韓草誌(J. Korean Grassl. Sci.) 18(3) : 195~204, 1998

Effects of *Bacillus subtilis* on Growth of Seedlings in Corn(*Zea mays* L.), White Clover(*Trifolium repens* L.) and Tall Fescue(*Festuca arundianacea* Schreb.)

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*Bacillus subtilis*가 Corn(*Zea mays* L.), White Clover(*Trifolium repens* L.) 및 Tall Fescue(*Festuca arundinacea* Schreb.) 유식물의 생육에 미치는 영향

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적 요

본 연구는 길항미생물인 *B. subtilis*가 '수원19호' corn(*Zea mays* L.), 'Califonia' white clover(*Trifolium repens* L.) 및 'Fawn' tall fescue(*Festuca arundianacea* Schreb.)의 유식물 생육에 미치는 영향을 조사하고자 목초 근권에서 분리한 *B. subtilis*를 이용하여 전남대학교 농과대학 부속 동물사육장내 vinyl house에서 pot로 수행하였다. White clover와 tall fescue의 건물중은 파종 후 36일째에, corn은 파종 후 50일째 조사하였다. 연작 및 비연작토양에서 corn, white clover 및 tall fescue의 지상부와 지하부의 건물중은 *B. subtilis*를 접종함으로써 증가하였다. 그리고 비연작토양에서 재배된 3 초종의 건물중은 연작토양에서 보다 현저하게 증가하는 경향을 나타냈다.

(Key words : *Bacillus subtilis*, Growth, Corn, Tall fescue, White clover)

Ⅰ. Introduction

The ruminant is supplied with its most important nutrients from forage. It is essential for the farmer to use repeated cultivation soil to produce a large quantity of forage. However, the repeated cultivation of forage soil results in the excess and deficiency of specific elements, the aggravation of physical and chemical properties in the soil, and the increase of forage diseases from soilborne pathogenic fungi and bacteria. Forage diseases occur in nearly every field in the world where forage is grown. These are usually caused by fungi, bacteria, nematodes, and viruses et al., which result in reduced crop stands and yields in the field. These pathogens are known to decrease plant yields by stunting and girdling the host roots(Marks and Mitchell, 1971). Most forage diseases are caused by soilborne pathogenic fungi(*Fusarium* spp, *Pythium*

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spp., *Phytophthora* spp. and *Rhizoctonia solani*), which induce a great loss on forage yields in every season (Falloon, 1985; Falloon and Fletcher, 1983; Skip and Christensen, 1983; Skip and Christensen. 1982). Soilborne pathogenic fungi cause seedling and root disease in poorly drained soil in many forage growing regions of the world. It is difficult to control these fungi because they have existed in the soil for a long time. Forage diseases which is caused by fungi were shown to be the serious soilborne diseases in grassland management(Falloon, 1987; Falloon, 1985; Falloon and Fletcher, 1983). Biological control has received much attention as a proper method to prevent or cure soilborn plant diseases(Lin and Sinclair, 1992; Phae et al., 1992; Turner and Backman, 1991; Callan et al., 1990; Savithiry and Gnanamanickam, 1987; Cook, 1985; Kloepper et al., 1980[b]; Kloepper and Schroth, 1981). The development of biological controls of soilborne pathogens and the effective use of beneficial microorganisms are very important in controling plant diseases and enhancing plant growth(Broadbent et. al., 1977, 1971). The workers suggested that bacterial substances, produced by *Bacillus* spp. and *Pseudomonas* spp. et al., play a major role in stimulating plant growth(Brown, 1972) in maintaining microbial equilibrium in soil, and serve as powerful agents for biological disease(Cook, 1985; Henis and Chet, 1975). Most microorganisms are known to produce various kinds of antifungal and/or bacterial antibiotics(Gueldner et al., 1988; Mckeen et al., 1986; Swinburne et al., 1975)

