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Isolation, Identification and Antagonisms of Rhizospheric Antagonists to Cucumber Wilt Pathogen, *Fusarium oxysporum* f. sp. *cucumerinum* Owen

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오이 덩굴쪼김病菌에 대한 오이 根圈拮抗微生物의 分離, 同定 및 拮抗作用

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ABSTRACT

Bacteria and fungi antagonistic to *Fusarium oxysporum* f. sp. *cucumerinum* Owen were effectively isolated with each of modified Triple Layer Agar (TLA) technique from rhizosphere soil where cucumber had been grown healthily in plastic film house. Three predominant bacterial isolates selected were identified as *Pseudomonas fluorescens*, and *P. putida*, *Serratia* sp. and three fungal isolates were *Gliocladium* sp. *Trichoderma harzianum*, and T. *viride*. Antagonistic bacteria inhibited 26-45% of germination and 41-56% of germ tube elongation of microconidia of F. *oxysporum* f. sp. *cucumerinum* on Water Agar (WA). *P. fluorescens* was the strongest inhibitor. Several mycoparasitisms were observed on dual culture of WA between antagonistic fungi and *F. oxysporum* f. sp. *cucumerinum* such as coiling, penetration, overgrowing, and lysis. Mycelial lysis of the pathogen was the most severe at pH 4.6, followed by 3.6, 5.6 and 6.6 of the medium in decreasing order. At pH 6.6, mycelia of the pathogen were not conspicuously damaged, however, the antagonistic fungi formed abundant chlamydospores especially *Gliocladium* sp. *T. harzianum* revealed the most excellent antagonism *in vitro*.

Key words: *Fusarium oxysporum* f. sp. *cucumerinum*, modified triple layer agar technique, antagonism of bacteria and fungi.

要　　約

晋州近郊와 琴山, 南旨 등의 17개 오이連作栽培地域에서 健全植物의 根圈土壤으로부터 Triple Layer A

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gar (TLA)法을 變形改良하여, 오이덩굴쪼김病菌에 대한 拮抗菌을 效果的으로 分離하였고, 豫備實驗(*in vitro, in vivo*)結果에서 拮抗力이 우수한 細菌(15 *isolates*)과 곰팡이(9 isolates)를 選別하여 同定하였다. 이들 중 *Serratia* sp., *Pseudomonas fluorescens*, *P. putida* 등 細菌 3균주와 *Gliocladium* sp., *Trichoderma harzianum*, *T. viride* 등 곰팡이 3균주를 供試하여 얻은 室內實驗의 結果는 다음과 같다. Water Agar (WA) 상에서 拮抗細菌에 依한 오이덩굴쪼김病原菌의 小形分生胞子의 發芽率은 26～45% 抑制 되었으며, 發芽管의 길이도 41～56% 抑制되었는데, *P. fluorescens* 가 그 중 가장 우수한 拮抗力을 나타내었다. WA 상의 對置培養에서 拮抗곰팡이의 菌糸가 本病原菌의 菌糸를 Coiling, Penetration, Overgrowing, Lysis 하는 등의 拮抗現象을 觀察할 수 있었으며, 그 중 *T. harzianum*이 가장 強力한 拮抗力을 나타내었다. 培養基의 pH別 拮抗곰팡이에 依한 오이덩굴쪼김病菌의 Lysis 정도는 pH 4.6에서 가장 높았으며 다음은 3.6, 5.6, 6.6의 順이었는데 pH 6.6에서는 拮抗現象이 잘 나타나지 않았으나, 拮抗곰팡이의 厚膜胞子가 多量 形成되었다.

INTRODUCTION

Cucumber wilt caused by *Fusarium oxysporum* f. sp. *cucumerinum* Owen is the most troublesome obstacle impeding high yield and good quality of the crop being cultivated especially in plastic film house (3, 4, 5, 12). This pathogen invades the host xylem of roots and stems and produces disease primarily by interfering with the upward movement of water through the xylem. Symptoms consist of epinasty, plugging and browning of xylem vessels, necrosis, wilting, and finally death of the plant (14).

Such a soil borne disease has been induced to epidemic level mainly by continuously intensive monocropping practices due to the shortage on arable land in Korea (3, 5, 20). Current commercial practices in soil disinfection are accomplished through such a drastic means as fumigation or steaming, but are limited by many negative side effects such as pesticide residues, need for complicate equipment and high cost, in addition to these, rapid reinfestation by the pathogen through the resultant antagonists-free biological vaccum (11, 16), which has further rendered favorable selectively for pathogen to proliferate by host cropping. Contrarily, biological control of soil borne disease is generally accepted offering answers to many serious problems and is an essential component of sustainable cultivation capable of continuing without interruption or diminution (1, 7, 20).

The approaches were made to develop the efficient isolation techniques of antagonists from soil, identify the antagonists, and determine antagonisms of the isolated antagonists *in vitro*.

