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Effect of Dual Inoculation with *Glomus mosseae* and *Bradyrhizobium* sp. R938 on the Nitrogenase Activity of *Arachis hypogeae* L.

Seo, Hyun-Chang[1], Chang-Jin Kim, Shin-Duk Kim and Ick-Dong Yoo*

Genetic Engineering Research Institute, Korea Institute of Science and Technology, P.O. Box 17, Daeduk Science Town, Daejeon 305-606, Korea

[1]Department of Food and Nutrition, Shingu Junior College, Sungnam 462-743, Kyonggi-do, Korea

*Glomus mosseae*와 *Bradyrhizobium* sp. R938의 동시 접종에 의한 땅콩의 질소고정력 증진효과

서현창[1] · 김창진 · 김신덕 · 유익동*

한국과학기술연구원 유전공학연구소 미생물생태연구실, [1]신구전문대학 식품영양과

Abstract — The effect of dual inoculation with vesicular-arbuscular (VA) mycorrhizal fungi, *Glomus mosseae*, and *Bradyrhizobium* sp. R938 on the nitrogenase activity of peanut plant (*Arachis hypogeae* L. cv. Youngho) was studied in pot experiment. As a result of the dual inoculation with *Glomus mosseae* and *Bradyrhizobium* on peanut plant, nitrogenase activity was increased by 1.5 times compared with single inoculation with *Bradyrhizobium* sp. R938. After 69 days growth of peanut plant, the size of nodules was also increased due to the mycorrhizal infection. The mean fresh weight per nodule was 6.7 mg in treatment with the dual inoculation, which is the 1.5 times increase compared with single inoculation with *Bradyrhizobium* sp. R938. These results suggested that there might be a hormonal effect of VA mycorrhizal fungi in addition to the effect of increase in nitrogen fixing ability through the enhancement of nutrient absorption.

Microorganisms such as *Rhizobium* sp., *Bradyrhizobium* sp., and vesicular-arbuscular (VA) mycorrhizal fungi are able to form symbiotic associations with their host plants. These symbiotic associations have been described to improve plant growth (1, 2).

In case of legume, fixation of atmospheric nitrogen by roots nodulated with *Bradyrhizobium* sp. can greatly enhance plant growth in soil low in available nitrogen. More than 10% increase in production was obtained in our previous field experiments on soybean plant using the selected *Bradyrihizobium japonicum* strain (1). In our pot experiment to determine the effect of selected *Bradyrhizobium* strains on growth of peanut plant, remarkable increase in plant growth was also revealed (3).

Inoculation of VA mycorrhizal fungi has also been suggested as a possible method for increasing nutrient absorption in soils of low fertility (2, 4). There have been many reports on the improved plant growth, both in the laboratory and the field experiment, due to the presence of these mycorrhizal associations (5, 6). Many investigators attribute this enhancement to increased uptake of phosphorous and other micronutrients (4, 7-9). Others indicate the possible enhancement through exchange of plant hormones (10, 11). In any case, these demonstrations have stimulated a great deal of recent scientific study and commercial interest in agriculture (12) in using VA mycorrhizal fungi as "biofertilizers".

VA mycorrhizal fungi and N_2 fixing bacteria form a three-membered association with legumes (13).

Key words: *Glomus mosseae, Bradyrhizobium* sp., nitrogen fixation, nodulation, *Arachis hypogeae*, peanut

*Corresponding author

Much attention has been given to this three-membered association (13-18). It was reported that all three members of this association were mutually influenced (14). Although direct interrelation between *Rhizobium* and VA mycorrhizal fungi is not known exactly, it is apparent that plant is a mediator of that interrelation (19). Therefore it can be said that VA mycorrhizal colonization can affect the nodulation and nitrogen fixation of nitrogen fixing bacteria, and conversely plant-rhizobium association can influence the formation of vesicle of VA mycorrhizal fungi in plant root.