Production of bacterial products is related to plant growth promotion and yield increase(Phae et al., 1992; Turner and Backman, 1991; Handelsman et al., 1990). Generally, the biological control of microorganisms is known to operate by antibiosis, competition, predation and lysis et al.. The researchers believe that the biological control of antagonistic bacteria is due in part to antibiosis in the rhizosphere, and subsequent displacement of root-colonizing microflora(Kloepper and Schroth, 1981; Kloepper et al., 1980[a]). Recently, forage disease research has been very interested in the biological control of soilborne forage pathogens (Handelsman et al., 1990; Callan et al., 1990; Rothrock and Gottlieb, 1981; Chang and Commedahl, 1968; Gregory et al., 1952). Inoculation of antagonistic bacteria may be an effective method for controling forage diseases caused by pathogenic fungi. Among various microorganisms observed, *B. subtilis* has been shown to have suppresive effect for bilogical control of several plant pathogens and has been successfully used as a seed inoculants(Phae et al., 1992; Turner and Backman, 1991; Merriman et al., 1974; Broadbent et al., 1971).

However, no report has been published on the effects of antagonistic microorgarnisms on forage growth in repeated cultivation soils. Therefore, this study was tried to investigate the effect of the antagonistic microorgarnism, Bacillus subtilis, on the seedling growth of 'Suwon 19' corn(*Zea mays* L.), 'California' white clover(*Trifolium repens* L.) and 'Fawn' tall fescue(*Festuca arundianacea* Schreb.) in repeated and unrepeated cultivation soils, respectively.

Ⅱ. Materials and Methods

1. Isolation and identificaton of antagonistic bacteria

Antagonistc bacteria were directly isolated from rhizosphere in repeated cultivation soil of forage in Korea. The selected bacteria with characteristics of

Bacillus spp. were purified by streaking(for 2 days, at 50℃) on B-2 medium(yeast extract 2.0g, sodium chlorude 70.0g, agar 15g in 1 liter, adjusted to pH 5.0)

The antagonistic effects on pathogenic fungi were as follow; the plugs of pathogenic fungi(3mm diameter) were placed in the center of each potato dextrose agar (PDA) plates and incubated for 3 days at 28℃. The selected isolates were pricked with sterilized toothpicks, three per plate, around the edge of the PDA plates. The plates were examined after 3 days. Antagonistic bacteria were selected by measuring the distance between the edges of bacterial colony and the fungal mycelium. The fungal pathogens used in this study was *R. solani* and *F. oxysporum* which cause forage diseases. The selected isolates were incubated for 24 hrs. in LB broth(tryptone 10g, yeast extract 5g, NaCl 10g in 1 liter, adjusted to pH 7.2) and cultures were stored at −75℃.

Of the selected antagonistic rhizobacteria, one isolate was finally selected as the most effective antagonist to pathogenic fungi and identified on the basis of biochemical, morphological and physiological characteristics. Identification of the isolate was conducted by the Prokaryates(Starr et al., 1981), Bergey's manual of Systematic Bacteriology(Krieg and Holt, 1984) and Microbiological method(Collins and Lyne, 1984).

2. The field experiment

The field experiment was carried out at the Animal Research Station, College of Agriculture, Chonnam National University. Forage was established by seeding into pots containing 1:1 mixture of soil and vermiculite with untreatment(control) and treatment (antagonistic rhizobacterium).

Small plastic pots, 11 cm in diameter and 9 cm in depth, were used for the cultivation of 'California' white clover(*Trifolium repens* L.) and 'Fawn' tall fescue(*Festuca arundianacea* Schreb.), and large plastic pots, 35 cm in diameter and 50 cm in depth, were used for cultivation of 'Suwon 19' corn(*Zea mays* L.).

Forage was established in a vinyl house under natural daylight conditions using repeated and unrepeated cultivation soils. Soils used in these experiments was collected from surface soil to a depth of about 10 cm, and sifted through a 10 mm screen. Repeated cultivation soils were collected from farms (Su-kwang farm, Young-am; Animal Research Station, Chonnam National Uni., Kwangju) in Korea which had a history of continuous forage cultivation for 5~6 years. Unrepeated cultivation soils were collected from the soil around pastures(Animal Research Station). The cemical properties of the experimental soils were shown in Table 1.

