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Effects of plant growth-promoting rhizobacteria and manure on yield and quality characteristics of Italian ryegrass under semi arid conditions

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Abstract

Plant growth-promoting rhizobacteria (PGPR) appeared as a promising alternative for organic fertilization. A field experiment was carried out to evaluate the effects of PGPR and solid cattle manure application on yield, quality and mineral concentrations of Italian ryegrass (*Lolium multiflorum* Lam.) under semi arid conditions in Turkey. The treatments included: three manure application rates (i.e. M1, 10; M2, 20; and M3, 30 Mg ha^{-1}), five PGPR strains (i.e. RC11, *Bacillus subtilis*; RC21, *Variovorax paradoxus*; RC105, *Paenibacillus polymyxa*; RP24/3, *Paenibacillus polymyxa*; and RF29/2, *Pseudomonas putida*), and a control (without bacteria or manure application). Manure application did not increase yield of Italian ryegrass, whereas the treatments with RC21 and RP24/3 rhizobacteria provided the highest dry matter yield. All PGPR and manure applications increased crude protein concentration of Italian ryegrass. The M3 manure rate and most treatments with PGPR (except RC11) produced greater crude protein yield than that of control. In addition, concentrations of P, S, Mg, Cu, Zn, and Fe in most PGPR and manure applications were greater than those of control.

Keywords: Cattle manure, Plant growth-promoting rhizobacteria, Crude protein, Organic fertilizer, Macronutrients, Micronutrients.
Abbreviations: ADF - Acid detergent fiber; NDF - Neutral detergent fiber; CP - crude protein; PGPR - plant growth-promoting rhizobacteria; RC21 - *Variovorax paradoxus*; RC11 - *Bacillus subtilis*; RC105 - *Paenibacillus polymyxa*; RP24/3 - *Paenibacillus polymyxa*; RF29/2 - *Pseudomonas putida*; M1 - 10 Mg ha^{-1} manure application; M2 - 20 Mg ha^{-1} manure application; M3 - 30 Mg ha^{-1} manure application.

Introduction

Organic forage production is one of the most important aspects for organic animal production. Diversity of crops cultivated for organic forage is limited in Turkey (Yolcu et al., 2009). Generally, lucerne (*Medicago sativa* L.), sainfoin (*Onobrychis sativa* L.), corn (*Zea mays* L.) and common vetch (*Vicia sativa* L.) are grown for organic forage. Thus, the diversity of cultivated crops for forage production should be increased for successful animal feeding in the context of organic production (Lithourgidis et al., 2011). Italian ryegrass (*Lolium multiflorum* Lam.) is an important forage crop that can be used to increase crop diversity (Yolcu et al., 2011). Ryegrasses produce forage of high quality for livestock with dry matter digestibility up to 80% in the early growth stages (Acıkgöz, 2001). Italian ryegrass usually provides greater overall productivity than other cool season grasses due to quick regrowth, early development in the spring, and prolonged growing period in the fall (Serin et al., 1996).

Application of manure and plant growth-promoting bacteria can play an important role in organic forage production. Manure applications improve the chemical, physical and biological characteristics of the soil and increase yield and quality characteristics of crops (Matsi et al., 2003; Lithourgidis et al., 2007; Yolcu et al., 2010). Biofertilizers are alternatives to mineral fertilizers that are used for increasing soil productivity and plant growth in sustainable agriculture (Canbolat et al., 2006a). Microorganisms are also important to promote the circulation of plant nutrients and reduce the need for chemical fertilizers as much as possible (Çakmakçı et al., 2006; Javaid and Mahmood, 2010). When effective cultures of microorganisms are applied to the soil they stimulate the decomposition of organic wastes and residues thereby releasing inorganic nutrients that become available for uptake by the plants (Javaid and Mahmood, 2010). Moreover, bacterial strains possess 1-aminocyclopropane-1-carboxylate (ACC) deaminase, an important trait of PGPR that stimulates root growth (Glick et al., 1998). Plant growth promoting bacteria can affect plant growth and yields because of their interaction with soil characteristics and nutrient availability (Yazdani et al., 2009).