MATERIALS AND METHODS

Collecting soil as a source of antagonists. Soils were collected from 17 commercial greenhouse fields of Jin-ju, Geum-san, and Nam-ji. Samplings were made from the rhizosphere at 5～15cm in depth, where cucumber plant were growing healthily in the plastic film house. The soil samples were stored in cotton bags at room temperature for later isolation of antagonists to *Fusarium oxysporum* f. sp. *cucumerinum* Owen.

Modified Triple Layer Agar (TLA) Technique for the isolation of antagonists. Initial isolation of antagonistic bacteria to *F. oxysporum* f. sp. *cucumerinum* was carried out through modified TLA technique (Fig. 1A) (20).

The procedures for the isolation of antagonistic fungi with developed modified (TLA) technique were as follows. Ten ml of Water Agar (WA) was poured onto the bottom of petri plate, 9 cm in diameter, as a foundation layer to maintain moisture for a long period of incubation and to allow even distribution of second layer. After WA was solidified, ten ml of Peptone Dextrose Rose-bengal Agar (PDRA) cooled to 50°C and 0.5 ml of 1: 10,000 soil dilutions were mixed thorougly prior to over-spread as a second layer. Colonies of the soil fungi were formed on the surface of PDRA in five days incubation at 25C. After fungal colonies were formed on PDRA, 10 ml of molten PDA containing

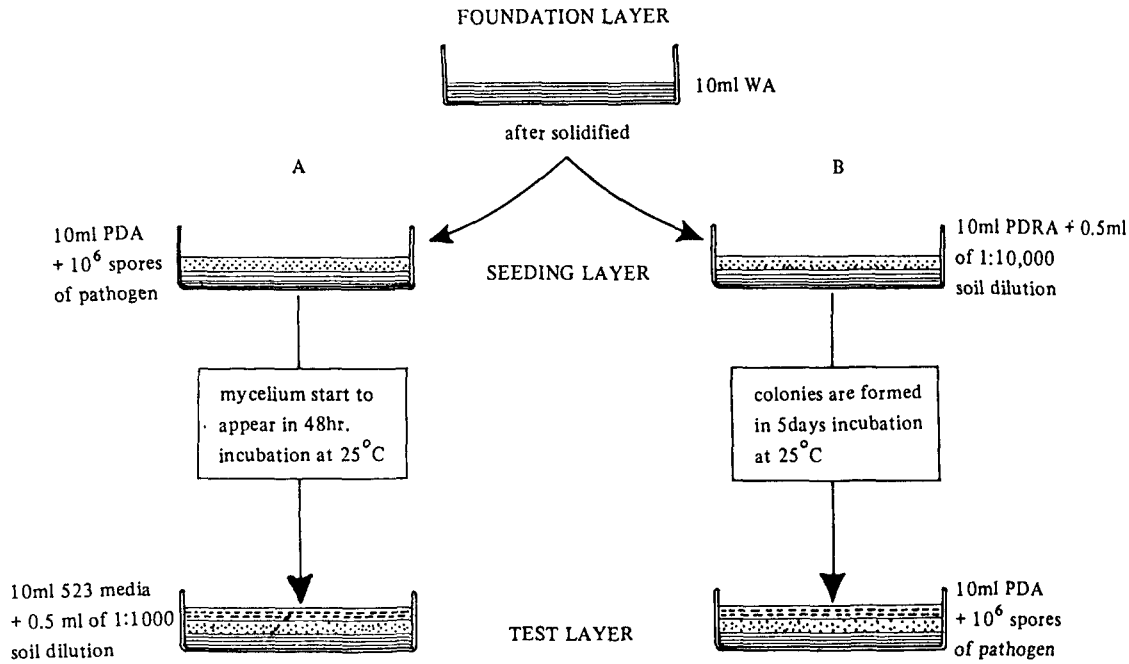

Fig. 1. Diagram of the technique used for the isolation of antagonists to *Fusarium oxysporum* f. sp. *cucumerinum* Owen. A: Method for antagonistic bacteria. B: Method for antagonistic fungi.

10^6 microconidia of *F. oxysporum* f. sp. *cucumerinum* was over-spread as a third layer (Figure 1.B). The mycelium of the soil fungi grew continuously and penetrated through the third layer and the mycelium of pathgen also grew on the surface of the medium. Some of antagonistic fungi apparently inhibited the mycelial growth or lyzed mycelia of the pathogen, resulting in clean zones around antagonistic fungi on the surface of the third test layer (Fig. 2 B). The antagonistic fungal colonies with clean zone around were isolated for later study.

Identification of antagonists. *Antagonistic bacteria:* Key characteristics of the antagonistic bacterial isolates were tested to characterize them taxonomically. The taxonomic schemes and criteria for identification of bacteria were followed Bergey's Manual of Systematic Bacteriology Vol. 1. (15). The recipes of media and detailed procedures for testing bacterial characteristics were mostly followed the method of Kado (10) and Collins and Lyne (6).

