Ecological roles of arbuscular mycorrhizal fungi in two wild legume plants

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Two wild legume plants, *Glycine soja* and *Cassia mimosoides* var. *nomame*, and a cultivated plant, soybean (*Glycine max*), were employed for a study of triple symbiosis with an inoculum of *Scutellispora heterogama* harvested from natural soils and an inoculum of their own rhizobial cells. The dry weight, colonization of arbuscular mycorrhizal fungus, nodule formation and N_2-fixation activity were estimated as the parameters of triple symbiosis. The two wild legume plants showed greater growth with colonization of arbuscular mycorrhizae than with nodulation, whereas the cultivated legume showed more nodulation than colonization of arbuscular mycorrhizae. Moreover, *S. heterogama* appeared to stimulate the triple symbiosis for the wild legume plants. The results suggested that spores of *S. heterogama* are important in disturbed soils in Korea.

Key Words——arbuscular mycorrhizae; N_2-fixation; *Scutellispora heterogama*; triple symbiosis; wild legume plants.

Introduction

Occasionally, many wild legume plants have been observed to inhabit nutrient-poor or disturbed areas in Korea. Two of these plants, *Glycine soja* Sieb. et Zucc. and *Cassia mimosoides* var. *nomame* (Sieb.) Makino, have often been found to be dominant species in disturbed areas and also to inhabit turfgrass areas in Korea. Further, the clover introduced during the Korean War as a competitor is considered to be a serious problem in golf-courses. A woody legume plant, *Pueraria thunbergiana* (Sieb. & Zucc.) Benth., has also been frequently observed near highway areas or in the forest and is considered to kill other economic plants, perhaps having an ecological niche. These legume plants are speculated to have several advantages in the ecosystem: symbiosis with rhizobial (R) cells for N_2-fixation (Moawad et al., 1984) and with arbuscular mycorrhizal (AM) fungi in the roots (Schenck and Smith, 1981). However, no one has yet demonstrated these abilities of AM fungi with the wild legume plants in disturbed areas.

The studies of the symbiosis of legume plants with R cells have progressed significantly (Moawad et al., 1984; Rainbird et al., 1983; Lee and Lee, 1992), and the results have recently been applied to particular inocula with resulting high yields (Chamber, 1983; Polonenko et al., 1987). The symbiosis of AM fungi with legume plant roots has also received attention over the past decade (Trappe, 1981; Hayman, 1978). AM fungi were reported to provide phosphate and other minerals to the legume plants (Harris et al., 1985). Recently, the association of

legume plants with both R cells and AM fungi has been called triple symbiosis (Pacovsky and Fuller, 1988; Pacovsky et al., 1991).

AM fungi were reported to receive energy nutrients from the plants, to colonize the plant roots (Harris et al., 1985), and then to affect the microbial flora in the rhizospheres (Meyer and Linderman, 1986). Various species of legume plant have been employed in studies of symbiosis with AM fungi: soybean (Ross and Harper, 1970; Carling et al., 1978; Pacovsky and Fuller, 1988), clover, *Phaseolus vulgaris* L. (Pacovsky et al., 1991), and alfalfa, soybean, and peanut (Daft and El Giahmi, 1975, 1976). The role of symbiosis with AM fungi was evaluated in terms of nodule formation, N_2-fixation activities, dry weight and absorption of phosphate in the plants. Symbiosis with AM fungi was reported to improve mineral nutrition in the legume plants more than symbiosis only with R cells (Barea and Aguilar, 1983; Powell and Daniel, 1978; Smith and Daft, 1977). Much attention has been paid to economic legume plants, but little to the wild legume plants, with regard to the ecological roles of AM fungi.

Our previous work revealed that many AM fungi might be involved in the rhizospheres of wild legume plants (Ahn et al., 1992). Also, the mycorrhizal floras in the soils of disturbed areas were observed to be more diverse than those in the soils of legume plants in undisturbed or natural areas (Ahn et al., 1992). Thus, this work was conducted to explore the ecological roles of AM fungi, especially for wild legume plants. AM fungi were demonstrated to stimulate nodule formation and

N₂-fixation, and to be related to the triple symbiosis observed in wild legume plants in Korea.

