International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 6, No. 3, p. 162-171, 2015

INNSPUB

RESEARCH PAPER OPEN ACCESS

Effect of phosphate fertilizer on quality and quantity of berseem clover forage under *Pseudomonas* strains inoculations

Mohammad Hossein Ansari[1*], Shahrokh Ghadimi[2]

[1]*Department of Agronomy and Plant Breeding, Rasht Branch, Islamic Azad University, Guilan, Iran*

[2]*Department of Agronomy and Plant Breeding, Guilan science and research Branch, Islamic Azad University, Guilan, Iran*

Key words: Berseem clover, phosphate fertilizers, Pseudomonas, forage yield.

http://dx.doi.org/10.12692/ijb/6.3.162-171 Article published on February 07, 2015

Abstract

Phosphorous is one of the essential elements which is highly needed by plants to grow and reproduce. Most of farming soils used for trifolium cultivation lack enough phosphorous. In such conditions, it is possible to recommend two main sources for the provision of phosphorous required for the clover plant: phosphorous biological fertilizers and phosphorous chemical fertilizers. In order to investigate the effects of phosphate solubilizing bacteria as biological phosphorous fertilizers and the different levels of phosphate fertilizers on the forage clover, an experiment using a factorial experiment conducted carried out based on completely randomized block design with three replications after rice harvest in Fuman region. The experimental factors included: 3 levels of phosphate (0, 50, 100 Kg/ha as superphosphate triple) and three strains of *Pseudomonas putida* (M21, M5, M168 strains and control). These factors were investigated on the berseem clover. The analysis of variance showed that the interaction of the bacteria in the phosphorous fertilizer on the fresh forage and the raw protein was significant. Furthermore, the mean comparison demonstrated that, at a level of 100 kg superphosphate fertilizer, the M5 strain produced the maximum yield in fresh forage and the raw protein.

* **Corresponding Author:** Mohammad Hossein Ansari ✉ ansary330@yahoo.com

Introduction

Berseem clover (*Trifolium alexandrinum* L.) belongs to leguminous family (Taylor,1985). This plant is among the most valuable forages available for stock feeding. The protein percentage of berseem clover has been reported as much as 20.96%. If the production of 30 tons of fresh forage was calculated per the basis hectare and only the resulting protein was estimated, as much as 650 kg pure protein would be obtained from the cultivation of one hectare of berseem clover in each period (Cassida *et al.*, 2000). The typical method of nutritional provision is using chemical fertilizers. Indiscriminate usage of chemical fertilizers not only does increase the production costs, but also leads to contamination of agricultural ecosystems. Therefore, this type of consumption is neither correspondent to sustainable development policies nor economical, since it results in energy loss in agricultural ecosystems. Utilization of Plant growth promoting rhizobacteria (PGPR) such as Azotobacter, Azospirillum, and Pseudomonas is an alternative strategy that has attracted a great deal of attention owing to increasing prices of chemical fertilizers (Molina *et al.*, 2000). PGPRs can enhance the plant growth and reduce pathogenic factors of the root by competitive elimination and through mechanisms such as production of plant growth-stimulating hormones which result in improved water absorption and other nutrients by the plant, improving germination and emergence of seedlings, production of some antibiotic compounds, removing pathogenic factors, and inducing defensive genes of the plant (Chen *et al.,* 2006). Therefore, identification of factors influencing growth of wheat with a more reasonable cost is necessary (Benizri *et al.,* 2001). A large number of studies have investigated the effect of PGPR bacteria on the growth and yield of farming plants. Most of studies have shown that growth-stimulating bacteria can enhance the growth of the plant in conditions devoid of microorganisms (He *et al.*, 2002). One of the indicators of the effect of plant growth-stimulating bacteria is the development of their roots, a phenomenon that has attracted the attention of many researchers. The root length development, the proliferation of peripheral and aberrant roots have been known to enhance the ability of the seedling to absorb water and nutrients and a improve posture in early stages of development (Molina *et al.*, 2000).

