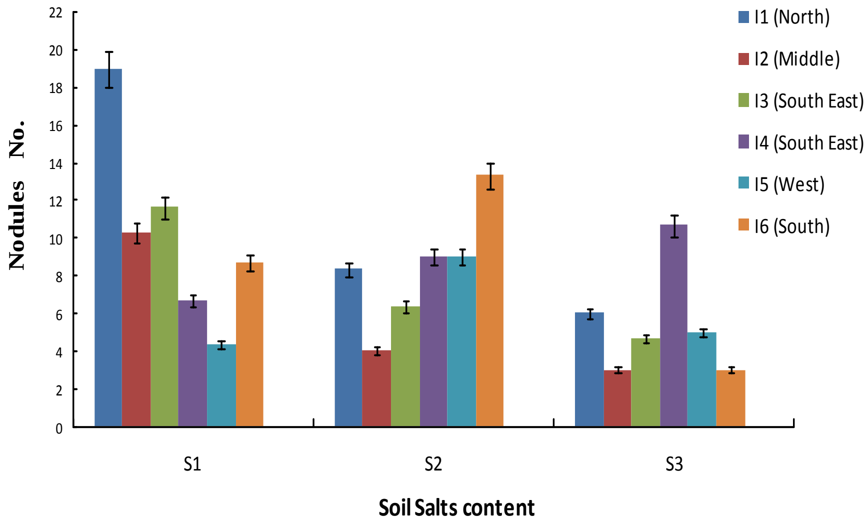

Figure (2.): Effect of soil salinity level on nodules number on cowpea plant under indicated local Bradyrhizobium strains.

Where : S1 = 3.0 ds.m^{-1} S2 = 6.0 ds.m^{-1} S3 = 9.0 ds.m^{-1}

At the second salinity level (6.0 dsm^{-1}), the highest nodules number was observed under strains number six and the least nodules number was observed under strain number 2. The highest nodules number under the third salinity level (9.0 dsm^{-1}) was observed with isolate 4. Average of total nodules number over the six isolates as affected by salt content is in the following order: level 1 > level 2 > level 3. This result is in agreement with those of Velagaleti *et al*. (1990); Zahran (1991a); Zahran and Sprent (1986) who found that legume-Rhizobium symbioses and nodule formation on legumes are more sensitive to salt or osmotic stress than are the rhizobia.

In fact, average of total nodules number at the first salinity level is 100% higher than that under the third salinity level. This result may be supported by the findings of Yelton *et al*. 1983 which stated that growth of a number of rhizobia was inhibited by 100 mM NaCl and in contrast with those of (Helemish *et al*., 1991; Mohammad *et al*., 1991; Embalomatis *et al*., 1994) who stated that some rhizobia, e.g., Rhizobium meliloti, are tolerant to 300 to 700 mM NaCl.

Percent of BNF as affected by salinity level is the highest at the first salinity level and it is the least under the third salinity levels.

Consequently, these results confirmed the previous conclusion that percent of BNF is not coincided with the nodules number.

Effect of soil salinity on plant Dry Weight

Figure (3.) shows the dry weight of cowpea under the six local isolates as affected by salinity levels. In general, plant dry weight under all isolates decrease linearly with the increase of soil salinity. That may be attributed to the determinant effect of soil salinity on plant growth and development. These results are in agreement with those reported by Patel *et al*. (2010) concerning the deleterious effect of soil salinity.

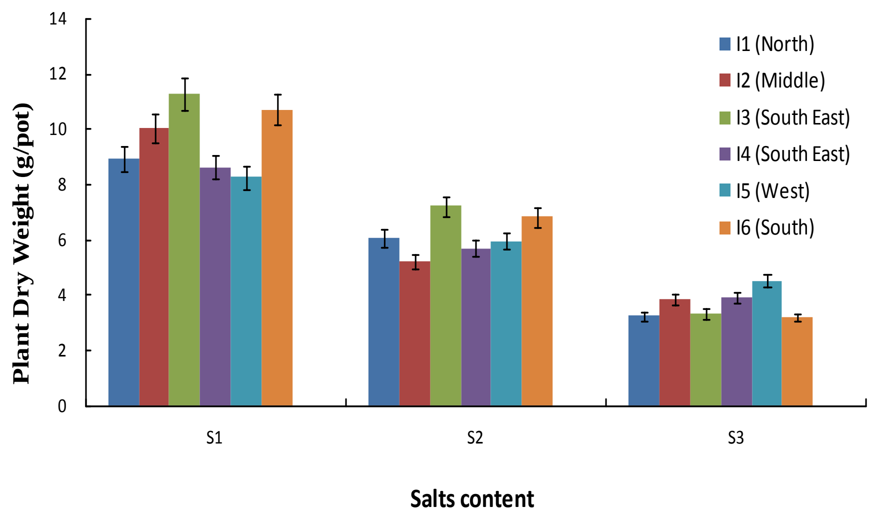

Figure (3.): Plant dry weight (g/pot) under local Bradyrhizobium isolates as affected by soil salinity level specified.

