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Impact of Rhizosphere Competence of Biocontrol Agents upon Diseases Suppression and Plant Growth Promotion

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ABSTRACT : Root colonization of biocontrol agents via seed treatment was investigated and a compatible combination, *Gliocladium virens* G872B and *Pseudomonas putida* Pf3, in colonizing cucumber rhizosphere was confirmed through the study. Much higher number of fungal and bacterial propagules were detected when two isolates were inoculated together. The presence of Pf3 in root system was greatly helpful to G872B to colonize at root tip. The mechanism of this phenomenon is partially elucidated through the results of *in vitro* experiments and the observations of scanning electron and fluorescence microscope. Addition of Pf3 cells resulted earlier germination of G872B conidia and increased mycelial growth. And the more number of germinated conidia on seed coat, the more vigorous hypal streching and sporulation on the root surface were observed in coinoculated treatment. The propagules of G872B on the cucumber root when they were challenged against the pathogenic *Fusarium oxysporum*, were even higher than that of G872 treated alone, and the magnitude of such a difference was getting greater toward the root tip and the population of *F. oxysporum* on the root was reduced by seed inoculation of G872B. The rhizosphere competence was obviously reflected to disease suppression and plant growth promotion that induced by the given isolate. Green house experiments revealed that the combined treatment provided long-term disease suppression with greater rate and the larger amount of fruit yield than single treatments. Through this study the low temperature growing *Pseudomonas fluorescens* M45 and MC07 were evaluated to apply them to the winter crops in field or plastic film house. *In vitro* tests reveal that M45 and MC07 inhibited the mycelial growth of *Pythium ultimum*, *Rhizoctonia solani* and *Phytophthora capsici* and enhanced growth of cucumber cotyledon in MS agar. This effect was more pronounced when the bacteria were incubated at 14℃ than at 27℃. And disease suppression and plant growth promotion in green house were also superior at low temperature condition. Seed treatment of M45 or soil treatment of MC07 brought successful control of damping-off and enhanced seedling growth of cucumber. The combined treatment of two isolates was more effective than any single treatment.

Over the past seventy years biological control of soil-borne plant diseases has been studied purposely (16). During most of that time it has not been considered commercially feasible. Only in the last two decades a renewed interest in biocontrol of diseases in horticultural and field crops evolved (3, 6, 17, 20, 32, 52). Currently, with increasing awareness of possible deleterious effects of agricultural chemicals on the ecosystem, the interests in clean and harmless agricultural products are growing (3, 23, 50, 59). But the alternative measures for controlling soil borne diseases are not practically available and breeding program for disease

resistance requires time consuming. Consequently the biological control of soil-borne diseases has received considerable attention (17, 32, 59).

Biological control of soil-borne plant pathogens can be achieved by treatment of antagonists to seeds (4, 6, 9, 21, 40). Seed treatment is an attractive method for delivering biocontrol agents to ecological niche. Cook and Baker (16) indicated that any root colonizing organisms given the advantage of being the first to colonize the root, as may occur with seed treatment, have the potential to preempt the nutrient supply of pathogen. Antagonists applied to seeds may have the opportunity to be the first colonizers of roots (6).

Weller and Cook (61) reported that treatment of winter wheat seeds with fluorescent pseudomonads, which protected against take-all in soils infested with *Gaeumannomyces graminis*, resulted in yield increases to 27% in field trials. Numerous instances in which bacteria inoculated onto plant seeds and roots have been reported to enhance plant growth (12, 23, 28, 34, 57, 60). Ahmad and Baker (1, 2) improved a method for seed application of *Trichoderma* isolate that previously rhizosphere incompetent on roots. However, compatible protection of the mature plant has not been realized because biocontrol agents were not rhizosphere competent (35, 44). Chao *et al.* (13) reported that the unsuccessful establishment of *T. harzianum* in pea rhizosphere. Papavizas (43) reported the failure of *Trichoderma* to establish in rhizosphere of pea and bean.

A major concern in the application of biocontrol agent to suppress soil-borne plant pathogens is the inconsistency of disease control. Level of disease control after application of biocontrol agent varies greatly from test to test. Inefficient root colonization by introduced biocontrol agent is generally accepted as a major factor for the inconsistency of experimental results under field condtions (53 ,61). Thus the importance of root colonization by introduced biocontrol agent for disease control was recently emphasized (10, 18, 29, 40).

Curl (2) and Rovira *et al.* (84) defined the concept of "the rhizosphere" as a narrow zone of soil subject to influence of living roots, which is the leakage or exudation of substance that promote or inhibit the microbial activity. The rhizosphere is the source of releasing a wide variety of organic materials which serve as a food source for the microorganism and competition for substrate utilization in intense (16, 18, 49). Many investigators (23, 45, 49, 60) suggested that bacteria, fungi and actinomycetes are commonly distributed more in the rhizosphere than soil away from roots. To describe root colonizing abilities of microbial strains, the terms colonization potential (6) and rhizosphere competence (1) are used to differentiate between strains. Schmidt (54) described the concept "rhizosphere competence" as an attribute of rhizobia characterized by their consistent association with legume root nodules, and suggested that the key property of antagonistic agent in biological control of soil-borne pathogen is rhizosphere competence. Ahmad and Baker (2) modified the concept "rhizosphere competence" to describe the ability of microorganism to grow and function in the rhizosphere and the concepts described the relative root-colonizing ablilty of a microorganism. Generally, both terms are used to compare strains on their root colonization by quantification of the numbers of colony forming units they establish on the root system

per unit of root, such as length, weight, number of roots, and etc.

Fluorescent *Pseudomonas* spp. are known to suppress a wide variety of plant pathogens, including deleterious microorganisms (53, 61). Interest in the application of fluorescent pseudomonads as biocontrol agents to root pathogens has been increasing during the past several years (10, 12, 14, 19). Also, fluorescent pseudomonads seem to contribute to plant growth promotion and yield increase (12, 17, 34). The possible mechanisms of disease supprression involved the competition for substrates (4, 12), or for iron by siderophores (35, 53), antibiotic production (9) and induced resistance (23).

Trichoderma spp. and *Gliocladium virens* are the typical fungal biocontrol agents that successfully inhibit *Pythium, Rhizoctonia, Sclerotium, Phytophthora.* and many reports indicated these fungi induced plant growth stimulation (14, 15, 24, 40, 56). Of antagonistic fungi, species of *Trichoderma* (2, 8, 14, 21, 40) and *Gliocladium* (21, 26, 43) are known as favorable biocontrol agents for the control of soil-borne disease. Sivan and Chet (56) reported that *T. harzianum* significantly reduced disease incidence of vascular wilt caused by various formae specialis of *F. oxysporum.* It was also effective against *R. solani* and *Sclerotium rolfsii* attacking seedlings. Recognized mechanisms for biocontrol of soil-borne pathogens by *Trichoderma* spp. and *Gliocladium* spp. were direct parasitism or hyperparasitism, production of antibiotics and competition for limiting nutrient or infection site (44, 56).

It is no doubt that knowledge of the colonization capabilities of biocontrol agents and scientific information on the stability of the populations in the rhizosphere are essential to understand microbial interaction and for targeting specific strains against specific pathogens.

Cultivation of vegetables and ornamental crops in Korea as well as many other countries in temperate zone have been changed in many ways for the social demands and the economical purposes (15, 17, 30, 50). During the winter time, the profitable crops are cultivated in plastic film house and in hot summer time, cruciferous families and some vegetable crops are planted in cool alpine area. This kind of farming practices become prevailing with development of agricultural technology. However, such a cropping system frequently result the accumulation of soil pathogens and lead to serious disease problems (17, 33, 50).