The purpose of this research was to determine the effect of dual inoculation with *Bradyrhizobium* sp. and *Glomus mosseae* on the nitrogen fixation parameters of peanut plant. And the mutual relationships between the VA mycorrhizal fungi and rhizobial activity during their symbiosis with peanut plant were also studied.

Materials and Methods

Experimental Design and Biological Materials

Peanut plants (*Arachis hypogeae* L. cv. Youngho) were grown with four basic treatments and 5 replicates: 1) non-inoculated control plants, 2) plants inoculated with VA mycorrhizal fungi, *Glomus mosseae*, 3) plants inoculated with N_2 fixing bateria, *Bradyrhizobium* sp. R938, 4) plants inoculated with both the endophytes simultaneously.

Glomus mosseae was supplied by Dr. Schenck of INVAM (International Culture Collection of VA Mycorrhizal Fungi), which was isolated from Anza-Borrego Desert State Park, San Diego County, California (20) and was used for production of mycorrhizal inoculum. For the production of VAM inoculum, Sudan grass (*Sorghum bicolor* L.) was used as a host plant. *Bradyrhizobium* strains were isolated from root nodules of peanut plants collected from Kochang-gun area, Chollabuk-do, Korea. The tested peanut cultivar was Youngho, which was obtained from Crops Experiment Station, RDA, Suwon.

Growth Conditions for Plants

Peanut (*Arachis hypogeae* L. cv. Youngho) seeds were surface sterilized, germinated, and selected for uniformity. Seedlings were transplanted into stylofoam cups containing 300g of an autoclaved sand and vermiculite mixture (1:1, v/v). At the time of transplanting, inoculum of VA mycorrhizal fungi was placed 2 cm below seedlings and 3 m*l* of a suspension containing 10^9 cells/m*l* of *Bradyrhizobium* sp. R938 were inoculated. Twenty gram of a thoroughly homogenized root-soil-fungus mixture was applied as an inoculum of VA mycorrhizal fungi. It consisted of spores, hyphae, and infected root segments and contained approximately 2 spores per g inoculum. Plants were fertilized with a modified Hoagland solution (plant nutrient solution) without nitrogen twice a week. Phosphorous concentration in the nutrient solution was adjusted to the level of 0.02g KH_2PO_4/l. Plants were grown on the greenhouse condition.

Measurements and Assays

Plants were harvested at 69 days after transplanting. At harvest, nodule was counted and the fresh weight was measured. Dry weight of stem and leaf was measured after drying at 80℃ in drying oven for two days. Mycorrhizal roots were stained with acid fuchsin-lactic acid staining solution (21). After staining, mycorrhizal infection in cortical tissue was determined microscopically. Nitrogenase activity of root nodule was measured as C_2H_2 reduction activity by gas chromatography (Varian 3700 series) using porapak R150 column (22). Purification for assaying plant growth regulators was performed as shown by Barea *et al.* (23).

The results were evaluated statistically by Anova and Duncan's multiple range test for multiple comparison.

Results and Discussion

Isolation and Selection of *Bradyrhizobium* strains

Bradyrhizobium strains were isolated from the root nodules of peanut plant collected from Kochang-gun area in Chollabuk-do, one of the largest production area of peanut in Korea. From 6 myeon in Kochang-gun, 49 strains of *Bradyrhizobium* sp.