Forage was irrigated three times in a week. Antagonistic bacterium was grown in late log phase at 37℃ with aeration in LB broth on a rotary shaker. The cultures were centrifuged at 12,000 × g for 20 min. at 4℃. Bacterial suspensions were made by suspending the pellets with sterile distilled water. The cell suspensions were mixed with the soil. The density of isolates ranged from 1 to 2×10^7 colony forming units per gram of dry soil in one pot.

Samples of white clover and tall fescue were taken from each pot at 36 days after seeding. Samples of corn were examined at 50 days after seeding. Roots of forage were separated from the shoots. The roots were washed with running water until free of soil. Shoots and roots of forage were dried at 65℃ for 48 hours and the dry weight was measured. Data were presented as the mean of three replications. The data were

Table 1. Chemical properties of the experimental soils

Soil	pH (1:5 H_2O)	CEC (me/100g)	Av.P_2O_5 (ppm)	OM (%)	Exch. cations(me/100g)		
					Ca	Ma	K
C*	5.6	11.3	49.5	2.45	4.11	0.83	1.53
T	5.3	11.6	29.2	1.97	4.92	3.09	2.46
W	5.3	9.3	62.2	2.32	3.38	1.02	1.56
N	5.2	9.5	13.5	0.58	4.02	2.80	1.59

* C : Continuous corn cultivation soils.
T : Continuous tall fescue cultivation soils.
W : Continuous white clover cultivation soils.
N : unrepeated cultivation soils.

analyzed by using the t-test analysis of variance

Ⅲ. Results

1. Selection and identification of antagonistic rhizobacteria

For the biological control of soilborne fungal diseases by antagonistic rhizobacteria, 326 isolates of rhizobacteria were originally obtained from rhizoshere soils in which forage was cultivated. The isolated bacteria were dual-cultured with the plant pathogenic fungi, *R. solani* and *F. oxysporum*. These were measured by inhibition zone. Among these isolates, only 10 isolates produced on inhibition zone of 10mm or more with both *F. oxysporum* and *R. solani* on PDA.

The most active antagonistic bacterium was selected and identified as *B. subtilis*(Choi et al., 1995), on basis of biochemical, morphological and physiological characteristics by the method of The Prokaryates, Bergey's Manual of Systematic Bacteriology and Microbiology.

2. Enhanced growth of forage due to bacterization

Corn

The dry weight of shoots and roots were measured in corn harvested at 50 days after seeding(seedling stage) in continuous corn cultivation soils(CCCS), and unrepeated cultivation soils(UCCS), respectively(Table 2).

The dry weight of shoots treated with *B. subtilis* was increased by 1.07g in CCCS and 0.88g in UCCS, and the dry weight of roots treated with *B. subtilis* increased by 0.21g in CCCS, and 0.27g, in UCCS, as compared with that of the control. The dry weight of shoots and roots were increased by inoculation with *B. subtilis* in both CCCS and UCCS, but they were not significantly different. The increase in dry weight of corn cultivated in UCCS was higher than that cultivated in CCCS.

White clover

The dry weight of shoots and roots were measured in white clover harvested at 36 days after seeding (seedling stage) in continuous white clover cultivation soils(CWCS), and unrepeated cultivation soils(UWCS),

Table 2. Effect of antagonistic bacterium on growth of corn at 50 days after seeding

Treatment	Dry weight (g/plant)			
	Shoot	Root	Shoot	Root
	..... CCCS		..... UCCS	
Control	4.98±0.361[a]	1.80±0.084[a]	5.79±0.457[a]	1.96±0.055[a]
B. subtilis	6.05±0.617[a]	2.01±0.083[a]	6.67±0.635[a]	2.23±0.251[a]

Mean±SE.
Same letter in the column is not significant difference at 5% level.
CCCS : continuous corn cultivation soils.
UCCS : unrepeated cultivation soils.

respectively(Table 3).

The dry weight of shoots treated with *B. subtilis* was increased by 0.0397g in CLCS and 0.0360g in ULCS, and the dry weight of roots treated with *B. subtilis* was inecreased by 0.0201g in CLCS and 0.0162g, in ULCS as compared with that of the control. The dry weight of shoots and roots were significantly increased by inoculation with *B. subtilis* into the soil as compared with the control in both CLCS and ULCS ($p < 0.05$). The reduction of dry weight was greater in CLCS than in ULCS.

