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THE *LEUCAENA-GLOMUS-RHIZOBIUM* SYMBIOSIS UNDER SALINE CONDITIONS: EFFECTS ON PLANT GROWTH, NODULATION AND TISSUE NITROGEN

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ABSTRACT

Healthy seeds of *Leucaena leucocephala* grown in small pouches were transferred to earthenware pots containing loamy soil irrigated with Sea salt solutions of 0.02%. 0.4%, 0.6%, 0.8% and 1.0% salinity, after 18 days of germination of seeds. Controls were irrigated with tap water only. Seedlings were irrigated with nitrogen free nutrient solution once a week of transplantation of seedlings into pots. Seedlings including control were inoculated with *Rhizobium* spp. and *Glomus* spp. Plants were harvested after 96 days of transplantation in pots. Root and shoot length, fresh and dry weights of roots and shoots, number, size, fresh and dry weights of nodules and percent tissue nitrogen showed a progressive decrease with increasing salinity levels. Root-shoot ratio showed a slight increase over control but the increase was statistically non-significant. Nodules were observed in all five salinity levels used in the experiment. In general, salt induced decrease in plant growth, biomass production and nitrogen fixing parameters. Results have been related to detrimental effects of salt on *Rhizobium* and AM populations in the pot soil.

Key-words: *Leucaena leucocephala*, *Rhizobium* spp. and *Glomus* spp. salinity, plant growth.

INTRODUCTION

Soil salinity has become a big challenge to agriculture in many parts of the world, particularly in areas with low rainfall (Sprent and Sprent, 1990). Salinity affects seriously all the major physiological and biochemical mechanisms operating in the plants (Ramoliya *et al*.; 2004). In the presence of excess salts in soil plants are subjected to osmotic as well as ionic stress (Munns, 2002; Benlloch-Gonzalez *et al*., 2005). The detrimental effect of salt is generally observed at the whole plant level. Salinity suppresses of growth in all plants but the tolerance levels of different species to high salinity shows considerable variations (Delgado *et al*; 1994, Rabie and Almadini; 2005, Lopez *et al*; 2008).

Growth of leguminous plants is affected by salt in particular since legumes meet their nitrogen requirement through symbiotic N-Fixation (Elsheikh and Wood, 1995). Salinity tolerance studies of nitrogen fixing legumes show that herbaceous grain and forage legumes have been examined in the past. Very few long term studies have been conducted with multipurpose nitrogen fixing tree (MNFT) species growing in the tropics. Tropical deep rooted trees that can fix atmospheric nitrogen have the additional advantage of adding natural fertilizer to the deep layers of the soil. (NFTL) therefore deserve more attention for growing them in stressed lands (Mahmood, 1999; Zahran, 2009).

*Leucaena leucocephala (*Lam.) de (Wit) is a mimosoid tree legume. It has been introduced in Pakistan as a fast growing tree with its potential uses as forage, firewood timber etc in the tropics (Brewbaker, 1987). *L leucocephala* leaves are highly valuable as fodder for ruminants. The palatable leaves of *L. leucocephala* contain 20-30 % protein and are 70% digestible (National Research Council, 1984). *L. leucocephala* also forms symbiotic association with *Rhizobium* (Mahmood, 1999). It has been found to have the highest rate of nitrogen fixation amongst trees which is estimated at 500 kg N. ha^{-1}. $Year^{-1}$ (Subba Rao, 1993). In addition to infection by rhizobium the fine root hairs of *Leucaena* are also usually infected by Arbuscular Mycorrhizae (AM). AM is known to enhance the uptake of phosphorus and many other nutrients such as Zn, Mo, CU etc. from the soil (Herrara *et al*., 1993). AM could enhance the ability of plants to cope with salt stress (Kumar et al., 2015). The benefits that plant obtained from the tripartite association with AM and nitrogen fixing bacteria many be greater than those obtained through association with nitrogen fixing bacteria alone (Lesuer and Sarr, 2008; Hashem *et al*., 2016). The benefits of tripartite association have also been documented under saline conditions (Dixon *et al*., 1993; Ahmed and Elsheik, 1998; Rabie and Almadini, 2005).

