Isolation, Identification, and Evaluation of Biocontrol Potentials of Rhizosphere Antagonists to *Rhizoctonia solani*

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金喜圭·盧明周：園藝作物 모잘록병(*Rhizoctonia solani Kühn*)의 發生에 관여하는 根圈拮抗菌의 分離, 同定 및 生物的 防除 檢討

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ABSTRACT Antagonistic microorganisms from rhizosphere soil were isolated, identified, and applied successfully as the biocontrol ageats of damping-off caused by *Rhizoctonia* spp. Rhizosphere antagonists isolated from rhizosphere soil were identified as *Trichoderma viride, T. harzianum, T. hamatum, T. polysporum, Gliocladium* sp., *Pseudomonas fluorescence, P. stutzeri, P. cepacia, Enterobacter* sp., *Serratia* sp. and *Erwinia herbicola.* Of these, the most promising ones *in vitro* were *T. virdie, T. harzianum, Gliocladium* sp., *Serratia* sp., *P. stutzeri,* and *P. cepacia.* These above six antagonists were efficient in reducing disease incidence to 40~70% when the reselected rhizosphere antagonists preparations were applied to the soil at 10^6 propagules per gram. Among six antagonists, *T. viride* was the most promising biocontrol agents against *R. solani* isolates in soil. The suppressive effect was more evident in steam-sterilized soil than in non-sterilized field soil.

INTRODUCTION

Damping-off of seeds and seedlings induced by *Rhizoctonia solani* Kühn is responsible for considerable losses in many crops, The literatures on biological control of *Rhizoctonia solani* Kühn are voluminous.[1,2]

Species of the genera *Trichoderma* and *Gliocladium* have been evaluated for efficacy in the biocontrol of fungal plant pathogens[9,18]. Of the *Trichoderma* spp., particulary T. *harzianum* and *T. viride* have been intensively investigated, including control of *Sclerotium rolfsii, Rhizoctonia solani,* and *Pythium debaryanum.*[3,6,10] Of the *Gliocladium* spp., particularly *G. virens* have been determined as promising biocontrol agent of *Sclerotinia sclerotiorum* and *R. solani.*[4,16,22] Many saprophytic soil bacteria involved in antagonism to plant pathogen or lysed the mycelium of pathogenic fungi.[1,11,12] *Bacillus* spp.[17], *Arthrobacter* spp.[23], and fluorescent pseudomonads[12] are frequently used for test of mycelial lysis and destruction of the dormant propagules of fungal pathogens.

Chet and Baker[6] collected naturally suppressive soil to *R. solani* from Columbia, South America. They further confirmed that the suppressive nature of soil contained 8×10^5 propagules of *T. hamatum.* Thus, the suppressive nature of this soil was transferred to conducive soils rendering them suppressive by inoculating conidia of this fungus at 10^6 propagules per gram. It is ecologically important to isolate antagonists from rhizosphere soil, where there is favorable condition for antagonist proliferation after introduction. Rhizosphere antagonists applied directly to soil may have the opportunity to be adapted to soil environment. Therefore, they may be effective in preventing damping-off caused by *Rhizoctonia* spp.

Our works on the AG identification[15] and pathogencicity[20] of *R. solani* isolates obtained from southern horticultural area were published previously.

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An attempt was made to isolate and identify antagonistic microorganisms from the rhizosphere soil to apply successfully as the biocontrol agents of damping-off caused by *Rhizoctonia solani*.

MATERIALS AND METHODS

Isolation of potential antagonists in rhizosphere soil

Soil samples were collected in 1985 from an open field or a vinyl house of conducive or suppressive rhizosphere soil where cucumber, red pepper, Chinese cabbage, and strawberry had been grown. The soil was a clay loam. It was blended, passed through a 4-mm sieve, and either used immediately for experiments, or stored in a vinyl bag at room temperature before use.