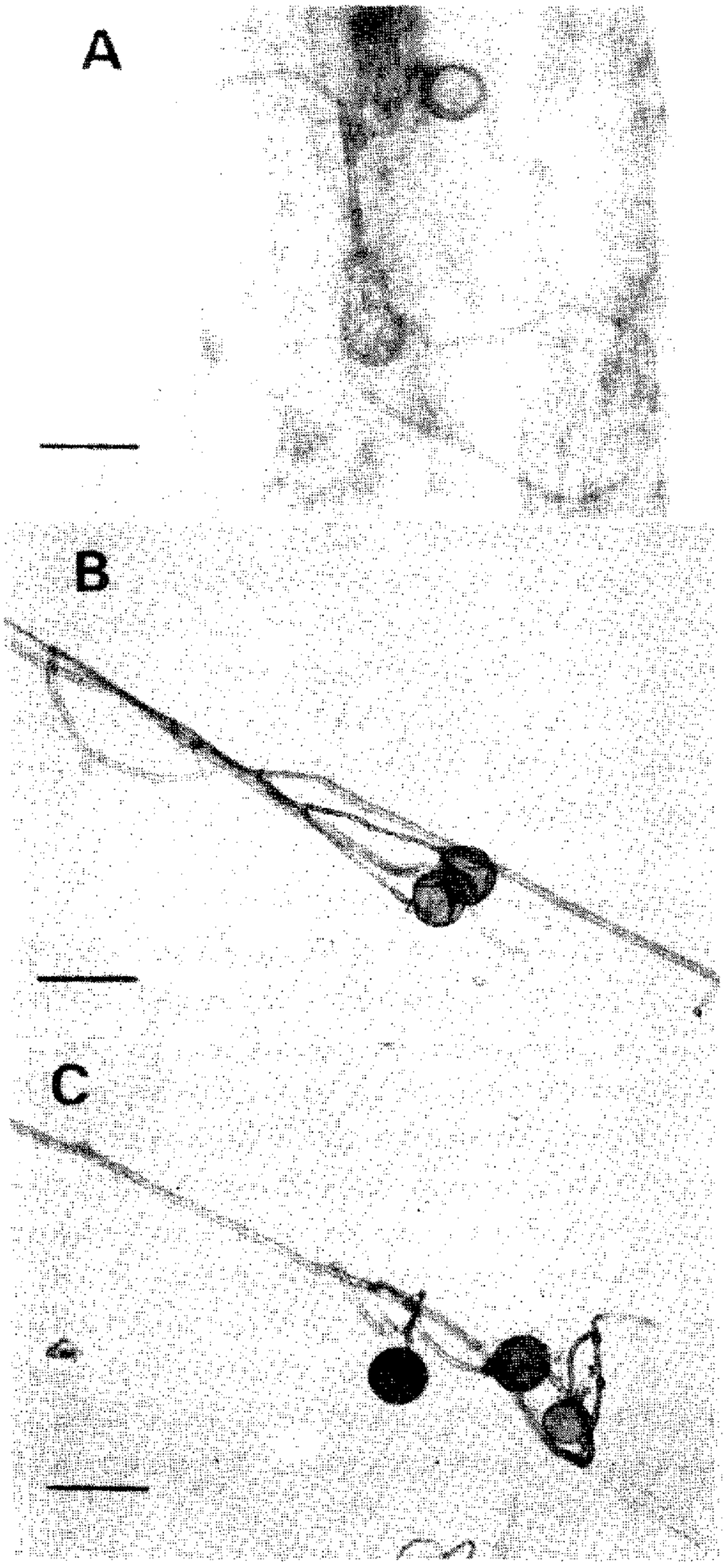

Fig. 1. Morphological characteristics of *Glomus mosseae* infected in peanut roots.
(A) Hyphae and chlamydospores of *Glomus mosseae*; (B) and (C) Extracellular hyphae and extracellular vesicles of *Glomus mosseae* (Bars represent 50 μm scale.)

were isolated and tested for the parameters, such as growth response, nodulation and nitrogenase activities. As a result of that plant test, *Bradyrhizobium* sp. R938 was finally selected as an inoculation strain.

