Research Article

Amelioration of Salt Stress in *Cicer arietinum* L. by Plant Growth Promoting Rhizobacteria

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Abstract | Salinity is a major abiotic stress that reduces the plant productivity. The plant growth promoting rhizobacteria (PGPRs) play important role in eliminating the effect of salinity. *Cicer arietinum* L. is an important legume crop having high quantity of proteins. In the present study, four varieties (CM44, CM91, CM98 and CM2000) were grown in the presence and absence of PGPR inoculated media and nitrogen in different salinity levels (20, 50, 100, 200 and 300mM NaCl). The biomass production of the varieties CM91 and CM98 increased at 20-100mM NaCl concentrations but drastically decreased at higher levels. While in varieties CM44 and CM2000 a gradual decrease of biomass with increasing salinity levels were observed. Number of pods, flowers and fresh weight of pods were not affected at salinity levels of 0-50mM and decreased at 100 mM NaCI in all the four varieties. In varieties CM91 and CM2000 nodules were formed at all levels of salinity treatments, while in varieties CM44 and CM98 nodules were formed at salinity levels of 0-50 mM NaCl and completely inhibited at 100mM NaCl. The total protein and nitrogen contents increased with concurrent increasing levels of NaCI. The plants treated with 50 mM NaCI had maximum amount of protein contents as compared to those at 0mM NaCI level. Varieties CM91 and CM2000 had higher protein contents than CM44 and CM98. At I/50 level of salinity the total protein contents were found more than inoculated control at 0mM NaCI. From this study, it is concluded that PGPRs can play an important role to mitigate the salt stress at different concentrations for better crop yield.

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Introduction

In nature, plants are continuously exposed to abiotic stresses. Abiotic stresses are caused by salinity, water deficit, extreme temperature, heavy metals and other pollutants. These stresses cause negative impacts on plant growth, development and yield. Increasing population of world demands more food. Extensive use of chemical fertilizer, weedicides and herbicides cause the major loss of microbial diversity from the soil (Bartels and Sunkar, 2005; Kumar and Verma, 2018; Rashid et al., 2018).

Under salinity stress, the plants change their morphology, physiology and molecular profile. The salinity stress reduces the plant biomass production,

chlorophyll and proteins contents (Molinari et al., 2007). The increased production of reactive oxygen species (ROS) is one of the major events that occur in mitochondria and chloroplast of plants (Parihar et al., 2015). This production of ROS decreased the plant proteins, photosynthetic machinery and important enzyme that are necessary to perform the important function in cell (Mahajan and Tuteja, 2005; Mantri et al., 2012).

To eliminate the negative impact of soil salinity, different strategies have been used. Among various approaches the use of plant growth promoting rhizobacteria (PGPR) is most effective and having low cost as compared to other approaches. PGPRs are important because they provide many benefits to plant such as nitrogen fixation from atmosphere, solubilization of mineral like phosphorus and other nutrients. These bacteria also act as biocontrol against plant pathogens. Under salinity stress, the PGPRs regulate the Na^+, K^+ and Ca^+ selectivity and as a result of this regulation the ratio of K^+/Na^+ increased. Nitric oxide and indole acetic acid (IAA) are produced by bacteria that promote lateral root development as result of this the root surface area is increased. The production of osmolytes such as glycine betaine and proline can act synergistically with plant osmolytes and speed up the osmotic adjustment (Dimkpa et al., 2009; Pérez-Montaño et al., 2014).