Antagonistic fungi: The taxonomic schemes and criteria for identification of antagonistic fungi were followed the method of Rifai (21) and Domsch and Grams (8). Isolates selected as antagonistic fungi were cultured on 7 ml of WA per plate, 9 cm in diameter, at 25C incubator under 60 watts cool white fluorescent light illumination for 5 days. The morphological characteristics examined were conidiophore branching system, side branch, phialide, phialospore size, and sterile hyphal elongation.

Germination and germ tube elongation of microconidia of *F. oxysporum* f. sp. *cucumerinum*. Three loopful of selected antagonistic bacteria were transferred into ten ml of distilled water containing 10^6 microconidia of the pathogen and mixed thoroughly by voltex mixer. Each of a 0.2 ml of the mixture was spread onto triplicate WA media and incubated for ten hours at 25C. Germination rate of the microconidia and germ tube length were examined under a light microscope at random ten microscopic fields per plate at 600x magnification.

Hyphal interactions between antagonistic fungi and F. *oxysporum* f. sp. *cucumerinum*. The cellophane (3 x 8 cm), disinfected with 95% ethanol, was placed on WA aseptically. Small portions of PDA block carrying each of antagonistic fungi and

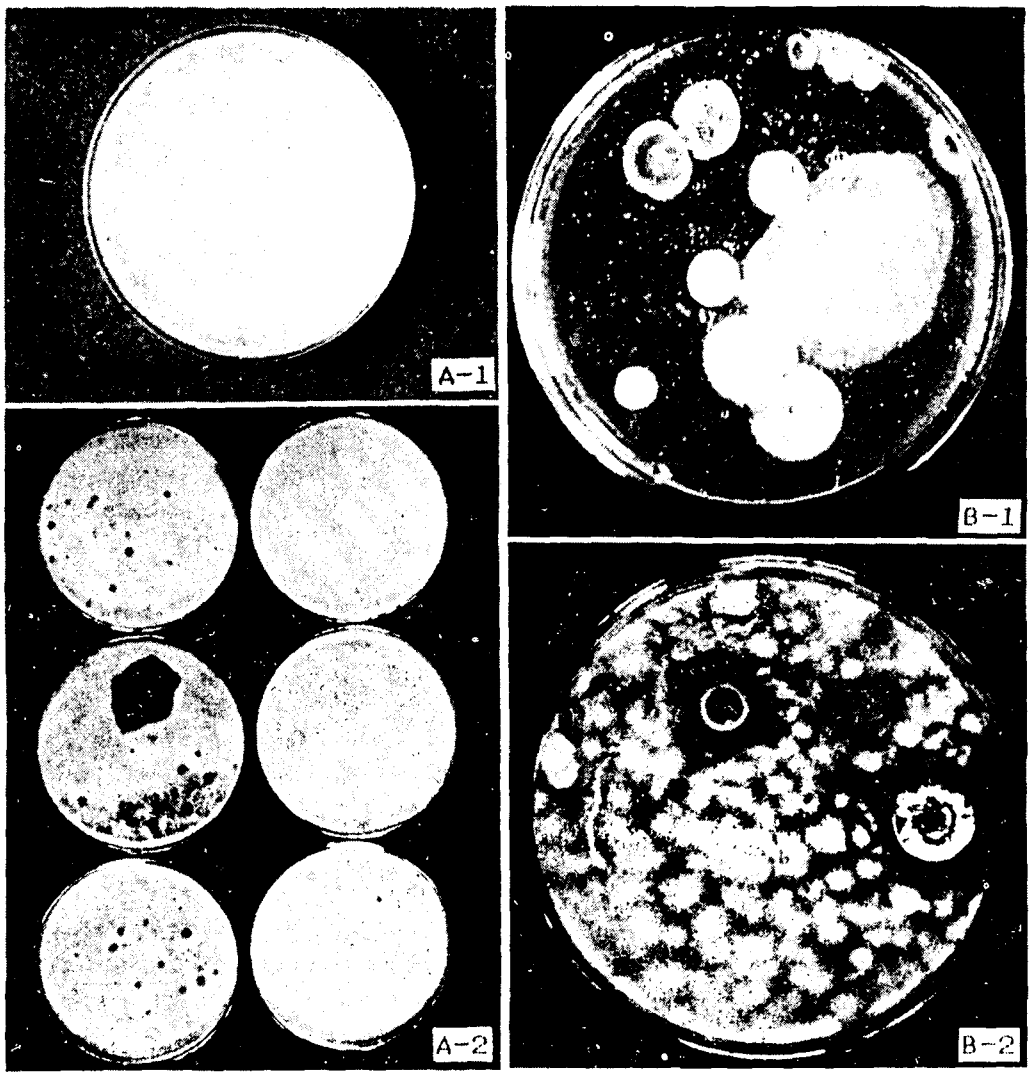

Fig. 2. A-1: Mycelium of *Fusarium oxysporum* f. sp. *cucumerinum* started to appear on the surface of the second layer agar. A-2: Antagonistic bacterial plaques formed on the surface of TLA. B-1: Colonies of the soil fungi formed on the surface of PDRA as the second layer. B-2: Clean zones formed around the antagonistic fungi on the surface of TLA.

cucumber wilt pathogen were placed on opposite sides for dual culture. After 7 days hyphal contacts of the two fungi, cellophane was transferred from WA medium to slide glass for microscopic examination. Hyphal interactions were clearly observed under the light microscope at 600x magnification.