Materials and Methods

Plants A crop plant, soybean (*Glycine max* (L.) Merr. cv. Kumgang-Sorip), and two wild plants, *Glycine soja* and *Cassia mimosoides* var. *nomame*, were employed for tests of triple symbiosis. Seeds of soybean, commonly used in agricultural fields, were obtained from a seed merchant in Seoul. Seeds of two wild plants were collected from disturbed areas near Korea National University of Education (KNUE, Gang Lae Myeon, Chung Won Kun Chung Puk, 363-791), and stored at 0°C until used. The seeds of *C. mimosoides* var. *nomame* were boiled for 2 min to induce germination. All seeds employed in this work were treated with 70% ethanol for 2-3 min, placed in 5% NaOCl for 3-5 min, then washed with sterile distilled water.

Inoculation of rhizobia R cells were collected from the nodules of their own host plants, *G. soja* and *C. mimosoides*, in disturbed fields and from soybean roots in the cultivated areas, and were cultured on YM agar by a streaking method (Lee and Lee, 1992). A colony on YM agar was randomly selected for inoculation of legume plants and cultivated at 28°C in YM broth for 4 days. The suspension of R cells was then centrifuged at 3,000 g for 5 min and diluted with sterile saline solution to give an absorbance of 0.5 at 680 nm. The R cells were inoculated on the legume plants before the plants were planted in pots. Three different R cells were inoculated on their own host legume plants.

Inoculum of AM fungal spores Spores of *Scutellispora heterogama* (Nicol. & Gerd.) Walker & Sanders emend. Koske & Walker (1985) identified in the previous work were collected from soils around the KNUE (Ka et al., 1990) and stored at 5°C for 3 weeks before use. *S. heterogama* was isolated by the wet sieving method (Eom and Lee, 1990). The spores collected were sterilized with 5% chloramine T and 20 µg/ml of streptomycin sulfate (Sigma) for 30 min, and washed 6 times with sterile distilled water. These spores were placed on water agar at 0°C for 5 days and the germination rate was determined (about 20%). Approximately fifty spores were mixed with the sterile soils composed of sand and silt (1 : 1 w/w). The silt soil employed here was tested for the composition of spores of AM fungi/10 g of soil: *Acaulospora laevis* (12.0), *Ac. spinosa* (5.0), *S. heterogama* (6.5), and *Glomus* WUM 1+3 (0.5 spores). The soils or mixed soils were autoclaved at 121°C for 2 h. The different spores of AM fungi were counted. Only *S. heterogama* was selected and inoculated in pots as an AM fungus.

Pot culture A soil mixture was made composed of sand collected from the Miho River near KNUE and silt from the forest near KNUE at the rate of 1 : 1 (w/w). The soil mixture was supplemented with humus soil at 3% level by weight, autoclaved for 2 h, and placed in polyethylene pots (15 cm diam × 20 cm depth). The compositions of spores of AM fungi were as previously mentioned, and

about fifty spores of *S. heterogama* were inoculated per pot. Four plants were transplanted per pot after germination. The legume plants treated with R cells, AM fungi or both were grown in a greenhouse from 20 July to 30 September, 1992.

Determination of N₂-fixation The reduction of acetylene to ethylene was used as an index of N₂-fixation (Hardy et al., 1973; Lee and Lee, 1992). Plant roots containing nodules were carefully washed and placed in 250-ml Erlenmeyer flasks, which were closed with black rubber stoppers (2.5 cm diam) equipped with a red septum (1 cm diam) in the center for needle injection. First, 10 ml of air was removed from the flask and replaced with acetylene gas at the pressure of 10 pounds per square inch. The flasks containing nodules and acetylene gas were incubated at 28°C for 1 h. Then 0.1 ml of gas was collected from the flask and injected into a gas chromatograph (Hewlett Packard 5800) equipped with Durapack columns (stainless steel 80/100, 6′ × 1/8″), N₂ gas flow rate was 50 ml/min, while H₂ gas and air were each fed at the rate of 30 ml/min for FID. The temperatures of injection port, oven, and detector were 60°C, 60°C, and 100°C, respectively.