Salinization of soil is a serious problem and at present out of 1.5 billion hectares of cultivated land around the world, about 77 million hectares is affected by excess salt contents (Sheng *et al*., 2008). Pakistan also has a large

area of salt affected land in Punjab and Sindh provinces. In Pakistan about 6.3 million ha are believed to be affected by salinity (Qureshi and Barrett–Lennard, 1998). The impact of salinity to agriculture in irrigated areas in which soil and water borne salts are accumulating during repeated cycles of water use is alarming. Agriculture lands could be damaged and degraded by secondary salinization through irrigation with water from the river Indus and its tributaries. In order to combat the threatening effects of salinity to agriculture lands, use of chemical fertilizers is in practice the world over including Pakistan since a long time (Chachar *et al*., 2008; Sharif and Khan, 2009; Javaid, 2009). Chemical fertilizers are hazardous and at the same time may not be economical and unaffordable in third world countries including Pakistan. Biofertilizers must therefore receive increasing attention. Biofertilizers (Nitrogen fixing bacteria and *Arbuscular Mycorrhizae* (AM) are an alternative source of enhancing nodulation and nitrogen fixation in saline soils. The purpose of the present study was to evaluate the effect of salinity on plant growth, nodulation and percent tissue nitrogen contents in *L. leucocephala* plants having dual inoculation with *Rhizobium* and *Glomus* species.

MATERIALS AND METHODS

Seeds of *Leucaena leucocephala* were collected from trees growing in the garden of Botany Department, University of Karachi. Healthy seeds were surface sterilized with 30% (M/V) mercuric chloride for 2 min. rinsed with sterilized water and germinated in the plastic pouches filled with sandy-loam soil. The pH of soil was 8.4; maximum water holding capacity 10.03 % and field capacity 7.08 %. Seedlings appeared after one week in pouches. Twenty four pots lined on the inside with polythene bags were prepared. Each pot had a hole at the bottom allowing adequate drainage. The hole was plugged with cotton wool. Each pot was filled with 3kg of air dried soil. The pots were arranged in a complete randomized design. Twenty four pots were used in the experiment (four replicates for each salinity treatment and a control). The pots were irrigated with 0.2%, 0.4%, 0.6%, 0.8% and 1% saline solutions. After attaining the required salinity levels in the pots, saturated extracts of soil samples from the pots were used to measure the electrical conductivity of the soil. The electrical conductivity values are given in Table 1.

Table 1. Electrical conductivity of the pot soil.

Percentage of sea salt solutions	**Electrical conductivity**
	dSm^{-1}
Tap water(control)	0.9
0.2%	5.18
0.4%	8.62
0.6%	10.2
0.8%	14.24
1.0%	16.22

After establishing required salinity level in pots three seedlings were transferred from plastic pouches to each pot. The desired salinity levels were maintained throughout the growing period of plants by fortification with saline water at regular weekly intervals, if necessary. The control sets were irrigated with tap water only. Seedlings were inoculated both with *Rhizobium* sp. and *Glomus* sp. Bacterial cultures were prepared by isolating rhizobia from root nodules of *L. leucocephala* plants growing in the garden of Botany department. The bacteria were grown on YEMA (Yeast Extract Mannitol Agar) medium following (Somasegaran and Hoben, 1984). Each pot including the control was irrigated with 100ml of bacterial culture near the root zone of the plants to ensure the root infection by rhizobia. The plants were irrigated with nitrogen free nutrient solution (Hoagland and Arnon, 1950) once a week of transplantation of seedlings in pots. The soil was saturated at field capacity to avoid leaching.