To examine the mycelial lysis of the pathogen by antagonistic fungi in response to pH of WA, following criteria were used to generalize the lysis degrees (Fig. 3).

RESULTS AND DISCUSSION

From the bacterial plaques and fungal colonies with clean zone around in cultures of cucumber wilt pathogen on modified TLA (Fig. 2 A, B), scores of bacteria and fungi antagonistic to *F. oxysporum* f. sp. *cucumerinum* were effectively isolated. The advantage of this technique was that one could work only with the isolates involved in antagonism or competition, eliminating numerous other microorganisms.

Table 1. Comparison of general characteristics between antagonistic bacterial isolates obtained from modified triple layer agar with three species of bacteria listed in Bergey's Manual of Systematic Bacteriology Vol. 1 (15)

Characteristics	*Serratia marcescens* vs.	B1	*Pseudomonas fluorescens* vs.	B6	*Pseudomonas putida* vs.	B7
Motility	+[a]	+	+	+	+	+
Cell shape	Rod	Rod	Rod	Rod	Rod	Rod
Gram reaction	−	−	−	−	−	−
O/F test	O/F	O/F	Oxid.	Oxid.	Oxid.	Oxid.
Pigment	Pink, Red	Red	Fluor.	Fluor.	Fluor.	Fluor.
Oxidase	−	−	+	+	+	+
Denitrification	+	+	d	−	−	−
Gelatin liquefaction	+	+	+	+	−	−
Catalase	+	+	+	+	+	+
Levan formation	−	+	d	+	−	−
Starch hydrolysis	−	−	−	−	−	−
Arginine hydrolysis	−	−	+	+	+	+
Utilization of						
Glucose	+	+	+	+	+	+
Lactose	−	−	−	−	−	−
Cellobiose	−	−	−	−	−	+
Sucrose	+	+	d	−	d	−
Trehalose	+	+	d	+	−	−
Arabinose	−	−	−	−	−	−
Ethanol	d	+	d	−	d	+
Mannitol	*	+	d	+	d	−
Galactose	*	+	d	+	d	+

[a] + : Positive reaction.
− : Negative reaction.
O/F : Oxidative and/or fermentative respiration.
d : Reactions are varied depending on biovar or isolates.
* : No mention.

Identification of antagonists. *Antagonistic bacteria:* Isolate B1 was gram-negative, straight rod, oxidase negative, and catalase positive. These were discriminative characteristics for separating Enterobacteriaceae from other families of Facultatively anaerobic gram-negative rods. The characteristics of red color pigment, positive liquefaction of gelatin at 22 C and utilization of sucrose, and many other tests so far carried out were consistent with those of *Serratia marcescens.* (Table 1). However, further and intensive studies should be conducted in order for this isolate B1 to be identified definitely as *S. marcescens.*

Isolates B6 and B7 diffused typical fluorescent pigment on King's B medium but did not produce any other pigment. This characteristic was definitely different from other families of Gram negative aerobic rods. The key characteristics to discriminate B6 and B7 from plant pathogenic fluorescent pseudomonads group were oxidase positive and arginine dehydrolysis positive (22).

The distinctive differences between *P. fluorescens* and *P. putida* are listed in Bergey's Manual of Systematic Bacteriology Vol. 1. (15) as follows: *P. fluorescens* has the ability of gelatin hydrolysis, levan formation from sucrose, and utilization of trehalose, but *P. putida* does not have those characters. According to Stanier et al. (24), no isolate of *P. putida* revealed denitrification, gelatin liquefaction,