Observations of colonization Plant roots were randomly collected from the soil mixtures, washed with the water, and cut into lengths of 10 mm. The pieces of root were stained as described by Koske and Gemma (1989) and randomly selected for mounting on slide glass. Thirty pieces of root per plant were observed for AM fungal colonization under 100 × magnification. The rate of AM fungal colonization on the roots was determined as the percentage of the length of the root colonized.

Results

Plant growth Plant growth was measured as dry weight per plant with six replications. No significant differences were found in three-week cultures, but significant differences occurred between plants with the three different inocula in six-week cultures (p<0.05). Dry weight of two wild legume plants showed difference as compared with others inoculated. AM fungus enhanced growth more than did R cells in the two wild legume plants, but not in the crop legume plant. However, the wild legume plants inoculated with both AM and R cells showed better growth of dry weight than those with a single inoculum or none (Table 1). The cultivated legume plants (soybean) inoculated with the R cells showed better growth of dry weight than those not inoculated or inoculated with AM fungus, but the differences were not significant. Thus, the two wild legume plants inoculated with both AM fungus and R cells grew better in six-week cultures than any of those inoculated with a single inoculum of AM fungus or R cells or those not inoculated.

Colonization of AM fungus on roots Colonization of AM fungus on soybean roots was not observed in three-week cultures, but it was observed at low level in six-week cultures (Table 2). This level of colonization was measured of the same order as that on the wild legume plant roots after three weeks of cultivation. The colonization of AM

Table 1. Dry weight of three legume plants inoculated with the arbuscular mycorrhizal spores, rhizobial cells or both on roots for three and six week cultivations.

Inoculated with[a]	Dry weight, mg/plant cultured for[b]					
	Glycine max		G. soja		Cassia mimosoides	
	3 weeks	6 weeks	3 weeks	6 weeks	3 weeks	6 weeks
Control	495.8	1072.0	86.3	186.4	45.2	117.1
M	583.6	1061.1	108.3	274.4	60.5	131.4
R	712.6	1368.2	90.2	256.2	51.8	108.7
MR	702.6	1216.4	104.1	359.8	50.8	171.0
LSD[c]	112.1	248.1	22.5	52.0	12.0	18.0

[a] Inoculated with the arbuscular mycorrhizal fungal spores (M, *Scutellispora heterogama*), rhizobial cells (R) or both (MR) before transplanting.

[b] All plants were grown for three and/or six weeks in pots under greenhouse conditions.

[c] Difference (p<0.05).

Table 2. Colonization rate of arbuscular mycorrhizal fungus (*Scutellispora heterogama*) on three different plant roots after three and six weeks of cultivation.

Inoculated with[a]	Colonization rate (%) of arbuscular mycorrhizal fungus[b]					
	Glycine max		G. soja		Cassia mimosoides	
	3 weeks	6 weeks	3 weeks	6 weeks	3 weeks	6 weeks
Control	0	0	0	0	0	0
M	0	16.5	13.0	65.5	15.0	39.5
R	0	0	0	0	0	0
MR	0	23.8	12.9	67.8	19.8	46.3

[a] Inoculated with the arbuscular mycorrhizal fungal spores (M, *S. heterogama*), rhizobial cells (R), or both (MR) before transplanting.

[b] Average colonization rate of arbuscular mycorrhizal hyphae on thirty segments of roots. Observed microscopically under 100×magnification.

fungus on *G. soja* roots was found to take place more quickly than on other legume plant roots. The colonization of AM fungus on the three legume plant roots is shown in Fig. 1. The colonization of AM fungus on soybean roots in six-week cultures was observed to be lower than on the two other plant roots at six weeks and to be similar to those in three week cultures of the two wild plants. Colonization of *S. heterogama* was not observed on the roots of soybean, but it was observed in the other legume plants at three weeks after inoculation. No colonization of AM fungus was observed in the uninoculated control pots or those inoculated with only R cells. The colonization rate of AM fungus was observed to be lower in the soybean than in other plants at six weeks (Table 2). AM fungus colonized the roots of wild legume plant roots better than those of cultivated legume plant roots, and infected the wild legume plant roots at higher levels. Some differences between the wild and cultivated legume plants were found in the colonization of AM fungus or fungal penetration on the host plants. However, *S. heterogama* was found to colonize the three legume plants without any host specificity.