The unfavorable environmental conditions in extra crop season, especially the low temperature, frequently cause more severe disease problems and predispose the plant more vulnerable to deleterious microorganisms than that of in ordinary crop season. Although some investigations of biological control or plant growth promotion had been conducted under the low temperature condition (11, 36, 60), most of the works have been demonstrated in warm weather or in greenhouse. Survival and proliferation of antagonist after introduction into the soil or host rhizosphere are prime requirements for effective biocontrol of soil-borne pathogens (5, 14, 29, 45). Therefore researchers have looked for the isolates which colonize and/or survive readily in the host root zone under the given environmental conditions.

In this paper, the author tried to elucidate the impactness of rhizosphere competence of antagonists for biological control of soil-borne diseases and the compatible colonization

of fungal and bacterial biocontrol agents that can improve the root colonizing potential of each isolate. The study also tried to describe the disease suppression and plant growth promotion induced by low temperature growing agents under the low temperature condition.

Results and Discussion

Assessment of rhizosphere competence of fungal biocontrol agents. The ability of rhizosphere colonization of fungal biocontrol agents was assayed by Ahmad and Baker's methods (1) and DLF method (5) which developed in our laboratory. Generally, the root colonizing patterns analyzed by two different methods were not greatly differ (Fig. 1). However, the population densities detected by DLF method were much higher than Ahmad and Baker's method regardless of root colonizing abilities of test isolates. When the rhizosphere incompetent isolate *Trichoderma hamatum* T8 was inoculated to cucumber seed, fungal population was detected only from upper 5 cm root segments. On the other hand, rhizosphere competent isolate *Trichoderma harzianum* T95 and *Gliocladium virens* G872B were detected from all depth of the root. Higher population densities were detected from the first 1-cm and the last 1-cm root segment and decreased in the midportions of the root, consequently formed a C-shaped curve in both assay methods (Fig. 1-A, B).

For the proper assessment of rhizosphere competence of fungal biocontrol agents, simple and convenient assay method is needed. Although Ahmad and Baker's method has provided fascinating approach to look into the real nature of rhizosphere competence (2, 30), it still does not allow to test a large number of promising biocontrol agents. Their method requires selective marker and media for enumerating population densities of test microorganisms in advance. Therefore, we have established a simple method which allows rapid screening

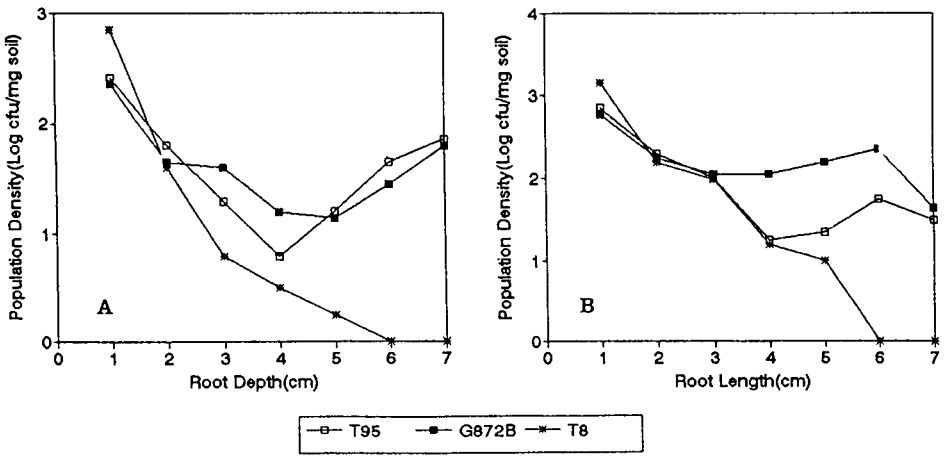

Fig. 1. Population densities of *Trichoderma harzianum* T95, *T. hamatum* T8 and *Gliocladium virens* G872B were detected by Ahmad & Baker's method (A) from 1 cm-root segment of cucumber rhizosphere soil and DLF method (B) from 1 cm segment of rhizoplane.

for massive microbial resources from nature. Selective marker and media are not required for enumerating propagules of test microorganisms in our method, which allows us to assay within only 78 hours compared to 8 days for Ahmad and Baker's method. The results obtained by double layered filter paper method (DLF method) revealed similar pattern with those by Ahmad and Baker's method. When two results were compared (Fig. 1), rhizosphere competent isolates revealed higher population densities, as many as 5 times at the top portions (1~3 cm) of root and 10 times at the root tip. Furthermore DLF method also allows to assess the root colonizing ability of bacterial biocontrol agents and combined treatment of fungal and bacterial agents.

Compatible colonization of *G. virens* G872B and *P. putida* Pf3. The compatible relationship between *G. virens* G872B and *Pseudomonas putida* Pf3 in root colonization was confirmed by DLF method. G872B and Pf3 were applied to the cucumber seeds and root colonizing populations were examined in terms of time sequence (Table 1) and spatial differences of root (Fig. 2). The population density of G872B in the treatment coinoculated with Pf3 was slightly higher than that of without Pf3 from the beginning. The differences between two treatments were getting greater with the lapse of time. The root colonizing population of Pf3 was rapidly increased after 48 hrs and the Pf3 population in the treatment of combined with G872B was sharply increased after 48 hrs, hence the population density at 72 hrs was over than double of Pf3 population that without inoculation of G872B (Table 1).

The root colonizing population densities of G872B and Pf3 at 72 hrs after seed inoculation were detected on the basis of spatial differences of cucumber root. Fig. 2-A shows the colonizing population of Pf3 alone and combined with G872B, and Fig. 2-B shows the colonizing population of G872B alone and combined with Pf3. At the all portions of cucumber root, the population density of combined treatment was much higher than single treatment of G872B or Pf3. Especially, the population densities of Pf3 and G872B at root tip were greatly increased in the treatment of coinoculation. The population density of Pf3 at root tip was higher as much as 3 log unit than that of single treatment. As same the manner, more than 100 cfu of G872B was detected from 1cm root tip in coinoculated treatment whereas no propagules were detected in single treatment (Fig. 2-B).

In this experiment, we have provided the evidence of synergistic effect in the co-treatment

Table 1. Population densities of G872B and Pf3 colonized on cucumber root detected by DLF method 3 days after seed inoculation of the isolate

Detection Time	G872B(cfu 10^3/root)		Pf3(cfu 10^4/root)	
	alone	combined	alone	combined
0 hr	14.6	15.2	28.6	24.6
24 hr	18.6	19.2	30.4	28.1
48 hr	18.6	21.2	99.1	117.9
72 hr	35.8	42.4	243.2	462.6

The initial population density of G872B was 10^5 conidia/seed, Pf3 was 10^6 cells/seed.