Regarding the importance of phosphorous in the plant and its role in many important compounds of plant cells such as nucleic acids, membrane phospholipids, and nucleotides involved in the plant energy metabolism, many studies have been conducted on the application of phosphorous and its effect on the developmental improvement of some farming plants. Phosphorous provision is possible through biological and chemical fertilizers (Kucey *et al.*, 1989). A large amount of phosphorus in chemical fertilizers becomes insoluble after mixing with soil and then it changes into insoluble compounds of calcium and magnesium in calcareous soils and into iron phosphate and aluminum in acidic soils (Khan *et al.*, 2007). Accordingly, the plant cannot use them anymore. Different forms of phosphorous in a simple solution include Hpo_4^{2-} , PO_4^{3-}, H_3PO_4, and $H_2PO_4^-$, whose frequency distribution depends on pH. If iron, aluminum calcium, and magnesium ions exist in the environment, the insoluble phosphates of iron and aluminum sediment in acidic conditions, while insoluble phosphates of calcium and magnesium (especially calcium phosphate) sediment in high pH. The pH of farming soils ranges from 5 to 8. In a pH lower than 5.5, iron and aluminum ions mix with phosphate and change into insoluble sediments. Likewise, in a pH higher than 7, insoluble compounds of calcium phosphate and magnesium are produced (Vassillev *et al.*, 2001).

Guilan province is a region where most of its farms cultivate berseem clover following rice harvest. The yield of clover is highly affected by environmental factors making farmers look for solutions for stability in production. Therefore, under the climatic conditions of Guilan province, utilization of these bacteria results in increased absorption of atmospheric nitrogen through the mentioned activities, whereby it could bring about enhanced yield of the plant forage. Accordingly, an experiment was done to evaluate the proliferation of phosphorous solubilizing bacteria and different levels of phosphorous fertilizer on qualitative and quantitative properties of berseem clover under second cultivation conditions.

Materials and methods

Field local

The experiment was conducted in a farm around Fuman city with an altitude of 34 m with a longitude of 20' 48°and a latitude of 19' 38°. The site of experiment, in terms of climatic conditions, is among mild-rainy regions. The average annual precipitation, based on the 30-year meteorological statistics, varies between 1400 to 1500 mm, where most of the precipitation is rain in fall and winter.

Soil analysis

The results obtained from soil decomposition in the experimental farm showed that at a depth of 0 to 30, the soil pH is 7.6, its electrical conductivity is about 0.83 $ds.m^{-1}$, its texture is of silt-loam type, and the percentage of silt, clay, and sand is 42, 36, and 26 percent, respectively. Full specifications of the soil are provided in Table 1.

Treatment and experimental design

The research was arranged in factorial experiment based on randomized complete block design with three replications.The experimental factors included: 3 levels of phosphate fertilizers (0, 50, 100 kg/ha as superphosphate triple) and *Pseudomonas putida* strains (strains of M21, M168, M5, and without inoculation). These factors were investigated on the berseem clover Hilali cultivar.

Agronomical activities during the season

Primary tillage, disk harrow and soil leveling were performed followed by the planting in autumn in plots containing 6 rows each 2 m long and 40 cm distance apart.

The seed of berseem clover used in this experiment was of crescent type provided by the forage plants sector of Alborz research center. In order to prepare treatments, seeds were smeared by an Arabic gum and the bacteria of interest were added to the seed mass. All of these bacteria were native to Iran and were separated and purified by the soil biology research sector of water and soil research institute. This center also provided their inoculum. For the inoculation of seeds, 7 g of inoculums (in each gram of which there are 10^7 active and alive bacteria), was used. The amount of consumption was based on the instruction provided by water and soil research center. Following seed inoculation and drying in shade, implantation was done according to provided recommendations. The plants were implanted by hand and by cultivation of 2 seeds per each mass. The farm was irrigated according to the humidity state of the soil and environmental conditions. Furthermore, to combat weeds, hand weeding was done in the course of developmental period. In this period, required note-taking and sampling were done to measure the traits of interest. To avoid the marginal effects, note-takings and samplings were done from 4 internal rows (2 of the 6 rows of lateral planting were considered as margins) and by omitting about 0.5 m from the beginning and end of each row.