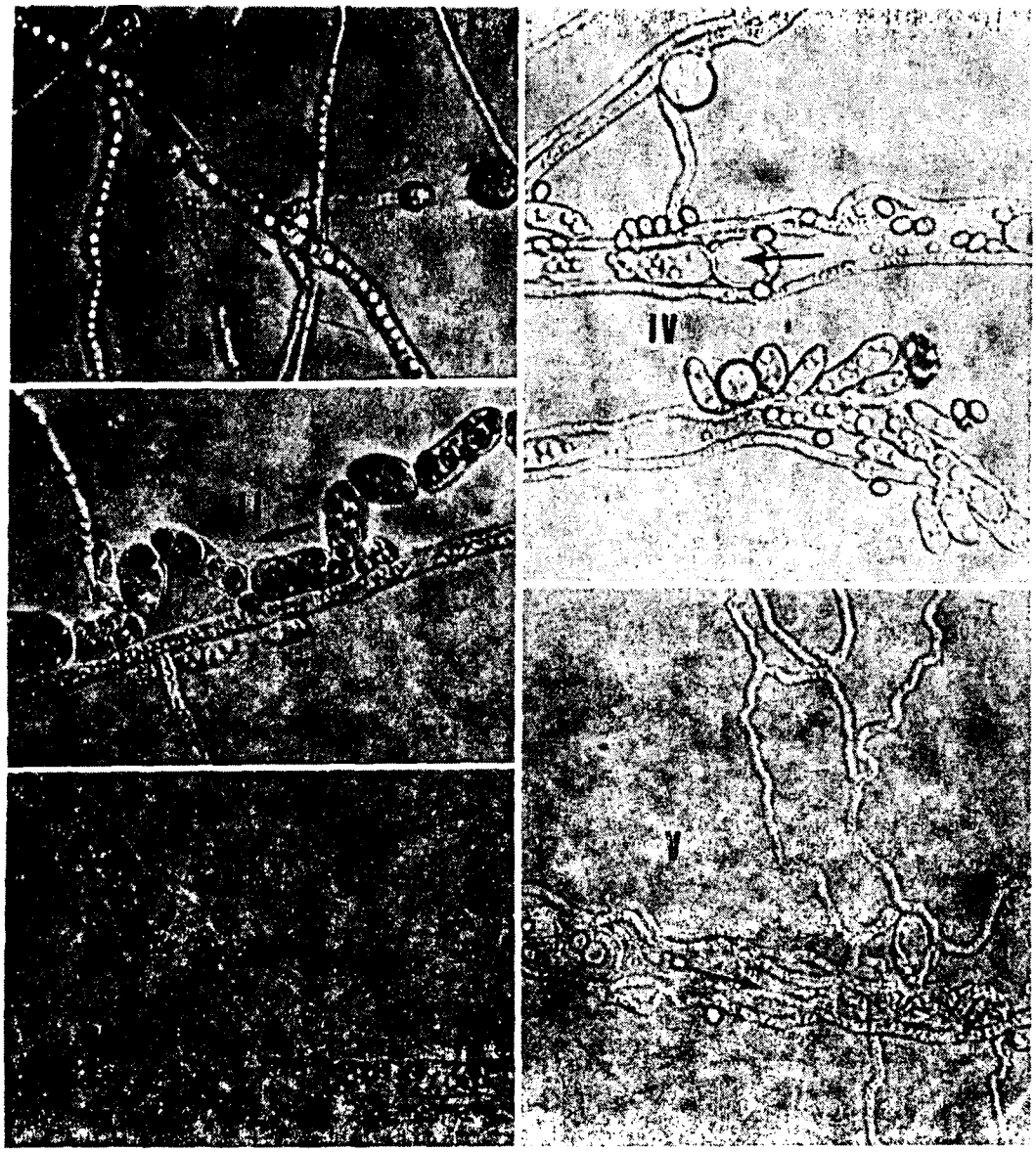

Fig. 3. Illustration of degrees of the mycelial lysis of *Fusarium oxysporum* f. sp. *cucumerinum* by antagonistic fungi (x400). I: Mycelia not lyzed. II: Not lyzed but damazed. III: Lysis ranged 0-30%. IV: Lysis ranged 30-70%. V: Mycelia lyzed over 70%. → : Hypha of cucumber wilt pathogen.

levan formation, and trehalose utilization, which were distintive characteristics for isolate B7 to be identified as *P. putida* in this study. The characteristics of B6 were well consistent with those *P. fluorescens* (Table 1).

As the results of above characterization procedures, three antagonistic bacteria selected were identified as *Serratia* sp. *P. fluorescens*, and *P. putida*, which had been known as bacterial antagonists and subjects of a lot of investigations for the control of soil-borne disease (1, 7).

Antagonistic fungi: The hyphomycete genus *Trichoderma* was characterized by fast growing hyaline colonies, later turning green, bearing branched conidiophores repeatedly in tufts with divergent, often irregularly bent, and flaskshaped phialides. Conidiophores were hyaline or green, short one celled globose, and less than 15 μm in diameter. Hyaline chlamydospores were usually present in the mycelium of older culture (21).