Cicer arietinum L. (Chickpea) is important legume crop valued for its nutritive seeds with protein content of 25.3–28.9%. Seeds are eaten fresh or as dry, parched, boiled, fried and in various dishes. Sprouted seeds are eaten as a vegetable or as salad. Young plants and green pods are eaten like spinach. Dhal is a split chickpea without seed coat, dried or cooked in to flour for snacks and sweet meats. Chickpea yield 21% starch suitable for textile sizing giving a light finish to silk wool and cotton cloth. Both Kabuli and Desi chickpea are grown in WANA, however, there is a predominance of Kabuli types in all the countries except Ethiopia, Iran and Pakistan, where Desi type dominates (Shiferaw et al., 2007). Excluding Pakistan, Kabuli chickpea accounts for nearly 99% of the total chickpea production in WANA (West Asia and North Africa) in contrast to Indian subcontinent where Kabuli chickpea accounts for less than 10% of the total chickpea production. Chickpea is important component of cropping systems of the dry rain fed areas of WANA. It can fix 8-120kg Nitrogen per hectare through symbiotic nitrogen fixation, thus increasing the input of combined nitrogen into the production system and reducing the depletion of soil nitrogen in comparison to continuous cereals (Garg and Singla, 2004; Tejera et al., 2006; Eyidogan and Öz, 2007; Rasool et al., 2013). Previous studies showed that in various crops such as wheat and chickpea, the biomass production was decreased in salinity stress (Grewal, 2010).

The present study was designed to investigate the effect of inoculum in salinity stress in chickpea varieties with free nitrogen fertilizer and un-inoculum with nitrogen fertilizer.

Materials and Methods

The experiment was conducted at Bio Park of Bahauddin Zakariya University Multan, Pakistan. The experiment was arranged in completely randomized design. Four varieties (CM44, CM91, CM98 and CM2000) of chickpea were taken from the NIAB (Nuclear Institute of Agriculture Biology), Faisalabad. Four replicates of each variety were sown in sand (clean and pure sand which at first was treated with 0.1 M hydrochloric acid and then carefully washed with tap water). Each pot was labeled as U/0, I/0, I/20, I/50, I/100, I/200 and I/300 indicating NaCI concentrations (mM), while the U strands for un-inoculated and I indicates the inoculated. Before sowing seed, nitrogen free nutrient media was applied to each pot (Shafique et al., 2019).

Four healthy seeds of similar size were sown in each pot. For this purpose, 1.25-inch-deep holes were made. Then seeds were poured into the hole and inoculated with 5ml of the inoculum suspension. Then the seed was covered with sand. The inoculum was given twice in week to inoculated pots. The nitrogen free media was supplied to inoculated plants where, the other group nitrogen containing media was applied. After two weeks of seed sowing, the salinity treatment was started (i.e.,0, 20, 50, 100, 200 and 300 mM).

After the salinity treatment, the plants were harvested according to following stages.

First physiological stage

First harvest was taken at flowering stage i.e. after 5 weeks of sowing.

Table 1: *Analysis of variance (ANOVA) for morphological and biometrical analysis of four varieties of Cicer arietinum L. grown in control, control inoculum and different level of NaCl concentration with inoculum.*

	D.F	FWS	FWR	DWS	DWR	NON	FWN	NOF	NOP	FWP	N %	PC %
Rep	2	28.36	56.74	22.36	19.13	14.97	14.95	10.28	8.11	25.13	25.13	27.10
Var	3	90.63	158.09	14.30	16.47	4.26	6.56	13.39	1.24	7.82	7.82	24.72
Trt	14	91.21	78.33	7.78	3.19	2.06	4.94	30.19	33.01	8.98	8.98	342.54
Var *Trt	42	14.22	17.80	1.02	0.84	0.45	0.95	4.39	1.30	0.19	0.19	4.63
Err	118	0.12	0.12	0.16	0.19	0.19	0.18	0.19	0.20	0.17	0.17	1.74
F Value		743.42**	638.47**	45.83**	16.71**	10.51**	26.66**	157.53**	159.72**	52.20**	52.18**	196.69**

*Rep: Replication; Var: Varieties; Trt: Treatment; Var*Trt: Interaction of Variety and Treatment; Err: Error; Level of significance at **: significant at 0.01; FWS: Fresh weight of shoot; FWR: Fresh weight of root; DWS: Dry weight of shoot; DWR: Dry weight of root; NON: Number of nodules; FWN: Fresh weight of nodules; NOF: Number of flowers; NOP: Number of pods; FWP: Fresh weight of pods; N%: Nitrogen percentage and PC%: Protein content percentage.*

Second physiological stage

Second harvest was taken at the beginning of pod growth stage i.e. after 7 weeks of sowing.

