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Response of forage quality in Persian clover upon co-inoculation with native *Rhizobium leguminosarum symbiovar (sv.) trifoli* RTB_3 and plant-growth promoting *Pseudomonas florescence* 11168 under different levels of chemical fertilizers

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The excessive use of chemical fertilizers has generated several environmental problems. In order to evaluate the effects of co-inoculation of *Pseudomonas*, native *Rhizobium* with nitrogen and phosphorus levels on forage quality in Persian clover (*Trifolium rosapinatum* L. cv.Br_{73}), this study was carried out in experimental fields of Lorestan Agricultural Research Center (Borujerd station) Lorestan province, Iran in 2011. A factorial experiment in the form of complete randomized block design with three replications was studied. Experimental treatments include: four levels of chemical fertilizers: $F_0 = N_0 + P_0$, $F_1 = N_{25} + P_{50}$ (25 Kg/ha Urea, 46% N_2) + 50 Kg/ha Super phosphate triple, 46% P_2O_5), $F_2 = N_{50} + P_{100}$ and $F_3 = N_{75} + P_{150}$ for sites 1 and 2. The biological fertilizers include: control (no bacterium), *Pseudomonas florescence*, native *Rhizobium* and co-inoculation (*Pseudomonas + native Rhizobium*). In these studies, some characteristics such as: crude protein (CP); dry matter digestible (DMD), water soluble carbohydrates (WSC), crude fiber (CF), acid detergent fiber (ADF), ash and neutral detergent fiber (NDF) were assessed. Results show that biological fertilizers were significantly ($p<0.05$) affected with regards to CP, WSC and ADF but chemical fertilizers were significantly ($p<0.01$) affected with regards to CP, DMD, ASH, CF, ADF and NDF. Interaction between chemical and biological fertilizers showed that CP ($\alpha=0.05$) and WSC ($\alpha=0.01$) were significant. Crude protein percentage tended ($r=-0.35$; $\alpha=0.1$) and ($r=-0.73$; $\alpha=0.01$) negatively correlated with ADF and CF percentages respectively. In this study, F_2S_3 (native rhizobium with reduced application of chemical fertilizers) treatment as compared to F_2S_1 (no application of biological fertilizers with recommended chemical fertilizers) increased positive indexes such as: CP (4.58%); DMD (4.38%) and ASH (3.25%) and decreased negative indexes such as CF (-0.77%) and NDF (-6.4%). Therefore, it is recommended for low input sustainable agriculture (LISA) and high quality of forage.

Key words: Low input sustainable agriculture (LISA), Persian clover, forage quality, *Pseudomonas*, *native Rhizobium*, chemical fertilizers.

INTRODUCTION

The diazotroph bacteria such as symbiotic bacteria (*Rhizobium sp*.) are beneficial microorganisms in the root zone of the legume being reported as very essential for plant establishment and growth, especially under unbalanced conditions (Braea, 1997).

The effect of fertilizers, bacteria *Azospirillum lipoferum* and *pseudomonas flouresence* on rice yield showed that application of looks/of nitrogen with *pseudomonas* and without *azospirillum* had the highest yield of 5733 kg/ha. In this study, nitrogen fertilizer with bacteria *Pseudomonas flouresence* and *A. lipoferum* had significant effect on harvest index at P<0.01 level (Khorshidi et al., 2011). Solubility of phosphorus is an important features as growth stimulant for this bacteria (Alipour and Malakouti, 2003; Kolb and Martin, 1998). Another useful role of stimulating growth bacteria for plants is to reduce or eliminate the harmful effects of pathogen agents, through the phenomenon of induced systemic resistance (ISR) in plants. Salicylic acid produced by plant growth stimulants bacteria could induce systemic resistance phenomenon in rhizobpher (Maurhofer et al., 1998).

The role of micro-organism such as plant- growth-promoting rhizobacteria (PGPR) as modifiers of soil fertility and facilitators of plant establishment is being considered (Requena et al., 1997). Application of PGPR to different crop-production systems has been proposed. Some PGPR have been described by several authors to promote emergence of host plants and have named emergence-promoting rhizobacteria (EPR) (de freitas and Germida, 1990). These effects may be of great use in encouraging plant emergence in soils with a poor structure, such as those in arid or semi-arid zones.

