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Salt Tolerance in Alfalfa Following Inoculation with *Pseudomonas*

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Abstract: This study aimed to investigate the effect of inoculation with plant growth-promoting *Rhizobium* and *Pseudomonas* species on NaCl-affected alfalfa. Two cultivars of alfalfa (cv. Bami and cv. Yazdi) selected on the basis of their yield potential were grown in pots under greenhouse conditions. Microorganisms were applied at seedling stage and salt stress was induced 21 days after sowing and maintained up to 50% flowering after 180 days of stress. The salt treatment caused a detrimental effect on growth and development of plants. The co-inoculation resulted in some positive adaptative responses of alfalfa plants under salinity. The salt tolerance from inoculation was generally mediated by an increase in osmoregulant (proline) production, maintenance of relative water content and selective uptake of K^+ ions. Generally, the microbial strain acted synergistically. Under stressed and unstressed conditions, *Pseudomonas* was more effective than *Rhizobium*. The alfalfa cv. Bami appeared to be more responsive to inoculation and was relatively tolerant to salt compared to that of cv. Yazdi

Key word: Alfalfa · Inoculation · *Rhizobium* · *Pseudomonas* · Salinity

INTRODUCTION

Salinity is one of the major stresses that limit crop productivity, particularly in arid and semi-arid regions with irregular pattern of rainfall [1,2]. Soil salinity significantly reduces plant nutrient uptake, especially phosphorus (P) because phosphate ions precipitate with Ca^{2+} ions in salt-stressed soil [3]. Phosphate-solubilizing bacteria can increase P availability to plants solubilizing insoluble phosphates [4] and this improved P nutrition can increase biological nitrogen fixation and the availability of other nutrients because these bacteria can produce plant growth-promoting substances [4].

The development of salt-tolerant crops is not an economical approach for sustainable agriculture, whereas microbial inoculation to alleviate salt stress is a better option because it minimizes production costs and environmental hazards [5, 6]. Use of PGPR inocula for cereals to alleviate salt stress has been reported whereas information is scanty on the role of PGPR in legumes. In this study, *Pseudomonas fluorescence* was selected because of its plant-promoting properties and because it can occur in soil before alfalfa cultivation.

Since, under salt stress, P fixation and P precipitation can occur in soil making the P unavailable to plant growth, we have studied the role of inoculated *Pseudomonas*-solubilizing P in alfalfa cropped soil alone or with inoculated *Rhizobium*. Generally, the effect has been studied in greenhouse and not under field conditions.

MATERIALS AND METHODS

Seeds of two cultivars of alfalfa (Bami and Yazdi) were obtained from The Seeds and Plant Improvement Institute, Karaj, Iran. Seeds were surface sterilized with 10% chlorox solution and grown in earthen pots (24×36 cm) containing a mixture of soil and sand (in the ratio of 3:1) under greenhouse conditions in 2012. Cultures of Rhizobium and phosphate-solubilizing bacteria (*Pseudomonads fluorescence*) were provided by the Institute of Soil and Water Research,Karaj, Iran. The seeds were soaked prior to sowing overnight in broth cultures of *Rhizobium* and *Pseudomonas* sp. alone or in combination (cell density of 10^6 cells/ml). Ten plants were grown in each pot. Salt solution (12 dSm^{-1}) was applied to plants 3 weeks after inoculation. Pots were irrigated according to their weight at 80% field capacity moisture.

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Plants were grown in the greenhouse under natural sunlight with temperatures of 25 - 30°C (day) and 20 - 23°C (night). After 15 days, thinning was carried out to leave five uniform seedlings in each pot. There were three replications for each treatment. The experiment was arranged as a factorial in complete randomized block design (CRBD).

Colony forming unit (cfu) of *Rhizobium* and *Pseudomonas* were determined on soil samples collected 10 days after salt stress or at flowering (180 days after sowing) in order to determine the short-term and long-term effects of salt on the survival of these two microbial species.

After 180 days of salt stress, plants were harvested at flowering stage and fresh weights of roots and shoots were determined. Both roots and shoots were dried at 72°C for 3 days in an oven until constant weight was obtained.

Phosphorus,nitrogen, Calcium, Potassium and Sodium Content: Plant were analyzed for the contents of P, N, Ca^{2+},K^+ and Na^+. Estimation of total N was done by kjeldahl·s method, while the total P content was measured by vanado - molybdate yellow color method [7]. Total Na^+ and K^+ were measured by flame photometric method [8] and Ca^{2+} with an atomic absorption spectrophotometry [9].