Morphological Characteristics of Infection

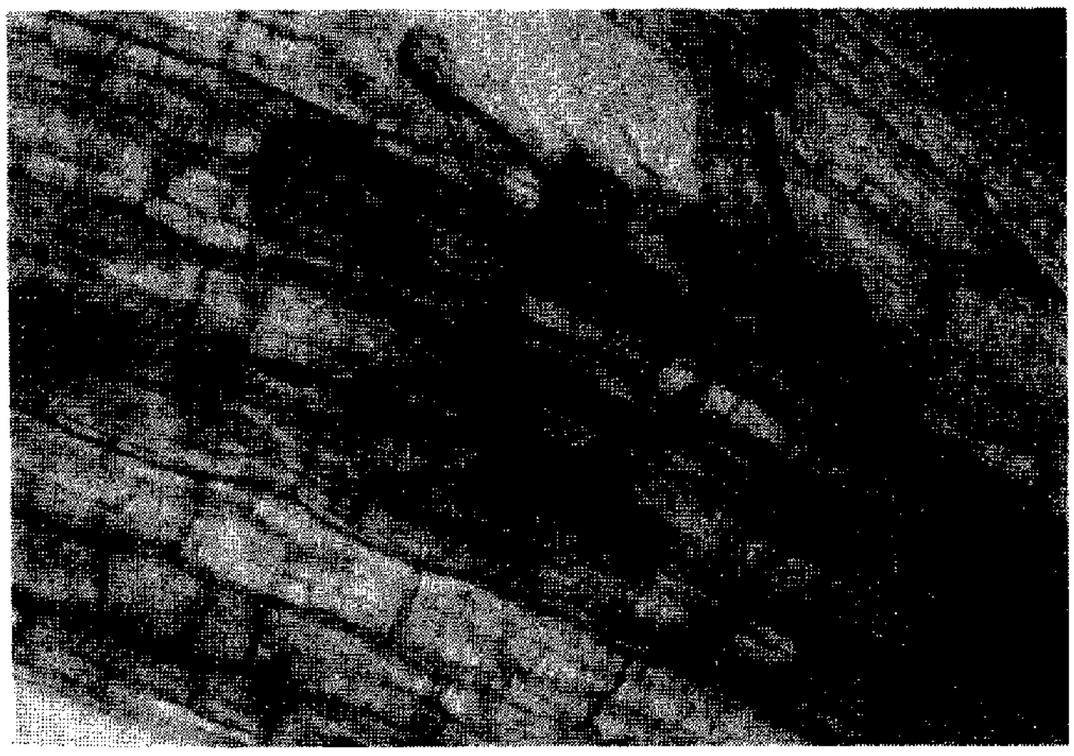

Fig. 2. Root segment of peanut plant showing well developed intercellular hyphae formed by *Glomus mosseae*.

Vesicular-arbuscular mycorrhizae is characterized by fungal vesicles and arbuscules. Because of the characteristic fungal vesicles and arbuscules, it is called as vesicular-arbuscular (VA) mycorrhizae.

As shown in Fig. 1-(B, C), extracellular vesicles were found after 69 days growth. Chlamydospores of *Glomus mosseae* were also found in Fig. -1(A). But at that period, arbuscules were not found. It was regarded as a result of autolysis of the arbuscules after the long period of growth time. VA mycorrhizae was also characterized by inter- and extracellular growth inside the roots of colonized plant and by extensive network of fungal hyphae in the soil (Fig. 1, 2).

The mixed inoculum applied was thought to be enough to infect the root. This extensive network was believed to be responsible for increasing nutrient uptake in plant inoculated with *Glomus* and *Bradyrhizobium*. It has been reported to comprise as much as 80 cm of fungal hyphae per centimeter of root tissue colonized (24). Therefore, the hyphae of VA mycorrhizal fungi can serve as extensions of the root systems and are thought to be more effective organs of absorption than the roots themselves.

Effect of Dual Inoculation on Nitrogen Fixation Parameters

At 69 days after seedling emergence, peanut plants were harvested. In our experimental condition, the harvest time was between the period of flowering and fruiting of peanut. It was characterized

Table 1. Number and fresh weight of root nodule of peanut plant inoculated with *Glomus mosseae* and *Bradyrhizobium* sp. R938

Parameter	Treatment	
	Bradyrhizobium	*Bradyrhizobium* + *Glomus*
Nodule number	171 a*	118 a
Nodule fresh weight (mg)	750 a	795 a
Mean fresh weight per nodule (mg)	4.4 b	6.7 a

*Means with the same letters are not significantly different at alpha=0.05 using Duncan Multiple Range Test.