AM spores were collected from the rhizosphere of *L. leucocephala* trees growing in the garden of Botany Department, University of Karachi by the method of Gerdemann and Nicolson (1963). Four grams of sieved soil was taken in a beaker and 50 AM spores were mixed in the soil. The mixture was placed in the center of the pot soil at a depth of six inches from the top. Then the seedlings were transferred in the pot in such a manner that the roots of the seedlings touched the AM- soil mixture. Estimation of mycorrhizal intensity was made from young roots cut into

small segments and stained with 0.5 % trypan blue in lectophenol as described by (Philips and Hayman, 1970). The percentage of AM infection was estimated following grid intersect method of (Giovannetti and Mosse, 1980). Plants were kept under natural environmental conditions.

Plants were harvested after 96 days of transplantation in the pots. They were uprooted taking great care not break the secondary and tertiary roots and nodules. Growth parameters such as root length, shoot length, fresh and dry weight of roots and shoots and number, size, fresh and dry weight of nodules were recorded. The dry weight of roots shoots and nodules were recorded after drying the samples in an electric oven for 72 hrs. at 70°C. Root-shoot ratio was determined by dividing root length with shoot length. Nitrogen estimation was carried out with the help of micro Jeldahl apparatus (Bergerson, 1980). Hundred grams of dry mass of shoots were used for determining tissue nitrogen.

RESULTS AND DISCUSSION

Effect of salinity on plant growth

Plant growth in terms of root length, shoot length and root/shoot ratio were markedly affected by salt (Table.2). Root and shoot lengths were maximum in control but decreased progressively with increasing salinity levels. Root and shoot length decreased respectively to 41% and 47% of the control when soil salinity was raised from 0.9dSm^{-1} to 16.2dSm^{-1} level. Decrease in shoot growth was more than in root growth. Shoot growth of plants growing under saline conditions is reduced because the ability of shoots to take up water is reduced in saline environment (Chachar *et al*., 2008). Akhtar and Hussain (2009) observed reduction in the root and shoot length in *vicia sativa* under salt stress.

Reduction in plant growth under saline conditions is a common phenomenon (Lopez *et al*.; 2008, Ben Salah *et al*; 2009, Al-Shaharani and shelta, 2011 and Mahmood *et al*; 2008, 2012). Salinity decreases plant growth depending upon the plant species, salinity level, duration of treatment and ionic composition of salts (Benlloch-Gonzales *et al*., 2005). For instance NaCl did not show any adverse affects on plant growth during the first seven weeks of treatment in *Leucaena leucocephala* plants (Anthraper and Du Bios, 2003) but after 14 weeks of treatment distinct differences appeared on growth, nodule formation and nitrogen fixation. NaCl concentration ≥ 0.025 mol/L showed the greatest decrease. Singleton and Bohlool (1984) recorded a depressive effect on growth of *Soybean* at NaCl concentration of 0.026 mol/L. A depressive effect of NaCl concentration ≥ 0.05 mol/L was observed in *Chickpea* (El-sheikh and Wood, 1995). Singla and Garg, (2005) found maximum depressive effects of NaCl on the growth of chickpea at 8dSm^{-1}. Reduction in growth was observed in fababean at 0.1 mol/L salinity (Delgado *et al*., 1994). Adverse effects on the growth of various organs of sugar beet and cabbage were reported by Jamil *et al*. (2007) at 150mM NaCl. Growth of *Medicago ciliaris* was effected at 100mM Nacl (Ben Salah, *et al*. 2009). Length of shoots and roots, and fresh weight of shoot and roots declined at 10 and 15dSm^{-1} salinity levels in *Vicia sativa* (Akhtar and Hussain, 2009). Salinity significantly reduced plant height, dry weight of shoots and roots and total plant biomass at 20.71mScm^{-1} salinity level in *Capparis decidua* and at 30.4mScm^{-1} salinity level in *Salvadora oleoides, Prosopis cineraria* and *Tamarix aphylla* (Sharif and Khan, 2009). Plant growth was severally affected at 3% NaCl in *Acacia ehrenbergiana* and *A. tortalis* (Al-Shaharani and Shelta, 2011).

Table 2. Effect of salinity treatment on root and shoot length and root-shoot ratio of *L. leucocephala* seedlings inoculated with *Rhizobium* and *Glomus* species.