External hyphae forming dark, as thick mycelia were observed around the roots, and internal hyphae were seen in the root tissue as a mass of dark "arbuscular"

spots in the cells. Several hyphae were observed to penetrate the plant tissues, but all hyphae appeared uniformly thick and constricted under the microscope. All fungal cells were observed to be relatively thick, long, heavily-stained with lactophenol cotton blue, and contained several granules (Fig. 1). Septa were found infrequently in long hyphae. The hyphal growth forms were observed to be similar in the roots of the legume plants employed here. *S. heterogama* penetrated the cells of the root cortex with the thick internal hyphae and formed arbuscules in each cell without any vesicles (Figs. 1-C~F).

N$_2$-fixation Nodulations were observed to be greater in roots of the two wild plants inoculated with R cells and AM fungus than those uninoculated or inoculated singly with R cells or AM fungus (Table 3). Nodulations in soybean plants were observed to be greater in the roots inoculated with R cells than those treated in other ways (Table 3). In the three legume plants, N$_2$-fixation was related to nodule formation, although no statistical analysis was conducted. In the two wild legume plants, the N$_2$-fixation was increased more by inoculation of both R cells and AM fungus than by a single inoculum of R cells or AM fungus. But the soybean plants inoculated with both R cells and AM fungus did not show any differences from
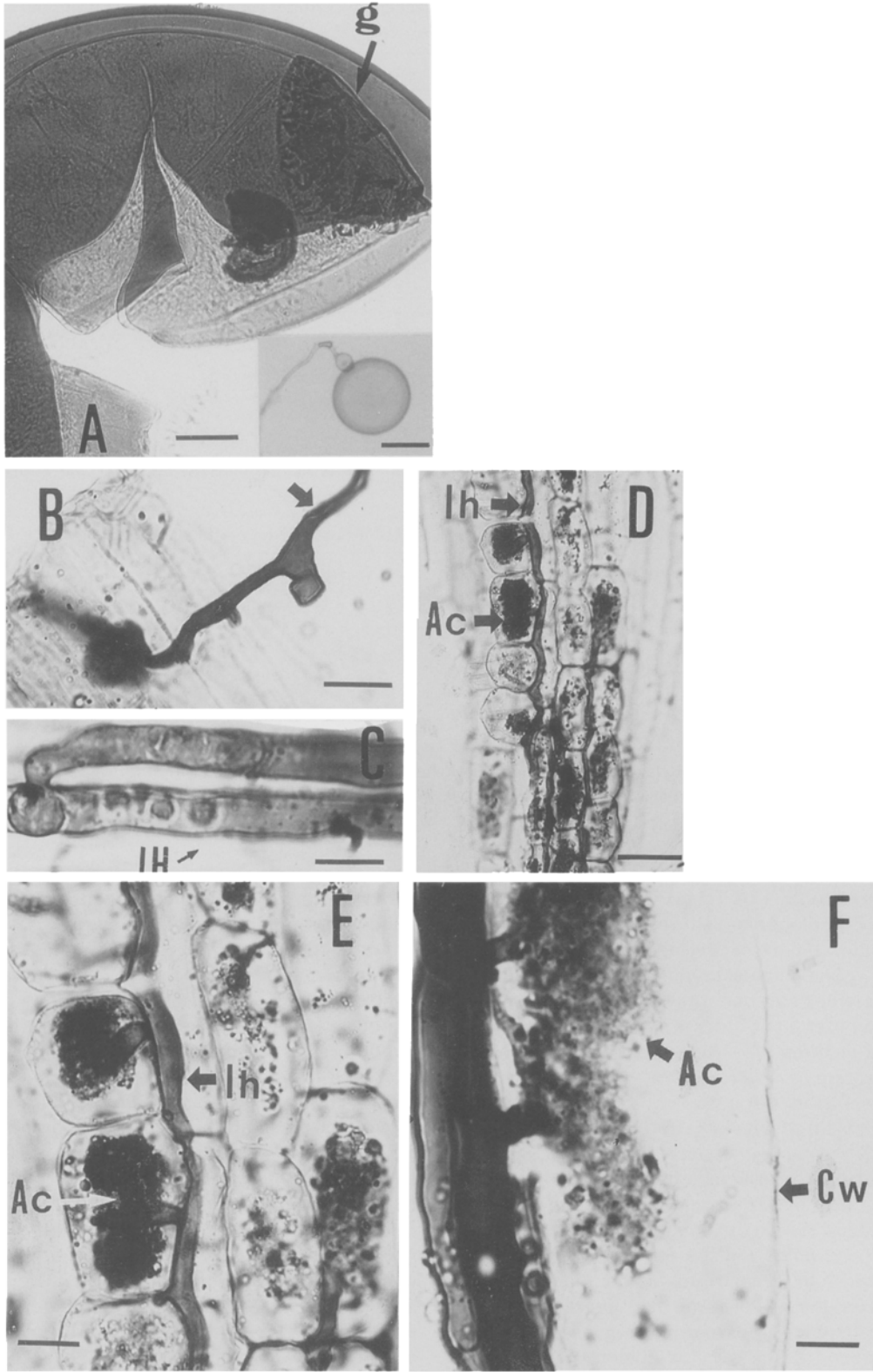
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Fig. 1. Colonizations of arbuscular mycorrhizal fungus and the roots infected with *Scutellispora heterogama* A. Broken spores show-ing the germination shield (g), and whole spores of *S. heterogama*. B. Early stage of external hyphae (arrow) in the roots of *Glycine soja* after three weeks of culture. C. Internal hyphae (IH) with branches on the infected roots of *Cassia mimosoides* (three-week culture). D. Internal hyphae (Ih) and arbuscules (Ac) in the roots of *C. mimosoides*. E. Arbuscules (Ac) in the roots of *G. soja*. F. A long internal hyphae (Ih) and arbuscules within the host root cell wall (Cw) of *G. max* after six weeks of culture. Scales: A, 50 μm for the broken spore, and 200 μm for the whole spore; B, 50 μm; C, 50 μm; D, 100 μm; E, 50 μm; F, 20 μm for hypha.