Table 2. Acetylene reduction activity of peanut root nodule inoculated with *Glomus mosseae* and *Bradyrhizobium* sp. R938

Parameter	Treatment	
	Bradyrhizobium	*Bradyrhizobium* + *Glomus*
Acetylene reduction activity (C_2H_4 nmole/h·plant)	2527 b*	4175 a
Acetylene reduction activity (C_2H_4 nmole/h·g nodule fresh weight)	3369 b	5251 a

*Means with the same letters are not significantly different at alpha=0.05 using Duncans multiple range test.

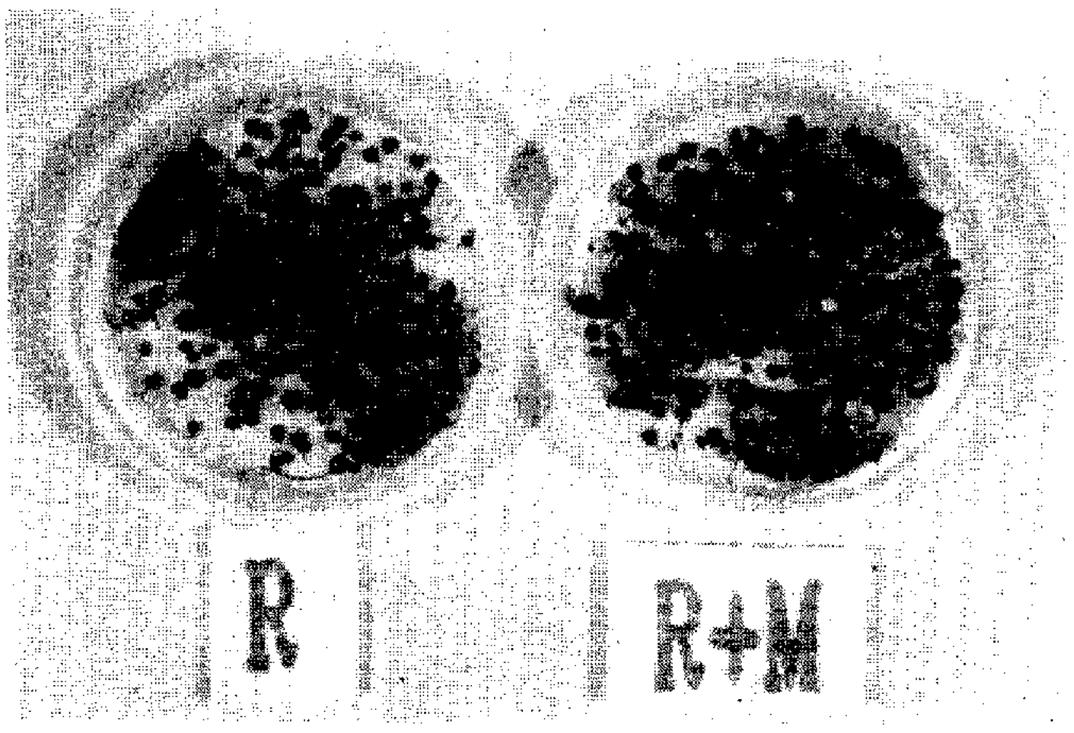

Fig. 3. Comparison of the size of root nodule of peanut plant inoculated with *Bradyrhizobium* sp. R938 (R) and *Bradyrhizobium* sp. R938 plus *Glomus mosseae* (R+M).

