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In Vitro and Greenhouse Evaluation of Cucumber Growth Enhanced by Rhizosphere Microorganisms

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실험실내와 비닐하우스에서 根圈 微生物에 의한 오이 生育增進의 檢定

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ABSTRACT : We developed an *in vitro* assay method for evaluating plant growth promotion and providing an evidence that the growth promotion is rendered by growth enhancing factors. The amendment of culture filtrates of *Trichoderma harzianum* T95 and *Gliocladium virens* G872 and G872B in Murashige and Skoog (MS) agar medium enhanced the cotyledon growth of cucumber in terms of fresh weight and primary leaf area of cucumber cotyledon cuttings, of which the treatment of *G. virens* G872B was the most effective. The mycelial culture filtrate of G872B was more effective in the growth promotion than its conidial germling filtrate. The addition of 1% sucrose in MS mineral medium with 0.1% culture filtrates of the antagonists (T95 and G872B) was optimum for enhancing the effect of the filtrates on the growth of cotyledon cuttings *in vitro*. When cucumber seeds treated with G-872B, *Pseudomonas putida* Pf3 or the G872B-Pf3 mixture were planted in a greenhouse, the rate of seed germination, biomass of shoot and root, and yield of cucumber fruits were increased, especially by G872B or the G872B-Pf3 mixture. Correspondingly, cucumber fruit yields in early to middle-cycles of harvest were significantly greater in the plots of G872B than the control and Pf3-treated plots, and the final yield was highest in the plots of the G-872B-Pf3 mixture application.

***Key words* : *Gliocladium virens* G872B, *Pseudomonas putida* Pf3.**

Besides microbial antagonists can control plant diseases, one of other attractive features is the stimulation of plant growth. There are numerous instances in which biocontrol agents, inoculated onto seeds and roots and into soil, enhanced plant growth (4, 7, 8). However, the mechanisms of the growth promotion by the agents have not been defined clearly yet.

Plant growth stimulation is hypothesized to be derived mainly from the suppression of harmful rhizosphere microorganisms, such as major or minor plant pathogens (11, 12, 14, 15). On the other hand, the growth responses induced by *Trichoderma* spp. or *Gliocladium virens* appear to be due to production of a growth regulating factor (4, 5, 9). Windham *et al.* (17) reported that when seeds of maize, tomato and tobacco were placed with *Trichoderma* spp. and separated by a cellophane barrier, seed germination started earlier by 1 to 2 days, compared with untreated checks. Recently, Jang *et al.* (9) also reported that the cucumber cotyledon grew well on Murashige and Skoog (MS) mineral agar medium supplemented with culture filtrates of *Trichoderma* spp. or *Gliocladium virens* compared with that without fungal filtrates.

The objective of this study is to examine the effects

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of rhizosphere microorganisms on plant growth promotion in cucumber. For evaluating the plant growth promotion, a new *in vitro* assay method was also developed in this study.

MATERIALS AND METHODS

Preparation of fungal filtrate and optimization of MS medium. Microbial isolates used in this test were *Trichoderma harzianum* T95 and *Gliocladium virens* G872 and G872B, which are described elsewhere (2, 3). Mycelial disks of T95, G872 and G872B were introduced to 100 ml of MS broth tissue culture medium in 250 ml Erlenmeyer flasks. Unless otherwise indicated, the original recipe of MS medium was modified to exclude myo-inositol, nicotinic acid, pyridoxine-HCl, thiamin-HCl and glycine throughout the experiment. Cultures of isolates were incubated at 26°C on a rotary shaker for 4 days. After incubation, the culture filtrate of each isolate was prepared by filtering through a sterile membrane filter (nitro-cellulose, 0.2 μm). The filtrate was added at 0.1% concentration to 30 ml of MS agar medium in a 100 ml Erlenmeyer flask.

To examine whether the fungal isolates excrete growth factors during conidial germination or mycelial growth, culture filtrates were prepared by conidial germling culture of 24 hr-incubation and mycelial culture of 4 day-incubation in MS broth medium at 26°C on a rotary shaker (50 rpm) prior to filtering through a membrane filter as above.

