Plant Growth Promoting Fungi from Zoysiagrass Rhizosphere as Potential Inducers of Systemic Resistance in Cucumbers

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ABSTRACT

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Sixteen fungal isolates from zoysiagrass rhizospheres that promoted the growth of a variety of crop plants were tested for their ability to induce systemic resistance in cucumber plants against *Colletotrichum orbiculare,* the anthracnose pathogen. Roots of cucumber plants were treated for different durations with these fungal isolates as barley grain inoculum, mycelial inoculum, or culture filtrate. The induced plants were challenge inoculated with *C. orbiculare* at three different spore concentrations: 10^4, 10^5, or 10^6 spores per milliliter. Among the 16 isolates, only 10 reduced the disease significantly and the protection obtained by these isolates varied among the methods of inducer application. However, isolates GU21-2, GU23-3, and GU24-3 produced the same effect when treated in all three forms and challenged with 10^4 spores per milliliter of the pathogen. Some isolates protected plants against high inoculum densities of the pathogen, while most of them were less effective under such pressure. The reduction in total lesion area and lesion number on leaves of plants induced by isolates GS6-1 and GU21-2 was similar to that induced by *C. orbiculare.* Among isolates that induced protection against anthracnose, isolates GS8-1, GS8-2, and GS8-3 colonized roots and significantly reduced the disease compared with the protection caused by noninduced control. The other isolates that were unable to colonize roots also offered protection. The degree of protection was highly dependent on the pathogen spore concentration and the inducer treatment period. A 24-h period was sufficient to initiate resistance; however, a 72-h duration proved to be more effective. The induction of systemic resistance in cucumber plants against *C. orbiculare* might be due not only to root colonization, but also to triggering of the host defense mechanism(s) by certain factors produced by fungi and their metabolites. When provided as barley grain inocula, most of the isolates increased the plant height and biomass significantly. Treatment of cucumber seeds with the mycelial inocula and culture filtrates of certain isolates also caused promotion of height and biomass of plants.

Additional keyword: *Colletotrichum lagenarium.*

The use of fungicides and breeding for host resistance have been the major strategies for the management of fungal diseases of vegetable crops. However, the breakdown of host resistance and the development of virulent populations of pathogens have forced breeders and producers to consider other alternatives for controlling plant diseases. In recent years, biological control has been seriously viewed as a potential strategy to control diseases.

Systemic resistance can be induced in a variety of crop plants using nonpathogenic and pathogenic microorganisms (4,8,13,23–25, 29,33,39) or their metabolites (38). Induction of systemic resistance has been shown to operate in cucumber (*Cucumis sativus* L.) when plants were induced by a foliar pathogen (*Colletotrichum orbiculare* (Berk. & Mont.) Arx = *C. lagenarium* (Pass.) Ellis & Halst.) against a root pathogen (*Fusarium oxysporum*) (14). Resistance was also induced by treating cucumber roots with a nonpathogenic form of *F. oxysporum* against foliar disease caused by *C. orbiculare* (20). When roots of watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai) were pretreated with either an incompatible pathogen or an avirulent strain of *Fusarium* species, plants were protected from virulent strain *F. oxysporum* Schlechtend.:Fr. f. sp. *niveum* (E. F. Sm.) W. C. Snyder & H. N. Hans when challenged after an interval of 24 or 72 h (3). Most of our knowledge of the induction of disease resistance has been gained by using cucumber and the anthracnose pathogen *C. orbiculare* as a classic model (22,42). Inducers of systemic resistance have also been shown to promote the growth of many crop plants (12,20,41,42). Certain plant growth promoting fungi (PGPF) are also reported to suppress disease effectively (9,31), but induction of systemic resistance was not reported. No information is available on the use of saprophytic nonsporulating fungi as inducers of systemic resistance in crop plants.