Where : S1 = 3.0 ds.m^{-1} S2 = 6.0 ds.m^{-1} S3 = 9.0 ds.m^{-1}

As the data revealed that, activity of isolates in term of nodule number and percent of BNF severely reduced as the soil salinity increase. This is in agreement with Patel *et al*. (2010) who confirmed the hazardous effect of salt on nodule number and BNF as well. In depth analysis of the data showed that different isolates behaved differently under the same salinity level as indicated in term of plant dry weight. This is in agreement with (Predeepa and Ravindran 2010).

Under the first salinity level, isolate number 3 showed the highest plant dry weight, while that under isolate number 5 is the least plant dry weight. The other 4 isolates are of close effect on dry weight.

Isolate 3 under salinity level 2 showed the highest plant dry weight compared to the other 5 isolates. Plant dry weight under salinity level 2 is in the following order: I_3> I_6> I_1> I_5> I_4>= I_2. This order is similar to that of BNF%.

Statistical analysis of the data showed that salinity level is of high significant effect on dry weight.

In conclusion, these result suggest adapting active breeding program to develop salt tolerant cowpea and more salt resistant rhizobia (Patel *et al*., 2010).

Percent of total N in plant as affected by salinity levels under local Isolates

Figure (4.) showed effect of soil salinity levels on the percent of total N in plant as affected by the six local isolates.

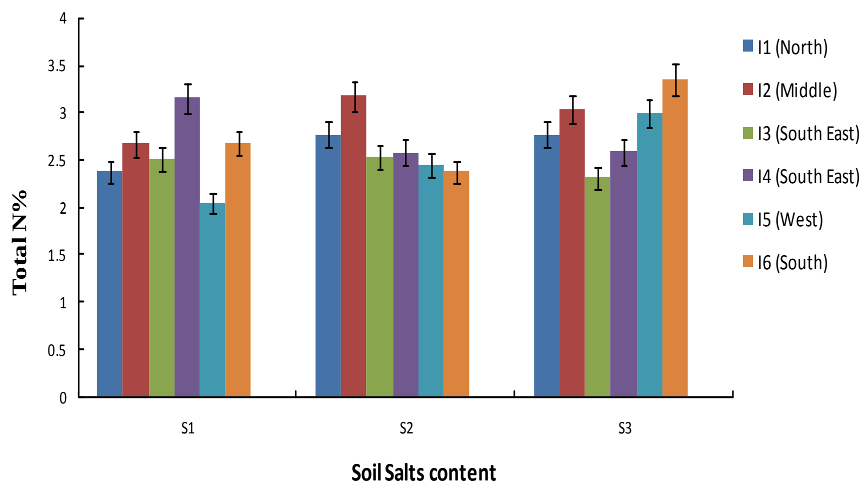

Figure (4.): Effect of soil salinity levels on percent of total N in plant under local Bradyrhizobium strains specified.

Where : S1 = 3.0 ds.m-1 S2 = 6.0 ds.m-1 S3 = 9.0 ds.m-1

Comparing these results to that of dry weight indicated that percent of total N in plant is relatively equal under different salinity levels. That may be attributed to the biological dilution of N stem from dry weight response to salinity.

In other word, that there are no significant differences among averages over isolates of the percent of total N in cowpea under different salinity levels.

Percent of total N in plant under the first salinity level was the higher under isolate 4 and the least was under isolate 5. Under 2nd salinity level the high percent of total plant N was under isolate 2. However, it decreased linearly with other isolate in the following order: Isolate 1 > Isolate 4 > Isolate 3 > Isolate 5 > Isolate 6. These results confirm that isolates respond differently to soil salinity as measured by percent of total N in plant. This is in agreement with the findings of Preedepa and Ravindran 2010.

When salinity level of soil increased to 9.0 dSm^{-1}, which is 3 fold that of first salinity level, the high percent of total N was observed under isolate number 6 and the least was observed under isolate 3. These results are not in the same trend of the plant dry weight, which indicates that salinity may affect other plant factor.

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