Tall fescue

The dry weight of shoots and roots was measured in tall fescue harvested at 36 days after seeding (seedling stage) in continuous tall fescue cultivation soils(CTCS) and unrepeated cultivation soils(UTCS), respectively(Table 4).

The dry weight of shoots treated with *B. subtilis* increased by 0.0372g in CTCS and 0.0388g in UTCS ($p < 0.05$), and the dry weight of roots treated with *B. subtilis* increased by 0.0365g in CTCS and 0.0558g, in UTCS($p < 0.05$) as compared with that of the control. Tall fescue had a lower dry weight in CTCS than in UTCS. The dry weight of shoots and roots in soil treated with *B. subtilis* was increased significantly rather than that of the control in both CTCS and UTCS($p < 0.05$).

Table 3. Effect of antagonistic bacterium on growth of white clover at 36 days after seeding (seedling stage)

Treatment	Dry weight (g/5 plants)			
	Shoot	Root	Shoot	Root
	..... CWCS		..... UWCS	
Control	0.1029±0.005[b]	0.0668±0.001[b]	0.1270±0.003[b]	0.1080±0.002[b]
B. subtilis	0.1426±0.005[a]	0.0869±0.002[a]	0.1630±0.004[a]	0.1242±0.003[a]

Mean±SE.
[a,b] means in the same column are significant difference at 5% level.
CWCS : continuous white clover cultivation soils.
UWCS : unrepeated cultivation soils.

Table 4. Effect of antagonistic bacterium on growth of tall fescue at 36 days after seeding

Treatment	Dry weight (g/5 plants)			
	Shoot	Root	Shoot	Root
	..... CTCS		..... UTCS	
Control	0.1070±0.004[b]	0.1024±0.001[b]	0.2278±0.013[b]	0.1649±0.008[b]
B. subtilis	0.1442±0.011[a]	0.1389±0.005[a]	0.2666±0.002[a]	0.2207±0.015[a]

Mean±SE

[a,b] means in the same column are significant difference at 5% level.

CTCS : continuous tall fescue cultivation soils.

UTCS : unrepeated cultivation soils.

Ⅳ. Discussion

B. subtilis was used in attempts to control plant pathogens and to increase forage growth and it showed a suppressive effects on fungal pathogens, not only *in vitro*, but also in the pot experiment. Forage was established and increased rapidly in repeated and unrepeated cultivation soils treated with *B. subtilis*. These was a markedly visible difference in the vigour of forage grown repeated and unrepeated cultivation soils. Also, forage grown in unrepeated cultivation soil grew taller, and appeared greener than that in the repeated cultivation soils. This means that the forage seedlings were stressed by soilborne pathogenic fungi and toxic materials from rotten forage which was remained in repeated cultivation soils. Forage was observed to have a increased vigour in response to the treatment with the rhizobacterium. *B. subtilis* was effective in protecting seedlings against pathogens living in the soil. The evidence in Table 1 to 3 clearly shows that the dry weight of forage seedlings grown in unrepeated cultivation soils was greater than that grown in repeated cultivation soils. Forage yield increases were obtained by employing the plant growth-promoting rhizobacterium(PGPR), *B. subtilis*, in repeated and unrepeated cultivation soil. Similar results were reported from other studies(Lin and Sinclair, 1992; Phae et al., 1992; Turner and Backman, 1991; Savithiry and Gnanamanickam, 1987; Kloepper et al., 1980[ab]). Handelsman et al.(1990) found that *B. cereus* UW 85 appeared to have potential as a biocontrol agent for alfalfa damping-off in a field infested with *Phytophthora magasperma* f. sp. *medicaginis*, and that coating seeds with UW85 significantly increased the emergence of alfalfa. *Bacillus* sp. B6 protected the seedling from alfalfa damping-off caused by *Pythium debrayanum* in natural soil, and that increased alfalfa yields without harming rhizobia(Gregory et al., 1952). Turner and Backman(1991) suggested that *B. subtilis* improved germination and emergence and root growth in peanut. The effect of *B. subtilis* on many crops was confirmed by the works of Clay(1986) and Jacks et al. (1985). Isolate A-13 of *B. subtilis* has been particulary useful in increasing yields and stimulating plant growth (Broadbent et al., 1974) and *B. megaterium* B153-2-2 has been shown to improve root growth, and is considered as a potential biocontrol agent of soybeans (Lin and Sinclair, 1992, 1991, 1990, 1989) Callen et al. (1990) reported that *Pseudomonas fluorescens* AB 254 was effective in protecting sh 2 sweet corn against