Table 3. Fresh weight of nodules produced by the plants and reduction of acetylene to ethylene by nodules collected from three different legume plants in six weeks of cultures.

Inoculated with[a]	Glycine max		G. soja		Cassia mimosoides	
	Nodules[b]	Activity[c]	Nodules	Activity	Nodules	Activity
Control	0	0.03	0	0.03	0	0.03
M	0	0.03	0	0.03	0	0.03
R	160	24.0	15	5.04	4.0	0.20
MR	124	0.57	47	12.61	18.0	6.05

[a] Inoculated with the arbuscular mycorrhizal fungal spores (M, Scutellispora heterogama), rhizobial cells (R), or both (MR) before transplanting.

[b] Fresh weight of nodules (mg/plant) from six replicates.

[c] Average reduction activities of acetylene to ethylene for the total nodules produced per plant (nmole/h/plant) from triple replicates.

Table 4. Spore numbers of Scutellispora heterogama collected from pot soils on three different roots after six weeks of cultivation.

Inoculated with[a]	Average of spore numbers of S. heterogama per pot[b]		
	Glycine max	G. soja	Cassia mimosoides
Control	0	0	0
M	212±18	612±25	452±24
R	0	0	0
MR	120±35	704±48	440±34

[a] Inoculated with the arbuscular mycorrhizal fungal spores (M, S. heterogama), rhizobial cells (R), or both (MR) before transplanting.

[b] Average values±standard deviation from triple replicates of arbuscular mycorrhizal fungal spores (S. heterogama) counted in the whole pot soils after six weeks of culture. Fifty spores of S. heterogama were inoculated in pot soil before transplanting.

those inoculated with a single inoculum of R cells, but fixed atmospheric N_2 with better than those inoculated with a single inoculum of AM fungus.

Production of AM fungal spores After three week as of culture, no spores were found in any pots of the three different plants. Therefore, the spores of S. heterogama were counted in six-week cultures (Table 4). G. soja produced the highest number of spores (1.76 spores/g of soil), when inoculated with both inocula. The crop plants produced a lesser number of spores (0.53 spores/g) when inoculated with only AM fungus. None of the plants employed produced any spores when not inoculated with AM fungal spores, as a result of the wet sieving and centrifuging methods. The spores produced by the legume plants reflected the infection rates of AM fungus on legume plant roots.