Furthermore, previous research has demonstrated that fungal isolates collected from the rhizospheres of different cultivated crops (*Capsicum annuum* L., *Solanum melongena* L., *Triticum aestivum* L., and *Zea mays* L.) and zoysiagrass (*Zoysia tenuifolia* Willd. ex Trin.) enhanced the growth of a variety of crop plants (18). The rhizosphere fungal isolates belonged to genera *Fusarium, Penicillium, Rhizopus*, and *Trichoderma.* A majority of isolates from zoysiagrass rhizosphere (ZR) and some from rhizospheres of other crop plants did not sporulate on several media tested nor did they produce clamp connections; they were therefore termed "sterile." These sterile, saprophytic fungal isolates promoted plant growth (17,34) and suppressed soilborne diseases (damping-off caused by species of *Fusarium* and *Pythium,* root rot caused by species of *Fusarium, Rhizoctonia,* and *Sclerotium* and take-all caused by *Gaeumannomyces graminis* (Sacc.) Arx & D. Olivier var. *tritici* J. Walker) of a number of crop plants (19). These studies revealed that simultaneous inoculation of soil with PGPF and pathogenic fungi or inoculation of PGPF into soil before inoculation with pathogenic fungi decreased the severity of certain soilborne diseases. Since these PGPF isolates effectively controlled soilborne diseases, experiments were planned to study whether these isolates could be used as inducers of systemic resistance. The classic model of cucumber and *Colletotrichum* was chosen. A preliminary report on the induction of resistance against *C. orbiculare* using barley grain inocula,

mycelial inocula, and culture filtrates of 24 PGPF isolates revealed that some isolates induced resistance in cucumber system (30). It was hypothesized that the defense mechanism might be initiated by fungal root colonization or chemical factors that might be secreted by PGPF isolates during their growth in soil or on roots. Experiments were conducted to analyze the potential of these isolates to induce resistance in plants by using barley grain inocula, mycelial inocula, and culture filtrates to induce resistance. The purpose of this paper is to present the dual role of ZR isolates in inducing resistance as well as in promoting growth of cucumber plants, and the importance of the type and duration of inducer treatment.

MATERIALS AND METHODS

Pathogen and the host. *Colletotrichum orbiculare* (= *C. lagenarium*) isolate 104 T was maintained on potato-dextrose agar (PDA, 3% agar) slants. The pathogen was transferred to petri plates containing the above medium and was incubated 7–10 days at 25 C for abundant sporulation. The cucumber cv. Gibai was used throughout these studies.

Preparation of inducer isolates. Sixteen PGPF isolates (GS6-1, GS6-2, GS6-4, GS7-3, GS7-4, GS8-1, GS8-2, GS8-3, GS8-6, GS10-1, GS10-2, GS12-2, GU21-2, GU23-3, GU24-3, and GU26-1) from zoysiagrass rhizospheres were screened for their effectiveness as inducers of resistance. Cucumber roots were treated with the following three forms of PGPF: barley grain inocula, mycelial inocula, or culture filtrates.

1. Barley grain inoculum (BGI). Autoclaved barley grains (115 g in 115 ml of water) were inoculated with 10–15 disks (5 mm) of PGPF from the actively growing margin of 7-day-old PDA (2% agar) cultures. After 10–12 days of incubation at 23–25 C, the completely colonized barley grains were air-dried at laboratory temperature (22–25 C)(35). The dried BGI was ground to 1- to 2-mm particle size and mixed (2%, w/w) with autoclaved (1 h, consecutively for 3 days) potting medium Star Bed (soil-less, peat-based potting medium, Kyodohiryo Co. Ltd., Japan), which contains humus, peat, rock phosphate, and composted plant materials (the exact composition of the potting medium was not revealed by the company). The nitrogen, phosphorus (P_2O_5), and potassium (K_2O) contents of the potting medium were 200:1500:200 mg L^{-1} of potting medium.