Many rhizobacteria have been shown to produce antibiotics that inhibit the growth of an antagonistic fungi (Shahverdi et al., 2012; Mirsheari et al., 2012) and bacterium *P. fluorescens* (Trevisan) Migula F113, for example, has been shown to control the soft rot potato pathogen *Erwinia carotovora* subspecies atroseptica by producing the antibiotic 2,4-diacetylphloroglucinol (DAPG) (Whipps 2001). Three glucanase-producing actinomycetes, when used separately or more effectively in combination, could significantly promote plant growth and therefore inhibit the growth of *Pythium aphanidermatum* (El Tarabily et al., 2009). Other major antibiotics produced by *Bacillus cereus* are phenazine- e-carboxylic acid and phenazine-1-carboxamide; 2, 4-diacetyl phloroglucinol (phl) (Dunne et al., 1998), pyoluteorin (Nowak-Thompson et al., 1999), zwittermicin A (Emmert et al., 2004), gluconic acid, 2-hexyl-5-propyl resorcinol (Cazorla et al., 2006) and kanosamine (Milner et al., 1996).

In forage crop such as Persian clover, total biomass used with animal, forage quality is important. In many reports, crude protein (CP), dry matter digestible (DMD) and metabolism energy (ME) have been mentioned as forage quality indexes that were assessment with nitrogen percentage and acid detergent fiber (ADF). Amount of protein in forage and protein yield have direct relationships with nitrogen in soil (Hasanvand et al., 2009). Pholsen (2004) reported that organic and chemical fertilizers had no affect on ADF, NDF and DMD percentages of sorghum forage. In this report, protein percentage had significant effect.

The effective strain of Rhizobia in alfalfa (Gholipoor et al., 2008) and bean (Khodshenas, 2006) was reported. These can affect economic production and decrease nitrogen fertilizer application.

Persian clover (*Trifolium rosapinatum* L.) is a native forage (Fabaceae) of Turkey, Iraq, Afghanistan and Iran (Taylor, 1985) that foragers consume with hay, silage and green yield. Total Persian clover-cropped area in Iran is about 42.000 hectares and about 45% belong to Lorestein province (West central in Iran). Clover plants require large amounts of mineral nutrients such as N, P and K for their growth and development (Yazdi et al., 1996; Zamanian et al., 2002; Fraser et al., 2009). The annual biological nitrogen fixation (BNF) rate, which is the result of symbiosis between clover and rhizobia ranges broadly from 85 to 360 kg.ha^{-1} (Russell et al., 1973; Ardakani et al., 2009a).

This study was carried out to integrate chemical and biological fertilizers (special native strain) for increasing forage quality, Low Input Sustainable Agriculture (LISA), safe agro ecosystem, environment and food in Persian clover as main forage in the region.

MATERIALS AND METHODS

The trials were conducted in Lorestan Agricultural research central at Borujerd Agricultural research station, Lorestan province, Iran (longitude: 48°55; E, latitude: 33°40 N, height 1476 m) in 2011. Climate of these area is characterized by moderate summers and cold and humid winter. The mean annual temperature is about 14°C, the average precipitation is about 400 mm and average evapotranspiration is about 1500 mm. Mean annual maximum air temperature was 39°C (in July) and minimum was -10°C (in January) Mirzae, 1995). Before planting, combined soil (samples to 0-30 and 30-60 cm depth were collected and their physical

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Abbreviation: CP, Percentages of crude protein; **DMD,** dry matter digestible; **WSC,** water soluble carbohydrates; **CF,** crude fiber; **ADF,** acid detergent fiber; **NDF,** ash and neutral detergent fiber.

Table 1. Some soil properties of the experimental fields.

Field	Silt (%)	Sand (%)	Clay (%)	Depth	K(av.) P.P.M.	P(av.) P.P.M.	O.C.	pH of paste	EC×10^3
Place 1	50	33	17	0-30	340	13.8	1.15	7.39	1.42
				60.30	140	2.8	0.66	7.60	0.69
Place 2	47	29	24	0-30	290	9.0	1.10	7.51	1.39
				30-60	160	5.4	0.49	7.65	0.86

Table 2. Analysis of variance for forage quality indexes.

S.O.V	d.f	CP	DMD	DMD	WSC	ADF	ASH	CF	NDF
Place	1	11.416	37.058	37.058	0.009	0.500	6.255*	93.183**	83.373
Place*Block	4	11.330	14.151	14.151	3.459	14.983*	0.954	3.919	13.937
Chemical Fertilizer	3	366.297**	141.924**	141.924**	1.066	22.101**	5.514**	117.250**	220.443**
Chemical Fertilizer* Place	3	2.578	3.234	3.234	0.405	4.567	1.290	11.842	19.848
ChemicalFertilizer* Place*Block	12	12.384	15.621	15.621	3.657	12.238**	0.717	9.026	17.538
Biological Fertilizer	3	59.168*	5.719	5.719	10.438*	14.800*	2.061	4.170	47.85
Chemical Fertilizer* Biological Fertilizer	9	10.697*	10.203	10.203	1.801**	10.635	1.671	20.036	38.162
Biological Fertilizer* Place	3	3.644	2.253	2.253	2.198	0.450	0.784	7.220	1.574
Biological Fertilizer* Place* Chemical Fertilizer	9	4.042	1.308	1.308	1.406	0.654	0.451	5.766	11.898
(CV%)		20.07	5.931	5.931	0.167	6.06	11.66	15.35	18.82