Third physiological stage

Third harvest was taken at the middle of pod growth stage i.e. after 9 weeks of sowing. At each harvest two/three pots for each treatment were randomly selected for chickpea. The sand was carefully washed off from the plants to get intact roots with nodules. The plants were enclosed in polythene bags. The polythene bags containing the harvested plants were taken to laboratory. In the laboratory plants were removed from polythene bags, and data was collected.

Number of flowers, pods and nodules per plant

Counted the total number of flowers, pods and nodules which were formed on each plant.

Fresh weight of shoot and root per plant

The shoots of the harvested plants were separated from the roots with sharp blade. The shoot and root were weighed on electrical balance.

Dry weight of shoot and root per plant

After measuring above parameters, the plant material was dried in oven at 80 °C for 48 hours. Dry weight of shoot and root were measured in grams.

Estimation of total nitrogen content

200mg of ground shoot was taken in a 25 ml digestion flask and added 10 ml concentrated H_2SO_4 along with 4ml $HCIO_4$/100mg CaSO (catalyst). Heated with occasional mixing until the dark color was become clear (approximately 24 hours). After cooling, the volume of the digested material was made up to 50ml with distal water. The sample was poured into a distillation flask. Few drops of methyl red indicator (0.5% in alcohol) were added along with 50ml of 40% NaOH (40 gm per 100ml H_20) into the distillation flask until it decolourized. The decolourization indicator in flask indicated the neutralization of acid by NaOH. 40ml of 0.1 M HCI was taken in a flask then added few drops of methyl red indicator. Then the flask was put beneath the condenser outlet. So, that the tip of the condenser outlet was just touching the contents of the flask. Heated the distillation flask so, that NH_3 was distilled along with steam into the flask containing acid and indicator. Distilled the sample until about 30ml of the distillation had collected. Titrated the excess HCI with standard 0.1 M NaOH with distillate.

Statistical analysis

Statistical analysis was done by Statistix 8.1. Mean was calculated by Ms Excel.

Results and Discussion

Fresh weight of shoot

The fresh weight of the shoot of CM44 was decreased with increasing salt concentrations as compared to the inoculated control. The fresh weight of the shoot of CM91 was decreased with increasing the salt concentrations as compared to the inoculated control except at I/20 level. However, the fresh weight of shoot of CM98 and CM2000 decreased gradually as compared to the inoculated control with increasing the salt level (Figure 1, Table 1). At second harvest, the all varieties were showed decline in the shoot fresh biomass. However, at third harvest the shoot fresh biomass of all the four varieties were decreased as compared to inoculated control, with increasing salt concentrations up to I/50 level. A sudden increase

in shoot fresh weight of CM91 was observed at I/50 level. At I/100 level of salinity plants do not survive in any of the four varieties and died due to the high level of NaCI concentration as shown in Figure 1, Table 1.

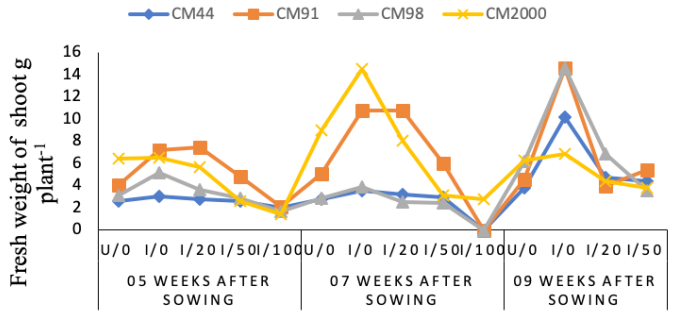

Figure 1: *Fresh weight of shoot of four varieties of Cicer arietinum L. grown in different level of NaCl concentration. U: Uninoculated, I: Inoculated.*