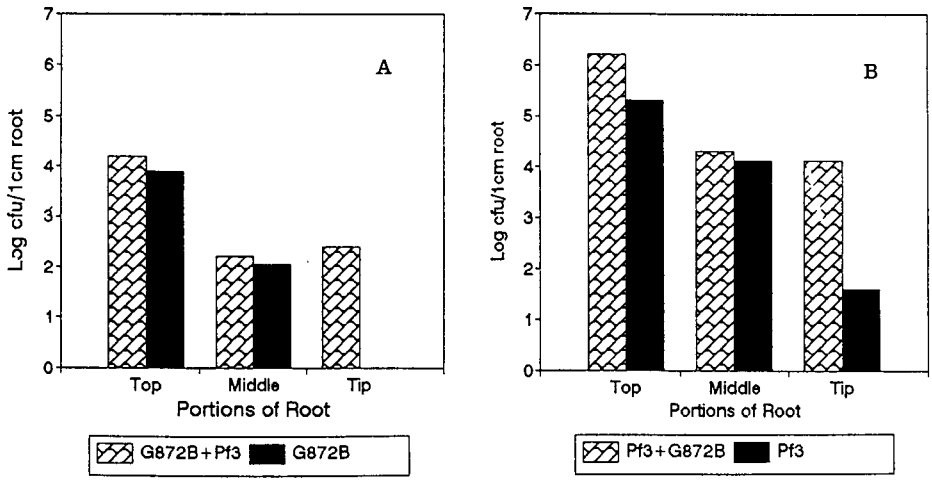

Fig. 2. Population densities of *P. putida* Pf3 and *G. virens* G872B in the rhizoplane of cucumber after the seed coated and incubated at 27℃ in the growth chamber for 72 hrs. A: G872B densities coinoculated with Pf3 and G872B alone, B: Pf3 population coinoculated with G872B and Pf3 alone. The initial population density of G872B and Pf3 was 10^5/seed and 10^8/seed, respectively.

Table 2. Population densities of G872B and Pf3 in the soil adhering to the last 1cm of cucumber root tip when the seeds were coated with either G872B, Pf3 alone or coinoculation of G872B and Pf3

Treatment	Population Density (cfu×10^3/g. soil)		
	1 day*	40 days	80 days*
Sigle Treatment			
G872B	7.11	1.41	1.04
Pf3 alone	28.90	27.10	24.00
Mixed treatment			
G872B	9.74	2.50	2.30
Pf3	26.30	33.12	31.00

*Population density was determined at the first day of transplanting, 40 days and 80 days after transplanting. The initial popualtion density of G872B was 10^5 conidia/seed, Pf3 was 10^6 cells/seed.

of G872B and Pf3. Many investigators (29, 41, 46, 47, 51) have studied on the rhizosphere colonizing ability of microorganism by theoretical calculations. However, little information is available regarding biocontrol agents in the rhizoplane. This study has demonstrated that G872B and Pf3 well colonized compatibly in the rhizoplane of cucumber. Fluorescent *Pseudomonas* Pf3 and *Gliocladium* G872B also colonized well in the rhizosphere of cucumber plant in the plastic film house experiment. But population density of G872B in the rhizosphere drastically reduced after 40 days from transplanting and Pf3 gradually declined with increase of cultivation period. Exceptionally, the population density of Pf3 that coinoculated with G872B was sustained at the initial density up to 80 days (Table 2). Generally the combined treatment of G872B and Pf3 revealed higher population than that of single treatment.

Proliferation along the developing rhizosphere is one of the most important traits for biocontrol agents applied to seeds. Many studies indicated that Pseudomonads (6, 10, 12, 23, 46, 58, 60) and few Trichoderma (2, 29) isolates can colonize the growing root but they are not competible (44). Our results, however, suggest that coinoculation of Pf3 and G872B brings the more root colonizing population than use them separately. *In vitro* tests revealed that Pf3 assists mycelial stretch and spore germiantion of G872B (30). Combination of compatible bacteria and fungi provided better sustaining of root colonizing population of each isolate than separate treatment. Consequently it may provide better control of root pathogens or enhancement of plant growth than either used alone.

Chao *et al.* (10) reported that competition has been suggested to have a profound impact on succession and the composition of microbial communities. They indicated that when the seeds treated with fungal antagonist were planted in sterile soil, more fungal propagules were found in the surrounding soil than in the rhiozosphere. Conversely, the bacteria tested were always found in greater numbers in the rhizosphere than in the surrounding soil. Our results suggest that populations of bacteria and fungi may avoid competition in the rhizosphere by colonizing different niches and/or by some degree of spatial separation within the rhizosphere.

Most of the exudates present in the rhizosphere are excreted from the root tip (18, 49). Thus preoccupation of this region by biocontrol agent must be important to reduce the infection of soil-borne pathogens. The root colonization abilities of introduced biocontrol agent should include their position towards the target organism. In most biocontrol systems, the biocontrol agent has to be present and active at the site where the pathogen attacks the plant (2, 5, 29). In this respect, many attempts have been made to monitor populations of introduced strains in the rhizosphere. Most studies have been performed under non-sterile conditions, in which the introduced strain had to be marked to differentiate it from the indigenous microflora (9, 41, 48). The combined treatment of G872B and Pf3 resulted not only increased the root colonizing population but also allowed the biocontrol agent to preoccupy the functional and active region of root.

Rhizosphere colonization by *G. virens* G872B and *F. oxysporum*. *In vitro* analysis of G872B population colonized on cucumber root either with or without introduction of *Fusarium oxysporum* was indicated in Fig. 3. When the microconidia of *F. oxysporum* (10^3 cfu/ml) to bottom layer of DLF filter paper seed coated G872B effectively colonized the rhizoplane of cucumber regardless of introduction of *F. oxysporum*. In fact little more population density of G872B was detected in the treatment of *F. oxysporum* inoculated than that of nontreated cucumber rhizoplane. *Furthermore*, the difference between two treatments was getting greater on the lower than 5 cm of the root segments. On the other hand, the popualtion density of *F. oxysporum* colonized on cucumber root reduced significantly at all portions of root segment by treatment of G872B seed inoculation.

The level of rhizosphere colonization by G872B was affected by the inoculum density of the pathogen in the root tip of cucumber (30). In our study, counts of *F. oxysporum*

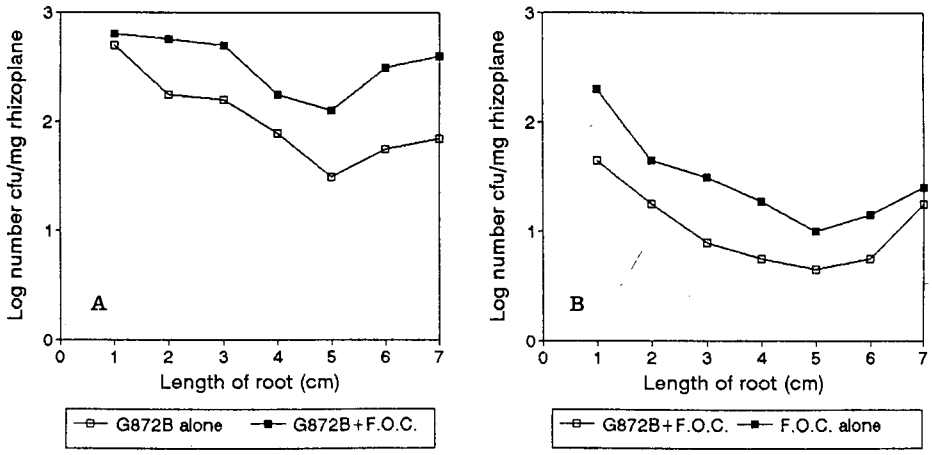

Fig. 3. Colonizing population of G872B(A) and *Fusarium oxysporum* (B) on cucumber rhizoplane when G872B was inoculated to seed(10^5 cfu/seed) and *F. oxysporum* was introduced by spraying microconidial suspension (10^6/ml) on the bottom layer of DLF filter paper.

decreased mainly on root tips on which the highest counts of the reduction of *F. oxysporum* population indicates that the antagonist is adapted to serve as an effective competitor on the rhizoplane. The reduction of the pathogen population in the rhizosphere could be the result of a lower proliferation rate of the pathogen in a rhizophere already colonized by the antagonist. In fact, Bae (5) reported that cucumber seeds treated with Pf3-G872B mixture significantly increased the rate of seed germination in greenhouse experiment. Most of the exudates present in the rhizosphere are excreted from the root tips. Thus, colonization of this region in the rhizosphere might reduce infection by Fusarium-like pathogens that penetrate the vascular system of their host through the undifferentiated xylem at the root tip (9, 17, 45, 54). Similarly, in the case of other important root pathogens, the juvenile root tip is the region in need of protection. Thus the antagonist is served as an effective competitor at infection site of the pathogen (29).