The effect of PGPR, as a complex process, depends on bacterial strains and population, plant-bacterial strain combination, plant genotype, growth parameters evaluated, and environmental conditions (Şahin et al., 2004; Çakmakçı et al., 2006, 2007a). Also, the effects of manure may depend on manure levels, plant type and environment (Matsi et al., 2003; Yolcu et al., 2010). However, adequate information about the effects of manure and PGPR on Italian ryegrass under field conditions is not available. The aim of the present study were to investigate the effectiveness of five PGPR and three solid cattle manure application rates on yield, quality and mineral

Table 1. Climatic data during the growing seasons of the experimentation and long-term average (1986-2006) at Kelkit, Turkey.

	J	F	M	A	M	J	J	A	S	O	N	D	Total/Mean
Years	Total Precipitation (mm) (Monthly)												
2008	40.8	23.3	38.4	51.4	28.4	35.8	2.6	20.0	30.3	35.2	21.1	34.4	361.7
2009	21.3	45.6	57.9	96.3	63.6	25.3	37.4	0.0	71.0	35.1	127.2	33.0	613.7
1986-2006	33.1	35.5	38.3	57.7	68.3	45.1	14.8	13.8	26.3	50.6	45.1	37.6	466.2
	Mean air temperature (°C) (Monthly)												
2008	-6.1	-4.4	8.1	11.6	11.7	16.6	20.1	21.5	17.1	11.9	6.6	-0.7	9.5
2009	-0.2	3.2	3.8	7.8	12.9	18.1	19.6	18.0	14.8	13.0	4.7	4.2	10.0
1986-2006	-1.8	-1.0	3.1	9.4	13.3	16.8	20.2	20.1	16.3	11.3	4.4	0.5	9.4

concentrations of Italian ryegrass under semi arid field conditions. This is the first report on the effects of N_2-fixing, P-solubilizing and ACC deaminase-containing PGPR on growth and yield of Italian ryegrass.

Result and Discussion

Dry matter yield

The ANOVA for the dry matter yield and the other characteristics showed that there was no treatment by harvest interaction. Thus, treatment means averaged across harvests are presented. The greatest dry matter yield was obtained after inoculation of Italian ryegrass seeds with PR24/3 (2853 kg ha^{-1}) followed by RC21 (2344 kg ha^{-1}), whereas the other PGPR treatments gave similar or lower yield than that of control (Table 4). Canbolat et al. (2006a) and Çakmakçı et al. (2007b) reported that inoculation of barley (*Hordeum vulgare* L.) with PGPR strains increased shoot weight up to 40% compared with control. Similarly, Wu et al. (2005) reported that the application of biofertilizer containing mycorrhizal fungus and three species of bacteria significantly increased corn growth. In contrast, Javaid and Mahmood (2010) found that the application only of commercial microorganisms did not significantly affect the yield of soybean (*Glycine max* L.). The first two levels of manure applications (M1 and M2) decreased biomass yield of ryegrass, whereas biomass yield with M3 rate did not differ significantly from that of control (Table 4). This could be because sometimes manure application may have negative effects on soils and plants (Chang et al., 1991) depending on many factors, such as application rates, crops, manure composition, quantity of soil available nutrient and the availability of manure nutrients (Matsi et al., 2003). On the other hand, many studies reported that the application of manure increased the forage and grain yield in many crops (Butler and Muir, 2006; Lanyasunya et al., 2007; Lithourgidis et al., 2007; Javaid and Mahmood, 2010; Yolcu et al., 2010).