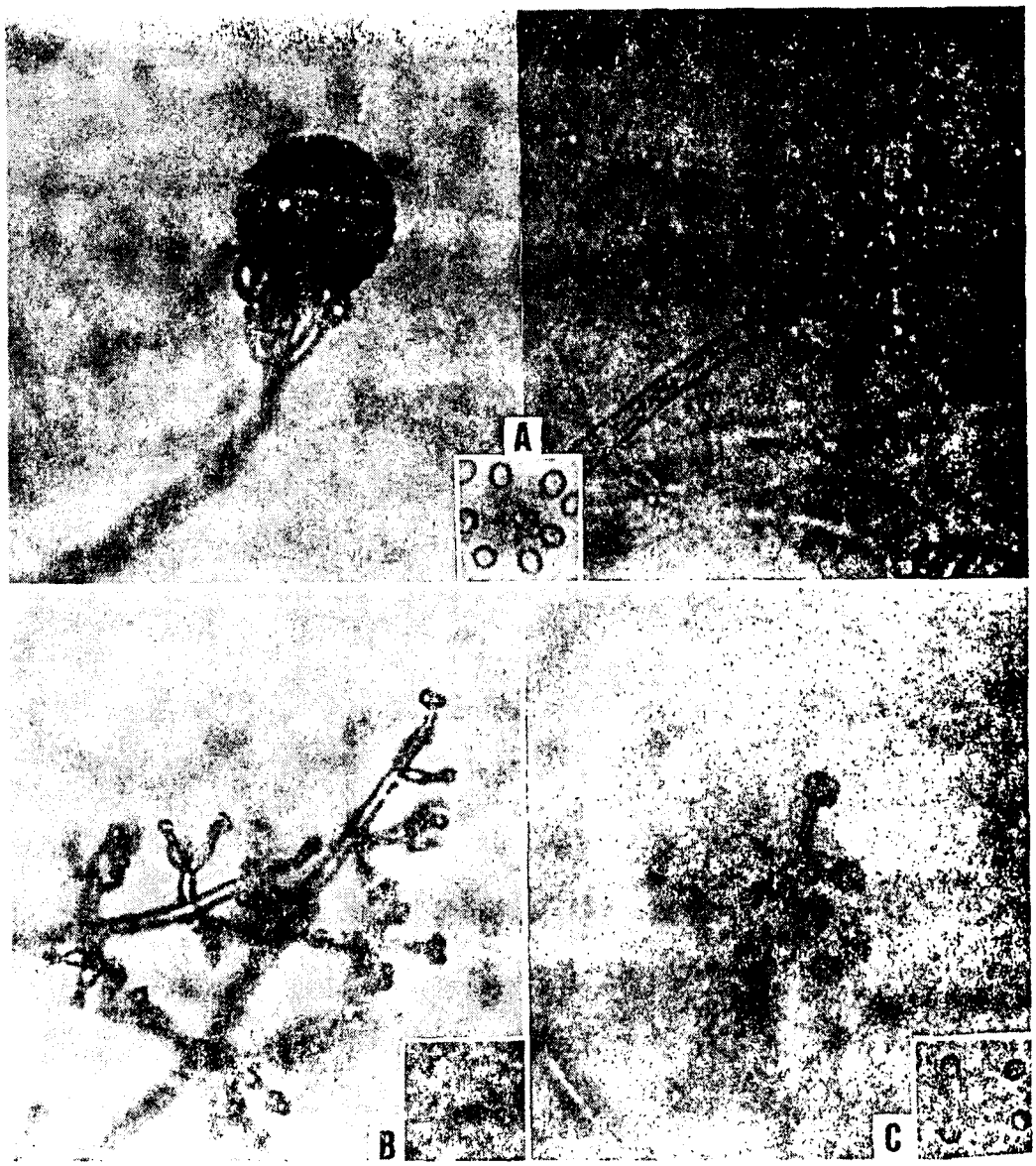

Fig. 4. Morphological characteristics of conidiophores with phialides and conidia of the three antagonistic fungi from modified TLA (x400). A: F3, *Gliocladium* sp. B: F8, *Trichoderma harzianum* C: F9, *T. viride*.

The antagonistic fungal isolate F8 was identified as *T. harzianum* with complicate dendroid branching system of conidiophores, regularly disposed phialides, and globose or short obvoid phialospores smaller in their size (21) (Fig. 4B).

Isolate F9 was identified as *T. viride* with long and slender conidiophores and their side branches without having elongated sterile hyphae, phialides not crowded but rather slender, and its coconut-like smell (21) (Fig. 4C).

Isolate F3 was identified as *Gliocladium* sp. with their discriminative features of densely penicillated conidiophores bearing slimy one celled hyaline, smooth walled conidia in heads. The colums with closely borne side branches supported a big conidial ball (Fig. 4A) (8). The genus *Gliocladium* was not delineated satisfactorily, because the species concept within the genes was not well estalished.