Enhancement of the germination and growth of G872B by Pf3 *in vitro*. The conidia of G872B started to germinate after 5 hrs in water agar at room temperature, and the germination rate reached well over than 80% after 9 hr in the treatment of G872B itself and coinoculation with 10^6 cells/ml of Pf3 (Fig. 4). The conidial germination of G872B was greatly increased by the combined inoculation with Pf3. The germination rate of in the plots that coinoculated with Pf3 was more than two times of that without Pf3, however the difference was not significant after 9 hrs incubation (Fig. 4-A). When the conidial concentration of G872B was increased to 10^9/ml, the stimulation effect of Pf3 on the conidial germination was nullified (Fig. 4-B). The hyphal growth of G872B with or without presence of Pf3 on the 1/10 PDA was examined by measuring the length from distal end septum to hyphal tip. An average length of marginal hyphal cell of G872B was much longer in the treatment of coinoculation of Pf3 than absence of Pf3 (Table 3). The hyphae of G872B grown on 1/10 PDA without Pf3 showed compact growth and smaller diameter of hyphae. On the

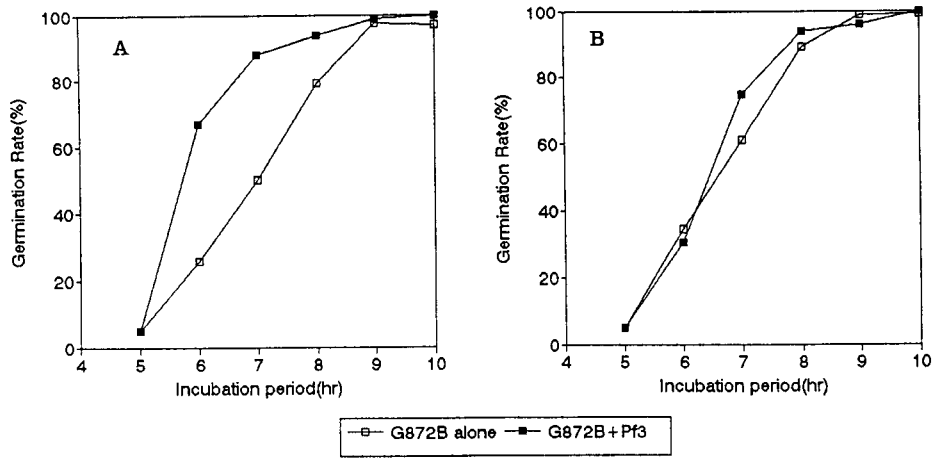

Fig. 4. Conidial germination of *G. virens* G872B on 1/10 PDA when the conidia were mixed with 10^8 cells/ml of *P. putida* Pf3. A: the concentration of conidia was adjusted to 10^6/ml, B: the concentration was adjusted to 10^7/ml.

Table 3. Effects of presence of *Pseudomonas putida* Pf3 on the mycelial growth of *Gliocladium virens* G872B

Treatment	Length of Marginal[a] Hyphal Tip (μm)	Dry[b] Weight (mg)
With Pf3	145.36± 39.21	36.9± 1.4
Without Pf3	85.94± 16.74	15.2± 0.7

[a] Lengths from last septum to hyphal tip of G872B were measured on 1/10 strength of PDA with or without supplement of Pf3.

[b] Dry mycelial weights of G872B were measured after 7 days culture in potato dextrose broth with or without cells of Pf3.

other hand, the hyphae of G872B combined with Pf3 was revealed sparse, streching out growth and thicker hyphae. The dry weight of mycelium of G872B cultured with Pf3 in the potato dextrose broth was almost twice much as that of G872B alone (Table 3).

Our result indicated that the conidial germination of *G. virens* was enhanced by *P. putida* (Fig. 4), which is the first available report so far. Mycelial weight of *G. virens* increased more than two times within 7 days by the addition of *P. putida.* Length of marginal hyphal tip was remarkably elongated (Table 3, Fig. 5). Dandurand and Knudsen (19) reported similar result by measuring colony radius of *T. harzianum* by addition of *P. fluorescens.*

Existing form of biocontrol agent G872B and Pf3 on the cucumber root. The existing form of G872B and Pf3 on the seed coat and root of cucumber was observed by means of fluorescent microscope and scanning electron microscope. Immediately after the biocontrol agents were treated to cucumber seeds, conidia of G872B or bacterial cells of Pf3 were existed separatly and scattered randomly on the surface of seed (Fig. 5). After 24 hrs, the conidia of G872B began to germinate on seed coat, and cells of Pf3 were partially multiplied. The more number of germinated conidia were observed on the surface of seed coat in

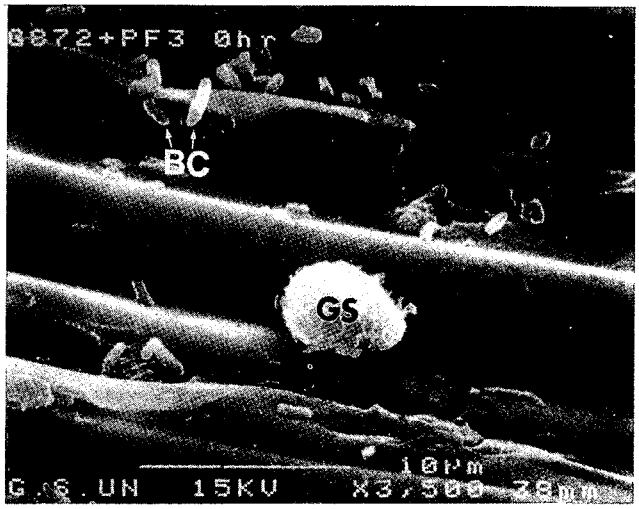

Fig. 5. Scanning electron microscopic observation of *G. virens* G872B and *P. putida* Pf3 on cucumber seed coat (×3.5 K), immediately after the bacterial cells and fungal spores were applicated into the seeds. The initial concentrations of G872B and Pf3 were 10^7 cfu/ml and 10^8 cfu/ml, respectively. BC: cells of Pf3, GS: spore of G872B.