Soil fungi were counted on soil-extract agar and potato-dextrose agar (PDA) supplemented with streptomycin at 0.3ppm. M-4 glucose medium[21] was used for isolation of soil bacteria and for their antagonism toward *R. solani*. *In vitro* antagonism of soil fungi against *R. solani* was examined by dual culture. Mycelial disks(5mm in diameter) from antagonistic fungi and *R. solani* were placed 4 cm apart on PDA. The plates were incubated at 25±2°C. *In vitro* antibiotic activity of soil bacteria toward *R. solani* was examined as follows: plates with M-4 medium were inoculated with potential antagonists, four in each petri dish, by depositing a loopful of the antagonist on an area approximately 1 cm spaced equidistant from each other and 1cm from the edge of the plate. After incubation for 2 days at 25°C, *R. solani* was introduced by placing a 2mm diameter mycelial disk from a yeast extract dextrose calcium carbonate agar (YDC) culture in the center of the plate. Measurements of area around each potential antagonist in which *R. solani* was unable to grow were recorded after an additional 3 days of incubation at 25°C.

Identification of antagonists

Fungi. Five antagonistic fungi were identified as genera *Trichoderma* and *Gliocladium*[7,19] All isolates were cultured on PDA. Each isolate was hyphal-tipped and conformed to the species of genera *Trichoderma* and *Gliocladium*.

The isolates were cultured on 2% water agar in 9-cm-diameter glass petri plates containing 7ml of the medium. The plates were incubated at 25±2°C. After incubation for 3 days, a disk was cut in the colony with an 11-mm-diameter cork borer. And then the disk was placed on a glass slide and the characteristic features were examined microscopically. Characterics used for identification of species were conidiophore, side branch, phialide, phialospore, and sterile hyphal elongation.

Bacteria. Key charcteristics of the bacterial isolates were tested to characterize them taxoomically. The taxonomic schemes and criteria for identification of the bacteria were followed the 8th edition of Bergey's manual of determinative bacteriology.[5] Compound microscope was employed for morphological observation. Staining character including Gram reaction was observed through bright field microscopy.

Nutrient agar (NA), 523, King's B (KB), and YDC agar were used for determining the culture character and pigment production. The recipes of media and detailed procedures for testing bacterial characteristics, sole carbon source utilization, levan formation, pigment production, starch hydrolysis, citrate utilization, and ammonia test, were mostly followed Kado's method.[14]

The effects of antgonists in greenhouse experiments

Three antagonistic fungi and bacteria were chosen for the most promising biocontrol

agents from *in vitro* tests of antagonism. To examine the effects of antagonists, they were applied to steam-sterilized and non-sterilized field soil at 10^6 propagules per gram(Fig. 2)

Inoculum was prepared for infestation of soil as in the previous report.[20] Inoculum, 1g per 400g dry weigh of both soil, was added and mixed thoroughly, and then 800g of soil was distributed each plastic pot(16×10×6.5 cm) Conidia of *Gliocladium* sp., *T. viride*, and *T. harzianum* were harvested from 7 days old cultures on PDA. The prepared suspension was added to both soil at concentrations of 10^6 conidia per gram. Bacterial suspension of *Serratia* sp., *Pseudomonas stutzeri*, and *P. cepacia* was prepared on KB broth in 1,000ml Erlenmeyer flasks. The flasks were shaked back and forth at 120 strokes per minute at 25°C. After incubatioa for 3 days, the suspension was centrifuged for 30 minutes at 2,744g. The suspension was centrifuged three times in three changes of distilled water. The preparations were added to both soil at concentration of 10^6 cell number per gram.

Two day old six seedlings of cucumber, Chinese cabbage, and radish were planted in each of triplicate pots. Twenty-one days after planting, seedlings were rated for suppression of damping-off compared with control.