To determine the yield of forage, cutting was done from two middle lines with a 0.5 omission from beginning and end of lines when the height of bushes reached as high as 50 cm, after which the harvested plants were weighted and fresh forage yield was determined. To evaluate the yield of dry forage, a 1-kg sample was chosen randomly. In the laboratory, up to a constant weight at 70±5 ^{0} C, the sample of interest was dried. Next, the yield of dry forage was investigated for each experimental unit and the humidity percentage of harvested plants was further determined. To determine the qualitative performance, a 200-g sample was chosen and powdered from dry forage of treatments, followed by raw fiber and nitrogen measurement in the laboratory. To calculate the protein content, after finding the nitrogen content in the leaves and stalk, it was multiplied by the constant number 6.25, giving the raw protein content.

Then, the raw protein content was multiplied by 0.93 and subtracted from 3.48. This value equaled the digestible protein. It was multiplied by dry forage yield and their weighted valued were obtained per hectare. In the stage of forage harvest time, from each plot a soil sample was selected from the areas around the root and sent to the laboratory. The soil pH was then determined for each experimental unit.

Statistical analysis

Data were subject to analysis by SAS and MSTATC procedures. Mean comparisons were done using LSD test at 5% probability level.

Results and discussion

Fresh forage yield

The results obtained from analysis of variance of the effects of different phosphorous fertilizer levels and phosphate solubilizing bacteria on the clover fresh forage yield showed that the impact of phosphorous fertilizer and bacteria on forage yield is significant at the probability level of 5% and 1%, respectively (Table 2).

The mean comparison of different levels of phosphorous fertilizer on the yield of clover fresh forage demonstrated that as the phosphorus fertilizer consumption increases, the fresh forage yield improves as the fertilizer consumption increased from zero to 50 kg, the forage yield elevated from 5302 kg/ha to 5445 kg/he. Similarly, from a 50-kg to 100-kg level, it reached 5619 kg/ha (Table 4).

Table 1. Physical and chemical properties of the experimental farm soil.

(%) clay	(%) silt	(%) sand	K	P	N (%)	OC (%)	pH	Ec (ds.m^{-1})	Depth (cm)
36	42	26	228	7.2	0.08	0.8	7.6	0.83	0-30
36	31	26	195	6.5	0.04	0.51	7.7	1.07	30-60

In investigating the effects of the application of different phosphorous fertilizer amounts on clover forage yield, Yadegari *et al* (2006) reported that the effect of phosphorus fertilizer on fresh forage yield is significant, where the highest yield of wert forage was obtained as much a 43.5 tons per hectare by consuming 145 kg/ha phosphorus. These results are congruent with the results of this experiment.

Table 2. Analysis of variances of measured traits in berseem clover.

S.O.V	df	Fresh forage yield	moisture content	Dry forage yield	raw protein	protein yield	raw fiber	soil pH	Digestible protein
repetition	2	92262ns	35.73ns	345221ns	2.841ns	13782ns	2.502ns	0.027ns	2.457ns
bacteria	3	800661**	13.04ns	601822*	39.73**	92371**	31.00**	0.0706ns	34.36**
phosphorus fertilizer	2	302348*	10.45ns	38401ns	0.211ns	269ns	6.29*	0.343*	0.182ns
bacteria*phosphorus fertilizer	6	47206ns	1.054ns	9723ns	2.514ns	25048*	1.972ns	0.0626ns	2.17ns
error	22	57235	20.57	144191	1.386	7039	1.83	0.063	1.198
cv		4.38	6.01	28.43	6.48	8.48	5.84	3.94	8.17

*NS, nonsignificant; **, significant at p≤1%; *, significant at p≤5%.*

The mean comparison of the effect of phosphate solubilizing bacteria on the trifolium showed that only the M21 strain had a significant dominance over the control treatment (no inoculation by bacteria) and all other strains were significantly different from the control treatment (Table 3). The impact of the application of pseudomonas bacteria on enhancing the fresh yield has also been reported in the results by Sundara *et al* (2000) and Javadi *et al* (2010).

Phosphorus often exists in soil in the form of low-soluble or insoluble mineral phosphates or as organic phosphorus which are not easily usable by plants. In other words, the low concentration of absorbable phosphates in farming soils has resulted in the fact that we have been made to add this element to soil in the form of phosphorus-containing fertilizers to fix the problem of phosphorus deficiency required for these plants since many years (Pant and Reddy, 2003). This element is found in soil in two forms of organic and inorganic. Its inorganic form is seen as different sorts of minerals including calcium, iron, aluminum, Fluor compounds, while its organic form includes phytin compounds in phospholipids and nucleic acids (Nautiyal, 2000).

