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Effects of Inoculation of Mycorrhiza and Rhizobium on N, P utilization and Vegetative Growth of Alfalfa (*Medicago sativa* L.)

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Mycorrhiza 및 Rhizobium 접종이 알팔파에 의한 질소와 인의 이용성 및 성장에 미치는 영향

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Abstract

To investigate the effect of Mycorrhiza (*Glomus intradics*) and Rhizobium inoculation on the N, P utilization and the growth of Alfalfa (*Medicago sativa* L.), four treatments (non-inoculation, control ; Mycorrhiza inoculation, M ; Rhizobium inoculation, R and Mycorrhiza and Rhizobium inoculation, M+R) were applied. The associated analyses were carried out on the early vegetative growth stage (DAS 56, 56 days after seeding) and on the early flowering stage (DAS 126). The decreased rate of total N and P content in soil, with advancing plant growth, was relatively higher in the mycorrhiza and/or rhizobium treatments than control. The content of available phosphorus in soil at DAS 126 increased by about 40 % in M and M+R treatment compared to control (141.0 mg P_2O_5/kg DM), while non-significant changes was observed in R treatment. Total N uptake and P uptake in the control at DAS 126 were 33.91 mg/plant and 2.42 mg/plant, respectively, about 21, 50 and 51 % of increases in total N uptake and 30, 11 and 47 % of increases in total P uptake were estimated in M, R and M+R treatment. Comparing to control, dry matter yield significantly increased by 8, 27 and 28 %, and crude protein yield also by 21, 42 and 39 %, respectively, in M, R and M+R treatment. The present data indicated that mycorrhiza or/and rhizobium inoculation improved N, P utilization, and consequently increased the yield of alfalfa.

(Key words : Alfalfa, Mycorrhiza, Rhizobium, N and P Utilization, Growth, Yield)

Ⅰ. INTRODUCTION

Available area for grassland development in Korea limits at the mountainous region. It has been documented that Korean mountainous soils have some problems such as low level of organic matter,

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deficiency of available P and N, low capacity of cation exchange. Therefore, the amendment of these soils are needed to make utilizable for grassland development. Special efforts of microbial inoculation have been made to improve nutrient utilization and stress tolerance. The reciprocal function of vesicular-arbuscular mycorrhiza (VAM) has been widely reported in various plant species (tomato, Kim et al., 1998; pearl millet, Raj et al., 1981; white clover, Nadian, 1997; alfalfa, Toro et al., 1998). VAM fungous colonization affected the translocation of nutrients into the root systems of associated plants (Chiariello et al., 1982; Francis et al., 1986; Bethlenfalvay et al., 1991). When inoculated PSB and *G. mosseae*, nitrogen and phosphorus contents in plant tissues were higher in dual inoculation treatment (Delorenzini et al., 1979). It has been reported that N and P uptake highly increased in *Medicago sativa* inoculated with arbuscular fungus (*G. mosseae*) (Azcon and Atrash, 1997).

Rhizobium of nodules leads an economic utilization of N fertilizer at a profit of N_2 fixation especially in infertile soils and enhances soil fertility. Thus rhizobium has been usually utilized for seed coating and soil inoculation. Recent physiological studies (Francis et al., 1986, Israel, 1993) suggested that P nutrition had a close influence on growth and N_2 fixation activity of nodules. Israel (1993) summarized the sequence of physiological responses to N and P utilization during recovery from P deficiency; 1) rapid uptake of phosphorus, 2) rapid increases in the P conentration of leaves and nodules, 3) enhanced growth and function of nodules 4) increased N concentrations in all plant organs and 5) enhanced plant growth.

From these results it is expected that the inoculation of mycorrhiza and rhizobium might improve N and P utilization in infertile soils as like mountainous soils in Korea. The objective of the study was to investigate the effect of mycorrhiza and/or rhizobium inoculation on the N, P utilization and the growth of forage legume.

Ⅱ. MATRIALS AND METHODS

1. Plant materials

Sterilized seeds of Alfalfa (*Medicago sativa* L.) were germinated on wet filter paper. 1 % (w/w) of rock phosphate and 3 % (w/w) of organic matter were added into the soils and then sterilized. Chemical characteristics of the soils utilized for this experiment are shown in Table 1. Germinated seedlings were transplanted to 3L pot (5 plants per pot). To inoculate mycorrhiza, about 1.7 % (w/w) of the soils abundantly infected with vesicular-arbuscular mycorrhiza (*Glomus intradics*) were mixed around the seedling transplanted. Rhizobium was separated from the nodules of wild alfalfa cultivar and incubated on the wright medium (Kim et al., 1992) at 30℃ for 3 days. 1 mL of incubated rhizobium (8.67×10^9 /ml) was injected into the soils around individual seedling.