Proline, Chlorophyll Content and Electrolyte Leakage: Proline content of shoots was estimated at flowering stage according to [10] and chlorophyll content of leaves was calculated as shown by [11] and modified by [12]. Electrolyte leakage was determined as shown by [13].

Survival of Rhizobium and Psuedomonas: The colony forming units (cfu) of *Rhizobium* were determined by plating decimal dilution of soil extract on yeast mannitol agar medium. The determination of phosphate-solubilizing bacteria was done by using the Pikovskaya's medium [14]. The number of bacteria per gram of soil was calculated from the colony forming units according to the following formula.

$$\text{Colony forming unit} = \text{number of colonies} \times \text{dilution factor} / \text{volume of inoculum}$$

Statistical Analysis: The data were analyzed by the analysis of variance and comparison among treatment means was made by Duncan's multiple range test using MSTAT-C version 1.4.2 [15].

RESULT

Salt stress adversely affected the growth of both alfalfa cultivars with relatively lower effects for the cv. Bmi than for cv. Yazdi. The co- inoculation treatment was more effective than the single inoculation in decreasing the adverse effects of salt.

The maximum electrolyte leakage was observed in salt- stressed plants of both cultivars and the minimum damage to cellular membranes was found in co- inoculated plants both under unstressed and salt-stressed conditions (Table 1). The decrease in electrolytic leakage was less in cv. yazdi than in the *other* cultivar.

Salt stress increased the osmotic potential of plants as compared to control (Table 1). Inoculation with Pseudomonas under stressed conditions maintained osmotic potential of plants at the level of the control in both cultivars, but inoculation with *Rhizobium* decreased the osmotic potential of both cultivars as compared to the control. Both microbial inocula increased the osmotic potential of plants of both cultivars under salt stress as compared to the values of control. The increase by *Pseudomonas* under stressed conditions was greater than that by Pseudomonas under unstressed conditions.

Salt stress significantly increased proline content of plants as compared to that of control (Table 1). Both Pseudomonas and *Rhizobium* used alone did not significantly affect the proline content under unstressed condition. Co-inoculation of *Rhizobium* and *Pseudomonas* significantly increased the plant proline content compared to the value of the control under salt stress.

Salt stress markedly decreased the relative water content (RWC) of plants in both varieties as compared to control (Table 2). The decrease was similar in both varieties. Both *Rhizobium* and *Pseudomonas* used separately were unable to overcome the adverse effects of salt on RWC of plants. The co-inoculation treatment significantly increased the plant relative water content in both varieties as compared to control and completely overcame the inhibitory effect of salt stress.

Salt stress decreased the plant height of both cultivars (Table 2). Plants of both cultivars inoculated with *Rhizobium* inoculation were markedly taller than that of control. Both microbes separately or together ameliorated the inhibitory effect of salt on plant height of Bami.

Table 1: Mean electrolyte leakage, osmotic potential and proline content in two varieties of alfalfa (Bami and Yazdi) under salt stress

	Electrolyte leakage (%)		Osmotic potential (osmol/kg)		Proline content (mg/g)	
Treatments	Bami	Yazdi	Bami	Yazdi	Bami	Yazdi
Control	43	46	0.48	0.4	163	145
Salt	74	81	0.63	0.52	243	200
Rhizobium	42	46	0.52	0.49	180	167
Rhizobium + salt	68	78	0.68	0.58	286	234
Pseudomonas	38	46	0.6	0.55	310	220
Pseudomonas + Salt	45	55	0.76	0.63	367	287
Rhizobium + Pseudomonas	30	33	0.7	0.68	325	248
Rhizobium + Pseudomonas + salt	44	51	0.8	0.73	430	3150
	LSD (0.05) 8.26		LSD (0.05) 0. 28		LSD (0.05) 69	

Values with different letters are significantly different (p>0.005)

Table 2: Mean plant height and Relative water content (%) in two varieties of alfalfa (Bami and Yazdi) under salt stress

	Relative water content (%)		Plant height (cm)	
Treatments	Bami	Yazdi	Bami	Yazdi
Control	32	29	54.92	53.8
Salt	18	16	45.7	42.5
Rhizobium	30	29	56.6	54.45
Rhizobium + salt	20	18	46.23	43.8
Pseudomonas	33	30	57.2	55.1
Pseudomonas + Salt	25	21	48.4	45.6
Rhizobium + Pseudomonas	46	38	59.6	57.2
Rhizobium + Pseudomonas + salt	35	31	50.7	48.3
	LSD(0.05) 7.16		LSD (0.05) 6.43	