Table 3. Comparison of means effects of *Pseudomonas putida* strains on some traits in berseem clover.

Pseudomonas putida strains	Fresh forage yield (Kg/ha)	Moisture content (%)	Dry forage yield (Kg/ha)	Raw protein (%)	protein yield (Kg/ha)	Raw fiber (%)	soil pH	Digestible protein (%)
Control	5314b	76.37a	1199b	16.20b	859c	25.04a	6.271a	11.58b
M21	5889a	71.06a	1722a	16.53b	975b	24.45a	6.300a	11.89b
M168	5399b	77.79a	1197b	20.42a	1103a	21.43b	6.428a	15.51a
M5	5218b	76.49a	1221b	19.43a	1016b	21.70b	6.446a	14.59a

In each column, values followed by the same letter are not significantly different as determined by LSD mean comparison test (p≤5%).

At the cellular level, what show the importance of phosphorus are energy vectors such as adenosine triphosphate which contains molecular phosphate. By releasing each phosphate, this compound releases a certain amount of energy and provides the energy for cellular activities. The natural growth and development of trifolium requires an adequate and complete source of phosphorus needed for the cell (Puchalka *et al.*, 2008).

Despite the abundance of total phosphorus amount in many soils of the country, the absorbable phosphorus is not sufficient for farming plants. Therefore, except for chemical fertilizers, it is also possible to use biological fertilizers which are in fact a set of microorganisms. The investigations have shown that several strains of soil-borne bacteria from bacilli and pseudomonases groups and also penicillium and aspergillus fungi, have demonstrated their ability in changing insoluble phosphate into soluble by producing organic acids (Timmis, 2002).

Table 4. Comparison of means effects of phosphorus fertilizer on some traits in berseem clover.

Phosphorus fertilizer	Fresh forage yield (Kg/ha)	Moisture content (%)	Dry forage yield (Kg/ha)	Raw protein (%)	protein yield (Kg/ha)	Raw fiber (%)	soil pH	Digestible protein (%)
P0	5302b	75.96a	1302a	18.22a	989a	22.32b	6.438a	13.46a
P50	5445ab	74.35a	1400a	18.22a	992a	23.48a	6.479a	13.47a
P100	5619a	75.96a	1302a	17.99a	983a	23.66a	6.167b	13.25a

In each column, values followed by the same letter are not significantly different as determined by LSD mean comparison test (p≤5%).

This group of bacteria is able to release the soil insoluble phosphorus as organic phosphorous acids and as light phosphorus and increase the mobility of this element in soil by changing the surrounding acidity and applying enzymatic processes (Puchalka *et al.*, 2008). These acids decrease the soil pH and eventually are effective in making phosphate soluble (Pandey *et al*, 2005). This increased phosphorus absorption results in improved growth and yield of the plant (Antoun *et al*, 1996).

Dry forage yield

The results obtained from analysis of variance for the effect of different levels of phosphorus fertilizer and phosphate solubilizing bacteria on the yield of clover dry forage showed that the effect of bacteria on the dry forage yield was significant at a 5% level, while the effect of phosphorus fertilizer was not significant (Table 2).

The mean comparison of the effect of phosphate solubilizing bacteria on the yield of clover dry forage demonstrated that only the M21 strain with an average of 1772 Kg/ha was significantly superior over the treatment strain, control (no inoculation by bacteria), with an average of 1199. Other strains were not different from the treatment control significantly. As the results imply, the lowest moisture content belongs to the M21 strain with an average of 71.06% (Table 3).