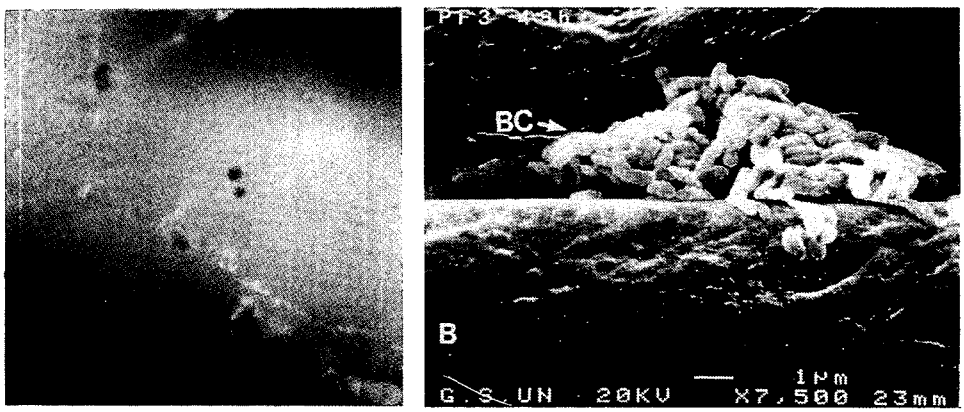

Fig. 6. Light microscopic observation of *G. virens* G872B(×400) on cucumber root systems 48 hrs after conidial inoculation. A is conidiophores and mycelium of G872B. B is bacterial mass of Pf3 multiplying on root surface.

the treatment coinoculated with Pf3 than G872B alone. After 48 hrs of inoculation, G872B formed the typical conidiophores with spore-balls (Fig. 6-A) and Pf3 was multiplied more vigorously and formed aggregated cell mass (Fig. 6-B). At 72 hrs, the propagated cells of Pf3 covered all over the surface of seed coat and rhizoplane, and mycelia of G872B grew around the root system, and mycelia grew linerly toward elongation root tip. In the single inoculation of G872B, many ungerminated conidia were observed and the hyphal growth was not vigorous and some of them were partially autolyzed. On the other hand, the bacterial cells of Pf3 were proliferated around the hyphae of G872B and the hyphal growth was

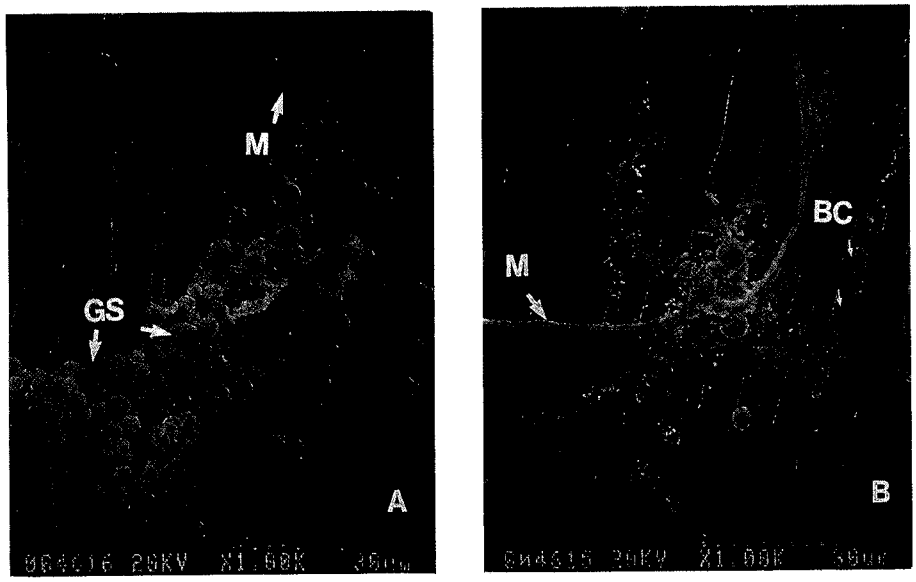

Fig. 7. Scanning electron microscopic observation of multiplying *G. virens* G872B and *P. putida* Pf3 on cucumber root. When the conidia of G872B were inoculated to cucumber seed separately (A) or coinoculated with Pf3 (B). M: mycelium of G872B, BC: bacterial cells of Pf3, GS: spores of G872B.

more vigorous in coinoculated treatment of G872B and Pf3 than single inoculation of G872B (Fig. 7). The ability of G872B and Pf3 being effective with small amounts as seed inoculation is associated with their ability to grow and sporulate on rhizoplane. Our results of scanning electron microscopy provided many supporting evidences. Both G872B and Pf3, upon coinoculation, are compatible and mutually benificial for germination, mycelial growth and proliferation of bacterial cells on the inoculated seed as well as in growing root system (Fig. 5, 7). Jeong (30) also demonstrated that rhizosphere competent isolate kept up with root growth by means of mycelial elongation.

Since beneficial rhizobacteria are often introduced as seed treatment they must proliferate rapidly in the spermosphere before they can begin to colonize the emerging root system. Seed also release exudates including sugars and amino acids during inhibition and germination (18, 23, 49). These organic substrates are potentially a rich source of nutrients for biocontrol agents in the spermosphere microflora if they are to benefit form the nutrients released from seeds (18). Pathogens such as *Rhizoctonia* and *Pythium* can attack seed within 6 hrs after planting, thus colonization of the spermosphere by beneficial rhizosphere microorganisms may be essential to protect the plant against seed rot or juvenile root diseases (11, 13, 21, 25). The effectiveness of biocontrol agents may depend partially on their ability to proliferate during a short period of favorable environmental conditions before they encounter plant pathogens. The earlier germination of G872B assissted by Pf3 may provide great advantage in proliferating in the host rhizosphere within short period of favorable environment. Therefore, we suggest that the rapid growth of mycelia is an important attribute of fungal biocontrol agent that potentially contribute to the rhizosphere competence as well as biological

control of soil-borne pathogens.

***In vitro* assay of abilities of low temperature growing biocontrol agents.** The low temperature growing biocontrol agents were collected from cool area and selected on the bases of their growth rate at 4℃ and 14℃, antagonistic activities against soil-borne pathogens and root colonization ability. Finally, two isolates, *Pseudomonas fluoresencens* M45 and MC07, were selected and their properties were characterized taxonomically. M45 and MC07 have the maker which is tolerant to 100 ppm rifampicin.

Culture filtrates of M45 and MC07 were obtained from the incubations at 27℃ and 14℃. Effect of the culture filtrates on mycelial growth of *Pythium ultimum, Rhizoctonia solani, Phytophthora capsici* were indicated on Table 4. The culture filtrate of MC07 suppressed the mycelial growth of pathogens more effectively than that of M45. Particulary, the culture filtrates obtained from the incubations at 14℃ were more effective than that obained from the incubation at 27℃ in inhibition of the mycelial growth of pathogenic fungi without regard to the isolate. The higher concentration (5%) of filtrate was more effective than lower one (1%).

Many workers reported *in vitro* inhibition of pathogenic fungi by culture filtrates of biocontrol agents (14, 25, 29, 36). However, it is hard to find from previous reports that inhibitory effect of culture filtrate is enhanced by low temperature incubation.

The growth of detached cucumber cotyledons in MS media with supplement of culture filtrate of M45 and MC07 were examined. Supplement of culture filtrate to MS media enhanced growth of cucumber cotyledon greatly compared to without filtrates. The culture filtrate of M45 was superior than MC07 at any concentration or any incubation temperature. The most significant growth enhancement was shown at 0.1% concentration of M45 culture filtrate obtained from incubation at 14℃. The fresh weight of root grown from the cotyledons that treated with M45 filtrate was more than 3 times of untreated plant root and the fresh weight of shoot and leaves developed from the cotyledons was 432 mg while that of control was 253 mg. Generally the culture filtrates of M45 and MC07 grown at 14℃ (Table 5)

Table 4. Inhibition of mycelial growth of plant pathogenic fungi on PDA by culture filtrates of M45 and MC07

Pathogens	Percent Inhibition of mycelial growth				
	Conc.	M45		MC07	
	(%)	27℃*	14℃	27℃	14℃
Pythium ultimum	1	16**	119	21	34
	5	33	48	46	79
Rhizoctonia solani	1	20	30	21	36
	5	37	49	50	65
Phytophthora capsici	1	23	33	26	40
	5	40	50	56	89

*Incubation temperature from which culture filtrate obtained from.