Fourty pots were allocated to four treatments [non-inoculation (C), mycorrhiza inoculation (M), rhizobium inoculation (R), mycorrhiza and rhizobium dual inoculation (M+R)] and two harvests [early vegetative growth (DAS 56), early flowering growth (DAS 126)] with 5 replications. A completed randomized block design was applied. The data obtained from 4 microbial inoculations and 2 harvest

Table 1. Chemical characteristics of soil utilized for the experiment

pH (1:5 H_2O)	TN (%)	OM (%)	P_2O_5 (ppm)	Exchangeable cation (Cmol/kg)				CEC (Cmol/kg)
				K	Ca	Mg	Na	
5.4	0.11	1.93	21.8	0.6	3.5	1.9	0.2	9.6

times were analyzed by using paired t-tests. Means were expressed ± standard error.

2. Chemical analysis

Total N was determined by ammonia microdiffusion on Conway dish as described by Kim and Kim (1996). The collected NH_4Cl reacted with Nesslers ammonium color reagent. The optic density (OD) was read at 410 nm.

For total P determination, appropriate samples were placed on a electronic furnace at 550℃ for 3 hrs, and digested on the mixture of 5 mL of $HClO_4$ and 5 mL of H_2O_2 on a hot plate. The aliquot was filtered after solubilizing with 5 mL of 1 N HCl, and adjusted to final volume of 50 mL. The prepared sample solution reacted with P-color reagent and incubated at 30℃ for 30 min. The OD was read at 470 nm.

3. Acetylene reduction activity

Acetylene (C_2H_2)-dependent ethylene (C_2H_4) production by nodulated roots was determined to measure the activity of N_2 fixation (Hardy at al., 1968). After removal of shoots, nodulated roots were gently collected free of soil. Roots were placed in 100 mL of sealed flasks. 10 mL of air were withdrawn and replaced with an equal volume of C_2H_2, and incubated at 30℃ for 60 min. The ethylene produced was measured using gas chromatography (Varian STAR 3400 CX, USA) equipped with a PLOT fused silica capillary column (CHROMPACK). The injector temperature was at 100℃ and the carrier gas was N_2 at a flow rate of 200 ml/min at 30 psi.

4. Determination of mycorrizal colonization

The mycorrhizal colonization of roots was assayed by modified method of Brundrett et al (1984). The roots of alfalfa were gently collected free of soil and rinsed with distilled water. Roots were immerged in FAA solution (the mixture of formalin/acetic acid/ethanol, 13/5/200, v/v/v) for 24 hrs (Sass, 1958). After washing and rinsing with distilled water several times, the mycorrhizal roots were placed into 20 ml vials and filled with 10 % KOH, and then incubated at 90℃ for 60 min. Mycorrhizal roots were washed with water and dyed with stain solution (400ml 85% lactic acid, 1.2g Chlorazole black E, 400ml glycerine, 400ml distilled water) at 50℃ overnight. The root samples were rinsed with water until clear and put into destain solution (50% glycerol) overnight. Mycorrhizal colonization was visualized using on a microscope (Olympus model BX 50) with 200 × magnification. Hypae and vesicular in plants were counted. Total mycorrhizal colonization rate (%) was expressed as the percentage of the number of colonized root against total number of examined roots as described by Read et al. (1976).

Ⅲ. RESULTS AND DISCUSSION

1. Mycorrhizal colonization and soil fertility

All micrographs of vesicular and hyphae colonized with the roots of non-inoculated (control) and inoculated alfalfa plants are presented in Fig. 1. Mycorrhizal colonization rate, vesicular and hyphae percentage at DAS 126 are shown in Table 2. The percentage of hyphae colonization was much higher than that of vesicular (36.7 and 34.1 % of hyphae; 8.3 and 3.3 % of vesicular in M and M+R plants). These results suggested that in alfalfa roots mycorrhiza were colonized mainly as the forms of hyphae. Mycorrhizal colonization rate of M and M+R treatment was about 45.7 and 37.4 %, respectively. These indicated that vesicular and hyphae were usefully colonized in rhizosphere of alfalfa by mycorrhizal inoculation at the infertile soils. Mycorrhizal colonization was closely associated with root mass of host plant in agreement with the result of Wallander and Nylund (1992). High mycorrhizal colonization was widely reported in