by the beginning of the emergence of fruiting roots. There were no nodule in non-inoculated control and inoculated only with *Glomus mosseae*. The nodule number and nodule fresh weight were not significantly different between single inoculation with *Bradyrhizobium* and dual inoculation with *Glomus* and *Bradyrhizobium* (alpha=0.05), but mean fresh weight per nodule was significantly different between the two treatments (Table 1). The mean fresh weight per nodule was 6.7 mg in treatment with the dual inoculation (Table 1). This was 1.5 times increase in weight compared with single inoculation with *Bradyrhizobium*. The tendancy of increase was similar to the increase after 160 days (at fruiting of peanut) growth of peanut plant (1.1 times increase in weight). Nodule size was remarkably larger in treatment with *Glomus* plus *Bradyrhizobium* than in treatment with *Bradyrhizobium* only after 69 days growth (Fig. 3). Between the two treatments, dry weight of shoot and leaf was not significantly different at harvest time of 69 days and 160 days, respectively (alpha=0.05). But in our experimental results after 160 days growth, the mean number of fruiting roots per plant was 30 in the single inoculation with *Bradyrhizobium* and 33 in the dual inoculation with *Glomus* and *Bradyrhizobium*. The similar tendancy was observed at the harvest time of 69 days. Because of the variance in the formation of fruiting root and of peanut, productivity difference between the two treatments was not so much. Ten percent increase in yield by mycorrhizal infection was recognized.

Nitrogenase activity measured as acetylene reduction rate was 1.5 times higher in treatment with dual inoculation than in treatment with single inoculation (Table 2), indicating that the nitrogenase activity was increased 1.5 times by mycorrhizal infection at the harvest time between the period of flowering and fruiting of peanut. In the experiment of alfalfa, after 69 days growth, the same of 1.5 times increase in their nitrogenase activity was obtained under the same conditions of this experiment (3). At harvest time during fruiting period of peanut (160 days after seedling emergence) nitrogenase activity was not significantly different (alpha=0.05) between the two treatments. It was thought that the differences were caused by the demand for carbohydrate and phosphorus in developing fruit.

After purification procedure for assaying plant growth regulators by extraction with ethyl acetate, TLC was performed using isopropanol-ammonia-water (10:1:1, v/v/v) as a developing solvent system. After that, we identified the difference of the UV spectra between nodulated mycorrhizal peanut and nodulated non-mycorrhizal peanut. The difference peak which was present only in mycorrhizal nodulated peanut was thought to be the peak of plant growth regulators. λ_{max}^{MeOH} of the peak was 262 nm (data not shown). Barea *et al.* proposed the presence of plant growth regulating substances in the axenic culture of *Glomus mosseae* (23).

As a result of mycorrhizal infection on peanut, the nodule size and mean fresh weight per nodule were remarkably increased. And nitrogenase activity was also increased as a result of mycorrhizal infection. These results suggested that the use of VA mycorrhizal fungi as a biofertilizer on peanut plant could be possible. The results of increased nodule size and difference UV spectrum of isolated plant growth regulators also suggested that there might be a hormonal effect of *Glomus mosseae* on the peanut plant, considering the carbon competition between VA mycorrhizal fungi and *Bradyrhizobium* sp..

Further research will be focused on the identification of the plant growth regulators and on the use of VA mycorrhizal fungi in agricultural field condition.

요 약

Bradyrhizobium sp. R938과 VA 내생 균근균인 *Glomus mosseae*의 동시 접종이 영호 땅콩의 질소고정력에 미치는 영향을 포트 실험을 통하여 조사하였다. 땅콩에 *Glomus mosseae*와 *Bradyrhizobium*을 동시 접종한 처리에서 질소고정력이 *Bradyrhizobium*만을 접종한 것보다 1.5배 증가되는 결과를 얻었다. 땅콩을 69일 동안 생육시킨 결과 근류의 크기도 내생균근균의 동시 접종으로 인해 증가되었다. 동시 접종 처리구에서 근류 하나의 평균 중량은 6.7 mg으로, *Bradyrhizobium*만을 접종한 것보다 1.5배 증가되는 결과를 보였다. 이상의 결과로부터 *Bradyrhizobium*과 *Glomus mosseae*의 동시 접종은 양분 흡수의 증진을 통한 질소고정력 증진효과 이외에도 VA 내생균근균으로 인한 호르몬 효과가 있으리라는 점을 추정할 수 있었다.

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