Fresh weight of root

The fresh weight of root of three varieties CM44, CM91 and CM98 showed a decline in the fresh weights of root at first harvest. The fresh biomass of the root was decreased with high levels of salinity. In CM2000, an increase in root fresh weight was observed at 1/20 level and then it decreased gradually as compared to inoculated control. At second harvest the fresh weight of root of CM44, CM91, CM98 and CM2000 was decreased with increasing the salt concentrations. The plants of the three varieties CM44, CM91, and CM98 could not survive at I/100 level of salt treatment, but CM2000 survived at this high salt concentration but its growth decreased as shown in Figure 2, Table 1.

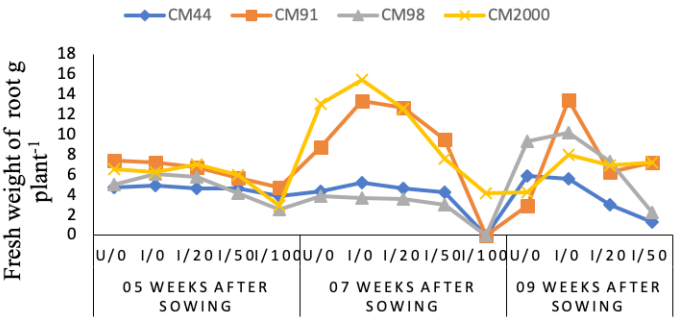

Figure 2: *Fresh weight of root of four varieties of Cicer arietinum L. grown in different level of NaCl concentration. U: Uninoculated, I: Inoculated.*

The fresh weight of root at third harvest of all the four varieties of *Cicer arietinum* L. decreased as compared to the inoculated control and again at I/100 level plants was not survived. In CM91 an irregular reduction in fresh weight of root was observed where it was increased from I/20 to I/50 and then decreased suddenly and approaches to minimum at 1/100 level of salinity. The fresh biomass of un-inoculated plants was less than inoculated plants in 0mM NaCI concentration as shown in Figure 2.

Dry weight of shoot and root

A gradual decline in the dry weights of shoots were observed in all varieties of chickpea at first harvest. The dry weight of root of all varieties at first harvest was decreased as compared to the inoculated control. as shown in Figure 3, Table 1. At 2nd harvest, the dry weights of the shoot were decreased as compared to the inoculated control in all varieties. The shoot dry biomass was decreased as compared to the inoculated control but in CM44, CM91 and CM 98, at I/100 level of salinity, the plants died. The variety CM2000 was showed normal growth at I/100 level of salinity. (Figure 3). However, at the 3rd harvest the shoot dry biomass of all the four varieties decreased as compared to the inoculated control but in all these four cultivars at I/100 level of salinity the plants were died due to high levels of salinity (Figure 3). Similarly, the dry biomass of fruit at third harvest also decreased as compared to the inoculated control of all the four varieties due to high level of NaCl as shown in Figure 3, Table 1.

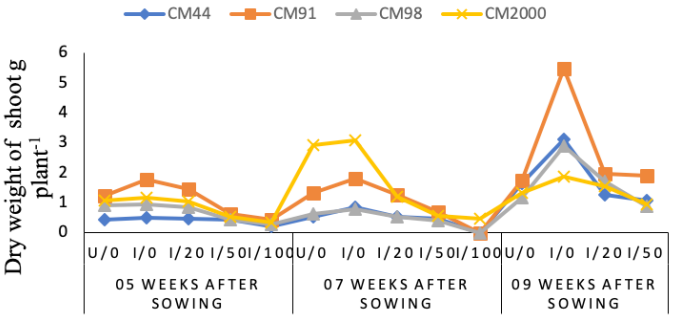

Figure 3: *Dry weight of shoot of four varieties of Cicer arietinum L. grown in different level of NaCl concentration. U: Uninoculated, I: Inoculated.*

A slight increase in the root dry weights was observed in CM91 at I/100 as compared to one previous level the same behavior was observed in CM2000 at I/50 level. The root dry biomass of all the four varieties CM44, CM91, CM98, and CM2000 was decreased as compared to the inoculated control but again at 2nd harvest no plant was survived at I/100 level of salinity in varieties CM44, CM91 and CM98 as shown in Figure 4, Table 1.