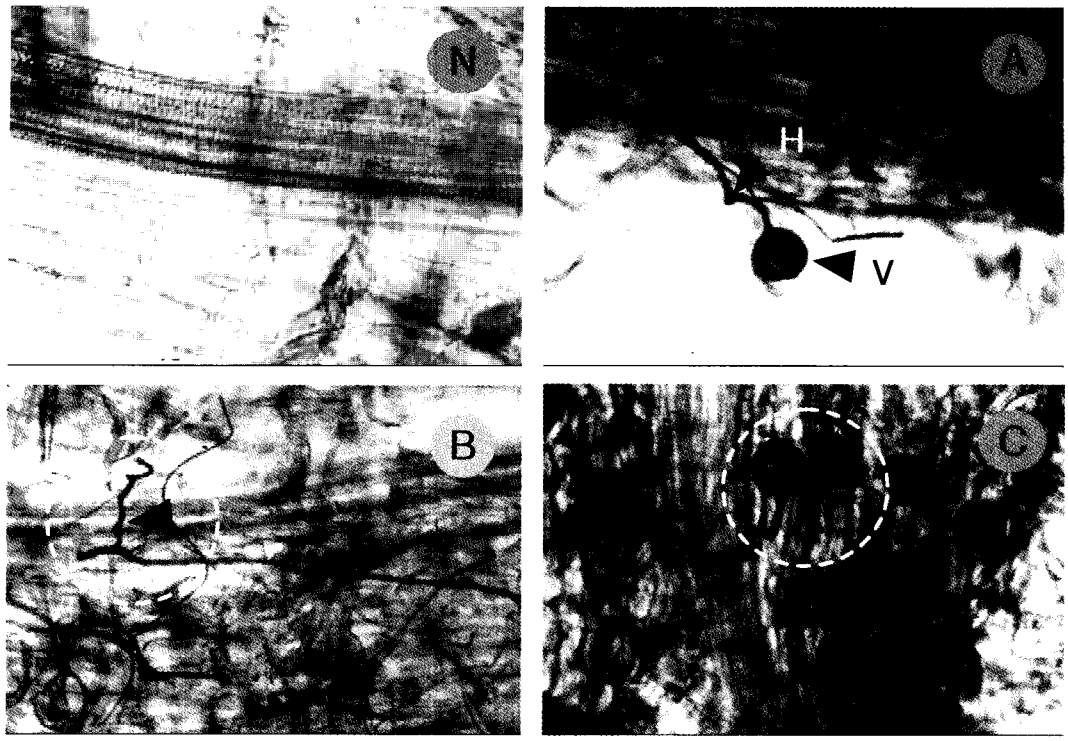

Fig. 1. All micrographs are oriented with the root of alfalfa (*Medicago sativa* L.).
N, Control ; A, vesicular(V) and hyphae(H) ; B, hyphae; C, vesicular. Magnification ca. 200 x.

Table 2. Mycorrhizal colonization on the alfalfa roots at the early flowering stage

Microbial inoculation	Vesicular	Hyphae	Colonization rate
	..... (%)		
Non-inoculation (Control)	0.0	5.4	5.4
Mycorrhiza (M)	8.3	36.7	45.7
Rhizobium (R)	0.0	2.6	2.6
Mycorrhiza + Rhizobium (M+R)	3.3	34.1	37.4

forage grasses and legumes such as *Zea mays* L. (Bethlenfalvay et al., 1991), *Sorghum vulgare* (Ibijbijen et al., 1996), *Lolium perenne* L. (Cliquet, et al., 1997), *Trifolium subterraneum* L. (Nadian et al., 1997) and *Medicago sativa* L. (Toro et al., 1998).

Alfalfa nodulation and relative activity of N_2 fixation as affected by rhizobium inoculation are shown in Table 3. Fresh weight of nodules in control and M treatment (non-rhizobium inoculation) was 202 and 213 mg/pot. Nodulation highly increased to about 4-fold in the R and R+M plants. Relative activity of N_2 fixation well reflected the nodule mass, showing 4.5-fold and 4.7-fold higher activity in the R and M+R plants compared to the control plants (non-inoculated). Azcon and El-Atrosh (1997) reported that N_2 fixation was closely associated with nodule formation and function of host plant.