2. Mycelial inoculum (MI). Two mycelial disks of each PGPF isolate obtained from the growing margin of a colony on PDA were transferred to 200-ml flasks containing 40 ml of potato-dextrose broth (pH 6.5). The isolates were cultured without shaking at 23–25 C for 10–12 days in darkness. The fungal mat was separated from the culture filtrate by using a few layers of cheesecloth. The mycelia were thoroughly washed with sterile distilled water to remove the remaining culture filtrate from the mycelial mat and placed on Whatman no. 1 filter papers to remove excess moisture. The mat was weighed and distilled water was added (1 g of mycelial mat/4 ml of water). Mycelia were then blended in a blender (Type H, Teraoka, Toyo Keisokuki Co. Ltd., Japan) at 5,000 rpm for 5 min.

3. Culture filtrate (CF). The crude culture filtrate was separated from mycelia and filtered three times through three layers (each time) of Whatman no. 2 filter paper. The CF was then filter sterilized (0.5-μm millipore filters, Millipore Products Division, Bedford, USA). One milliliter of the CF was added to 4 ml of sterile distilled water and used for root treatment.

Procedures for induction. One hundred grams of the potting medium/inoculum mixture was placed in each sterilized plastic pot (autoclavable, 6 × 7.5 cm). The mixture was sown with cucumber seeds (one seed per pot) that were previously surface sterilized with 0.5% NaOCl for 2 min. The plants were grown at 25 C and 75% RH for 21 days in growth chambers. The plants received 12/12 h light/darkness cycle (cool fluorescent daylight tubes, 300 $\mu E\ m^{-2}\ s^{-1}$). The growing conditions of plants in growth chamber were the same throughout the present study, unless specifically mentioned. Two sets of plants were maintained for each treatment; one set was pretreated with PGPF and challenge inoculated, while the other set was only challenge inoculated. Each treatment consisted of four replicates, with three plants in each replicate. Sterile plastic pots containing potting medium amended with 2% (w/w) autoclaved, uninfested barley grains served as treated control.

Paper pot sets (Nippon Beet Sugar Co. Ltd., Japan) containing 162 separable pots (each pot 3.8-cm wide × 5.0-cm long) were autoclaved and filled with approximately 70 g of autoclaved potting medium and sown with an individual surface-sterilized cucumber seed. The plants were grown in growth chambers for 21 days or until the second leaf was fully expanded. The paper pots were separated into individual pots, each with a plant. The bottom portion of the pot was partially cleared of the potting medium to expose tips of roots. After trimming the root tips, plant roots were dipped into either 4 ml of MI, 20 ml of CF, or 20 ml of water contained in sterile plastic pots. After 24 h of incubation, the treated roots were covered with the autoclaved potting medium and then watered with sterile distilled water. Plants were challenge inoculated after 24 and 72 h of incubation. Treatments and replications were as described in the BGI experiment.

Challenge inoculation. Spores of *C. orbiculare* were scraped with a sterile scalpel from a culture and a spore suspension was prepared in sterile water and filtered through eight layers of cheesecloth. The spore concentrations were adjusted based on spore density measured with a hemacytometer. Plants were inoculated with three spore concentrations (10^4, 10^5, or 10^6 spores per milliliter) by placing 20 drops (10 μl) of the spore suspension on the second true leaf. A 6-mm diameter disk of lens paper was placed on each drop to prevent runoff and to maintain a uniform distribution of spores on the leaf surface. The inoculated plants were incubated at 25 C for 30 h in a dark, humid chamber (85–90% RH) and were transferred back to the growth chamber for another 6 days before analyzing the disease. Experiments were conducted three times. The total number of lesions per leaf and total area of lesions per leaf caused by *C. orbiculare* in protected and unprotected plants were measured.