Nodulation

At first harvest in varieties CM44 and CM98 nodules were formed at I/0, I/20, and 1/50 levels of salinity

but no nodules were formed at 1/100 level of salinity but in CM91 and CM2000 nodules were formed at all levels of salinity. A gradual decrease in number of nodules were observed in all the four varieties with gradual increase in salt concentration. At 2nd harvest in varieties of *Cicer arietinum* L. CM 44, CM91, CM98 and CM 2000 nodules were formed up to 50mM of salinity. Although only CM2000 cultivar survived at I/100mM yet no nodules were formed in it. At third harvest in all the varieties, nodules were formed at 0-50mM and I/50 levels of NaCI but at 100mM level of salinity no nodules were formed, as all plants had died at this high salt concentration (Figure 5, Table 1).

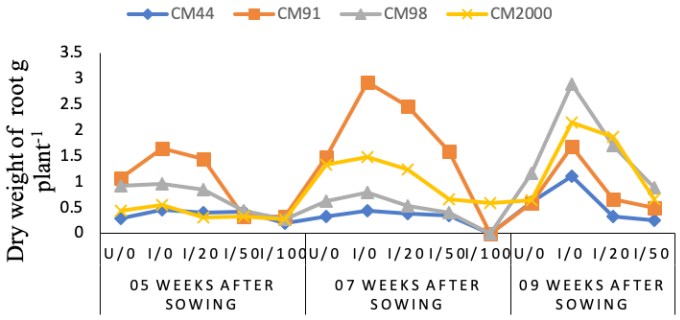

Figure 4: *Dry weight of root of four varieties of Cicer arietinum L. grown in different level of NaCl concentration. U: Uninoculated, I: Inoculated.*

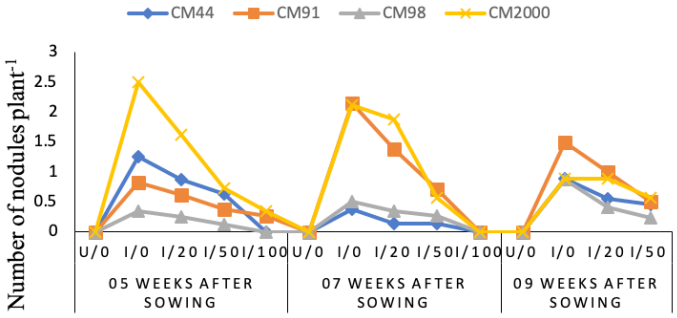

Figure 5: *Number of nodules of four varieties of Cicer arietinum L. grown in different level of NaCl concentration. U: Uninoculated, I: Inoculated.*

The fresh weight of nodules decreased abruptly at different salinity levels from inoculated control to 100mM NaCI level in all the four varieties i.e. CM44, CM98, CM91, and CM2000 at 1st harvest. In CM44 and CM98 nodules were not observed at 100mM NaCl level.

The fresh weight of nodules decreased from inoculated control up to I/50 at 2nd harvest. In all these varieties no nodules were formed at 100mM NaCI level except in CM2000 where a very small number of nodules was formed. At third harvest the fresh weight of nodules, in of all varieties was decreased with increased in salt concentration as compared to the inoculated control. At the I/100 level of NaCl, the plants were died, therefore no nodule were present at this level (Figures 5 and 6, Table 1).

