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RESPONSE OF BERSEEM CLOVER (*TRIFOLIUM ALEXANDRINUM* L.) TO CHEMICAL, BIOLOGICAL AND INTEGRATED USE OF FERTILIZERS

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ABSTRACT. To evaluate the effect of different fertilizer types on the vegetative growth characteristics, yield and forage quality of berseem clover (*Trifolium alexandrinum*), the present experiment was conducted in Mahidasht Agriculture and Natural Resources Research Station (Kermanshah, Iran), during 2011 and 2012 growing seasons. The experimental treatments consisted of control (no fertilizer), chemical fertilizer, biological fertilizer and different combinations of chemical and biological fertilizing systems. A complete randomized block design with three replicates was employed for analysis of the data for each year. A combined analysis of variance was conducted to compare the data from the two years of the experiment. The results showed that the highest forage yield (172.1 g/m^2) was produced in integrated fertilizer application (urea chemical fertilizer + mycorrhiza treatment). The highest crude protein content of 25% was obtained from integrated biological fertilizer treatment (nitrogen-fixing bacteria + phosphorus-solubilizing bacteria treatment). The superiority of integrated fertilizer application for higher forage production and biological fertilizer application for higher forage quality in berseem clover could be recommended by the results of this experiment. Application of integrated fertilizing treatments not only optimized the chemical fertilizer application (consequently reducing the environmental pollutions), but it also enhanced forage quality in terms of higher macro a micronutrients concentrations. According to the results of this study it could be concluded that integrated fertilizing treatments may be accounted more efficient in dry farming than in irrigated agroecosystems.

Key words: Berseem clover; Fertilizer types; Forage yield; Forage quality.

Abreviations: DMD- digestive dry matter, CP- crude protein, WSC-water soluble carbohydrate, ADF - acid detergent fiber, NDF- neutral detergent fiber, ASH- ash, CF - crude fibre

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Co - control (no fertilizer application); Nch + Pch - chemical fertilizer; Nch + Pbio - urea chemical fertilizer + phosphorus - solubilizing bacteria; Nch + Mbio - urea chemical fertilizer + mycorrhiza; Nch + Pbio + Mbio - urea chemical fertilizer + phosphorus - solubilizing bacteria + mycorrhiza; Nbio + Pch – nitrogen - fixing bacteria +triple superphosphate fertilizer; Nbio + Pbio – nitrogen -fixing bacteria + phosphorus - solubilizing bacteria; Nbio + Mbio – nitrogen - fixing bacteria + mycorrhiza; Nbio + Mbio + Pbio – nitrogen - fixing bacteria + phosphorus solubilizing bacteria + mycorrhiza

INTRODUCTION

Worldwide reductions in production resources compared to increasing growth rate of world population are encouraging means for sustainable crop production in current and future farming systems. Sustainable agriculture, especially organic agriculture, is a low input system that implies the efficient use of biological resources. Transition from high input to low input agriculture requires information to solve the problems of transition period.

Nutrient availability is one the most important factors during plant development. The use of biological fertilizers is a critical component to crop production in sustainable farming systems (Canbolat *et al.* 2006). In such a system, fertilizing with organic fertilizers such as nitrogenous and phosphorous bio-fertilizers (phosphate solubilizing microorganisms) is considered as major contribution to sustainable crop production. However, there are evidences that yield in organic farming systems is less than conventional production systems, especially in areas with soils of low organic matter content (Dawson *et al.,* 2008). So, food security for increasing population will be at risk in countries with low soil organic matter.

There are scientific evidences supporting the idea that the application rate of chemical fertilizers could be reduced (to achieve optimum yield levels) if they were applied along with organic fertilizers (Berecz *et al.* 2005). Even in some cases the yield of cotton was higher if chemical fertilizer was applied along with organic fertilizers (Blaise *et al.* 2006).

Bio-fertilizers contain various types of free-living microorganisms that could convert unavailable nutrients to available forms through biological processes which enhance better root development and seed germination (Chen, 2006; Rajendran and Devarj, 2004; Vessey, 2003). Phosphorous solubilizing bacteria are one of the most useful bio-fertilizers to provide soil phosphorus in optimum level. Şahin *et al.* (2004) in an experiment studied the influence of inoculation with phosphorus solubilizing and free nitrogen fixing bacteria on barely and sugar beet. He claimed that bio-fertilizer possibly improved the amount of nitrogen accessibility for plants, resulting in better conditions for growth and increased the yield quality.