As our preliminary investigation revealed that the growth response of cotyledon cuttings *in vitro* was influenced by sucrose concentration, the effect of sucrose concentration on the cucumber cotyledon growth was also studied. Sucrose concentration in the modified MS agar medium was adjusted to 0%, 1%, 2%, 3%, and accordingly the effect of culture filtrates of T95 and G-872B, prepared as mentioned above, on the growth of cotyledon cuttings was examined.

Tissue culture of cucumber cotyledon. Cucumber seeds were surface-disinfected for 5 min in 1% sodium hypochlorite solution and washed three times in sterile distilled water. The seeds were allowed to germinate on moist filter paper in a petri dish at 28°C. After cotyledons emerged from seed coat, the cotyledons with 3 mm of hypocotyl were aseptically cut off from young seedlings. The cuttings were disinfected again for 1 min in 1% sodium hypochlorite solution, washed three times in sterile distilled water, and blot on filter papers. The cuttings were then placed upright on the surface of the MS agar medium with various supplementation, and were incubated at 28°C in a growth chamber for 10 days. After 10 day incubation the primary leaf area and fresh weight of seedlings were measured. The experiment was repeated twice.

Greenhouse experiment. The greenhouse experiments, including application of seed-coating with G-872B and *Pseudomonas putida* Pf3 and plot management, were described (3). Briefly, cucumber seeds (cv. Sa-yeup-Oi, Jungang Seed Co.) were surface disinfected for 5 min in 1% sodium hypochlorite solution, washed and air-dried for 1 hr at room temperature. Two grams of seeds were treated either with either 1 ml of the conidial suspension of G872B (cultured on potato dextrose agar medium) or the suspension of Pf3 (cultured in King's B broth medium) in 0.1% methyl-cellulose solution and air-dried for 1 hr. For the combined treatment of both G872B and Pf3, both conidial and bacterial suspensions were mixed to 1 : 1 (v/v) prior to seed treatment as above. Non-treated seeds were dipped in 0.1% methyl-cellulose solution. Rate of seed germination was examined 8 days after seeding. The fresh weights of shoot and root were measured twice (15 days after seeding and 40 days after transplanting). Fruit yield was examined 50 days after transplanting up to 80 days. Only the fruits longer than 25 cm were picked every other day, at a total of eight times during the experiment.

RESULTS

Effect of culture filtrate on the cucumber cotyledon cuttings in MS agar medium. Culture filtrate amendment enhanced remarkably fresh weight and primary leaf area of cucumber cotyledon in MS agar medium (Table 1). Especially, the treatment of culture filtrate of *G. virens* G872B resulted in a significant growth enhancement.

The growth of cucumber cotyledon cuttings in the MS agar medium supplemented with the mycelial filtrate of G872B was much higher than that amended with the conidial germling filtrate in terms of fresh weight and primary leaf area (Fig. 1). The growth in the media added with conidial germling filtrate was not significantly different from the untreated check. This result indicates that a growth stimulating factor was excreted during mycelial growth.

Sucrose concentration was also an important factor

Table 1. Effect of culture filtrates of fungal isolates T95, G872, and G872B on the growth of cucumber cotyledon cuttings in MS agar medium for 10 days at 28°C

Treatment[a]	Fresh weight (mg)	Primary leaf area (cm^2)
Untreated	392±21[b]	5.60±0.46
T95	495±36	6.63±0.55
G872	495±42	7.58±0.53
G872B	576±75	7.54±0.68

[a] The culture filtrate of each isolate was supplemented at 0.1% concentration in MS medium. T95 : *Trichoderma harzianum*, G872 : *Gliocladium virens*, G872B : a mutant of *G. virens* G872 tolerant to benomyl.

[b] Each datum is the mean and standard error of four replications with two plants each.