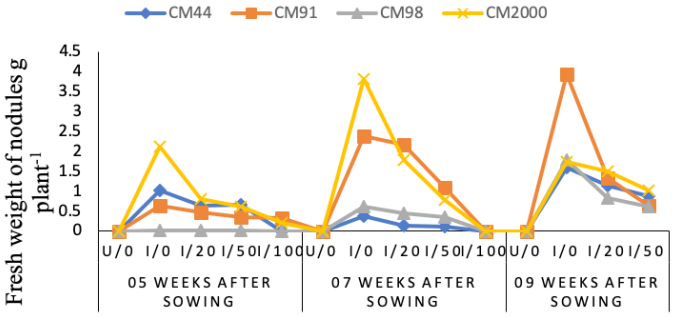

Figure 6: *Fresh weight of nodules of four varieties of Cicer arietinum L. grown in different level of NaCl concentration. U: Uninoculated, I: Inoculated.*

Numbers of flowers

The flower formation was observed at 0-50mM NaCl concentration and inhibited at 100mM NaCl concentration in the 1st harvest. In variety CM44, the formation of flowers was not occurred at 50mM NaCI level of salinity (Figure 7, Table 1). At 2nd harvest in variety CM2000 flowers were present at all levels of NaCI concentration but in varieties CM91, CM98, and CM44, the flowers were present at to 1/50 levels of salinity and at 1/100 level of salinity no flowers were available to collect in these varieties as plants died high level of salinity. Flowering was completely inhibited at third harvest in all the varieties of *Cicer arietinum* L. at all the levels of salinity as shown in Figure 7, Table 1.

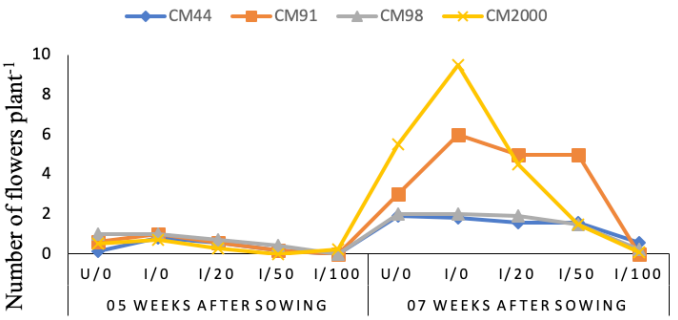

Figure 7: *Number of flowers of four varieties of Cicer arietinum L. grown in different level of NaCl concentration. U: Uninoculated, I: Inoculated.*

Pods

Pods were present at 2nd harvest at 0-50mM levels of NaCI concentration but at 100mM NaCl level of salinity no pods were observed. All plants of varieties CM44, CM91 and CM98 died at I/100 level of salinity. Only variety CM2000 survived at this high level of salinity but pods were not present at this concentration. But at 3rd harvest the pods were formed

at I/0, I/20 and I/50 levels of salt but at I/100 level of salt no pods were collected as plants died before taking 3rd harvestable in all varieties. Number of Pods was smaller in un-inoculated plants as compared to inoculated plants with 0mM NaCI level of salinity in all the varieties (Figure 8, Table 1).

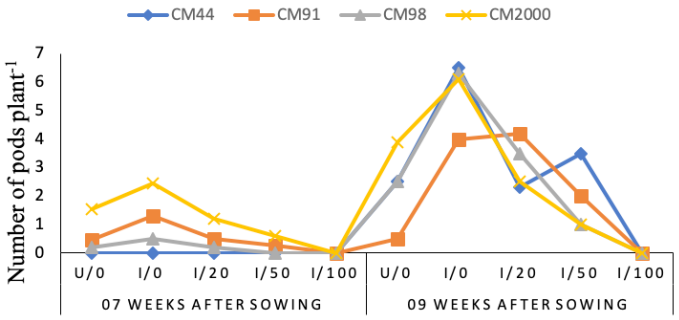

Figure 8: *Number of pods of four varieties of Cicer arietinum L. grown in different level of NaCl concentration. U: Uninoculated, I: Inoculated.*

The fresh weight of pods of all the four varieties. CM44, CM91, CM98 and CM2000 showed a gradual decline from inoculated control to increasing salt concentrations at different levels.