Pythium preemergence damping-off in natually infested soil. Seedling growth was increased by AB 254 treatment, and at least, 1×10^7 cfu per seed of AB 254 was needed to achieve maximum protection from fungal pathogens. Savithiry and Gnanamanickam(1987) found that bacterization of peanuts with *Pseudomonas fluorescens* result in increased yields, and reduced stem-rot disease caused by R, solani. Kloepper and Schroth (1981, 1980a) reported that PGPR increased plant growth indirectly by interacting with the native roots, rather than directly, by producing growth-promoting substances. The mechanism by which *B. subtilis* stimulates root growth is unknown. Kloepper and Schroth(1981) suggested that the elaboration of bacterial products is related to plant growth promotion, and yield increase is consistant with the idea that bacterial products play an important role in stimulating plant growth(Kloepper et al., 1980[ab]). Many workers suggested that plant hormones produced by *Bacillus* spp. and *Pseudomonas* spp. et al., increase plant growth, and that plant growth promotion by PGPR is related to reductions in the population of pathogens in the rhizosphere(Brown, 1972; Eklund, 1970; Brown and Burlington 1968; Hussain and Vancura, 1970; Sobieszczanski, 1966; Katznelson and Cole, 1965). Bacilli and *B. subtilis* were known to produce various kind of antifungal and bacterial antibiotics(Gupta and Utkheda, 1986; Gueldner et al., 1988; Swinburne et al., 1975). Phae et al.(1992) identified a biologically active substance secreted by *B. subtilis* NB 22 as the cyclic peptidolipidic antibiotic substance, iturin. Whether the bacteria elaborate products directly influence plant growth and/or affect the composition of root microflora by antagonism remains to be discerned(Lin and Sinclair, 1992; Phae et al., 1992; Turner and Backman, 1991). This experiment clearly shows that forage growth was promoted by *B. subtilis* and was severely affected by different soils. Bacterization with native strains has proves very valuable for efficient forage management. The Results of this experiment suggest that forage diseases caused by fungal pathogens can be reduced by bacterization with efficient strain of *B. subtilis*, which have potential as PGPR and as a biocontrol agent for forage disease.

V. Summary

This study was designed to investigate the effects of antagonistic microorganism, *Bacillus subtilis*, on the growth of forage seedlings in repeated cultivation soils and unrepeated cultivation soils. The field experiment was conducted in pots in a vinyl house using repeated and unrepeated cultivation soils. Forage types were 'Suwon 19' corn(*Zea mays* L.), 'California' white clover(*Trifolium repens* L.) and 'Fawn' tall fescue (*Festuca arundianacea* Schreb.).

Samples of white clover and tall fescue were taken from each pot at 36 days after seeding. Samples of corn were examined at 50 days after seeding. The most active antagonistic bacterium was isolated from forage rhizosphere soil, and selected by reference to it's antagonistic ability on the growth of pathogenic fungi, *Rhizoctonia solani* and *Fusarium oxysporum*, and it was identified as *Bacillus subtilis*. This strain strongly suppressed the growth of fungal pathogens among isolated rhizobacteria. The dry weight of forage shoots and roots cultivated in unrepeated cultivation soils was higher than that cultivated in repeated cultivation soils. The dry weight of forage was positively affected by the inoculation of the antagonistic bacterium, *Bacillus subtilis*, in both repeated cultivation soils and unrepeated cultivation soils. In conclusion, the growth

of forage was more affected by the inoculation of the antagonistic bacterium in unrepeated cultivation soils than that in repeated cultivation soils, and bacterization of forage with *B. subtilis* resulted in an increased yield.

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