Inhibition of germination and germs tube elonag-

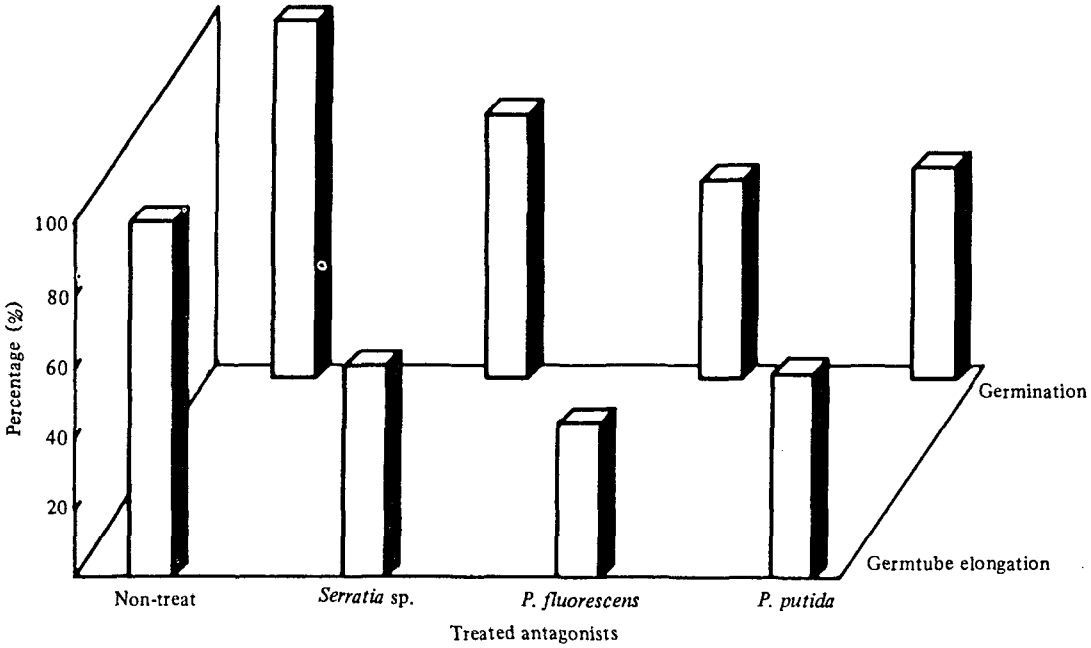

Fig. 5. Relative value of germ tube elongation and germination rate of the microconidia of *Fusarium oxysporum* f. sp. *cucumerinum* combined with antagonistic bacteria on the water agar after ten hours incubation at 27 C.

tion of microconidia by antagonistic bacteria. Germination and germ tube elongation of microconidia of *F. oxysporum* f. sp. *cucumerinum* were greatly inhibited by three isolates of antagonistic bacteria. Figure 5 indicates the relative values of inhibition in germination and germ tube elongation. *P. fluorescens* revealed the most strong inhibition both in germination and in germ tube elongation about 50 percents on WA medium in ten hours incubation at 27 C.

Baker and Cook (1) mentioned the importance of bacteria as antagonists especially against Fusarium and others that produced germ tube and caused root rot by multiple infection. Fluorescent Pseudomonads might intercept sufficient numbers of pathogen germlings by means of producing siderophores and other substances which were thought to inhibit the pathogen and the deleterious rhizobacteria (13, 23), rendering ecosystem to be suppressive to disease incidence.

Mycoparasitism of antagonistic fungi. After 7 days of hyphal contact between antagonistic fungi and *F. oxysporum* f. sp. *cucumerinum* on dual culture of WA, various hyphal interactions were observed under the light microscope at 600 x magnification.

Coiling (Fig. 6A): Main pathogenic fungal hyphae was tightly coiled by two or more slend hyphae of *Trichoderma harzianum.*

Penetration (Fig. 6B): Slender hyphae of *viride* penetrated directly into the thick pathogen fungal hyphae and formed haustorium.

Lysis (Fig. 6C): Slender parasitic hyphae of *harzianum* were grown along with host hypha breaking down and disolving the host hyphal cel by secreting some antifungal substances.

Over growing (Fig. 6D): Parasitic hyphae of *harzianum* were closely adhered to and growin along with the host hyphae, sometimes develope side branches penetrating the host hyphae.

In types of hyphal interactions described a above, there were not much dissimilarity betwee three antagonistic fungi and the pathogen. Howeve the modes of mycoparasitism such as coilling, pene tration, and overgrowing were not often observed on WA, while lysis was frequently observed. Therefore, it was suggested that those mycoparastisms were influenced by nutritional conditions.