Mycorrhiza increases the ability of host plant to uptake insoluble nutrients, particularly phosphorus and some microelements. Shabani *et al.*

(2011) showed that by application of proper fertilizer on annual medic (*M. scutellata* cv. Robinson) while harvesting a forage yield at early flowering stage, not only a considerable amount of forage could be produced, but also an active soil seed bank could be achieved. He also concluded that by integrated fertilizer (urea chemical fertilizer+phosphorus solubilizing bacteria + mycorrhiza) application, the highest vegetative growth was achieved, however, due to the lack of sufficient seed production, seed bank was poor. In a ley farming system, the natural reestablishment of annual medic pasture is the main goal which is achieved by application of integrated biological fertilizer (nitrogen fixing bacteria+phosphorus solubilizing bacteria) application (Shabani *et al.*, 2011).

The increase in the phosphorus uptake happens in the symbiotic plants by developing the absorption contact surface of the root system, hence it increases the phosphorous discharging area (Zaidi *et al.,* 2004).

Mycorrhiza are multifunction organisms in agroecosystems that improve soil physical, chemical and biological properties by developing mycelium, increasing nutrient absorption and soil nutrients network, respectively (Cardoso and Kuyper, 2006). Nadian *et al.* (1998) reported that dry matter of berseem clover (*Trifolium alexandrinum*) inoculated with mycorrhiza was significantly more than control treatment. According to the same report, co-inoculation by rhizobium bacteria and mycorrhiza increased berseem clover shoot dry weight and leaf area index by five times, compared to control. Co-inoculation of medic with *Glomus intraradices* fungi and *Sinorhizobium meliloti* bacteria significantly increased the shoot dry weight and total phosphorus and nitrogen content in the plant tissues (Stancheva *et al.,* 2008). Co-inoculation of medic under the phosphorus deficiency conditions led to higher forage weight, fixation of N_2 and phosphorus content in plant tissues, compared to separately inoculated treatments (Stancheva *et al.* 2008). Maleki Farahani *et al.* (2011) showed that by nitrogen bio-fertilizer application, phosphorous concentration in barley grains was significantly higher than those in other treatments. Shoghi-Kalkhoran *et al.* (2013) showed that integrated fertilizers application in sunflower significantly increased leaf area index, plant height, grain production, biomass, oil yield, and protein content compared to sole organic or chemical fertilizers application.

Proper nutrient management in crop production not only improve and stabilize the yield, but also will improve the environmental health. Shabani *et al*. (2011) working on the effect of different fertilizer types on annual medics, reported that integrated nutrition system, not only reduced chemical fertilizer application, resulting in reduced environmental pollution, but also improved forage quality characteristics. They also reported that co-inoculation of annual medic seeds with *Rhizobium* and phosphorus solubilizing bacteria improved seed number in soil seed bank, pods per plant, phosphorus percentage in pods and phosphorus absorption in plant as well as plant dry matter. They finally recommended the integrated application of different bio-inoculants to achieve the best results in annual medics. Since there is not enough evidence on the effect of bio-fertilizer application on quantity and quality of berseem clover yield, the current experiment was conducted to investigate the effect of different nutritional systems on forage quality and quantity in berseem clover.

MATERIALS AND METHODS

This experiment was conducted during two cropping seasons of 2011-2012 in Mahidasht Soil Fertility Research Station, Kermanshah, Iran. The geographic position of the site was 46°50′ N and 24°16′ E, with elevation of 1380 meters above the sea level. Soil samples were collected before the commencement of the experiment. The experiment was conducted based on a randomized complete block design with three replicates. The experimental treatments consisted of control (without fertilizer), chemical, biological and integrated fertilizing systems as follows:

(Co): control (no fertilizer application);
Nch+Pch: chemical nitrogen (urea) and phosphorous (triple superphosphate) fertilizer*;
Nch+Pbio: chemical nitrogen fertilizer+phosphorus solubilizing bacteria;
Nch+Mbio: chemical nitrogen fertilizer+mycorrhiza;
Nch+Pbio+Mbio: chemical nitrogen fertilizer+phosphorus solubilizing bacteria+mycorrhiza;
Nbio+Pch: nitrogen-fixing bacteria+chemical phosphorous fertilizer (triple superphosphate);
Nbio+Pbio: nitrogen-fixing bacteria+phosphorus solubilizing bacteria;
Nbio+Mbio: nitrogen-fixing bacteria+mycorrhiza;
Nbio+Pbio+Mbio: nitrogen-fixing bacteria+phosphorus solubilizing bacteria+mycorrhiza;
* Chemical fertilizers of triple superphosphate and urea were applied according to soil test to fulfill the requirements of the crop in each year.