Comparison of induction abilities of PGPF isolates and *C. orbiculare*. This experiment was conducted to compare the protection of plants induced with five selected PGPF isolates (GS6-1, GS8-1, GS8-2, GS10-1, and GU21-2) with the protection of plants induced with *C. orbiculare*. The roots of cucumber plants were induced with PGPF isolates applied as either BGI, MI, or CF, as described earlier. Plants were challenge inoculated by placing twenty 10 μl drops of 10^5 spores per milliliter of the pathogen. For induction with *C. orbiculare*, plants were grown in potting medium contained in sterile pots for 15 days in a growth chamber. Plants were induced by placing twenty 10 μl drops of 10^5 spores per milliliter of the pathogen on the first true leaf and, after 6 days, plants were challenge inoculated with the same spore density of the pathogen. The incubation conditions after inducer and challenge inoculations were as described earlier.

Root colonization ability. Sixteen PGPF isolates were amended separately to autoclaved potting medium as BGI (2%, w/w) and surface-sterilized cucumber seeds were sown. Seedlings with intact roots were removed from the medium at 7-day intervals for 21 days. Roots were thoroughly washed in running tap water for 30 min and finally rinsed with distilled water. To test the effect of disinfection on the viability of the fungal isolates, some roots were surface disinfected (0.25% NaOCl for 1 min), while surfaces of some other roots were not disinfected. Roots were cut into three main segments: upper (toward the stem); middle; and lower (toward the root tip). Each segment was further cut into 1-cm segments. The segments were placed on PDA amended with chloramphenicol (200 $\mu g\ L^{-1}$) and incubated for 2 days. The fungal colonies growing from the root segments were compared with the original culture and the colony-forming segments counted. Fungi from roots were reisolated and grown on fresh PDA plates; their colonies were compared with original PGPF colonies for the confirmation of identity. Cultural characteristics such as the color and growth pattern of mycelia and the pigment produced were considered as identification parameters. Three replicates were

used for each treatment and the experiment was conducted three times.

The aboveground parts such as petioles, leaves, and stems of plants grown in PGPF amended and unamended potting medium were cut into 1-cm pieces, surface disinfected, and incubated on PDA to determine whether the PGPF isolates also invaded these regions.

In vitro interaction. To test whether PGPF isolates were antagonistic to *C. orbiculare* in culture, 5-mm disks of each PGPF isolate and the pathogen cultured on PDA (3% agar) for 5 days were placed simultaneouly on the same medium contained in petri plates at a distance of 5 cm from each other. Plates were incubated at 25 C for 10 days. Growth rate of these two fungi were measured. The zone of inhibition, if any, was also recorded.

Testing for plant growth promotion. All 16 PGPF isolates were tested for their ability to promote growth of cucumber plants. The autoclaved potting medium (100 g) in sterile pots was amended with the powdered BGI (2%, w/w) of the candidate PGPF isolate and surface-sterilized cucumber seeds were sown into the potting medium/inoculum mixture (one seed per pot). Autoclaved potting medium unamended or amended with autoclaved barley grains (2%, w/w) served as untreated and treated controls, respectively. Plants were grown in a growth chamber for 21 days as described earlier. The shoot height and plant top dry biomass (dried in an oven for 24 h at 100 C) were determined.

To determine the effect of MI and CF of PGPF isolates on the plant growth, seven isolates (GS6-1, GS6-2, GS8-1, GS8-2, GS8-3, GU21-2, and GU23-3) were selected that were effective in reducing the lesion number and lesion diameter when used as any of the three forms of inoculum. The isolates were cultured in potato-dextrose broth for 10 days and mycelial mat and CF were separated. The preparation of the MI and CF for treatment was as described earlier. Ten milliter of MI, CF, or broth/sterile water were placed in sterile petri dishes and surface-disinfected cucumber seeds were immersed for 72 h. The treated seeds were sown in autoclaved potting medium contained in sterile plastic pots. The plants were grown in a growth chamber for 21 days. The plant height and dry biomass were determined. The experiment was conducted three times.