RESULTS AND DISCUSSION

Identification of antagonists

Fungi. Rifai[19] distinguished nine species "aggregates" based on microscopic chcracters. Four *Trichoderma* isolates selected in this study were identified as *T. viride*, *T. harzianum*, *T. hamatum*, and *T. polysporum*(Fig. 1). *T. hamatum* and *T. polysporum* often formed elongated sterile hyphae. The sterile hypha of *T. hamatum* were curved and hooked(Fig. 1a), and these of *T. polysporum* were flexuous (Fig. 1b). *T. viride* was characterized as conidiophores and their side branches being slender and long, without sterile hyphal elongations; phialides not crowded, rather slender; conidia globose and large, but roughness of walls were not clarified in this study(Fig. 1c). *T. harzianum* was identified for its conidiophores with complicated dendroid branching system; phialides quite regularly disposed; phialospores globose or subglobose or short obvoid(Fig. 1d).

Gliocladium sp. was identified in this study for its side branches approaching their bearer rather closely and producing adpressed phialides at their apices so that on the whole the typical branching systems had obconical or obpyramidal outline and appeared like a small brush which ultimately supported a big conidial ball(Fig. 1e).

Bacteria. Among 50 isolates tested previously, 6 isolates were selected on the basis of their inhibitory effects to the pathogenic fungi. The physiological characteristics of such promising antagonistic bacteria were examined to manifest their taxonomic positions. To determine the identity in detail, the characteristics of six selected isolates were compared with authentic descriptions[5] of related bacteria. The related isolates were grouped according to their physiological charteristics.

1) Fluoresent pseudomonads

The selected isolate belonged to fluorescens pseudomonads was R1. The important physiological characteristics of this isolate were listed in Table 1. This bacterium produced diffused typical green fluorescent pigment on KB but not formed any other pigment. This isolate was oxidase and arginine dihydrolysis positive which were the key character to discriminate plant pathogenic fluorescent pseudomonads group. Among the fluorescens group, *P. aeruginosa*, *P. fluorescens* and *P. putida* are very closely related[21]. The possibility of isolate being *P. putida* could be eliminated

Table 1. Comparison of general characteristics between antagonistic bacteria isolates with six species in Bergey's Mannual of Determinative Bacteriology

Characteistics	*Pseudomonas fluorescens*		*Pseudomonas stutzeri*		*Pseudomonas cepacia*	
	VS.	R1	VS.	R2	VS.	R3
Motility	+	+	+	+	+	+
Cell shape	Rod	Rod	Rod	Rod	Rod	Rod
Gram reaction	−	−	−	−	−	−
O/F tast	Oxi.	Oxi.	Oxi.	Oxi.	Oxi.	Oxi.
Pigment	Fluor.	Fluor.	−•	−•	−•	−•
Oxidase	+	+	+	+	+	+
Gelatin liquefaction	+	+	−	−	d	+
Catalase	+	+	+	+	+	+
Denitrification	−	−	+	+	−	−
Levan formation	d	−	−	−	−	−
starch hydrolysis	−	−	+	+	−	−
Arginine hydrolysis	+	+	−	−	−	−
Utilization of Trehalose	d	−	−	−	−	−
Galactose	d	−	*	+	*	+
Cellobiose	−	−	*	+	*	+
Arabinose	−	−	*	−	*	−
Motitly	+	+	+	+	+	+
Cell shape	Rod	Rod	Rod	Rod	Rod	Rod
Gram reaction	−	−	−	−	−	−
O/F test	O/F	O/F	O/F	O/F	O/F	O/F
Pigment	yel.	yel.	red or pink	red	yel.	yel.
Oxidase	−	−	−	−	d	−
Gelatin liquefaction	d	−	+	+	+	+
Catalase	d	+	+	+	*	+
Levan formation	d	−	−	+	*	−
starch hydrolysis	d	−	−	−	+	+
Arginine hydrolysis	+	+	−	−	*	−
Ammonia test	d	+	−•	−•	*	+
Citrate utilization	+	+	−•	−•	+	+
Methyl red test	−	−	−	−	*	+
Urease test	−	−	−•	−•	+	+
KCN test	+	+	−•	−•	d	+
Indol production	−	−	−•	−•	−	−
Utilization of Trehalose	+	+	+	+	+	−
Galactose	d	+	*	+	*	−
Celllobiose	+	+	−	−	−	−
Arabinose	+	+	−	−	+	+

by positive in gelatin liquefaction and that of *P. aeruginosa* could be ruled out by the negative denitrification. Therefore, the isolate designated as R1 was identified *P. fluorescens*.