Land preparation including plowing (cultivation), disking and roller was done before planting in first half of April in each experimental year. Each experimental plot consisted of six planting rows 25 cm apart with 5 m length. Clover was planted with the density of 20 kg seed per hectare. Between experimental plots four rows were left unplanted. Also 2 m space was left between replicates. Before planting, according to soil test (*Table 1*), 100 kg/ha of phosphorus and 150 kg/ha of urea were applied to the soil in chemical fertilizer treatment plots. All required chemical phosphorous fertilizer was applied as band fertilizer at land preparation time. Half of urea fertilizer was applied in planting time and the rest was used as top dressing at 4-leaf growth stage.

Forage quality properties (digestive dry matter, crude protein percentage, water soluble carbohydrate percent, acid detergent fiber percentage, neutral detergent fiber percentage, total ash percentage and crude fiber percentage) were measured by near-infrared spectroscopy system (Jafari *et al*., 2003).

Table 1 - Selected physical and chemical characteristics of soil (0-30 cm depth) in two years

Characteristic	Year	
	2011	2012
Organic carbon (%)	1.01	0.74
Olsen phosphorus (mg kg^{-1})	8.00	11.6
Available potassium (mg kg^{-1})	530	400
DTPA extractab Zn (mg kg^{-1})	0.38	0.84
DTPA extractab Cu (mg kg^{-1})	0.7	2.64
DTPA extractab Fe (mg kg^{-1})	2	10.22
DTPA extractab Mn (mg kg^{-1})	2.42	7.80

Homogeneity of variance and observation normalization test was prepared and then analysis of variance and mean comparison was done. Analysis of variance was done for each year separately as well as combined analysis for both years were performed. Statistical analysis was performed with SAS software. All mean comparisons were done at 1 and 5% probability levels, according to Duncan's domains test.

RESULTS AND DISCUSSION

Quantitative traits

The results of combined analysis of variance are shown in *Table 2* (for two years) showed that plant height, forage dry weight and leaf to stem ratios was significantly ($p<0.05$) affected by fertilizing systems. Integrated biological and chemical fertilizer (Nch+Pbio) application significantly increased the biomass production across both years of experimental period. The maximum forage dry weight (172.1 g/ m^2) was obtained by application of chemical nitrogen fertilizer+phosphorus solubilizing bacteria (Nch+Pbio). However, no significant difference was observed among the other fertilizing treatments (*Table 3*). This result could be explained by beneficial effect of integrated fertilizer application which led to increased nutrient supply; improved photosynthesis and ultimately provided by better plant growing conditions. The superiority of integrated chemical nitrogen fertilizer+phosphorus solubilizing bacteria (Nch+Pbio) application in higher biomass production could also be probably explained by better and earlier growth due to higher temperature and soil moisture content which provided the proper environment for microorganisms to grow and function more efficiently. This result is supported by Zaidi *et al.* (2004), who reported a significant increase in dry matter production in mung bean when the seeds were inoculated by an integrated bio-fertilizer consisting of free N fixing bacteria and mycorrhiza. Shabani *et al.* (2011) reported that application of integrated nutrient system application in annual medic had a synergetic effect on yield improvement.

Because of earlier and more growth due to favorable soil conditions, the biomass production was higher in 2012, compared to 2011 (*Table 1*). Desired climatic and soil conditions in second year caused clove to grow well and have a better performance than the first year (over 25% more dry matter production). William (2000) implied that better soil texture, soil pH, soil temperature and moisture affected the potential production of forage in clover. Longer growing period and more favorable environmental conditions could also explain higher biomass production in 2012, compared to 2011.

Table 2 - The Anova of berseem clover (*Trifolium alexandrinum*) agronomic characteristics as affected by different fertilizer types in during 2011 and 2012 growing seasons

SOV	Df	Plant height (m)	Forage dry weight (g/m^2)	Ratio of leaf to stem
REP	2	0.041ns	0.001ns	0.002ns
Treatment (T)	8	0.049 *	0.007**	0.009**
EROR1	16	0.015	0.0006	0.0009
YEAR(Y)	1	0.053 **	0.169*	0.002*
(Y) × (T)	8	0.001 ns	0.0008*	0.001*
EROR2	18	0.001	0.0003	0.0004
CV		2.08	0.78	1.12

Table 3 - Agronomic characteristics as affected by different fertilizer types in during 2011 and 2012 growing seasons