Soil Salinity Ec_e (dSm^{-1})	Shoot length (cm)	Root length (cm)	Root-Shoot ratio
0.9(control)	34.92±3.63(a)	43.18±5.38(a)	1.23
5.18	30.48±1.27(a)	41.40±1.93(a)	1.35
8.62	29.21±0.71(a)	38.10±1.44(a)	1.30
10.2	26.16±3.98(b)	35.56±3.35(b)	1.35
14.24	20.32±1.27(b)	27.94±0.71(c)	1.37
16.22	18.41±2.79(c)	25.40±5.48(d)	1.37

Means sharing the same letters are not significantly different at p = 0.05 according to Duncan's multiple range test.

Effect of irrigation water salinity may be different on root and shoot growth in different plants. For instance Keck *et al*. (1984) have reported that salinity had greater effect on shoot growth than root growth in *alfalfa*. Sousssi *et al*. (1998) observed greater reduction in shoot growth than root growth in chickpea. Similar observations were

made by Tejara *et al*. (2007) in *Phaseolus vulgaris*, by Jamil *et al*. (2007) in beet and cabbage and (Sharif and Khan, 2009) in *Salvadora oleoides,Pprosopis cineraria, Capparis decidua* and *Tamarix aphylla*. On the other hand Singla and Garg (2005) and Abdelmajid (2009) observed that root growth was more adversely affected than shoot growth in chickpea. Mahmood *et al*., (2008) observed greater inhibition of root growth than shoot growth in *Sesbania sesban*. Chachar *et al*. (2008) noticed a depressive effect of salinity on root than shoot growth in *Gossypium hirsutum*. Our results corroborate with (Keck *et al*., 1984; Jamil *et al*., 2007; Sharif and Khan 2009).

Effect of salinity on plant biomass

Fresh and dry weights of root and shoot showed a gradual decrease with increasing salinity levels (Table3). Fresh weight of root and shoot decreased to 71 % and 60 % of the control while dry weight of root and shoot decreased to 62 % and 45 % of the control when soil salinity was increased from 0.9dSm^{-1} level to 16.2dSm^{-1}. Hameed *et al*. (2006) reported decline in fresh and dry weights of shoots in coastal halophytes grown under saline conductions. Akhtar and Hussain (2008) have shown that fresh weight of roots and shoots in *Vicia sativa* declined at 10dSm^{-1} and 15 dSm^{-1} salinity levels. Decrease in dry weight of roots and shoots in plants growing in saline conditions has been reported by (Tajera *et al*; 2005; Lopez *et al*., 2008; Ben Salah *et al*., 2009; Al-Shaharani and Shelta, 2011 and Mahmood *et al*., 2012).

Table 3. Effect of Sea salt salinity treatment on fresh and dry weights of shoots and roots of *L. leucocephala* plants inoculated with *Rhizobium* and *Glomus* species.

Ec_e (dSm^{-1})	Fresh Weight of Shoot(g)	Dry Weight of Shoot (g)	Fresh Weight of Root (g)	Dry Weight of Root (g)
0.9(control)	11.61±1.2 (a)	4.6±0.66 (a)	8.32±1.49 (a)	3.95±1.06 (a)
0.18	9.5±0.57 (a)	4.5±1.15 (a)	7.12±1.09 (a)	3.4±0.54 (a)
8.62	8.97±1.12 (b)	3.8±0.52 (b)	5.5±0.45 (b)	3.2±0.5 (b)
10.2	8.3±0.81 (b)	3.25±0.75 (b)	4.5±0.64 (b)	2.0±0.4 (b)
14.24	4.8±1.06 (c)	2.26±0.41 (c)	2.4±0.65 (c)	1.7±0.47 (c)
16.22	4.65±0.09 (c)	2.50±0.5 (c)	2.25±0.5 (c)	1.47±0.33 (c)

Means sharing the same letters are not significantly different at p = 0.05, according to Duncan's multiple range test.