2) Nonfluorescent pseudomonads

Isolates R2 and R3 were Gram negative, strict aerobic rod. They turned out to be nonfluorescent pigment by the irradiation of UV light in dark room. R2 was related to *P. stutzeri* and R3 was to *P. cepacia*(Table 1). The isolates R2 and R3 did not have the ability of levan formation and araginine hydrolysis. From these results, it could be assumed that isolate R3 was not *P. alcaligenes*. The distinctive differences between *P. stutzeri* and *P. cepacia* were as follows: *P. stutzeri*

had the ability of denitrification from nitrate and starch hydrolysis, but *P. cepacia* did not have those characters. From these results, it was concluded that isolates R2 and R3 were identified as *P. stutzeri* and *P. cepacia*, respectively.

3) Enterobacteriaceae

Isolates R4, R5 and R6 were Gram negative, straight rod, oxidase negative, and catalase positive. These were the discriminative characteristics for separating Enterobacteriaceae from the other families of Gram negative facultative anaerobic rods that were listed in 8th edition of Bergey's manual. The genera of Entrobacteriaceae are not easily distinguishable each other with one or two distinctive biochemical properties.

The isolates R4, R5, and R6 were related to *Enterobacter* sp., *Serratia* sp., and *Erwinia herbicola*, respectively. Isolates R4 and R5 were presumed to be group II of Entrobacteriaceae for negative reaction of methyl red test(Table 1). The reactions of methyl red reduction and Voges-proskauer test in other groups of Entrobacteriaceae were mostly opposite to group II. Distinctive characters to classifying *Klebsiella*, *Enterobacter*, *Hafnia* and *Serratia*, the genera belonged to group II, were acid formation from arabinose and lactose, H_2S gas from triple sugar iron media within 5 days[5]. From the reaction of the isolate to utilizattion of arabinose, it was concluded that isolate R5 was belonged to the species of *Serratia*. In 8th edition of Bergey's manual, only one species, *S. marcescens*, is listed in genus *Serratia*. However, further and intensive studies should be conducted in order for this isolate R5 to be identified as *S. marcescsns* definitely. From the reactions of the isolate to utilization of arabinose and arginine hydrolysis, it was concluded that isolate R4 belonged to the species of *Enterobacter*. *E. cloacae* and *E. sakasakii* had the ability of arginine hydrolysis. But, *E. sakasakii* was only one represented by diffused yellow pigment.[5] Thus, R4 isolate was assumed to be *E. saksakii*. Diffused yellow pigment on NA was the key characteristics of *E. herbicola* group in the genus *Erwinia*. Gelatin liquefaction positive and no indol productions were another criteria to eliminate another species reading herbicola group. Thus, the possibility of isolate R6 being *E. uredovora* and *E. herbicola* var. *ananas* could be eliminated by negative in utilization of cellobiose.

The effects of antagonists preparation on damping-off disease under green house condition

T. viride, *T. harzianum*, *Gliocladium* sp., *Serrtia* sp., *P. stutzeri*, and *P. cepacia* were re-selected from *in vitro* tests of antagonism, for they were promising biocontrol agents against *R. solani* isolates. Those antagonists were added to steam-sterilized and non-sterilized field soil at 10^6 propagules per gram. The soil used was infested with *Rhizoctonia* isolates obtained from four host sources before treatment. Four *Rhizoctonia* isolates were selected because they were highly virulent to cucumber, Chinse cabbage and radish.