Treatment (T)	Plant height (m)			Forage dry weight (g/m^2)			Ratio of leaf to stem		
	year		mean	year		mean	year		mean
	2011	2012		2011	2012		2011	2012	
Co	21.4	27.3	24.4c	110.6	158.3	134.5d	43.3	44	43.6d
Nch+Pch	31.3	35.3	34.8abc	130.0	174.3	152.1c	52.6	53.6	53.0dc
Nch+Pbio	38.7	42.6	40.7a	142.3	184.0	163.6ab	53.0	53.6	53.3dc
Nch+Mbio	39.3	45	42.1a	133.3	191.0	172.1a	60.3	53.6	57.0abc
Nch+Pbio+Mbio	36.3	40.6	38.5ab	142.3	190.3	166.3ab	57.6	54.3	56.0ab
Nbio+Pch	25.4	31.3	28.4bc	163.3	185.0	160.6bc	62.6	54.3	60.1a
Nbio+Pbio	41.7	52.6	34.7a	141.6	181.0	161.6ab	59.3	61	51.5dc
Nbio+Mbio	30.0	33.8	31.9abc	132.3	184.6	158.5c	51.6	51.3	53.0bcd
Nbio+Mbio+Pbio	38.4	41.3	39.8b	151.3	182.6	167.0ab	55.0	55	55.0bcd
Mean	33.0b	38.0a		135.5b	181.2a		55.0a	53.4a	

The highest plants as well as the most dry matter weight were obtained in integrated bio+chemical fertilizers application, while the highest leaf/stem ratio was observed in the integrated biological fertilizer treatments. Combined analysis of variance (*Table 3*) showed that the most yield variation of 30% in two years of the study was observed, when chemical nitrogen fertilizer + mycorrhiza (Nch+Mbio) treatment was applied. Also the least variation of only 17% was related to nitrogen fixing bacteria + mycorrhiza + phosphorus solubilizing bacteria. It seems that Nbio+Mbio+Pbio fertilizer application caused more stable forage yield during the course of the experiment. These results are supported by Zandieh *et al.* (2012) in their research on the influence of different fertilizer types on two barley cultivars.

Qualitative traits

The effect of the year for some traits such as total ash and NDF was significant ($p<0.01$). Different fertilizer types had significant effects on NDF and crude protein percentage (*Table 4*).

Table 4 - The Anova of berseem clover (*Trifolium alexandrinum*) quality characteristics as affected by different fertilizer types in during 2011 and 2012 growing seasons

SOV	Df	DMD	CP	WSC	ADF	ASH	CF	NDF
REP	2	0.0005ns	0.0008ns	0.0006ns	0.0001ns	0.0008ns	0.0007ns	0.0002ns
Treatment (T)	8	0.0003ns	0.0004ns	0.0004ns	0.00007ns	0.0002ns	0.0009	0.0007*
EROR1	16	0.0004	0.0004	0.0003	0.0001	0.0004	0.0008	0.00005
YEAR(Y)	1	0.00004ns	0.0002ns	0.001ns	0.00001ns	0.007**	0.005**	0.001**
(Y) × (T)	8	0.00018ns	0.0003ns	0.0006ns	0.00007ns	0.0002	0.00004ns	0.00005ns
EROR2	18	0.0002	0.0004	0.0004	0.0001	0.0002	0.00008	0.00005
CV		0.86	1.36	1.84	0.76	3.61	2.62	0.41

The highest crude protein percent (25%) was obtained in Nbio+Pbio and the highest NDF value of 54.9% was observed in Nbio+Pch (*Table 5*). The significant increment in crude protein at integrated biological fertilizer application of nitrogen fixing bacteria+phosphorus solubilizing bacteria (Nbio+Pbio) could probably be explained by more nitrogen and phosphorous availability for roots in rhizosphere environment. One of the important roles of nitrogen is its contribution in protein production (Gholamhosiani *et al.,* 2012). The results of Ebrahim-Ghoch *et al*. (2012) experiment on the effect of fertilizer types on corn forage quality proved that application of a combination of chemical and manure fertilizer could decrease chemical fertilizer utilization without any substantial reduction in forage quality. Co-inoculation of alfalfa seed with different biological fertilizers in a phosphorus deficient soil resulted in increased forage dry matter, more N_2 fixation and better phosphorus content, compared to inoculation with single bacteria (Stancheva *et al.,* 2008).