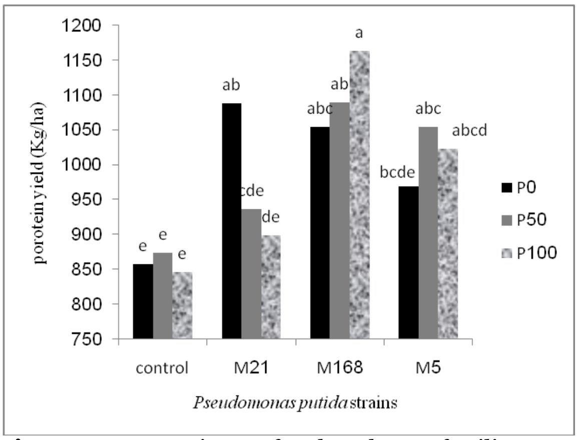

Fig. 1. Interaction of phosphate fertilizer × pseudomonas strains on protein yield.

Increased phosphorus absorption, improved plant nutrition, and probably the further development of root in soil, are the reasons of further growth of *Trifolium alexandrium* stalk. Unkovich and Pate (2000) have reported similar results for different farming plants. Aghaalikhani and Ehtshami (2008) also reported elevated biomass by *Pseudomonas fluorescent* and Mycorrhizal fungus compared to non-inoculated plants due to nutrient provision by this group of bacteria.

Raw protein

The results obtained from analysis of variance for the effect of different levels of phosphorus fertilizer and phosphate solubilizing bacteria on the raw protein of trifolium demonstrated that the effect of bacteria on raw protein is significant at a 1% level, while the effect of phosphorus fertilizer was not significant on the raw protein (Table 2).

The mean comparison of the effect of phosphate solubilizing bacteria on clover raw protein revealed that the M168 and M5 strains with an average of 20.42 and 19.43% has a significant dominance compared to control treatment (no inoculation by bacteria) with a 16.20% average, and the M21 strain (average of 16.53) was not significantly different from the control treatment (Table 3).

Generally, plants inoculated by Plant growth promoting rhizobacteria possess higher nitrogen content that non-inoculated plants (Lawlor *et al.*, 2001). It has been reported that an inoculation by *Azospirillum brasilense* elevates PO_4^{-3} and NH_4^+ absorption in the common bean plant (German *et al.*, 2000).

Nogales *et al* (*2008*) have stated that *pseudomonas putida*, the NtrC strain, can adjust the soil nitrogen for the root and provide the root with amino-acidic compounds. Betlach *et al* (1981) reported that the fluorescence pseudomonas bacterium can absorb nitrate and convert it to ammonium and transfer it actively.

Protein yield

The results obtained from analysis of variance for the effect of different levels of phosphorus fertilizer and phosphate solubilizing bacteria on the protein yield showed that the interactive effect of bacteria and phosphorus fertilizer on protein yield is significant at a 5% level (Table 2).

The mean comparison of the interactive effect of bacteria and phosphorus fertilizer on the protein yield demonstrated that at a phosphorus fertilizer level of zero, the M21 strain showed highest protein yield with a mean of 1088 Kg/ha. At 50 and 100 kg/ha phosphorus, the M168 strain showed a higher protein yield with an average of 1090 and 1164 kg/ha, respectively compared with other strains. It should be noted that in all of the three phosphorus fertilizer levels, inoculated treatments were significantly superior than the control treatment (no inoculation). The protein yield which is dependent on the percentage of protein and were forage yield, is an indicator of the amount of raw protein obtained from harvested forage (figure 1).

Shaharoona *et al* (2008) showed that the total nitrogen of the leaf is related to the intensity of CO_2 assimilation and the activity of metabolic enzymes of ribolose 1,5-biphosphate carboxylase/oxygenase (Rubisco) and Phosphoenolpyruvate (PEP) carboxylase. Lawlor *et al* (2001) reported that 40 to 50% of nitrogen dissolved in C_4 plant leaves can be found in the structure of these two enzymes plus pyruvate orthophosphate dikinase. Since phosphorus exists in many compounds of plant cells such as amino acids and nucleic acids along with phospholipids, therefore it seems that one of the symptoms of its deficiency is slow growth and diminished protein content. Indeed, as phosphorus diminishes in the plant due to photosynthesis disorders, the growth of vegetative organs will be difficult. Furthermore, extra phosphorus will also cause some problems such as disproportionate growth of stalk and leaves in the plant. Therefore, the existence of proper amount of available phosphorus results in enhanced development and elevated protein, thereby improved protein yield (Wang *et al.*, 2006). In addition to obtaining similar results, Yanni *et al* (1997) reported that Plant growth promoting rhizobacteria such as pseudomonas can increase the plant's nitrogen in an appropriate manner through elevating the number of knots of the berseem clover roots. This elevation leads to an increase in the forage protein and can provide the plant with required nitrogen in alternation with rice.