Damping-off of cucumber, Chinese cabbage and radish was successfully controlled by applying the preparations of six antagonsists to the infested soil(Fig. 2). Six antagonists suppressed damping-off of cucumber, Chinese cabbage and radish seedlings caused by highly virulent isolate of cucumber root by 30~50%. Moreover, they suppressed damping-off by moderately virulent isolates of rice sheath blight, red papper fruit and red pepper root by 60~80%(Fig. 2). The suppressive effect of a given antagonist to each isolate of *R. solani* ranged from moderate to strong. The responses of a given pathogen isolate to a

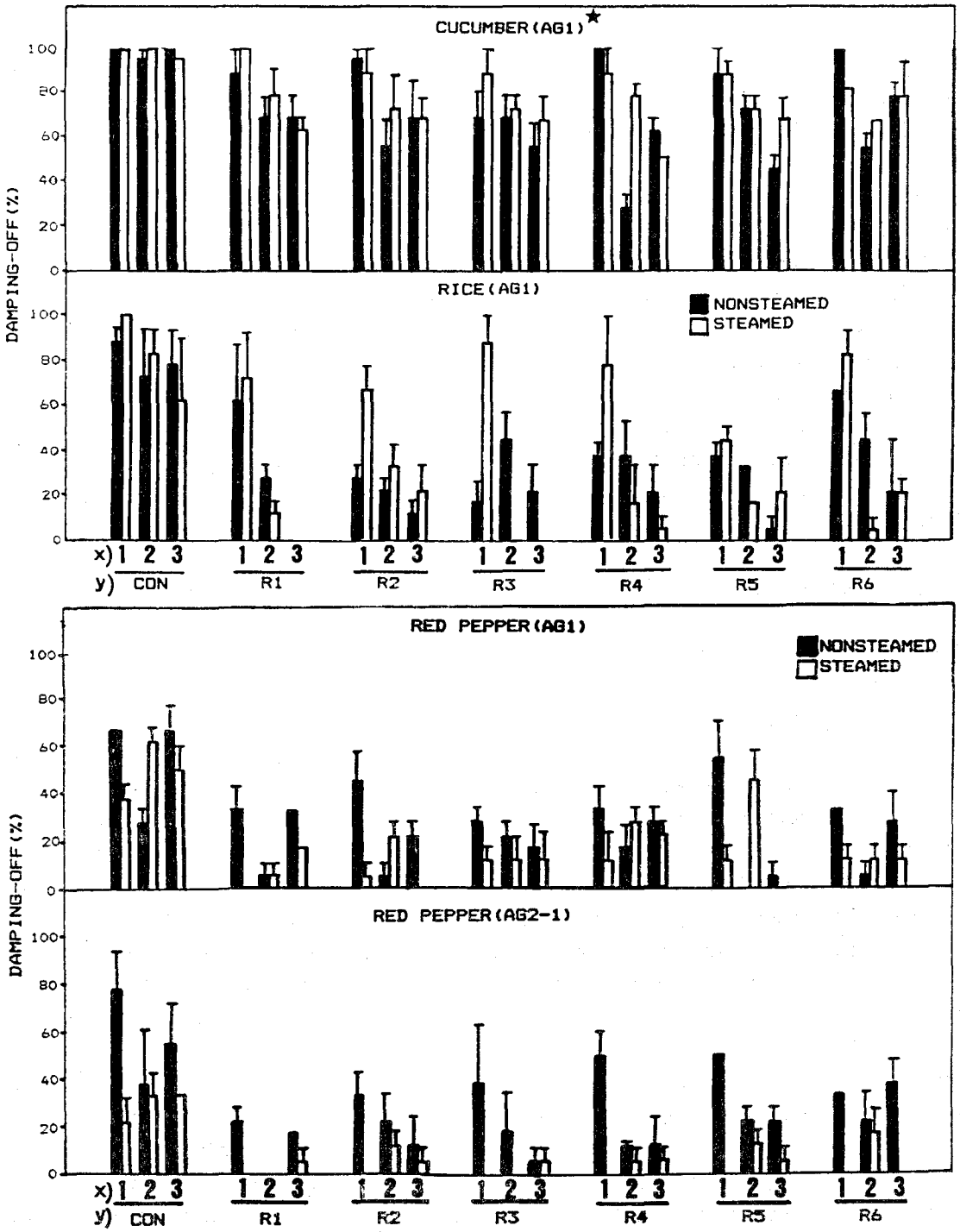

Fig. 2. The effects of antagonists added to steam sterilized and natural soil at 10^6 propagules per gram on suppression of damping-off in three host in soil infested with Rhizoctonia isolates obtained from four host source(*).
There were six seeds in each treatment in three replications. The standard error (SE), shown as vertical bars, was computed from original data.
x : Host tested(1, Cucumber; 2, Chinese Cabbage; 3, Radish)
y : Antagonists applied(Con, Control; R1, *Trichoderna viride*: R2, *T. harzianum*; R3, *Gliocladium* sp.; R4, *Serratia* sp.; R5, *Pseudomonas stutzeri*; R6, *Pseudomonas cepacia*).

couple of antagonists were also variable from very susceptible to fairly tolerant. Above result indicated the diverse nature of ecosystem of soil microbes. Therefore, it was assumed that any promising antagonist against certain *R. solani* isolate could not necessarily be equally suppressive to all host isolates. Efforts should be directed toward the potential application and further investigation to find microbes with broad spectrum antagonism.