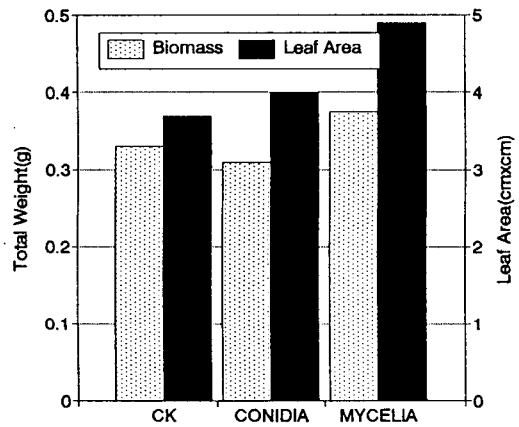

Fig. 1. Effect of culture filtrates of conidial germlings and mycelia of *Gliocladium virens* G872B on the growth of cucumber cotyledon in MS agar medium at 28°C for 10 days in a growth chamber. Conidia germling cultures were prepared by incubating for 24 hours and mycelial cultures were incubated for 4 days in MS broth at 27°C on a rotary shaker.

affecting the growth of cotyledon cuttings in tissue culture medium (Fig. 2). The supplement of 1% sucrose was the optimum for enhancing the effect of culture filtrate on the growth of cotyledon cuttings. The results herein provided an *in vitro* technique to assay the effect of the culture filtrates of the isolates on the growth and development of cotyledon cuttings to seedlings.

Enhanced cucumber growth and increased yield. The total emergence of cucumber seedlings was significantly higher in the plots of G872B or the G872B-Pf3 mixture than Pf3 alone or untreated plots (Fig. 3). Cucumber seeds treated with G872B or the G872B-Pf3

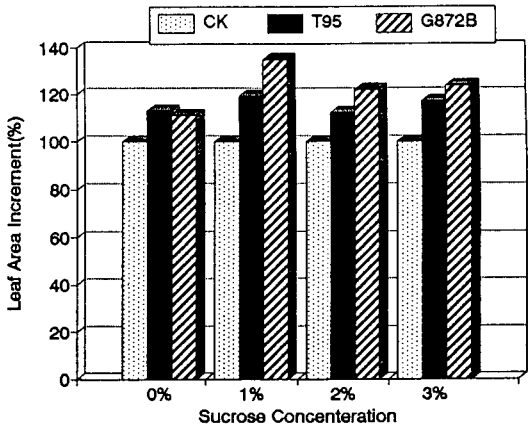

Fig. 2. Effect of sucrose concentrations in MS agar medium supplemented with fungal mycelial culture filtrates on the growth-enhancing of cucumber cotyledon cuttings. Cucumber cotyledon cuttings were incubated at 28°C for 10 days. Sucrose concentrations in MS agar medium were adjusted to 0%, 1%, 2%, and 3%.

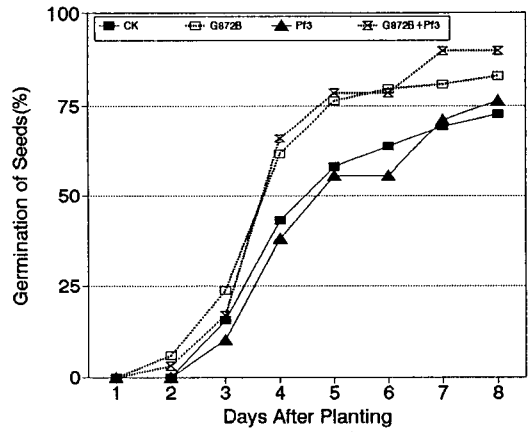

Fig. 3. Percentage of cucumber seed germination in the seed-coating applications with either G872B alone, Pf3 alone or G872B-Pf3 mixture in greenhouse potting soil. Prior to seed treatment (2 g seeds vs. 1 ml suspension), suspensions of G872B with 10^9 conidia per ml and Pf3 with 10^8 cells per ml were prepared and used alone or mixed together with equal volumes of each isolate suspension. CK : untreated check, G872B : *G. virens*, Pf3 : *P. putida*, G872B+Pf3 : *G. virens-P. putida* mixture.