Digestible protein

The results obtained from analysis of variance for the effect of different levels of phosphorus fertilizer and phosphate solubilizing bacteria on the digestible protein revealed that the effect of bacteria on the dry forage yield was significant at a 1% level and the effect of phosphorus fertilizer on digestible protein was not significant (Table 2). The mean comparison of the effect of phosphate solubilizing bacteria on the digestible protein showed that the M168 and M5 strains were significantly dominant by an average of 15.51 and 14.59%, respectively compared to the control treatment (no inoculation by bacteria) with an 11.58% average. Similarly, the M21 strain with an average of 11.89 was not significantly different from the control treatment (Table 3).

Soil pH

The results obtained from analysis of variance for the effect of different levels of phosphorus fertilizer and phosphate solubilizing bacteria on the soil pH revealed that the impact of phosphorus fertilizer on the soil pH was significant at a 5% level, while the effect of bacterium fertilizer was not significant on the soil pH (Table 2).

The mean comparison of the effect of phosphorus on the soil pH exhibited that phosphorus fertilizer consumption as much as 100 Kg/ha increased the soil pH compared to the control. However, a 50 Kg/ha phosphorus was significantly different from that of the control (Table 4).

Different forms of phosphorus in soil are controlled by natural properties of the soil including its pH, soluble and exchangeable cations (Mg^{+2}, Ca^{+2}, Fe^{+2}), the type of particles and their levels. A large amount of phosphorus available in chemical fertilizers becomes insoluble when mixed with soil, as in Calcareous soils changes into insoluble calcium and magnesium compounds and into iron phosphate and aluminum in acidic soils, so becomes unavailable to the plant. Different forms of phosphorus in a simple solution include: Po_4^{3-}, Hpo_4^{2-}, $H_2po_4^{-2}$, and H_3PO_4, where their frequency distribution depends on pH. If iron, aluminum, calcium, and magnesium ions are present in the reaction environment, insoluble phosphates of iron and aluminum sediment in acidic conditions and insoluble calcium and magnesium phosphates (especially calcium phosphate) sediment at higher pH (Rashid *et al*, 2004). Increased content of phosphorus fertilizer in soil itself is one of the pH-elevating factors in normal soil conditions (Arshad *et* *al*., 2008). In an experiment, Raju *et al* (1990) reported that the effect of phosphate solubilizing bacteria and mycorhizal fungi strains is influenced by the impact of chemical fertilizers and it decreases as the consumption of phosphorus-containing fertilizers increases.

Raw fiber

The results obtained from analysis of variance for the effect of different levels of phosphorus fertilizer and phosphate solubilizing bacteria on the amount of raw fiber of clover manifested that the effects of phosphorus fertilizer and bacteria on the amount of raw fiber were significant at a probability level of 5% and 1%, respectively (Table 2).

The mean comparison of the effect of phosphorus on the amount of raw fiber of clover showed that both levels of phosphorus fertilizer revealed higher raw fiber content compared with the case with no phosphorus fertilizer. However, between a level of 50 and 100 kg of phosphorus fertilizer there was no significant difference (Table 4).

The mean comparison of the effect of phosphate solubilizing bacteria on the amount of raw fiber showed that control and M21 strains had no significant difference with averages of 25.4 and 24.45%, respectively. However, the M168 and M5 strains were dominant by an average of 21.43 and 21.70%, respectively (Table 3). Yanni *et al* (1997) reported that when the phosphorus content decreases, the cellular wall starts producing fiber (increased fiber) and protein, thereby reducing the digestibility of the plant. Thereafter, they considered the role of bacteria as important in timely provision of phosphorus and in protein elevation and in fiber reduction of berseem clover leaves. Based on the report by Karsli *et al* (1999), the amount of raw protein in berseem clover is 22% on average with 24% fiber content. According to the reports by Clark (2007), berseem clover has an 18 to 28% raw protein.

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