Crude protein, ADF and NDF concentrations

Crude protein concentration of forage is one of the most important criteria for the evaluation of forage quality. In this study, all PGPR and manure applications had higher CP concentration than that of control (Table 4), whereas the CP concentration was the highest in M3 manure rate, followed by the M2, RC21 and RF29/2 applications. Crude protein yield is a measure of how much nitrogen is taken up by the crop and also how much nitrogen is removed from the field with harvest. The CP yield in most treatments (with the exception of RC11, M1 and M2) was higher than that of control. However, despite the fact that M3 had the highest CP concentration, it did not produce the highest CP yield. On the other hand, the PR24/3, with low CP concentration, had the highest CP yield, because of its highest forage yield (Table 4). Similarly, Lanyasunya et al. (2007) reported that Columbus grass (*Sorghum almum*) had much higher CP yield after manure application than that of control. In addition, Butler and Muir (2006) reported that CP of tall wheatgrass (*Thinopyrum ponticum* Podp.) was greatest at the two highest rates of dairy manure compost only in one of the two years of the experimentation. On the contrary, Yolcu et al. (2010) found that CP yield of common vetch-barley mixtures was not affected by the application of solid or liquid manure in the first year of the experimentation. On the other hand, Singh et al. (2010) reported a significant improvement in the crude protein content of corn and wheat following inoculation with PGPR. In terms of ADF and NDF concentrations, no significant differences were found among treatments (Table 4). However, Yolcu et al. (2010) reported that ADF, NDF, DMI, and DDM concentrations of

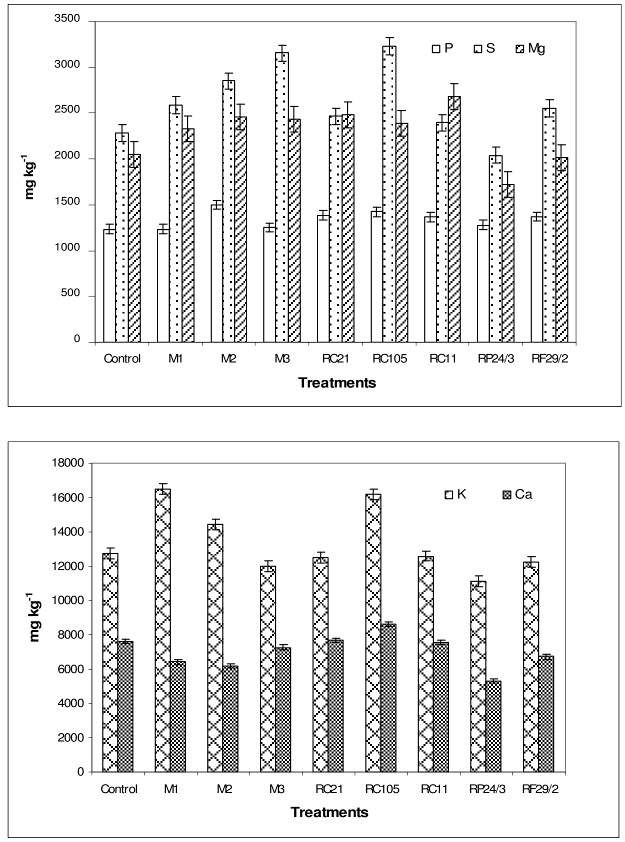


Fig 1. Effect of plant growth-promoting rhizobacteria (PGPR) and solid cattle manure applications on macro-nutrient concentrations in Italian ryegrass.

Table 2. Selected chemical characteristics of the experimental field soil before Italian ryegrass sowing.