Table 5 - Quality characteristics as affected by different fertilizer types in during 2011 and 2012 growing seasons

Treatment	DMD			CP			WSC			ADF			ASH			CF			NDF		
	year		mean	year		mean	year		mean	year		mean	year		mean	year		mean	year		mean
	2011	2012		2011	2012		2011	2012		2011	2012		2011	2012		2011	2012		2011	2012	
CO	49.1	49.7	49.4a	23.2	23.7	23.5b	10.5	1.5	10.5a	35.0	35.2	35.1a	8.2	8.8	8.5a	29.4	30.5	30.1a	56.4	58.0	57.2ab
Nch+Pch	49.8	47.3	48.5a	23.5	24.4	23.9ab	10.5	10.4	10.4a	35.2	35.0	35.6a	8.1	8.6	8.4a	30.2	31.3	30.7a	56.6	57.5	57.1ab
Nch+Pbio	50.8	51.0	50.9a	24.1	23.8	23.9ab	11.0	11.0	11.0a	36.5	34.3	34.8a	8.1	9.0	8.5a	30.3	32.0	31.2a	56.5	57.8	57.2ab
Nch+Mbio	50.4	49.8	50.0a	24.0	24.7	24.7ab	10.8	10.4	10.4a	35.3	52.6	35.6a	8.3	8.5	8.4a	29.4	31.8	31.3a	55.6	55.5	54.9bc
Nch+Pbio+ Mbio	49.9	49.2	49.4a	23.0	25.1	24.5ab	10.4	10.9	10.7a	35.3	35.6	35.4a	8.3	8.7	8.5a	29.8	30.9	30.4a	53.0	55.6	54.3bc
Nbio+Pch	50.1	51.3	50.7a	24.4	23.9	24.1ab	1.2	10.9	10.5a	35.4	35.5	35.4a	8.0	8.5	8.2a	30.7	32.8	31.7a	56.6	59.3	54.9a
Nbio+Pbio	50.4	50.0	50.2a	24.6	25.5	25.0a	10.3	11.5	10.9a	35.4	34.8	35.1a	8.2	8.3	8.3a	30.3	31.8	31.0a	54.9	56.1	52.2bc
Nbio+Mbio	50.4	51.0	50.7a	24.7	24.4	24.2ab	10.8	10.4	10.6a	35.3	35.1	35.2a	8.3	8.8	8.6a	29.4	31.7	30.2a	55.6	56.8	54.1c
Nbio+Mbio+ Pch	50.0	51.4	50.7a	25.0	24.0	24.5ab	10.8	10.9	10.8a	34.9	35.3	35.1a	8.2	8.6	8.4a	28.3	29.5	28.9a	54.0	54.2	
Mean	50.1a	49.9a		24.0a	24.3a		10.5a	10.7a		35.2a	35.1a		8.5a	8.6b		29.7a	31.3b		55.6a	56.7b	

The highest protein yield of clover was obtained by Nbio+Pbio application. This increment showed that providing with required P for clover is an essential mean to improve the produced protein per hectare. A positive effect of P fertilizer on protein yield of turnip (Türk *et al.*, 2009) and artichoke (Salamah, 1997) has been reported earlier. Since the correlation between forage yield and protein yield was strong, then by increasing the forage yield, the protein yield was also increased. It was concluded that the protein yield of clover was more related to forage yield than CP percentage.

At this experimental condition, integrated fertilizing systems (containing biologic nitrogen fertilizer) were more effective in annual medic forage improvement (quantity and quality), compared to other fertilizer types. Integrated biological nitrogen+chemical or biologic phosphorus fertilizers, caused improved forage quality as well as nutrient absorption efficiency. On the other hand, the integrated fertilizer systems such as both chemical and biological fertilizers and integrated biologic fertilizers improved forage yield. It should be considered that in these systems, increased yield quantity and quality was achieved while less inputs of chemical fertilizing systems were necessary. These results led to significant decrease in production cost and guaranteed more beneficial effects on social and environmental health.

CONCLUSIONS

It seems that improving the chemical, biological and especially physical properties, with the aid of a proper combination of the biological and chemical fertilizers causes the appropriate nutritional conditions and increases the nutrients uptake and leads to growth improvement. Considering the concentration and the magnitude of nutrients uptake in the annual medics, it was highlighted that the highest concentration and nutrient uptake was achieved by integrated fertilizing treatments. Application of integrated fertilizing treatments not only optimized and moderated sole chemical fertilizer application (consequently reducing the environmental pollutions), but it also enhanced the features of the forage quality in terms of higher macro and micro nutrients concentration in plant tissues. According to the results of this study it could be concluded that integrated fertilizing treatments may be accounted more efficient in dry farming than in irrigated agro-ecosystems.

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