mixture germinated earlier by about 1 day and sizes of emerged seedlings in both plots were uniform compared to those of the untreated plots. A similar tendency was also noted for the growth of cucumber in

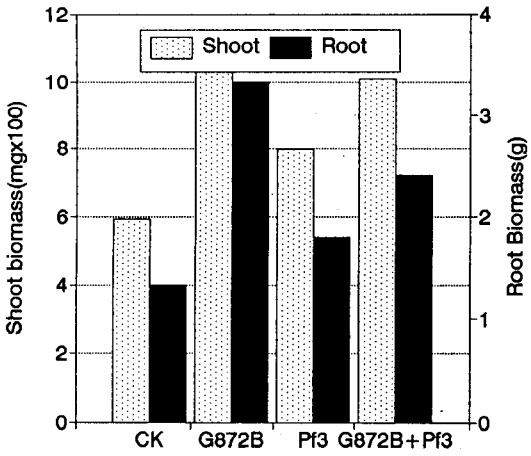

Fig. 4. Biomass of root and shoot of cucumber seedlings grown in greenhouse potting soil for 15 days after sowing seeds with the biocontrol agents treated. Each value is the mean of six replications with five plants each. G872B : *G. virens*, Pf3 : *P. putida*.

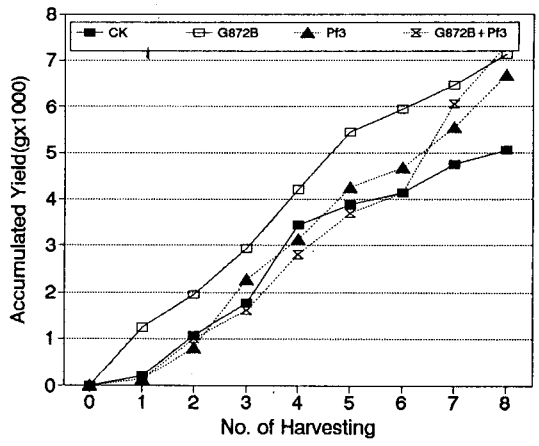

Fig. 5. Accumulated weight of cucumber fruits harvested from the plants grown out of seeds with the antagonistic microorganisms treated. Cucumber plants were grown in greenhouse soil for 80 days after transplanting. Cucumber fruits were picked every other day. CK : untreated check, G872B : *G. virens*, Pf3 : *P. putida*, G872B+Pf3 : *G. virens* - *P. putida* mixture.

terms of shoot and root weight 15 days after transplanting (Fig. 4). Remarkable growth enhancement by G872B was observed for root weight rather than for shoot weight (Fig. 4).

Cucumber fruit yield from the plots of G872B alone was significantly greater than those of any other treatments throughout the harvesting-period (Fig. 5). The accumulated weight of cucumber fruits in the plots of Pf3 alone or G872B-Pf3 mixture was not significantly different from the untreated plots until the 6th harvesting, and thereafter increased greatly compared to the untreated plots. The final yield was highest for the plots of the G872B-Pf3 mixture application.

DISCUSSION

Two mechanisms have been demonstrated most frequently to explain the nature of the increased plant growth response induced by certain microorganisms. The first hypothesis is that the enhanced growth mainly results from the control of harmful rhizosphere microorganisms (6, 12, 13, 15, 16). The other is that a microbial agent produces substances that stimulate plant growth (9, 17). In this study, we have concentrated on the substances relevant to plant growth stimulation. The culture filtrates of the isolates used in this experiment enhanced the growth and development of the cucumber cotyledon cuttings (Table 1). The growth of cotyledon cuttings in the MS agar medium supplemented with mycelial filtrates also was much higher than that with conidial germling filtrates in terms of fresh weight and primary leaf area (Fig. 1). This result indicates that growth-stimulating factor was excreted during mycelial growth. We have previously suggested that a rhizosphere-competent isolate reveals more rapid mycelial growth than conidial production (2). Jeong *et al.* (10) also demonstrated that a rhizosphere-competent isolate kept up with root growth by means of mycelial elongation. Therefore, we suggest that the rapid growth of mycelia is the most essential attribute of the organism that potentially contributes to the plant growth promotion as well as the rhizosphere competence.