Soil Properties	Units	0-30 cm depth	30-60 cm depth
Cation exchangeable capacity	$cmol_c$ kg^{-1}	27.7±1.30*	27.8±1.10
Total N	mg kg^{-1}	20.4±0.08	12.6±0.04
pH	(1:2 soil:water)	7.6±0.3	7.7± 0.19
Organic matter	g kg^{-1}	2.8±0.10	1.7±0.10
$CaCO_3$	g kg^{-1}	24.0±0.10	23.9±0.20
Available P	mg kg^{-1}	15.3±1.80	10.7±0.70
Exchangeable Ca	$cmol_c$ kg^{-1}	21.1±3.50	21.0±1.10
Exchangeable Mg	$cmol_c$ kg^{-1}	5.5±0.70	5.4±0.56
Exchangeable K	$cmol_c$ kg^{-1}	8.8±0.90	8.7±0.60
Exchangeable Na	$cmol_c$ kg^{-1}	1.1±0.04	1.3±0.08
Available Fe	mg kg^{-1}	2.10±0.40	1.5±0.50
Available Mn	mg kg^{-1}	1.5±0.09	1.0±0.08
Available Zn	mg kg^{-1}	3.2±0.18	2.3±0.19
Available Cu	mg kg^{-1}	1.20±0.23	0.9±0.20

*Mean and standard deviation of four replications

Table 3. Some chemical characteristics and nutrient content of the solid cattle manure.

Cattle manure properties	Units	Value
pH	(1/5) (s/w)	7.6 ± 0.10*
Moisture	%	88.9 ± 3.40
Dry matter	%	11.1 ± 1.35
Organic matter	%	29.1 ± 2.60
N	g kg^{-1}	4.2 ± 1.11
P	g kg^{-1}	1.6 ± 0.05
Ca	g kg^{-1}	4.7 ± 0.02
Mg	g kg^{-1}	2.3 ± 0.10
K	g kg^{-1}	1.1 ± 0.05
Na	g kg^{-1}	1.0 ± 0.04
Fe	mg kg^{-1}	524 ± 5.10
Mn	mg kg^{-1}	248 ± 3.09
Zn	mg kg^{-1}	431 ± 4.18
Cd	mg kg^{-1}	5.48 ± 0.10
Cu	mg kg^{-1}	28 ± 0.23
Pb	mg kg^{-1}	24.3 ± 2.18

*Mean and standard deviation of four replications

common vetch-barley mixtures were significantly affected by manure application.

Macro- and micro-nutrient concentrations

Macro- and micro-nutrient concentrations in the biomass of Italian ryegrass showed significant variation after solid cattle manure or bacteria inoculants applications (Fig. 1, 2). In most cases, concentrations of P, K, S and Mg were higher after the three manure application rates compared with those of control, whereas Ca concentration was lower than that of control. Similarly, Motavalli et al. (1989) reported that the uptake of P and K by corn plants increased after liquid dairy cattle manure applications for more than 2 years. Butler and Muir (2006) reported that tall wheatgrass P and K concentrations increased linearly as rates of manure increased. Moreover, application of farm yard manure decreased Mg and Ca concentrations in oat (*Avena sativa* L.) (Dahiya and Singh, 1980; Singh and Dahiya, 1980). All tested P-solubilizing bacteria significantly increased P, S and Mg concentration of Italian ryegrass, with the exception of non-phosphate solubilizing RP24/3 strain (Fig. 1). Similarly, Çakmakçı et al. (2007a; 2009) reported that phosphate solubilizing and N_2-fixing PGPR increased the uptake of P and Mg in spinach and wheat plants. A strain of *Mesorhizobium mediterraneum* promoted growth and increased phosphorous content in chickpea and barley plants in a soil with and without the addition of phosphates (Peix et al., 2001). Most of the rhizobacteria (except RC105) provided lower or similar K and Ca concentrations to those of control (Fig. 1). Similarly, Elsheikh and Mohamedzein (1998) reported that the Ca content of groundnut seed did not show any response to *Bradyrhizobium* and/or VA mycorrhiza. Moreover, Singh et al. (2010) reported that *Bacillus mucilaginosus* resulted in significantly higher mobilization of K than *Azotobacter chroococcum* and *Rhizobium* spp. in corn and wheat plants. On the other hand, Ibrahim et al. (2008) reported that fertilization of soybean with chicken manure increased considerably Ca and Mg content in the seeds, whereas the inoculation of seeds with *Bradyrhizobium* caused further increase in Ca and Mg content. The differential responses to such treatments could be attributed to differences among cultivars as well as to differences in the growing environment. In most cases, the M3 manure application (highest rate) provided higher concentration of micronutrients compared with the other treatments (Fig. 2). Also, M1 and M2 manure applications had lower or similar concentrations of Cu, Mn, B and Na to those of control. Nikoli and Matsi (2011) reported that the concentrations of Cu, Zn, Fe, Mn, and B micronutrients in corn biomass remained unchanged with liquid cattle manure for a period of nine years. On the contrary, several researchers reported increased micronutrient concentrations in the tissues of oat, corn, sugar beet (*Beta vulgaris* L.), cotton (*Gossypium hirsutum* L.), barley, and soybean, probably because manure application enriches the soil in nutrient elements (Singh and Dahiya, 1980; Kapur and Kanwar, 1989; Mitchell and Tu, 2005; Ibrahim, et al., 2008; Yolcu et al., 2010). All PGPR used in this study resulted in a higher Fe concentration than that of control (Fig. 2). All manure rates increased Zn concentrations with those of control. In addition, most of them provided higher or similar micronutrients concentrations than those of control. Similarly, Canbolat et al. (2006a, b) and Çakmakçı et al. (2007a, b, 2009) reported that phosphate solubilizing and N_2-fixing PGPR increased the uptake of Fe, Cu, Mn and Zn in barley, spinach and wheat plants. In addition, Dursun et al. (2010) found that bacterial (BA-142, MFD-2, CD-1, and FF) applications increased Na, Cu, Mn and Fe content in tomato (*Lycopersicum esculentum* L.) and cucumber (*Cucumis sativus* L.). Moreover, Ibrahim et al. (2008) reported that