ns, *, **no significant at 5 and 1% probability levels based on Duncan, respectively. CP: Crude protein percentage; DMD: Dry Matter Digestible Percentage, ADF; Acid Detergent Fiber Percentage, CF: Crude Fiber Percentage, NDF: Natural Detergent Fiber Percentage, WSC: Water Soluble Carbonhydrates Percentage, ASH: ASH Percentage.

and chemical properties tested. Specifically, our tests included determination of soil texture using the hydrometry method (Gee and Bauder, 1986), pH and salinity of a saturated paste (Rhoades, 1982), organic C (wet oxidation method) (Nelson and Sommers, 1973), the concentration of available P (sodium bicarbonate extraction method) (Olsen, 1954) and available K (flam photometer method, emmition spectrophotometry) (Knudsen et al., 1982) were determined (Table 1).

The study was conducted in a factorial arrangement in the form of randomized complete block design (RCBD) with three times. Experimental treatments including: Four levels of chemical fertilizers: $F_0 = N_0 + P_0$, $F_1 = N_{25} + P_{50}$ (25 kg/hUrea, 46% N_2) + 50 Kg/h super phosphate triple, 46% P_2O_5), $F_2 = N_{50} + P_{100}$ and $F_3 = N_{75} + P_{150}$. The biological fertilizers include: control (no bacterium), *Pseudomonas florescence* 11168 strain (plant growth promoting rhizobacteria-PGPR), native *Rhizobium* (*Leguminosarum symbiovar (sv.) trifoli* RTB3 strain) and co-inoculation (*Pseudomonas* + native *Rhizobium*).

The *P. flurescence* was prepared from gene bank of Soil and Water Research Institute of Tehran, Iran and native *Rhizobium* was isolated from Lorestan province fields under Persian clover by the Biology Department of Tehran Water and Soil Institution with a population of 10^8 CFU.ml^{-1}. Before inoculation, the seeds surface was mixed with 10% sugar completely for more adhesion of inoculums. Finally, the seeds were inoculated and mixed thoroughly with inoculants. Plot consisted of 4 rows in 5 m long with 50 cm spaced between rows and 15 kg.ha^{-1} seed rates. In each place, the crops were harvested three times during the experiment (3 cut of forage were carried out). In the second cutting (about 10% flowering), the two middle rows were used for sampling and measured parameters such as forage quality was assessed. Forage quality indexes included: percentage of crude protein (CP); dry matter digestible (DMD), water soluble carbohydrates (WSC), crude fiber (CF), acid detergent fiber (ADF), ash and neutral detergent fiber (NDF). These indexes were assessed by Near Infrared Reflectance spectroscopy method (NIR) (Jafari et al., 2003; Hofman et al., 1999).

Chemical nitrogen fertilizers were included (urea 46%N) and triple super phosphate (46%P_2O_5). Field was irrigated due to environment condition and soil moisture. Weeding was done by hand. Data was analyzed with SAS statistical software and using Duncan's multiple range test (DMRT) for mean comparison.

RESULTS AND DISCUSSION

Result of this study showed that there was affect only on CF (a=0.01) and ASH (a=0.05) (Table 2). The amount of CF was 25.99 and 24.02% in site 1 and 2, respectively. Also, ASH in site 1 was more than the other place.

In some reports, the effect of climate on forage quality was not significant while soil and fertility effects were significant. It was related to water contents and maintenance in these soils (Arzani et al., 2010; Zaboli et al., 2010).

The biological fertilizers could affect significantly (α=5%) CF, WSC and ADF. The data indicated that the highest CP (22.84%) and DMD (65.94%) belong to native

Table 3. Comparison of mean values of forage quality indexes.