The preliminary investigation revealed that the growth of cotyledon cuttings in MS agar medium was significantly influenced by the sucrose concentration in the medium. The addition of 1% sucrose was optimum for enhancing the effect of culture filtrates on the growth of cotyledon cuttings (Fig. 2).

Based on these results obtained, the concentration of 0.1% culture filtrate in modified MS agar medium with 1% sucrose is recommended as a standard in vitro technique to screen promising microbial resources for growth-enhancing substances and to assay the effect of culture filtrates of isolates on the growth and development of cotyledon cuttings to seedlings.

Windham *et al.* (17) reported that the rate and/or the total emergence of tobacco and tomato seedlings in

soil treated with *Trichoderma* spp. increased significantly. Jang *et al.* (18) also reported that application of *Trichoderma* spp. and *G. virens* to cucumber seeds enhanced significantly the growth of shoot and root of cucumber plants. Ahmad and Baker (1) reported that *Trichoderma* spp. produced significantly higher fruit weight and higher dry weights of cucumber plants. In this study, we also demonstrated that seed treatment with G872B or the G872B-Pf3 mixture significantly increased the rate of seed germination (Fig. 3).

Cucumber fruit yield from the plots of G872B alone was significantly greater than those of any other treatments throughout the harvesting-period (Fig. 5). However, the disease suppression by this isolate was rather limited relatively to the early growth stage; the final yield was highest in the plots of the mixture of G-872B and Pf3. Although Pf3 was less effective in the growth promotion and yield of cucumber than G872B, consequently leading to less cucumber fruit production, its population in the cucumber rhizosphere endures up to 80 days after treatment (3), suggesting that the wilt incidence might be suppressed in a sufficiently long period of time. Moreover, G872B and Pf3 are compatible in the colonization of the cucumber rhizosphere (3). Accordingly, they are mutually compatible, providing synergistic effects that lead to successful disease control and enhance fruit production.

요 약

근권정착능력이 우수한 균주를 선발하여, 식물 생육증진 관련 물질에 대한 실내 검정 방법을 개선하고, 이들 균주를 오이 종자에 처리하여 온실 조건하에서 그 효과를 검정하였다. 무처리와 sucrose 첨가 MS액체 배지에 *Trichoderma harzianum* T95, *Gliocladium virens* G872와 G872B를 4일간 배양한 후, 그 배양 여액을 MS 한천배지에 0.1% 첨가하여 오이 자엽절편을 치상하였다. 무처리구에 비해 배양 여액을 첨가한 처리군에서 오이 생육촉진이 뚜렷하였으며, 처리 균주 중 G872B의 효과가 가장 높았다. 또한, 식물 생육 증진물질이 균사생육시 분비되는지 또는 포자발아시 분비되는지를 조사한 바, 균사 배양여액을 처리했을 때 오이 자엽의 생육이 현저히 증가하였다. MS 배지에 sucrose 농도를 조절한 후 오이 자엽의 생육을 조사한 바, sucrose 농도 1%에서 균의 배양여액 효과가 무처리구 및 2~3%에 비하여 뚜렷한 차이를 나타냈다. G872B와 Pf3를 종자에 단독 또는 혼합처리시 오이의 생육을 조사했을 때, G872B 단독과 G872B-Pf3 혼합처리구에서 종자 발아력 및 초기생육, 중간생육이 증진되었으며, 특히 G872B 단독처리구에서 초기 및 중간의 오이 열매 수량이 현저히 증가하였으며, 최종수확량은 G872B-Pf3 혼합처리구에서 가장 높았다.

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