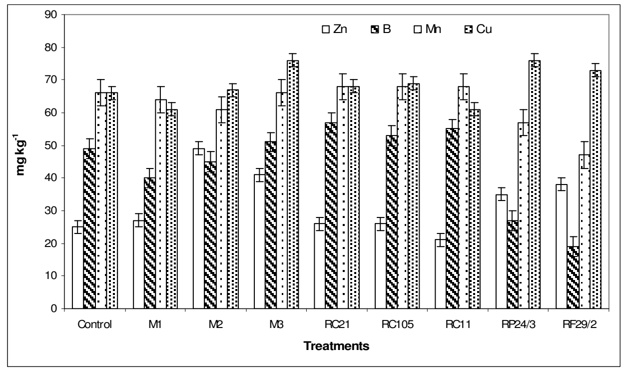
Table 4. Dry matter yield and quality components of Italian ryegrass in response to inoculation with PGPR and solid cattle manure applications under semi arid conditions.

Treatments	DM yield (kg ha^{-1})	Crude protein content (%)	Crude protein yield (kg ha^{-1})	ADF (%)	NDF (%)
Control	1901 cd*	12.1 f	230 d	37.2	54.4
M1	1601 e	13.1 e	210 de	35.1	55.4
M2	1509 ef	14.3 b	215 d	36.3	53.1
M3	1827 d	15.0 a	274 c	35.4	53.1
RC21	2344 b	14.0 b	330 b	34.7	55.2
RC105	2066 c	13.6 cd	281 c	35.1	51.3
RC11	1361 f	13.2 de	180 e	34.7	52.8
RP24/3	2853 a	13.6 cd	387 a	36.6	55.5
RF29/2	1961 cd	13.9 bc	273 c	33.5	54.3

*Different letters within each column indicate statistically significant differences at P=0.01 (Duncan's test)

fertilization of soybean with chicken manure increased considerably the content of micronutrients in the seeds, whereas inoculation of seeds with *Bradyrhizobium* caused further increase in mineral contents.