**Data indicate inhibition rates of myclial growth induced by culture filtrate.

Table 5. Effect of culture filtrates of M45 and MC07 on the growth of cucumber cotyledon in MS media

Isolates	Concentration (%)	Fresh weight (mg)	
		Root	Shoot
M45	1	170a	390a
	0.1	244a	432a
MC07	1	109b	344b
	0.1	103b	351b
CK	1	85c	293c
	0.1	78c	253c

The culture filtrates were obtained from the incubations at 14℃. Data in each colum with diffrent letter are significantly different (P=0.05).

Table 6. Effect of culture filtrates of M45 and MC07 on the growth of cucumber cotyledon in MS media

Isolates	Concentration (%)	Fresh weight (mg)	
		Root	Shoot
M45	1	142a	316a
	0.1	179a	354a
MC07	1	103b	327b
	0.1	91b	356a
	1	85b	293c
	0.1	78c	252c

The culture filtrates were obtained from the incubations at 27℃. Data in each colum with diffrent letter are significantly different (p=0.05).

were enhanced the growth of cucumber cotyledon significantly more than that of grown at 27℃ (Table 6) and 0.1% concentration was much better than 1%. The culture filtrate of M45 promoted root growth better than shoot growth while that of MC07 enhanced the shoot growth more than root.

Jang *et al.* (28) reported that the culture filtrate of Trichoderma and Gliocladium isolates stimulated the growth and development of the cucumber cotyledon cuttings.

Various mechanisms that related to inducing plant growth promotion by *Pseudomonas* spp. have been reported. In the absence of pathogens, fluorescent *Pseudomonas* spp. are able to exert direct effects on growth regulation (10, 23, 54). It could be presumed that the growth stimulating factor may play an important role in early growth of plants especially for the crops growing under the unfavorable condition. Because growth stimulatory effects of M45 and MC07 were better at low temperature condition, it might be suitable for winter crops or plastic film house crops growing in winter time.

Root and soil colonization of low temperature growing *Pseudomonas* spp. M45 and MC07.

The population densities of low temperature growing isolates, M45 and MC07 that colonized in the cucumber root tip were measured by Ahmad & Baker's method. The population density of MC07 detected from the last 1-cm-root segment was continuously reduced without

regard to their growing temperature either 27C or 14℃. However, the population density of M45 slighly decreased in the first 2 days, then sustained initial population density in the plot which is incubated at 27℃ incubation. When M45 seed treated plants were grown at 14℃, the population density slightly increased up to first 60 hrs then decreased, but the population denisty was little higher than initial density up to 140 hrs (Fig. 8). The root colonizing ability of M45 was quite distinctive from that of MC07 especially at 14℃.

To evaluate the survivability of M45 and MC07 in soil, cell suspension (10^9/g soil) of M45 or MC07 were added to natural field soil and the moisture content of soil was adjusted 18% (w/w) with bacterial suspension then enumerated the bacterial population in soil with King B medium containing 100 ppm rifampicin. There were significant differences in survival densities between M45 and MC07 at 14℃. Population density of M45 was slightly increased at first then reduced drastically, about 4 log-unit up to 60 days. But MC07 population continuously increased up to 9 days and then sustained initial inoculation density up to 60 days (Fig.9). From these results, low temperature growing isolate M45 was clarified as rhizosphere colonizer and the isolate MC07 was turned out as soil colonizer.

Lifshitz *et al.* (37) reported that diazotrophic bacterial strains can fix nitrogen and aggressively colonize roots at low temperatures. Loper *et al.* (35) also demonstrated that populations of low temperature growing *Pseudomonas* estimated from the rhizosphere at 12℃ were generally more stable than those at 18℃ or 24℃ suggesting that the survival ability of bacterial cells was maximized at low temperature. The results obtained in this investigation also revealed that the low temperature growing biocontrol agents were more compatible on the rhizosphere at low temperature condition.

Enhancement of cucumber growth and yield increase by G872B and Pf3. The growth enhan-

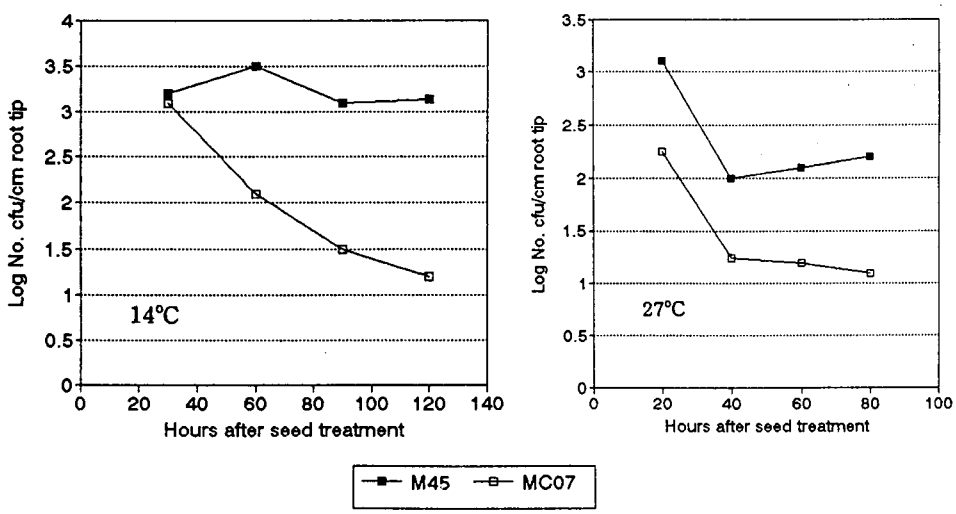

Fig. 8. Population densities of low temperature growing isolates, M45 and MC07, colonized on the root tip of cucumber grown at 14 (left) and at 27℃ (right). The initial concentration of each isolate was 10^5 cfu/seed.

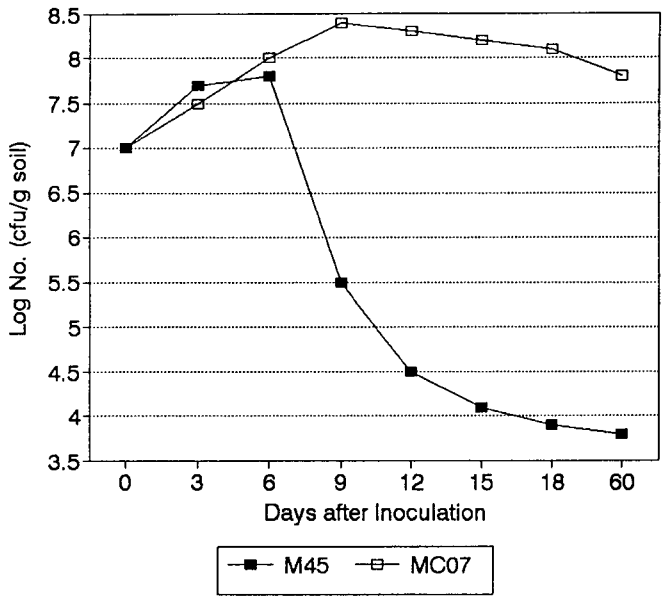

Fig. 9. Survival densities of *P. fluorescens*, M45 and MC07, in natural soil. The initial density of each isolate was adjusted to 10^7 cfu/g soil.