At third harvest, the fresh weight of pods was decreased from the inoculated control to increasing levels of salt treatment up to I/50 in all the varieties. An irregular increase in fresh weight of pod was observed at I/50 in CM44, but again at I/100 levels of salinity no pods were formed in all the varieties as shown in Figure 9, Table 1.

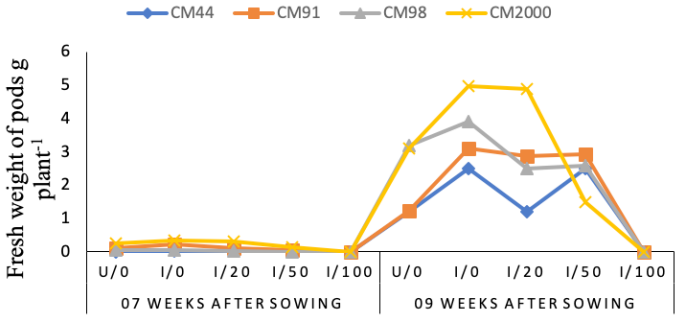

Figure 9: *Fresh weight of pods of four varieties of Cicer arietinum L. grown in different level of NaCl concentration. U: Uninoculated, I: Inoculated.*

Percentage nitrogen and total protein contents

Figures 10 and 11 shows the percentage of nitrogen and total protein contents in shoot dry weight of *Cicer arietinum* L. varietiesCM44, CM91, CM98 and CM2000 without inoculation and with inoculation along with a range of NaCI treatments at three harvests. From figures shoot dry weight of inoculated control had about 8-24% higher nitrogen and total protein contents in shoot and seed. At 20 mM NaCI concentration in CM44 and CM91 the shoot nitrogen and total protein contents were 17-24% increased as compared to inoculated control in different varieties of *C. arietinum* L. Whereas, at 100mM NaCI the seed nitrogen and total protein contents was 30-40% reduced as compared to inoculated control in four different varieties of *C. arietinum* L. as shown in Figures 10 and 11, Table 1.

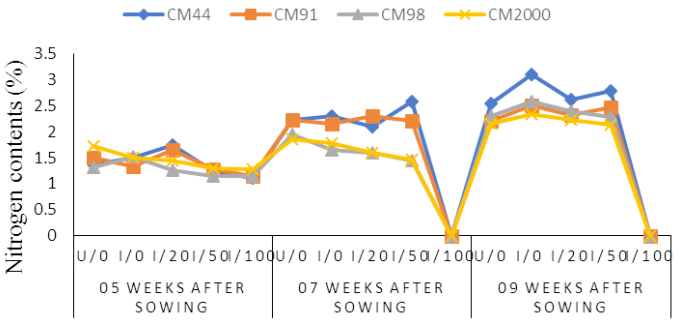

Figure 10: *Nitrogen contents (%) of four varieties of Cicer arietinum L. grown in different level of NaCl concentration. U: Uninoculated, I: Inoculated.*

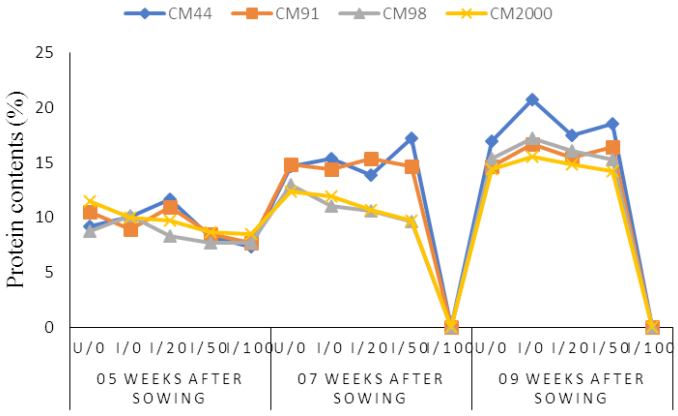

Figure 11: *Protein contents (%) of four varieties of Cicer arietinum L. grown in different level of NaCl concentration. U: Uninoculated, I: Inoculated.*