For crops grown for forage it is important to produce great forage yields and high nutritional quality. High forage yield is very important for the producers, but for livestock enterprises it is also important to produce forage of high quality. In this study it was found that high forage yield and high forage quality characteristics for animal nutrition could be obtained after the inoculation of ryegrass seeds with RP24/3 and RC21 rhizobacteria. However, further research would be useful to verify the effects of manure application and inoculation with growth-promoting rhizobacteria strains on growth, yield and nutrient uptake of Italian ryegrass.



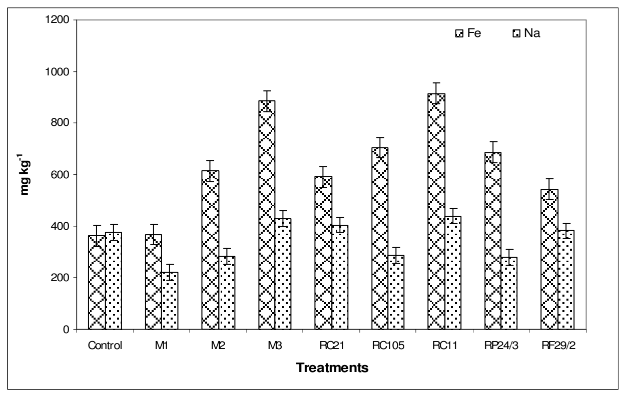


Fig 2. Effect of plant growth-promoting rhizobacteria (PGPR) and solid cattle manure applications on micro-nutrient concentrations in Italian ryegrass.

Material and methods

Isolation and characterisation of bacterial strains

Five plant growth-promoting rhizobacteria, *Bacillus subtilis* (RC11), *Variovorax paradoxus* (RC21), *Paenibacillus polymyxa* (RC105), *Paenibacillus polymyxa* (RP24/3) and *Pseudomonas putida* (RF29/2) were used in the experiment. The RC11, RC21, and RC105 rhizobacteria were isolated from the rhizosphere of wild red raspberries (*Rubus idaeus* L.) from an organic soil of Yedigöl valley in the south-western side of Kackar Mountain (Çakmakçı et al., 2008), and characterised as PGPR that could promote plant growth, solubilize P, and fix N_2. The RP24/3 and RF29/2 were originally isolated from the acidic tea (*Camellia sinensis* L.) rhizosphere at Ataturk University of Turkey (Çakmakçı et al., 2010). The RC11 and RC21 rhizobacteria had positive effects on the activity of 6-phosphogluconate dehydrogenase enzyme and growth parameters of wheat and spinach and also they increased activities of the enzyme glutathione reductase and glutathione S-transferase (Çakmakçı et al., 2007a, 2009).

Field experiment and growth conditions

An experiment was carried out at the Kelkit Aydin Dogan Vocational Training School Research Station of Gumushane University in north east Turkey (40° 08' N, 39º 25' E). Italian ryegrass (cv Peleton) was sown at 21 April 2008 at a seeding rate of 30 kg ha^{-1}. Seedbed preparation included moldboard plough, disk harrowing, and scrapper. Manure was incorporated into the soil (0-5 cm) in all plots during seedbed preparation. Before Italian ryegrass sowing, seeds were surface-sterilised in 70% (v/v) ethanol for 2 min, and rinsed ten times in sterile water. For this application, pure cultures were grown in NB at 28°C and diluted to a final concentration of 10^9 colony-forming units (cfu) ml^{-1} in sterile distilled water containing 0.025% (v/v) Tween-20. Surface-sterile seeds were inoculated by immersion in the appropriate PGPR suspension (at 10^9 cfu ml^{-1}) for 2 h on a rotary shaker at 81 rpm, air-dried, and sown immediately. The cell densities in the PGPR suspensions were adjusted to a final density of approximately 10^8 cfu seed^{-1}.