Values with different letters are significantly different (p>0.005)

Table 3: Mean dry weight of shoot and root in two varieties (Bami and Yazdi) of alfalfa under salt stress

	Dry weight of shoot (g)		Dry weight of root (g)	
Treatments	Bami	Yazdi	Bami	Yazdi
Control	1.26	1.23	0.83	0.76
Salt	1.14	0.97	0.64	0.54
Rhizobium	1.36	1.3	0.94	0.85
Rhizobium + salt	1.27	1.1	0.72	0.6
Pseudomonas	1.46	1.38	1.06	0.98
Pseudomonas + Salt	1.35	1.25	0.85	0.72
Rhizobium + Pseudomonas	1.55	1.4	1.12	1.02
Rhizobium + Pseudomonas + salt	1.45	1.35	0.98	0.83
	LSD (0.05) 0. 27		LSD (0.05) 0. 18	

Values with different letters are significantly different (p>0.005)

Table 4: Mean Na^+,K^+ content and Na^+/K^+ ratio in two varieties (Bami and Yazdi) of alfalfa under salt stress

	Na^+ (mg kg^{-1})		K^+ (mg kg^{-1})		K^+/ Na^+ (mg kg^{-1})	
Treatments	Bami	Yazdi	Bami	Yazdi	Bami	Yazdi
Control	2.11	2.42	32.64	30.23	15.47	12.49
Salt	4.25	5.12	26.23	21.3	6.17	4.16
Rhizobium	1.7	1.85	34.14	31.26	20.08	16.9
Rhizobium + salt	3.18	4.1	28.14	24.6	8.85	6
Pseudomonas	1.3	1.6	37.7	33.2	29	20.75
Pseudomonas + Salt	2.45	2.75	35.8	31.65	14.61	11.51
Rhizobium + Pseudomonas	1.25	1.57	40.9	37.7	32.72	24.01
Rhizobium + Pseudomonas + salt	1.9	2.25	37.6	35.23	19.79	15.66
	LSD (0.05) 1.26		LSD (0.05) 2.7		LSD (0.05) 4. 38	

Values with different letters are significantly different (p>0.005)

Under salt stress, both cultivars showed similar decreases in dry weight of shoot (Table 3). Co-inoculation increased the dry weight of shoots in both unstressed and salt-stressed conditions.

Salt stress decreased the dry weight of roots in both cultivars (Table 3). Single inoculation with *Rhizobium* and *Pseudomonas* was less effective than co-inoculation in increasing the dry weight of root in both cultivars under salt stress. The maximum increase was observed with cv.Bami. Under unstressed conditions, single inoculation with *Rhizobium* or *Pseudomonas* sp. increased the dry weight of roots as compared to control.

The K^+ content was greater in cv. Yazdi than in cv. bami. Salt stress decreased the K+ content in both cultivars (Table 4). Inoculation with *Rhizobium* and *Pseudomonas* increased significantly the K^+ uptake of cultivar Bami as compared to control. A similar pattern was observed following co-inoculation in cv. Yazdi.

Co-inoculation showed less accumulation of Na^+ in cv. Bami than Yazdi under both conditions (Table 4). The maximum accumulation of Na^+ was recorded in control plants under salt stress with a greater effect in cv. Yazdi than in cv. Bami. Both microbes were not effective in reducing the Na^+ content under unstressed conditions. Under salt stress, *Pseudomonas* decreased the stimulatory effect of salt stress on Na^+ content.

Plants inoculated with both Pseudomonas and *Rhizobium* showed the maximum K^+/Na^+ ratio in cv. Bami under salt stress condition. Inoculation with *Rhizobium* showed a lowest effect on the K^+/Na^+ ratio in cv.Yazdi than in Bami under salt condition.the highest ratio (Table 4).

The cv. Bami had a higher level of P accumulation than cv. Yazdi (Table 5). The former cultivar inoculated with *Pseudomonas* showed 19% greater uptake of P than the treatment with *Rhizobium*. Co-inoculation showed the maximum P accumulation under salt stress.

Salinity decreased the Ca uptake in alfalfa plants. Under unstressed conditions, inoculation treatments decreased Ca^{2+} content, whereas co-inoculation treatment significantly increased the Ca^{2+}content of plant under salt stress over control (Table 5).