Field	CP	DMD	WSC	ADF	ASH	CF	NDF
Place 1	21.2634[a]	64.3608[a]	19.0418[a]	34.5867[a]	8.8740[a]	24.0227[b]	41.815[a]
Place 2	20.9538[a]	60.9538[a]	19.0227[a]	34.4424[a]	8.3635[b]	25.9931[a]	39.957[a]
B1	20.436[ab]	65.034[a]	19.5196[ab]	34.3094[b]	8.6240[a]	25.262[a]	41.00[a]
B2	19.538[b]	64.698[a]	19.6288[a]	33.8408[b]	8.4060[a]	25.282[a]	38.857[a]
B3	22.847[a]	65.948[a]	18.7394[b]c	34.2583[b]	8.4137[a]	25.092[a]	41.983[a]
B4	22.373[a]	64.549[a]	18.2412c	35.6496[a]	9.0314[a]	24.396[a]	41.692[a]
F1	19.666[b]	63.539[b]	19.454[a]	34.4850[ab]	8.2081[b]	26.217[a]	36.980[b]
F2	20.09[b]	64.847[b]	19.1931[a]	34.3314[b]	8.4969[b]	25.780[a]	42.832[a]
F3	18.382[b]	63.088[b]	18.7310[a]	35.7839[a]	8.4585[b]	26.323[a]	40.41[ab]
F4	27.056[a]	68.454[a]	19.1594[a]	33.4577[b]	9.3117[a]	21.711[b]	43.678[a]

CP, Crude protein percentage; DMD, dry matter digestible percentage; ADF, acid detergent fiber percentage; CF, crude fiber percentage; NDF, natural detergent fiber percentage; WSC, water soluble carbonhydrates percentage; ASH, ASH percentage; F, chemical fertilizers; B, biological fertilizers .

Table 4. Ortogonal comparisons in forage quality Indexes.

NDF	CF	ASH	ADF	WSC	DMD	CP	%
38.22	34.61	8.06	33.98	19.08	65.18	22.23	B1F3
39.8	32.89	7.84	33.98	18.66	64.69	22.77	B2F2
38.91	34.16	8.94	34.73	18.74	65.03	24.13	B3F2
44.93	36.62	8.36	34.13	19.16	64.93	23.13	B4F2
*	ns	ns	ns	ns	ns	ns	Prob.

CP, Crude protein percentage; DMD, dry matter digestible percentage; ADF, acid detergent fiber percentage; CF, crude fiber percentage; NDF, natural detergent fiber.

Rhizobium and lowest negative indexes such as ADF(34.25%) and NDF(38.85%) belong to *Pseudomonas* (data not shown). Also the ortogonal comparisons in forage quality Indexes showed that no significant differences between B1F3 and B2F2, B3F2 and B4F2 treatments except in NDF index (Table 4).

The above mentioned amounts comparison to control treatment increased (9.18% in CP and 1.38% in DMD respectively). While ADF decreased (-5.63%) in comparison with the control treatment. In many reports, CP and DMD percentages are positive indexes of forage quality. Most of researchers suggested that CP and DMD percentages were quality indexes in forage crop that were affected by organic and chemical fertilizers (Ahmed et al., 2012).

The effects of biological fertilizers on these indexes indicated that the highest positive indexes such as CP, DMD, WSC and ASH as compared to control (S1) were 10.55 and 1.38% (in native *rhizobium*); 0.56 (in *Pseudomonas*) and 4.55% (in co-inoculation) while negative indexes such as ADF, CF and NDF belonged to S2, S4 and S2 which were -1.35%, -3.56% and -5.53%, respectively (Table 3). Interaction between biological × chemical fertilizers showed that CP (α = 0.05) and WSC (α = 0.01) were significant (Table 2). The highest CP (28.91%) was observed in F_4S_3. Mentioned treatment had 21.9% more than F_1S_1 (control). The other characteristics such as DMD; ASH; NDF; CF and ADF were 9.9; 3.5, 7.83, 20.65, 16.58 and -10.5%, respectively. The antigenic and synergic effects of co-inoculation were observed in this study. The antigenic effect was observed on WSC. The amounts of this effect were 18.24% while it was 18.73% in native *rhizobioum* and 19.62% in *Psudomonas.* The synergic effect was observed on ADF too (Table 3).

Rodeles (1999) reported that co-inoculation with *rhizobium* and *azotobacter* in *Vicia faba* L. as compared to *Rhizobium* had 100% increase on action of nitrogenas

Table 5. Correlation between forage quality Indexes.