Total contents of nitrogen and phosphorus in soil

Table 3. Fresh weight of nodules and relative activity of acetylene reduction as affected by microbial inoculation at the early flowering stage. Relative acetylene reduction activity was expressed as percentage against the control plants. The absolute activity of acetylene reduction in the control plants was 15.2 μmol.C_2H_2/g FW/hour. Each value is mean±s.e. for n=5

Microbial inoculation	Nodule F.W (mg/pot)	Relative activity
Non-inoculation (Control)	202±15	100
Mycorrhiza (M)	213± 9	102
Rhizobium (R)	812±36	449
Mycorrhiza + Rhizobium (M+R)	936±40	468

at 56 [early vegetative stage (DAS 56)] and 126 days after seeding [early flowering stage (DAS 126)] are shown in Table 4. At the start of experiment, total N in soil was 3.30±0.21 g/pot. At DAS 56 total N contents of soil were significantly decreased in all treatment compared to initial content. The decreased rate until this growth stage was relatively higher in the M (26 %) and M+R (29 %) treatments than control (19 %) and R (21 %) treatment. Total N contents in soil at DAS 126 were 2.49, 2.29, 2.53 and 2.46 g/pot in the control, M, R and M+R treatment, respectively. The decreased rate in total N, until this growth stage, was the highest in the M (31 %) treatment. These indicated that nitrogen utilization was improved by mycorrhiza inoculation rather than rhizobium inoculation.

At the start of experiment, total P contents in soil was 2.07±0.12 g/pot. At DAS 56, total P content in soil was significantly decreased in all treatments. The decreased rate of total P during this period was relatively higher in the M, R and M+R treatment. The decreased rate until the early flowering stage (DAS 126) was also significantly higher in the M, R and M+R treatment than control. The data obtained in this experiment suggested that mycorrhizal inoculation enhanced of phosphorus utilization. The content of available phosphorus in soil (Table 5) well consist with this suggestion. The content of available phosphorus in control soil was the lowest throughout entire experimental period. Comparing to the control soils (129.2 mg P_2O_5/kg DM) at DAS 56, available phosphorus in soils was not significantly changed in the R treatment, but M and M+R inoculation increased about 72 % and 86 %, respectively. At DAS 126, the available P content was also significantly higher in the M and M+R treatment (about 66 % and 59 % of increase) than control (141.0 mg P_2O_5/kg DM). These results suggested that mycorrhiza infection leaded to increase P availability from P substances having low solubility and mobility as like rock phosphate, which was applied in this experiment. Azcon et al. (1976) reported that dual inoculation of mycorrhiza and phosphate solubilizing bacteria increased P utilization from rock phosphate.

2. N, P uptake

Total uptake of nitrogen and phosphorus as affected by microbial inoculation during experimental period are shown in Fig. 2. At DAS 56, total N uptake in control (non-inoculation) was the lowest at 4.93 mg/plant. It significantly increased by 57 % and 58 % in the M and R amended soils, respectively. An additional increase of N uptake was observed in M+R amended soils. At DAS 126, total N uptake in control soils was also the lowest at 33.91 mg/plant. It significantly increased by 21 %, 50 % and 51 % in the M, R and M+R amended soils, respectively. However non-additional increase

Table 4. Total content of nitrogen and phosphorus in soil at the early vegetative stage (56 days after seeding ; DAS 56) and the early flowering stage (126 days after seeding ; DAS 126). Each value is mean±s.e. for n=3

Microbiol inoculation	Day after seeding			
	DAS 56	DAS 126	DAS 56	DAS 126
	····· Nitrogen (g/pot) ·····		····· Phosphorus (g/pot) ·····	
Non-inoculation (Control)	2.67±0.13	2.49±0.10	1.78±0.11	1.61±0.12
Mycorrhiza (M)	2.43±0.12	2.29±0.15	1.63±0.06	1.43±0.11
Rhizobium (R)	2.61±0.20	2.53±0.17	1.69±0.07	1.48±0.09
Mycorrhiza + Rhizobium (M+R)	2.35±0.11	2.46±0.21	1.60±0.10	1.46±0.05

cf : total contents of N and P in soil at the start of experimental were 3.3 and 2.07 g/pot, respectively.