Six antagonists decreased damping-off more in stean-sterilized soil than in non-sterilized field soil. With the exception, *R. solani* isolate from rice sheath bligh tresulted in decrease damping-off in non-sterilized field soil than in steam-sterilized soil(Fig. 2). From this observation, the rice sheath blight pathogen appears to be the most aggressive saprophytically of the most Rhizoctonia isolates investigated. Cucumber plant suffers rather severely from cucumber wilt in sterilized soil, since the pathogen outcompetes saprophytically the antagonists in sterilized environment.[13]

In greenhouse experiments, damping-off caused by *R. solani* isolates from four host sources was controlled by *T. virdie, T. harzianum, Gliocladium* sp., *Serratia*, sp., *P. stutzeri*, and *P. cepacia*. Among six antagonists, *T. viride* was the most promising biocontrol agent against *R. solani* isolates in soil.

摘 要

南部地方에 栽培되는 고추, 오이, 배추, 딸기 等의 根圈土壤에서 分離, 選拔된 拮抗微生物을 利用하여 Rhizoctonia病의 生物的 防除를 檢討한 結果는 다음과 같다.

根圈土壤에서 分離, 選拔된 拮抗菌은 *Trichoderma viride, T. harzianum, T. hamatum, T. polysporum, Gliocladium* sp., *Pseudomonas fluorescens, P. stutzeri, P. cepacia, Enterobacter* sp., *Serratia* sp., *Erwinia herbicola*等으로 同定되었고, *in vitro*에서 優秀한 拮抗菌은 *T. viride, T. harzianum, Gliocladium* sp., *P. stutzeri, P. cepacia, Serratia* sp. 等이었다.

*In vivo*에서의 拮抗效果는 寄主와 菌株에 따라 다소 差異가 있었고, 殺菌土에서 非殺菌土보다 拮抗效果가 더 좋은 傾向을 보였다. 再選拔된 6가지 拮抗菌을 土壤內에 接種(10^6cfu/g soil) 했을 때, 拮抗效果가 가장 優秀한 것은 *T. viride* 이었다.

病原性이 가장 强한 오이 뿌리에서 分離된 菌株(AG 1)를 오이, 배추, 무우等에 處理하여 拮抗菌을 接種하였을 때는, 病原性이 强하여 拮抗效果가 弱한 傾向(40%)이었으나, 고추(AG 1), 고추(AG 2-1), 수도(AG 1)에서 分離된 菌株에 대해서는 全般的으로 無處理에 비해 各各 70% 정도의 發病 抑制效果가 있었다.