Soil salinity is major problem of arid and semi-arid regions. The soil salinity imposes the negative impact on plant health (Shrivastava and Kumar, 2015). It reduces the plant growth, development and yield. Thus, it has challenge to food, feed and fodder crops. To overcome the salinity stress, plant adopts different strategies such as morphological, physio-chemical and molecular changes. The exogenous application of plant growth promoting rhizobacteria is the most important strategy to minimize toxic effect of the NaCl stress. They provide the enzymes, proteins and mineral nutrients to maintain their growth under salinity stress. They are helpful for storing of sodium ions in vacuole (Harper and Harmon, 2005; Liang et al., 2008; Chaves

et al., 2009; Cotsaftis et al., 2012; Chunthaburee et al., 2016; Cura et al., 2017; He et al., 2017).

From the present study, it has been shown that under salinity stress reduces the fresh weight of shoot and root in all varieties of chickpea. The performance of Inoculated plants with no salinity stress was better than the control plant with no inoculum (Figures 1 and 2). Similarly, the dry weight of shoot and root was decreased under salinity stress (Figures 3 and 4). In another study carried out on chickpea under 0.1M, 0.2M and 0.5M NaCl concentration, the fresh and dry weight of shoot and root was decreased. The increasing amount of salinity decreased the biomass production (Eyidogan and Öz, 2007). The decrease of shoot and root weight may be due to the less availability of water and sodium chloride toxicity to plant (Singla and Garg, 2005). Similarly, previous studies carried out on canola, wheat and chickpea, the biomass production was decreased under salinity stress (Grewal, 2010). The soil salinity also decreased the biomass production, chlorophyll contents and photosynthesis activity in Lettuce plants (Han and Lee, 2005).

The number of nodules and fresh weight of nodules was zero at U/0 and I/100 level in all varieties in present study. (Figures 5 and 6). Similarly, the number of flowers were maximum in I/0 (Figure 7). The number of pods and fresh weight was maximum at I/0 and I/20 levels (Figures 8 and 9). The nitrogen and proteins contents were increased with increasing level of salinity (Figures 10 and 11). The nitrogen contents were better under normal salinity. The nitrogen contents were better due to fixation of nitrogen in soil in salinity stress. The highest level of salinity inhibits the formation of symbiotic association in legumes crops. The nodules and fresh weight of nodules decreased under serve salinity stress (Rao et al., 2002). The nitrogen contents were increased with inoculum with bacteria in other chickpea cultivars (Tejera et al., 2006). The salinity affects the nodulation and number of nodules in chickpea. The decrease number of nodulation and fresh weight of nodules may be due to NaCl promote the senescence as result of this senescence the leghemoglobin and its concentration was decreased that decrease the nodules formation and their fresh weight of nodules in salinity stress condition. As result of this process, the nitrogen fixation is reduced in chickpea (Al-Mutawa, 2003; Flowers et al., 2010).

The PGPR enhanced the fresh and dry weight of shoot and root, nodulation, number of flowers, pods, total proteins contents and percentage of nitrogen in chickpea under different level of salinity stress. Thus, it is concluded that the PGPR may be helpful for the salinity tolerance in chickpea. The other strains of PGPRs may be applied for crop improvement in the presence and absence of salinity stress

Novelty Statement

This research indicated that the salinity plays negative impact on chickpea plant by decreasing the growth and development. The PGPRs play an effective role against this salinity stress and increase the plant growth and development.

Author's Contribution

Nosheen Noor Elahi designed the experiment; Muhammad Shafiq performed the experiment; Umer Farooq analysed the data, Nosheen Noor Elahi, Muhammad Rashid and Muhammad Imtiaz prepared the manuscript.

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