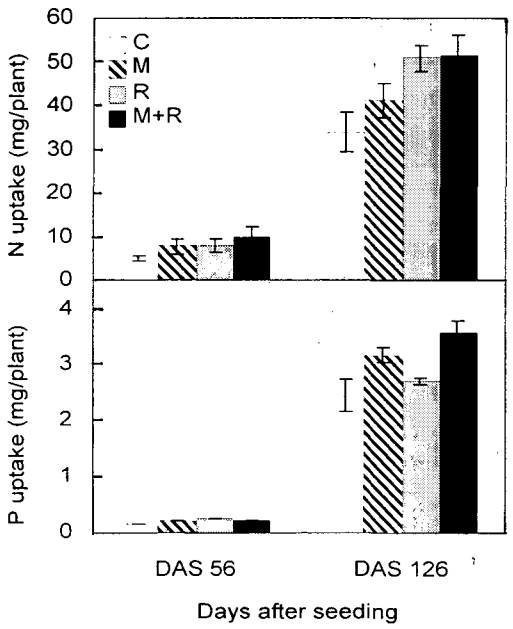

Fig. 2. The effects of microbial inoculation on the total uptake of nitrogen and phosphorus during the early vegetative stage (DAS 56) and the early flowering stage (DAS 126). Each value is mean±s.e. for n=5.

by dual (M+R) inoculation occurred when compared to R single inoculation. These showed that mycorrhizal inoculation significantly enhanced nitrogen uptake, and well agreed with the data of soil nitrogen analysis, which showed the improvement of nitrogen utilization in the plants inoculated with mycorrhiza (Table 4). Several reports of studies using isotopically labelled N indicated that mycorrhizal infection could increase plant N uptake from soils and soil N availavility (Barea et al., 1989; Ibijbijen et al., 1996). Therefore it could be assumed that mycorrhizal hyphae can translocate nitrogenous compounds from soil to plant tissues (Ames et al., 1983; Tobar et al., 1994) or even transfer N from one plant to another plant (Haystead et al., 1988).

The absolute value for P uptake was much lower than the N uptake throughout experimental period. At DAS 56, total P uptake was the lowest at 0.15 mg/plant in the control plants. It slightly increased to 0.22, 0.23 and 0.22 mg/plant in the M, R and M+R treatment. Total P uptake at DAS 126 was also the lowest (2.42 mg/plant) in the control plants. It was significantly increased by 30 % and 47 % in the M and M+R treatment, while non-significant increase occurred in the R treatment (2.69 mg/plant). The similar results on the increase of N and P uptake by VAM or AM fungus inoculation were reported in various species; pine (Reid et al., 1983 ; Rousseau, 1990), spruce (Eltrop and Marschner, 1996), white clover (Nadian et al., 1997) and alfalfa (Toro et al., 1998). In addition, the direct evidences of the active role of VAM fungus in the translocation of nitrogen (Van Kessel et al., 1985) and phosphorus (Ritz and Newman, 1984) from rhizosphere were obtained by

Table 5. Concentration of available phosphorus in soil (mg P_2O_5/kg DM) as affected by microbial treatment at the early vegetative stage (56 days after seeding ; DAS 56) and the early flowering stage (126 days after seeding ; DAS 126). Each value is mean±s.e. for n=3

Microbial inoculation	DAS 56	DAS 126
Non-inoculation (Control)	129.2± 9.2	141.0± 8.5
Mycorrhiza (M)	221.9±14.6	234.1±14.3
Rhizobium (R)	146.7±11.2	153.2±14.8
Mycorrhiza + Rhizobium (M+R)	239.7±16.6	224.5±12.4

isotopic technique.

3. Growth characteristics and productivity

Growth characteristics of alfalfa plants as affected by microbial inoculation are shown in Table 6. Plant height, root diameter and number of branch by microbial inoculation were not significant different. SPAD value (chlorophyll content measurement) in the control leaves was the lowest at 23.0. It significantly increased to 30.5, 29.5 and 32.8 in the M, R and M+R inoculated plant, respectively.

Dry matter and crude protein yield as affected by microbial inoculation are shown in Fig. 3. Dry matter in the control was the lowest at 366 kg/10a. It significantly increased by about 9 %, 27 % and 28 % in the M, R and M+R treatment, respectively, compared to control plants.