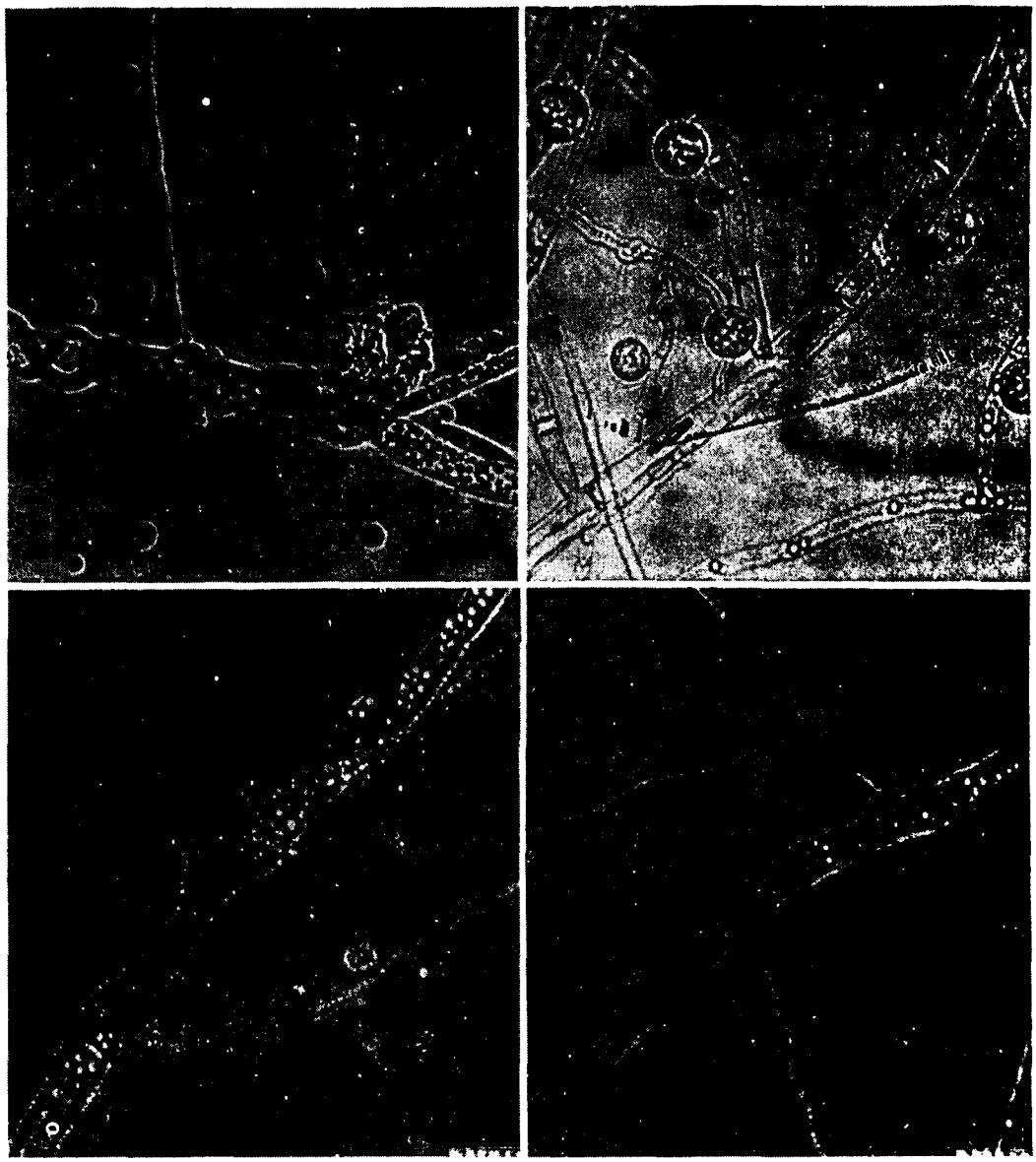

Fig. 6. Mycoparasitism of antagonistic fungi to *Fusarium oxysporum* f. sp. *cucumerinum* (x400). A: Coiling B: Penetration, C: Lysis, D: Overgrowing. *—: Hypha of cucumber wilt pathogen.

Mycelial lysis of *F. oxysporum* f. sp. *cucumerinum*. *T. harzianum* revealed the strongest antagonistic effect on agar medium out of three species, followed by *T. viride* and *Gliocladium* sp. in decreasing order. Degree of mycelium lysis by three antagonistic fungi was dependent on pH of cultural media. Mycelia of the cucumber wilt pathogen were most severely lyzed at pH 4.6, almost equivalent to or slightly less at pH 3.6. Only a small portion of lysis was observed at pH 5.6, and no lysis or slight damaged mycelium at pH 6.6 (Table 2). However, at this pH the antagonistic fungi produced abundant chlamydospores, especially for *Gliocladium* sp.

Many investigators indicated that the efficiency of biological control with antagonists was largely influenced by soil pH. Opgenorth and Endo (18) and Yuen et al. (25) observed slightly alkaline pH was favorable to disease control in soil and inhibited mycelial growth of *Fusarium oxysporum* by an-

Table 2. Mycelial lysis degrees of *Fusarium oxysporum* f. sp. *cucumerinum* by antagonistic fungi in response to pH on dual culture of water agar at 7 days after hyphal contact[a]

pH of WA	Antagonists		
	Gliocladium sp.	*T. harzianum*	*T. viride*
3.6	II, III[b]	III, IV	III, IV
4.6	III	IV, V	IV
5.6	II	III	III
6.6	I	I, II	I, II

[a] Index of mycelial lysis representing 100 random observations at 600x magnification.

[b] I : Mycelia not lyzed, approximately normal condition.
II : Mycelia not lyzed but damaged; just prior to lysis.
III : A small portion of lyzed mycelia; ranged from 0% to 30%
IV : A great portion of lyzed mycelia; ranged from 30% to 70%.
V : Approximately all lyzed mycelia; over 70% lysis.

tagonistic bacteria *in vivo*. On the other hand, the activity of antagonistic fungi, *Trichoderma* spp. was affected by acid conditions in reducing disease incidense (2, 9, 17).

Therefore, it was considered that the severe mycelial lysis of the pathogen at pH 4.6 was the favorable pH for the growth of antagonistic fungi. The lysis was not observed at pH 6.6, which was favorable condition to the pathogen but might not have been acidic enough for the antagonists to be effective.

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