cement of plant growth by seed treatment of biocontrol agent G872B and Pf3 was evaluated in plastic film house. In general, the treatment of G872B and Pf3 increased the cucumber growth remarkably to compare the untreated control. Especially, the treatments of G872B and Pf3 resulted significant increase of root and shoot weights at early stage. Cucumber fruits harvested from the treatment of G872B itself was greater than those from any other treatment throughout the experiments (Fig. 10). The accumulated weight of cucumber fruits in the plots of Pf3 treatment or coinoculation of G872B and Pf3 was not significantly different from the control plots until the 6th harvesting. However the fruit yield was remarkably increased thereafter compared to other treatments. The final yield was the highest in the plots of coinoculation of G872B and Pf3. In this study, we also have demonstrated that seed treatment with G872B or coinoculation of G872B and Pf3 significantly increased cucumber shoot and root growth as well as fruit yeild. The previous paper (29) indicated that the disease suppression by rhizosphere incompetent isolates was limited only to the early stage of growth. Such results could be explained that cucumber plants became vulnerable to Fusarium wilt when the plants begin to bearing fruits. On the other hand, the rhizosphere competent isolates, such as G872B or Pf3 were effective up to end of 30 days. Consequently the treatment of G872B or Pf3 support health root system and increase the growth and fruit yield. Moreover, G872B and Pf3 are mutually complementary in cucumber rhizosphere leading to successful disease control and enhanced fruit production by synergistic effect.

Enhancement of seedling growth by low temperature growing M45 and MC07. The cucumber seeds inoculated with M45 or MC07 were sown in pots containing natural field soil and examined the rate of emergence and first foliage leaf expansion. The total rate of germinated

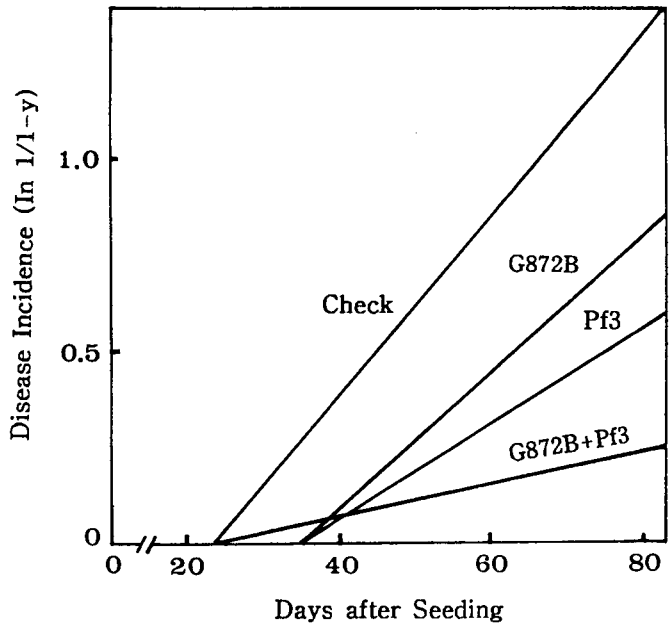

Fig. 10. Regression lines of disease progress of cucumber fusarium wilt resulted by seed treatment of biocontrol agents G872B and Pf3, and coinoculation of two isolates.

plants was not remarkably different among the treatments or nontreated control. However, the rate of completely expanded cotyledon and the rate of first foliage leaf expansion were significantly different from treated seed and untreated seed (Fig. 11). The seed treatment of M45 brought remarkable enhancement of cotyledon expansion as well as first foliage leaf expansion. When the experiment was conducted in autoclaved soil, there were no noticeable differences in emergence rates between the treatments and control but the fresh weight of seedling in each treatment was different statistically. M45 was more effective than MC07 to enhance the seedling growth. In natural field soil, all of the items of growth measurment including emergency rate were significantly different between treatments. The difference in fresh weight between bacteria treated and nontreated seeds was more pronounced in natural soil than in autoclaved soil (Table 7).

Kloepper *et al.* (34) suggested that potato growth promotion caused by PGPR in the early season was followed by significant yield increases up to 17% compared to untreated controls in four of five harvested field. Suslow and Schroth (59) reported that significant increases in dry and fresh weights of seedling roots of sugar beet grown in the ranged from 20 to 85%. It could be assumed that the isolates employed by Kloepper or Suslow might be low temperature tolerant pseudomonads because potato and sugar beet are grown under relatively low temperature. However, their experiments with potato or sugar beet were carried out in regular growing season of those crops. The growth conditions of the crops in plastic film house during the winter time are quite different from that of growing in ordinary season. Therefore we would suggest that the low temperature growing biocontrol agent is

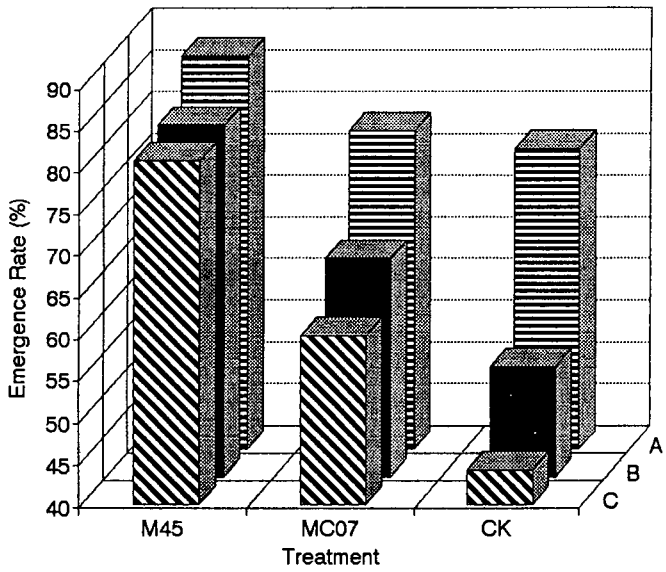

Fig. 11. Enhancement of seedling emergence of cucumber by seed treatment of M45 and soil treatment of MC07 at 14℃ growth chamber. A: total emergence rate. B: complete emergence rate C: first foliage leaf expanding rate.

Table 7. Effect of seed treatment of M45 and soil treatment of MC07 on the growth of cucumber seedlings in autoclaved and natural soil at low temperature

Treatment	Autoclaved soil		Natural soil	
	Emergence (%)	Fresh wt. (mg)	Emergence (%)	Fresh wt. (mg)
M45	95	694± 33	94	680± 28
MC07	95	610± 24	90	570± 25
CK	90	574± 10	82	497± 48

*Treatment: Isolates M45 was inoculated to cucumber seeds and MC07 was inoculated to soil, the initial concentrations of each isolates were 10^8/seed and 10^8/g soil, respectively.

required to enhance the plant growth growing under low temperature condition. *Pseudomonas fluorescens* M45 and MC07 developed in this study would be recommendable for the above purpose.