Table 6. Growth characteristics as affect by microbial inoculation at the early flowering stage. Each value is mean±s.e. for n=15

Microbial inoculation	Plant height(cm)	Root diameter (mm)	No. of Branch	SPAD value
Non-inoculation (Control)	36.7±2.6	5.9±0.3	6.0±0.7	23.0±3.8
Mycorrhiza (M)	36.8±3.4	5.9±0.1	6.8±0.6	30.5±5.0
Rhizobium (R)	37.3±3.5	6.1±0.3	6.7±0.8	29.5±4.0
Mycorrhiza + Rhizobium (M+R)	37.8±4.3	6.1±0.3	7.2±1.2	32.8±3.2

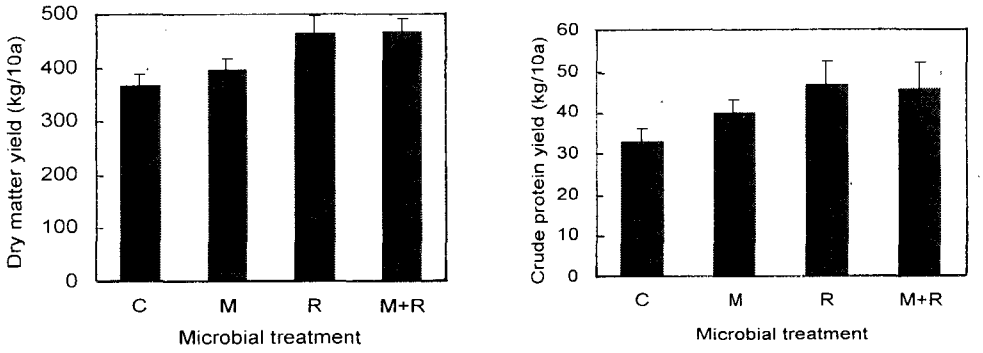

Fig. 3. Dry matter yield and crude protein as affected by microbial inoculation at the early flowering satge. Each value is mean±s.e. for n=5.

Crude protein yield in the control was also the lowest at 33 kg/10a. About 21 %, 42 % and 39 % of increase in crude protein yield was observed in the M, R and M+R treatment.

The overall data obtained in this experiment showed that mycorrhiza or/and rhizobium inoculation improved N, P utilization, and consequently increased the yield of alfalfa. Thus the inoculation of mycorrhiza and rhizobium is assumed to be a low-input technical practice, which might lead to the development of sustainable soil-plant systems. It has a particular significance in the amendment of infertile soils, as like mountainous soils in Korea.

Ⅳ. ACKNOWLEDGMENTS

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Ⅴ. 적 요

Mycorrhiza 및 Rhizobium 접종이 알팔파에 의한 질소와 인의 이용성 및 성장에 미치는 영향을 조사하고자, 4가지 처리구 [미생물 비접종구 (대조구), Mycorrhiza 접종구 (M), Rhizobium 접종구 (R) 그리고 Mycorrhiza+Rhizobium 동시접종구 (M+R)]에서 초기영양생장기(DAS 56)과 개화초기(DAS 126)에 화학적 성분, 성장 및 생산성을 각각 비교하였다. 토양내 총질소와 인의 함량은 식물이 성장함에 따라 유의적으로 감소하였다. 그 감소 비율은 Mycorrhiza와 Rhizobium 접종구에서 대조구보다 상대적으로 높았다. 개화초기(DAS 126)에 토양내 유효인산농도는 대조구 (141.0 mg P_2O_5/kg DM)와 비교할 때 Mycorrhiza 접종구와 Mycorrhiza+Rhizobium 동시접종구에서 각각 약 40 % 증가하였으나, Rhizobium 접종구에서는 유의적인 차이가 없었다. 대조구와 비교할 때 질소 총 흡수량은 Mycorrhiza 접종구, Rhizobium 접종구 및 Mycorrhiza +Rhizobium 동시접종구에서 21, 50 및 51 % 증가하였고, 인의 총 흡수량은 30, 11 및 47 % 각각 증가하였다. 건물 수량과 조단백질 수량은 대조구와 비교할 때 Mycorrhiza 접종구, Rhizobium 접종구 그리고, Mycorrhiza+Rhizobium 동시접종구에서 각각 8, 27 및 28 %와 21, 42 및 39 %가 증가하였다. 이상의 결과들은 Mycorrhiza와 Rhizobium 접종에 의해 질소와 인의 이용성을 향상시키며, 알팔파 성장 및 수량의 증가를 가져옴을 잘 보여 주었다.

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