LITERATURES CITED

1. Baker, K.F. and R.J. Cook. 1974. Biological control of plant pathogens. W.H. Freeman, San Fransico. 433p.
2. Baker, R. and C.A. Martinson. 1970. Epidemiology of disease caused by *Rhizoctonia solani*. Pages 172~188 in: J.R. Parmeter, Jr., ed. *Rhizoctonia solani*, biology and pathology. Univ. Press, Berkeley. 255p.
3. Beagle-Ristaino, J.E. and G.C. Papavizas. 1985. Biological control of Rhizoctonia stem canker and black scurf of potato. Phytopathology 75 : 560~564.
4. Beagle-Ristaino, J.E. and G.C. Papavizas. 1985. Survival and proliferation of propagules of *Trichoderma* spp. and *Gliocladium virens* in soil and in plant rhizospheres. Phytopathology 75 : 729~732.
5. Buchanan, R.E. and N.E. Gibbons, eds. 1974. Bergey's manual of determinative bacteriology, 8th ed. Williams and Wilkins Co., Baltimore, 1246pp.
6. Chet, I. and R. Baker. 1981. Isolation and biocontrol potential of *Trichoderma hamatum* from soil naturally suppressive to

Rhizoctonia solani. Phytopathology 71 : 286 ~290.

7. Domsch, K.H. and W. Grams, eds. 1980. Compendium of soil fungi, vol I. Academic Press, London, New york, Toronto, Sydney, San Francisco. 859pp.
8. Hadar, Y., I. Chet and Y. Henis. 1979. Biological control of *Rhizoctonia solani* damping-off with wheat bran culture of *Trichoderuma harzianum*. Phytopathology 69 : 64~68.
9. Harman, G.E., I. Chet and R. Baker. 1980. *Trichoderma hamatum* effects on seed and seedling disease induced in radish and pea by *Pythium* spp. or *Rhizoctonia solani*. Phytopathology 70 : 1167~1172.
10. Henis, Y., A. Ghaffer and R. Baker. 1978. Integrated control of *Rhizoctionia solani* damping-off of radish: Effect of successive planting, PCNB, and *Trichoderma harzianum* on pathogen and disease. Phytopathology 68 : 900~907.
11. Homma, Y. 1984. Perforation and lysis of hyphae of *Rhizoctonia solani* and conidia of *Cochliobolus miyabeanus* by soil myxobacteria. Phytopathology 74 : 1234~1239.
12. Howell, C.R., and R.D., Stipanovic. 1979. Control of *Rhizoctonia solani* on cotton seedling with *Pseudomonas fluorescens* and with an antibiotic produced by the bacterium. Phytopathology 69 : 480~482.
13. Jee, H.J. 1986. Evaluation of biocontrol potentials with rhizosperic antagonists to *Fusarium oxysporum* f. sp. *cucumerinum* Owen. In English. M.S. Thesis. Gyeongsag National University. 74p.
14. Kado, C.I. 1973. Methods in plant bacteriology, a laboratory manual. Univ. Calif. Davis. Davis. Calif.
15. Kim, H.K., M.J. Roh and D.I. Kim. 1986. Characterization of *Rhizoctonia solani* Kühn from southern horticultural area in Korea. J. Inst. Agr. Res. Util. Gyeongsang Natl. Univ. 20 : 59~63.
16. Lewis, J.A. and G.C. Papavizas. 1985. Effect of mycelial preparations of *Trichoderma* and *Gliocladium* on populations of *Rhizoctonia solani* and the incidence of damping-off. Phytopathology 75 : 812~817.
17. Okazaki, H. 1975. Factors affecting the germination of chlamydospore of *Fusarium oxysporum* f. sp. *raphani*. Ann. Phytopath. Soc. Japan 41 : 314~320.
18. Papavizas, G.C. 1985. *Trichoderma* and *Gliocladium:* Biology, ecology and the potential for biocontrol. Ann. Rev. Phytopathol. 23 : 23~54.
19. Rifai, M.A. 1969. A revision of the Genus *Trichoderma*. Mycological papers 116 : 1 ~56.
20. Roh, M.J. and H.K. Kim. 1987. Pathogenicity of *Rhizoctiona* isolates from southern horticultural area in Korea. Korean J. Plant Pathol. 3(3) : In press
21. Stainer, R.Y., E.A. Adelberg and J.L. Ingraham. 1976. The Microbial World. 4th ed. Prentice-Hall Inc., Englewood Cliffs. NJ. 871pp.
22. Tu, J.C. 1980. *Gliocladium virens*, a desiructive mycoparasite of *Sclerotinia sclerotiorum*. Phytopathology 70 : 670~674.
23. Weller, D.M., and R.J. Cook. 1981. Control of take all of wheat with fluorescent pseudomonads. Abstr. Phytopathology 81: 1007.