Discussion

The growth forms of hyphae of Scutellispora heterogama on the roots were considered to be very restricted, having a constricted hyphae containing granules, and to be similar to those of Entomophthora sepulchalis Thaxter (Alexopoulos and Mims, 1979). This was consistent with those observed by Jayasundara et al. (1992), who reported that vesicles are not formed on roots infected by species of Gigasporaceae and that relatively thick hyphae penetrate internally with connections of arbuscules in the roots. The results obtained indicated that only S. heterogama successfully colonizes the roots of the legume

plants and grows within the roots of the these plants. The thick and heavily stained hyphae in the cells were considered to be those formed by S. heterogama. Also, this fungus quickly colonizes the roots of wild legume plants and produces spores at least three weeks after inoculation. In this work, the fungal hyphae are reported to be thick, long, dark-stained and to contain a number of granules with septa.

Plant growth has been increased by inoculating the plants with AM fungus (Mosse et al., 1976; Abbott and Robson, 1978; Powell, 1976; Kahn, 1975; Black and Tinker, 1977; Jackson et al., 1972). When inoculated with AM fungus (Glomus mosseae Nicol & Gerd.), soybean plants were evaluated to increase their N_2-fixation, nodule formation, and dry weight (Ross and Harper, 1970; Carling et al., 1978; Asimi et all., 1980). However, no increase of growth was observed when soybean was inoculated with AM fungus (Ross and Harper, 1970). This is consistent with the change in dry weight of soybean found here. It is possible that the soybean plant is either not involved in the triple symbiosis or not a suitable root host for S. heterogama. However, two wild legume plants, G soja and C. mimosoides var. nomame, showed significantly grow with inocula of both AM and R cells than with a single inoculum of R cells or AM fungus in six-week cultures. AM fungus was concluded to be needed for the growth of the two wild legume plants. The effects of AM fungus were thus clearly observed for the wild legume plants but not for the crop legume plant. Also, AM fugus might play an im-

portant role in wild legume plants in disturbed areas.

Pacovsky and Fuller (1986) observed the penetration of *Glomus fasciculatum Thaxter seusu Gerd.* and *Glomus mosseae* var. *nomame* hyphae nine weeks after inoculation. However, *S. heterogama* colonized the roots of wild legume plants more rapidly and produced spores at a higher level per pot (see Table 4) than has been found in other works (Kahn, 1975; Black and Tinker, 1977; Pacovsky and Fuller, 1988). This difference is considered to be due to the different plants or AM fungus or in the different systems involved. It is speculated that this fungus would be suitable for spore production for the application of AM fungus. In this work, two wild legume plants were demonstrated to participate in triple symbiosis in natural ecosystems. The spores of *S. heterogama* employed here were collected from silt soils with natural vegetation of the two wild legume plants. The increases in growth of the wild legume plants were consistent with the measurements of N_2-fixation activities and nodule formation. Nevertheless, the wild legume plants seemed to first require the colonization of AM fungus more than the nodulations formed by the R cells. In other words, phosphate is a greater limiting factor than nitrogen compounds in the disturbed soils. Thus, the wild legume plants were concluded to solve the limitation of phosphate nutrients by fungal colonization rather than that of nitrogen compounds by nodulation. Also, the wild legume plants seemed to need the triple symbiosis for growth, at least in the disturbed areas or soils.

Symbiotic associations of legume plants with the R cells have attracted attention for the last two decades. It was not questionable that the wild legume plants are dominant on the natural or the disturbed vegetations because of nitrogen nutrients. However, our experiments indicate that the role of AM fungus is more important than that of N_2-fixation on the roots. This is, we believe, the first report on the importance of the ecological role of AM fungus in a disturbed ecosystem. Much interest in the AM fungus relates to its role of enhancing nutrient uptake. The activity of AM fungus is an important factor, not only in regulating the cycling of nutrients in natural ecosystems, but also in assessing the potential for managing this symbiosis in disturbed ecosystems.

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