The experimental design was a randomized complete block with nine treatments replicated three times. Plot size was 3.0 m x 1.68 m, with 24 cm row spacing. Plots were separated by a 2.5 m buffer zone. The treatments were: three solid cattle manure application rates (i.e., M1, 10 Mg ha^{-1}; M2, 20 Mg ha^{-1}; and M3, 30 Mg ha^{-1}), five bacteria inoculations (i.e., RC21, RC105, RC11, RP24/3, and RF29/2), and a control (without bacteria or manure applications). The manure was taken by Dogan Organic Dairy Farm and was certified by IMO. The field was irrigated once in the early stage of crop growth each year. For better establishment of the plants and rhizobacteria, Italian ryegrass was not harvested in the first year. Therefore, evaluations were made only in the second year (2009) of the experimentation.

Generally, climatic conditions of the research location in Kelkit are dry during the summer and cold and snowy during the winter. Climatic data during the two growing seasons of the experimentation and long term means are shown in Table 1.

Plant and soil analysis

Italian ryegrass was harvested in a 1 m^2 area of each plot twice (at the beginning of anthesis of annual ryegrass) for determination of fresh weight. Samples were dried in the oven at 68 ^oC for 48 h for dry matter determination. For forage quality at harvest dried samples were ground with a Wiley mill to pass a 1-mm screen and analyzed for nitrogen. Total N was determined using the Kjeldahl method and CP was calculated by multiplying the N content by 6.25 (Bremner, 1996). ADF and NDF concentrations were determined according to Van Soest (1963). Macro- (P, K, S, Ca and Mg) and micro-elements (Cu, Zn, Fe, Mn, B and Na) were determined after wet digestion of dried and ground sub-samples using a HNO_3-H_2O_2 acid mixture (2:3 v/v) in three steps (first step: 145ºC, 75%RF, 5 min; second step: 180ºC, 90%RF, 10 min and third step: 100ºC, 40%RF, 10 min) in microwave (Bergof Speedwave Microwave Digestion Equipment MWS-2) (Mertens, 2005a), and Inductively Couple Plasma spectrophotometer (Perkin-Elmer, Optima 2100 DV, ICP/OES, Shelton, CT 06484-4794, USA) (Mertens, 2005b).

Soil samples (0-30 cm and 30-60 cm) were collected prior to crop seeding and air-dried, crushed, and passed through a 2-mm sieve and analyzed for selected chemical characteristics (Table 2). Samples were analyzed for cation exchange capacity (CEC) (Sumner and Miller, 1996), Kjeldahl-N (Bremner, 1996), Olsen-P (Olsen and Sommers, 1982), pH (1:2.5 H_2O w/v), organic matter (Nelson and Sommers, 1982) exchangeable Ca, Mg, K, and Na (Rhoades, 1982) and extractable Fe, Mn, Zn, and Cu (Lindsay and Norvel, 1978). Certain chemical characteristics and nutrient content of solid cattle manure are presented in Table 3.

Statistical analysis

Dry matter yield, crude protein, ADF, NDF and nutrients data were subjected to analysis of variance (ANOVA) using a two-way ANOVA approach with nine treatments and three replications. Treatment means were compared by Duncan's multiple range test using SPSS software package (SPSS 11.5 for windows) (SPSS, 2004).

Conclusion

The results of this study showed clearly that application of manure and inoculation of seeds with plant growth-promoting rhizobacteria can affect the dry matter yield, crude protein, macro and micronutrients concentration of Italian ryegrass. The RP24/3 and RC21 rhizobacteria provided the highest dry matter yield, whereas all the rhizobacteria and manure applications increased the crude protein concentration of Italian ryegrass. Also, the mineral concentrations of Italian ryegrass in most PGPR and manure applications were greater or similar to those of control. The results suggest that the effective PGPR strains tested in this study have a potential to be formulated and used as inoculants for ryegrass. Taking into account the high cost of chemical fertilizer and the environmental pollution from the excessive chemical fertilizer use, the bacteria tested in this study may well be used to promote plant growth for sustainable and organic forage production under semi arid conditions.

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