Salt stress conditions decreased the chlorophyll content of leaves of both cultivars. Inoculation treatments counteracted the adverse effect of salt on leaf chlorophyll of both cultivars so that no differences were observed between this treatment and the control (Table 5). *Rhizobium* was less effective. The *Rhizobium* treatment under unstressed conditions increased the chlorophyll content of cv. Bami but not in cv. Yazdi, whereas the *Pseudomonas* increased the chlorophyll content of leaves in both the cultivars. The co-inoculation treatment either under unstressed or salt-stressed conditions increased the chlorophyll content more than the salt stress.

The counts of both *Rhizobium* and *Pseudomonas* were higher under unstressed than salt-stressed conditions and were higher with *Pseudomonas* than *Rhizobium* treatment both under unstressed and stressed conditions (Table 6). There were no differences in the cfu counts of *Rhizobium* by comparing the inoculation and the co-inoculation treatments, whereas the *Psuedomonas* counts were stimulated by the co-inoculation treatment. The cfu counts of the co-inoculated treatment were not affected by the salt stress.

Table 5: Mean P and Ca^{2+}content and Chlorophyll content (ig/ml) of two varieties (Bami and Yazdi) of alfalfa under salt stress

	Phosphorus (mg kg^{-1})		Ca^{2+}(mg kg^{-1})		Chlorophyll content (ig/ml)	
Treatments	Bami	Yazdi	Bami	Yazdi	Bami	Yazdi
Control	3.1	2.23	26.67	23	41	38
Salt	2.2	1.76	15	12	16	12
Rhizobium	3.67	3.13	28	24.3	43	40
Rhizobium + salt	2.83	2.15	25.1	21.3	25	16
Pseudomonas	4.14	3.86	29.3	26.2	51	44
Pseudomonas + Salt	3.8	3.42	26.3	24.5	28	21
Rhizobium + Pseudomonas	4.34	4.15	30.47	27.4	55	45
Rhizobium + Pseudomonas + salt	4.1	3.6	28.6	25.8	33	26
	LSD (0.05) 1.23		LSD (0.05) 2.12		LSD (0.05) 8.32	

Values with different letters are significantly different (p>0.005)

Table 6: Colony forming unit (cfu) for Rhizobium and Pseudomonas per gram of rhizosphere soil

Treatments	After 10 days	At harvest
Rhizobium	3.3×10^6	3.5×10^6
Rhizobium + salt	2.43×10^6	2.85×10^6
Pseudomonas	3.7×10^4	3.92×10^4
Pseudomonas + Salt	$3.58.9\times10^5$	3.67×10^5
Rhizobium + Pseudomonas	(3.3×10^6) + $(3.83.0\times10^4)$	(3.55×10^6) + (4.0×10^4)
Rhizobium + Pseudomonas + salt	(3.1×10^6) + (3.6×10^4)	(3.2×10^6) + (3.66×10^4)
	LSD (0.05) 0.53	LSD (0.05) 0.26

Values with different letters are significantly different (p>0.005)

DISCUSSION

The accumulation of proteins under stress is an adaptation mechanism as they bound to membranes, regulating membrane water permeability in cells and influencing water movement among tissue and organ [16]; in addition, they can prevent and reduce the denaturation of other cellular micromolecules under dehydrative conditions [9]. The increased accumulation of proline depends on the stimulation in osmotic potential of plants as has been reported earlier [17] and it correlated with osmotic stress tolerance.

The observed increased accumulation in proline content in the co-inoculation treatment particularly under salt stress may contribute to cellular adaptation to salt stress as reported by [18]. Increased Ca^{2+} accumulation also affects the osmoregulation capacity by increasing the content of proline, leading to a higher water potential gradient and thereby improving the water uptake and growth under stress [19].

The present study showed that stress sensitivity of two alfalfa cultivars was related to the variation in accumulation of proline and hence osmoregulation mechanism as reported in some crops [20, 21]. Salinity interferes with chlorophyll synthesis in un-inoculated plants and co- inoculation effectively ameliorated the inhibitory effect of salt. Noteworthy, chlorophyll content was increased by co- inoculation treatment under unstressed and stressed condition.