Root – Shoot Ratio

Root-shoot ratio in control plants was 1.23 which increased with increasing salinity levels (Table 2). However increase in root-shoot ratio was statistically in significant. Sharif and Khan (2009) also registered an increase in root-shoot ratio over control but it was not statistically significant Keck *et al*. (1984). Observed an increase in root-shoot ratio in *Cicer arietimum* growing under salinity stress but results were non significant, statistically. Root-shoot ratio decreased with salinity in *Cicer arietinum* (Abdelmajid, 2009). An increase in root-shoot ratio results due to high sensitivity of shoot than roots to salt (Tejara *et al*., 2005).

Effect of salinity on nodulation and nitrogen fixation

Saline conditions effect nodule initiation, nodule development and growth of the host plant (Sprent and Sprent, 1990). Fresh and dry weights of nodules, nodule number, nodule size and percent tissue nitrogen were maximum in controls but showed a gradual decrease with increasing salinity levels (Table 4). Fresh and dry weight of nodules decreased to 66 % and 92% respectively of the control when soil salinity was raised from 0.9dSm^{-1} (control) to 16.2dSm^{-1} (maximum) salinity level. Depressive effect of NaCl on nodulation and nitrogen fixation is well documented in the literature. Reduction in dry weights of nodules has been reported by Tajera *et al*. (2005); Ben Salah *et al*. (2009); Al-Shaharani and Shelta (2011) and Mahmood *et al*. (2012). On the other hand Fernandez Pascual *et al*. (1996) in white lupin nodules and Lopez *et al*. (2008) in *Lotus japonicus* nodules have observed that nodule dry weight was not affected by salinity. This has been related to higher content of leghaemoglobin and higher rate of respiration in the bacteroid tissue of the nodules. Soussi *et al*. (1999) recorded that salt tolerant cultivar of *chickpea* reflected an increase in nodule dry weight under salt stress. This has been related to increase in nodule number and nitrogen activity of the cultivar under salt stress. Nodules were found in all treatments used in the present studies which implies that growth of *rhizobia* in the rhizosphere, process of root hair infection and nodule development were not completely inhibited by salt. Similar results were obtained by Roomi *et al*. (2002) with *Acacia ampliceps* and (Tejara *et al*., 2005) in common been plants, growing under saline conditions. Nodule number decreased progressively with increasing salinity levels (Table 4). Nodule number decreased to 94 % of control at 16.2dSm^{-1}. Decrease in nodule number with increasing salinity levels recorded in present investigation supports

earlier workers (Roomi *et al*., 2002; Anthraper and DuBois, 2003; Singla and Garg, 2005; Tejara *et al*; 2005 and Lopez *et al*., 2008). Nodule number was not affected by salinity in *Medicago ciliaris* which means that salt did not inhibit the development of new nodules in *Medicago ciliaris*. Decline in nodule number under saline conditions is most likely due to impact on the infection process itself. Root hair infection and infection thread formation is negatively influenced under stress conditions (Graham *et al*., 1992).

Table 4. Effect of salinity on number of nodules, size of nodules, fresh dry weights of nodules and tissue nitrogen.

Ec_e (dSm^{-1})	Number of nodules	Nodule Size (mm)	Fresh Weight of nodules (g)	Dry Weight of Nodules (g)	Tissue Nitrogen (%)
0.9 (control)	190±0.42 (a)	1.55±0.17 (a)	3.45±0.21 (a)	2.07±0.02(a)	5.72±0.75 (a)
0.18	183±0.34 (a)	0.9±0.21 (b)	1.83±0.20 (b)	1.15±0.04(b)	4.80±0.04 (b)
8.62	170±0.86 (a)	0.67±0.11 (c)	1.67±0.10 (b)	1.15±0.40(b)	4.80 ± 0.04 (b)
10.2	52.5±0.61 (b)	0.60±0.2 (c)	1.15±0.03 (c)	0.85±0.03(b)	4.75 ± 0.03 (b)
14.24	22±0.41 (c)	0.40±0.24 (d)	1.17±0.20 (c)	0.42±0.13(c)	4.05±0.02 (b)
16.22	10.5±1.06 (d)	0.15±0.17 (c)	1.12±0.31 (c)	0.15±0.17(c)	2.95±0.35 (b)

Means sharing the same letters are not significantly different at p = 0.05 according to Duncan's multiple range test.