	CP	DMD	WSC	ADF	ASH	CF	NDF
DMD	0.69706**	1.0000					
WSC	-0.27941	0.14412	1.0000				
ADF	-0.35588	-0.73824**	-0.23529	1.0000			
ASH	0.56176**	0.38529	-0.15882	-0.06176	1.0000		
CF	-0.73824**	-0.46765	0.14706	0.22059	-0.82941**	1.0000	
NDF	0.52059	0.73824**	-0.10588	-0.39706	0.63529**	-0.48235	1.0000

CP, Crude protein percentage; DMD, dry matter digestible percentage; ADF, acid detergent fiber percentage; CF, crude fiber percentage; NDF, natural detergent fiber percentage; WSC, water soluble carbonhydrates percentage; ASH, ASH percentage.

enzyme. He added that co-inoculation with *Rhizobium* + *Azosprillium* and *Rhizobium* + *Azotobacter* changed concentration and distribution of micro and macro elements such as: Mg, Ca, K, P, Fe, Cu, Zn, Mn and B as compared to *Rhizobium* application alone. Also, Tilak (2004) reported the synergic effect of *Rhizobium* + *Pseodomonas* on numbers of nodule in peagon pea and added that co-inoculate with these bacteria increased to 85% in nodulation while it was 50% in *Rhizobium* application alone. In many researches, CP; Phosphoruse content in forage (P), cell wall content in forage; DMD; CF; In vitro digestible of forage; crude energy and energy of metabolism (Em) were mentioned for assessment of forage quality. In this study, chemical fertilizers were more effective on indexes. Moreover, in these indexes, CP is the most important that was affected by chemical and biological fertilizers; because it is related to nitrogen content in soil (Ali et al., 2009; Poormoradi et al., 2010).

The influence of PGPR on dry matter accumulation in chick pea *(C. arietinum* L.) yield under field conditions has been thoroughly studied (Rokhzadi et al., 2008). Studies have shown that a combined inoculation of *Azospirillum* spp., *Azospirillum chroococcum* 5, *Mesorhizobium ciceri SWR17* and *Pseudomonas fluorescens* P_{21} improved nodulation, increased dry matter accumulation in roots and shoots, grain yields, biomass and protein yield of chick-pea by a significant margin. Parmar and Dadarwal (1999) studied co-inoculation of the rhizobacteria with effective Rhizobium strains of chickpea and observed a significant increase in nodule weight, root and shoot biomass and total plant nitrogen when grown either in sterilized chillum jars or under pot culture conditions. The *Rhizobium* stimulatory *Pseudomonas sp.* “CRP55b” showed maximum increase in all the symbiotic parameters.

Ahmed et al. (2004) and Cox et al. (1998) reported contradictory effects of chemical and organic fertilizers on qualities of forge crop with multi-cutting. In this crop, chemical fertilizers effect was seen on forage quality only in first cutting but organic fertilizers had effect on throughout plant growth period (throughout cutting). This is related to leaching and fixation of chemical fertilizers, they added that by application of organic and chemical fertilizers in grass and legume, amount of the CF in grass did not any change while it decreased in legume by organic fertilizer.

Most researchers believed that increase of yield is due to increase of photosynthesis rate and photosynthesis matter translocation time. In other reports, increase in P absorption was related to biological fertilizers application too (Ali et al., 2009; Mirzakhani et al., 2009; Ardakani et al., 2000b; Hazarika et al., 2000).

Correlation coefficients among variables are presented in Table 5. Crude protein percentage tended (r=-0.35; α=0.1) and (r=-0.73; α=0.01) negatively correlated with ADF and CF percentages respectively. In addition, CP was positively correlated with DMD (r=0.69; α=0.002) and ASH (r=0.56; α=0.02). Gadberry et al. (2005) reported that ADF tended to be negatively correlated with CP (α=0.08). Also, negative correlation of CP with WSC indicated that with increase of fertility nitrogen and phosphorus caused improvement in growth of plant and protein content. Negative correlation between DMD and CP with ADF and also CF with CP, ASH and DMD indicated the efficiency of integrated chemical and biological fertilizers application. Others reported these results in application of organic fertilizers integrated with chemical fertilizers, for example Gadberry et al. (2005) in Bermuda grass and James (1997) in alfalfa hay; vegetative hay of corn and tall fescue hays.

Conclusions

In this study, F_2S_3 (native *Rhizobium* with reduced application of chemical fertilizers) treatment as compared to F_2S_1 (no application of biological fertilizers with recommended chemical fertilizers) increased positive indexes such as: CP (4.58%); DMD (4.38%) and ASH (3.25%) and decreased negative indexes such as CF (-0.77) and NDF (-6.4%). Also, application of F_2S_3 was decreased, 1050 ton/year Urea, and 2100 ton/year. Superphosphate triple in Persian clover in Iran, so F_2S_3 is recommended for low input sustainable agriculture (LISA) and high quality of forage.

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