Sodium has an antagonistic effect on Mg absorption [22] which is an important structural constituent in chlorophyll synthesis. [23] statedthat chlorophyll loss and cellular membrane stability evaluated by electrolyte leakage are indicators of oxidative damage. [24] demonstrated that balanced nutrition increased the salt-tolerance capacity of plants. [1] reported that accumulate of Na^+ was strongly influenced by storage of other cations, particularly K^+. Increased K+ concentration under saline conditions may help to decrease Na+ uptake and this can indirectly maintain the chlorophyll content of the plant [25].

The highest P accumulation exhibited by the co-inoculation treatment may be important in mitigating salt stress by overcoming the scarce P availability in saline soils. The Pseudomonas has been shown to have a positive influence on plant nutrition under salt-stressed conditions [26, 25] reported that,in saline soil, higher absorption of P in inoculated plants may improve their growth rate and salt tolerance and suppress the adverse effect of salinity stress. The higher K^+ uptake in the tolerant cultivar Bami may demonstrate the role of K in salt tolerance. The effect of co-inoculation in alleviating salt stress may partly be assigned to reduced Na^+ uptake by root-and translocation-to-shoot tissues and low Na^+/K^+ ratio. As already mentioned, increased K^+ [1] and Ca^{2+} concentration under saline conditions may decrease Na^+ uptake, which is required for maintaining the osmoticbalance [27]. High K^+/Na^+ selectivityis an important selection criterion for salt tolerance as suggested by [16, 28, 29] reported that roots of salt-tolerant plants had a greater affinity for K^+ than did the salt-sensitive plant.

The maintenance of calcium uptake acquisition and transport under salt stress is an important determinant of salinity tolerance [30, 31]. Indeed, supplied Ca^{2+} reduces the toxic effects of NaCl, presumably by facilitating higher K^+ versus Na^+ selectivity [12]. Our results confirm previous findings that inoculated plants grow better and had higher biomass of plants than non-inoculated plants under salt stress conditions [26, 32-34].

The decrease in shoot:root biomass ratio under salt stress is an index of salt tolerance. An increased functional root biomass has been reported to strengthen the root Na^+ detoxification capability [35]. The co- inoculation of stressed plants markedly stimulated shoot and root growth as compared to that of co-inoculated unstressed plants probably because it increased the synthesis of the photosynthesizing pigment, i.e. chlorophyll and increased production of growth-promoting hormones.

The stimulatory effect of *Rhizobium* and *Pseudomonas* inoculation on the plant height resulted in taller plants, probably due to an increase in cell division and cell elongation. The greater stem diameter following the co-inoculation treatment may be attributed to an increased assimilate distribution in stem under both stress and unstressed conditions.

Tolerance limits of rhizobia were reported to vary from 0.09 to 340 mM NaCl [36]. Plant Growth Promoting Rizobacteria (PGPR) has evolved a variety of adaptive mechanisms in order to restore the cell turgor pressure and reduce the osmotic potential between the cell and the environment. Among these mechanisms, one response of *Rhizobium* to salt stress is the transient adjustment in ionic balance and changes in the metabolism of cytoplasmic low molecular weight compounds [37, 38] reported that reduced plant growth height and dry matter content of root and shoot under continuous irrigation with 160 mM NaCl was ameliorated by inoculation with gfp-tagged *Azospirillum brasilense*.

The increase in cfu counts of both *Rhizobium* and *Pseudomonas* observed in soil samples in co-inoculation treatments exposed to salt stress indicates that *Rhizobium* and *Pseudomonas* act synergistically. Possibly *Pseudomonas* increased P availability to plants, subsequently increasing plant growth, which in turn provided better nutrient supply to *Rhizobium* symbionts. [39] reported that P plays a key role in improving the density of *Rhizobium* in the soils surrounding the root.

Under stressed and unstressed conditions, *Pseudomonas* was more effective than *Rhizobium*. The mechanism of growth stimulation by *Pseudomonas* differs from that of *Rhizobium* as indicated by the greater membrane stability (less electrolyte leakage) and more P accumulation.

CONCLUSION

Both *Rhizobium* and *Pseudomonas* can combat salt stress in alfalfa by increasing the total uptake of K^+, P and Ca^{2+} without affecting Na^+ accumulation as compared to salt treatment and the effect depends on the plant cultivar. Generally, co- inoculation was better than single inoculation. Their mode of action appears to affect production of proline as osmoregulants, reduction in electrolyte leakage and increase in plant height, even if exceptions are observed. However, further research need to be conducted to have an insight into the phytohormone production by these microbes, the production of salt- induced protein and the antioxidants.

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