Percent Tissue Nitrogen

Percent tissue nitrogen decreased gradually with increasing salinity levels (Table 4). It decreased to 60 % of the control when salinity was raised from $0.9dSm^{-1}$ to $16.2dSm^{-1}$ level. Decrease in nitrogen fixation under saline conditions has been reported by Hopman's *et al*. (1983); Tejera *et al*. (2005); Abdelmajid *et al*. (2009); Al-Shaharani and Shelta (2009). Salt inhibited nodulation and nitrogen fixation even at the lowest NaCl concentration (50mM) in *chickpea* (Soussi *et al*., 1998) where as plant growth was inhibited at the highest salt concentration (100mM). This shows that nodulation and nitrogen fixation are more sensitive to salt than plant growth. Similar observations have been made by Elsheikh and Wood (1995); Lopez *et al*. (2008); Ben Salah *et al*. (2009). In general salinity reduces the number, weight the activity of nodules and consequently nitrogen fixation (Yousef and Sprent, 1983; Elsheikh and Wood, 1995; Frechill *et al*., 2001).

High salinity creates water stress in nodules as a result of which water is withdrawn osmotically from the nodules, resulting in low water potential in the nodules (Sprent and Sprent, 1990). Low water potential developed in the nodules on the one hand may directly reduce the nitrogen fixing ability of nodules with accompanying reduction in nodule respiration (Pankhurst and Sprent, 1975) and on the other hand transport of fixed nitrogen out of nodules is decreased (Minchin and Pate, 1975). Reduction in N-fixing ability of legumes under salt stress has been attributed to a decline in dry weight and nitrogen contents in shoot (Cordovilla *et al*., 1995) and due to detrimental effect on soil microbial populations as a result of direct toxicity as well as through osmotic stress (Tate, 1995).

Nodule size also showed a progressive decrease with increasing salinity levels (Table 4). Nodule size showed a 90 % decrease over control at $16.2dSm^{-1}$ salinity. Decrease in nodule size under saline conditions may occur due to severe water stress causing irreversible damage to simplistic connections between nodule cells (Sprent, 1971).

Direct effect of salinity on the growth of *Rhizobium* and *Glomus* species.

Rhizobium and *Glomus* species subjected directly to salt treatment showed that number of rhizobial colonies and percentage of VAM infection were highest in controls but showed a progressive decrease with increasing salinity levels showing maximum decrease at 16.2 dSm^{-1}(Table 5).

Table 5. Effect of Sea salt salinity on *Rhizobium* and VAM growth.

Salinity Ec_e (dSm^{-1}) Control	Number of Rhizobial colonies	VAM Infection Percent
0.09(control)	307±42 (a)	50.1 ±2.13% (a)
5.18	281±15 (a)	23.28±3.20% (b)
8.62	263±13.5 (b)	19.3±3.20% (b)
10.2	185±11 (c)	12.5±4.56% (c)
14.24	73±5 (c)	10.5±0.62%(d)
16.22	25±1.81(d)	6.85±3.14%(c)

Means sharing the same letters are not significantly different at P=0.05 according to Duncan's multiple range test.

Direct effects of salt on the growth of *Rhizobium* and AM have also been observed in the past. For instance salt reduced the hyphal length of AM (Mizukami and Yamamoto, 1991; Jahromi *et al*., 2008) and salinity reduced overall mycorrhizal infection percent (Dixon *et al*; 1993). Salt has detrimental effects on soil microbial populations (Tate 1985; Zahran 2009) , which results in reduced nitrogen fixation. Thus, it may be concluded that the benefits of dual inoculation with Rhizobium and AM on the growth and nitrogen fixation parameters of *L. leucocephala* plants were suppressed under saline conditions.

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