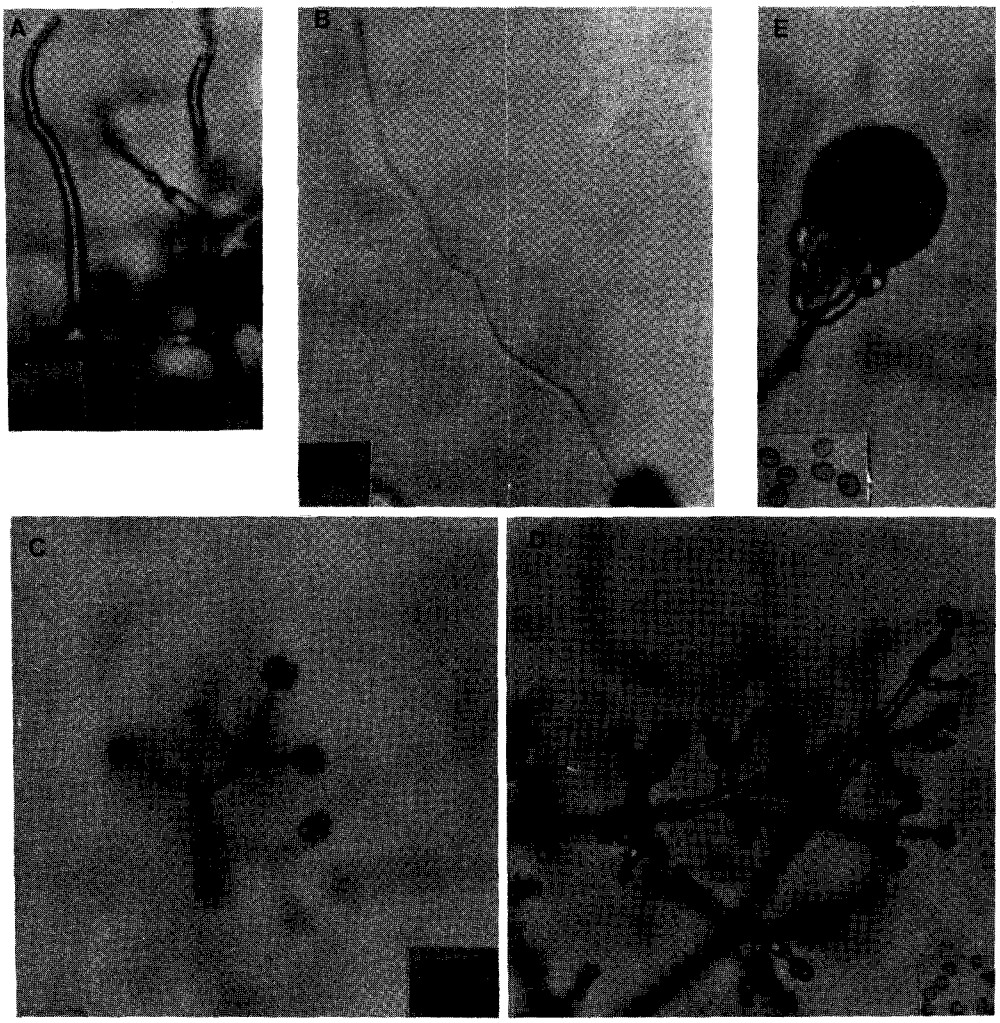

Fig. 1. *Trichoderma* spp. and *Gliocladium* sp. conidiophores with phialides and conidia (×400).
a : *T. hamatum*, b : *T. polysporum*, c : *T. viride*, d : *T. harzianum*, e : *Gliocladium* sp.