Biocontrol of cucumber wilt by G872B and Pf3 in plastic film house. The biological control of fusarium wilt of cucumber by seed treatment of G872B and Pf3 was presented in Fig. 12. The disease progress of each treatment was represented as natural-log converted regression lines. Comparing the disease incidence to untreated control, all of the biocontrol agents treated to seeds evidently suppressed fusarium wilt of cucumber. The bacterial biocontrol agent Pf3 was superior to fungal agent G872B. The remarkable result notified in this experiment was coinoculations of Pf3 and G872B at the same seed resulted more effective in disease suppression than separate treatment of each isolate. The coinoculation of Pf3 and

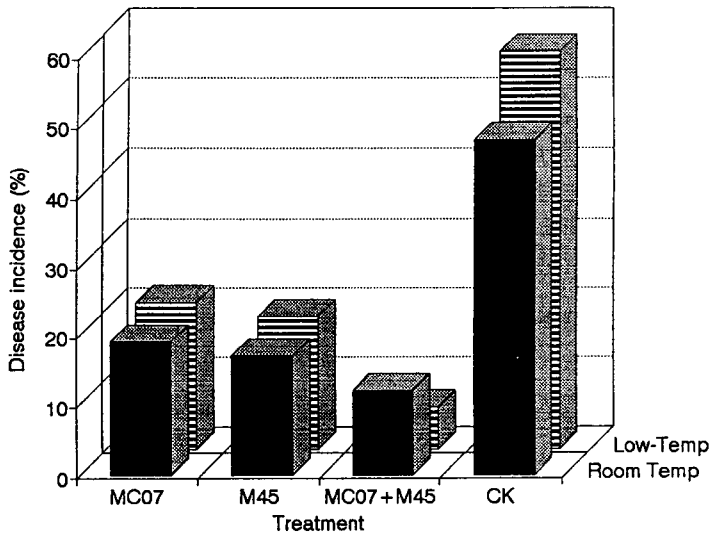

Fig. 12. Suppression of cucumber damping off caused by *Rhizoctonia solani* resulted by seed treatment of M45 and soil treatment of MC07. Room temperature trials were set to 25℃ with continuous light. Low temperature trials were set to 21℃ with light for 14 hrs and 12℃ without light for 10 hrs.

G872B successfully control the fusarium wilt of cucumber up to 80 days after seeding.

The most case of biological control have used a single isolate of antagonist. However, control by mixed strains was greater than by any single strain. Burr and Schroth (12) reported that mixtures of two or more rhizobacteria resulted significant reductions in daughter tuber infection by deleterious microcorganisms.

Our results indicated synergistic effects of rhizosphere competent *G. virens* G872B and fluorescent *Pseudomonas* Pf3 in combination for the control of cucumber wilt (Fig. 2). Hubbard *et al.* (43) observed that fluorescent *Pseudomonads* did reduce the biocontrol ability of *T. harmatum* in soil and the efficacy was reduced further containing steamed soil in the treatment of coinoculaion. They elucidated that Trichoderma or Gliocladium were suppressed by fluorescent *Pseudomonas* because the available iron was deprived by siderophore producing bacteria. But iron availability in plastic film house soil is mostly sufficient and the soil pH is generally low in Korea (15, 30, 50). Our result is agreeable to the recent findings of Dandurand and Knuden (23) that *P. fluorescens* strain 2-79RN10 did not have a inhibitory effect on the biocontrol activity of *T. harzianum* in the spermosphere and rhizosphere of pea.

From the results of this study it would be suggested that the disease control of soil-borne pathogens can be enhanced by the combined treatment of compatible biocontrol agents fluorescent pseudomonads and *Trichoderma* or *Gliocladium*.

Biocontrol of cucumber damping-off by low temperature growing M45 and MC07. Low temperature growing biocontrol agents, M45 and MC07, tested in this experiments effectively suppressed the seedling diseases of cucumber. Coating M45 to seed, adding MC07 to soil

and combined two treatment were evaluated in the soil that artificially infested with *Rhizoctonia solani*. The combined treatment of M45 and MC07 revealed most effective disease suppression either at room temperature or at low temperature in growth chamber. While the disease incidence in non-treated control was 48% and 57% at room and low temperature respectively, it was 7% and 4% in combined treatment of two strains; 12% and 17% in M45 seed treatment and 16% and 21% in MC07 soil treatment (Fig. 13). *In vitro* experiment revealed that the soil colonizing isolate MC07 was more effective than root colonizing isolate M45 in inhibition of soil-borne pathogens such as *Pythium, Rhizoctonia* and *Phytophthora* (Table 4). However, M45 was more effective than MC07 in suppression of damping-off disease in green house experiments (Fig. 13).

It is remarkable that suppression of soil-borne pathogen and enhancement of plant growth are more pronounced when the experiments were conducted in low temperature. The suppressive effect of M45 and MC07 to *Rhizoctonia,* and *Pythium* (data not shown) was greater at low temperature than at room temperature (Fig. 13). The reason why low temperature made the bacteria more effective to enhance the plant growth or to suppress the soil-borne pathogen could not be elucidated in this investigation. However it was recognized that the M45 and MC07 produced more fluorescent pigment, which is assumed as an antifungal substance, at low temperature condition (Data not included).

The enhancement of biological control of Rhizoctonia damping-off could be achieved by the inoculation of root colonizing M45 to seed and soil colonizing MC07 to soil. The root colonizing isolate M45 protected the germinating seed, following root system and young

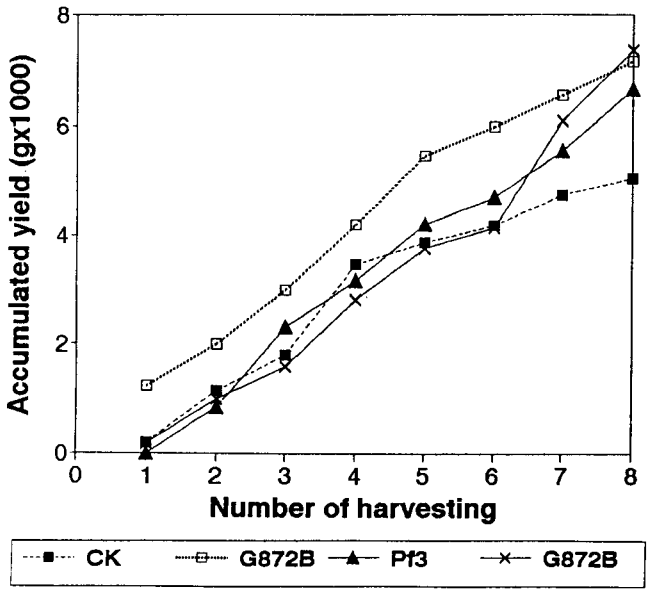

Fig. 13. Accumulated fruit yield of cucumber in vinyl house experiments for 80 days resulted by seed treatment of G872B and Pf3 and coinoculation of two isolates,

seedling. At the same time the indigenous soil-borne pathogens are inhibited by soil colonizing isolate, MC07. Burrr and Schroth (12) suggested that the treatment of plant with combination of rhizobacteria antagonistic to various soil-borne plant pathogens and quasipathogens could have a marked effect on reducing root disease if the rhizobacteria was not mutually inhibitory. Park *et al.* (45) reported that fluorescent pseudomonas and nonpathogenic isolates of *F. oxysporum* were effective on inducing suppressiveness to Fusarium wilt of cucumber when added to soil together, but were ineffective when added separately.

We have proposed and provided the evidences that rhizosphere competence, competibility with other agents and their synergistic activities are most important attributes of biocontrol agents. These are principal and essential characters contributing to high biocontrol efficacy and plant growth enhancement.

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