Statistical procedures. Experiments were conducted in a split-plot design (BGI) and split-split-plot design (MI and CF) depending on the number of variables involved. Inducer (PGPF) treatments were considered as main plot factor, while spore concentration of the pathogen and inducer treatment duration were considered as sub- and sub-sub-plot factors, respectively. Data from the repeated trials were pooled, since variances among trials were homogeneous. The results of experiments involving inducer treatment with BGI, MI, and CF revealed that only 11 out of 16 PGPF isolates reduced disease. Hence, 11 inducer treatments along with a control were considered for the analysis of variance. Data of total lesion number and total lesion area were analyzed separately. Significant interactions among inducer treatment, pathogen spore concentration, and inducer treatment duration were often present. The treatment means were separated by Fisher's least significant difference test (LSD, $P = 0.05$, 0.01) or by Duncan's multiple range test (DMRT, $P = 0.05$).

The experiment for root colonization ability was also set up in the split-split-plot design with PGPF isolates (three treatments) as main plot factors, with root regions and days of isolation as sub- and sub-sub-plot factors, respectively. The experiment for testing the growth promotion ability of fungal isolates was set up in a completely randomized design consisting of 16 treatments and two controls with four replicates. The experiment involving seed treatment with MI or CF was also set up as above with seven PGPF treatments and four replicates. The treatment means were separated by LSD ($P = 0.05$, 0.01) and DMRT ($P = 0.05$).

RESULTS

The results indicated that some PGPF isolates were effective as BGI only, others as MI or CF. However, certain isolates were effective in all three forms.

Barley grain inoculum. Among the 11 isolates selected, only eight isolates reduced the total number of lesions significantly ($P = 0.05$) in plants challenge inoculated with 10^4 spores per milliliter of the pathogen, when compared with the control. Isolates GS8-1, GS8-2, and GU23-3 were the most effective against this spore concentration in reducing the total lesion number by 80–90% (Table 1). Isolates GU21-2, GU24-3, GS10-1, and GS6-4 were less effective, but still gave a significant control. A similar trend in the reduction of total lesion area was observed. However, isolates GS6-2 and GS7-4, which were not capable of reducing the total lesion number, reduced the total lesion area significantly ($P \leq 0.05$) compared with control. Isolate GS6-1 was not effective in reducing the total lesion area.

However, at 10^5 spores per milliliter of the pathogen, only isolates GS8-1, GS8-2, GU21- 2, GU23-3, and GU24-3 reduced both total lesion number and area. At 10^6 spores per milliliter, GS8-1, GS8-2, GU21-2, and GU23-3 reduced the total lesion number and area significantly ($P = 0.05$) compared with control while the other isolates failed to reduce disease.

Significant interactions ($P \leq 0.05$) were obtained between the inducer treatment and pathogen spore concentration, which indicated that PGPF isolates, when used as BGI, became less effective with an increase in pathogen spore concentration (Table 1).

Mycelial inoculum. The MI of six isolates (GS6-1, GS6-2, GS7-4, GU21-2, GU23-3, and GU24-3) reduced the total lesion number significantly ($P = 0.05$) when incubated for 24 and 72 h, against 10^4 spores per milliliter spore concentration of the pathogen (Table 2). The remainder of isolates were not effective. However, out of six isolates that reduced the total number of lesions, four (GS6-1, GS6-2, GU21-2, and GU24-3) significantly ($P = 0.05$) reduced the total area of lesions after 24 and 72 h of incubation (Table 3). Isolates GS8-1, GS8-2, GS8-3, and GS10-1, which did not reduce the total number of lesions, reduced the total area of lesions. The total lesion number and area of plants treated with MI of GS6-1 and GU21-2 was reduced by 64 and 77%, respectively.

Isolates GU21-2 and GU23-3 were highly effective in reducing the total lesion number after 24 and 72 h of incubation against 10^5 spores per milliliter of the pathogen. GS6-1, GS6-2, GS7-4, GS8-2, and GU24-3 were next in their effectiveness. With an increase in incubation period of up to 72 h, isolates GS6-1 and GU24-3 reduced the total number of lesions (Table 2). Isolates that reduced the total lesion area against the lower pathogen inoculum of 10^4 spores per milliliter were also effective against

TABLE 1. Total lesion number and lesion area on leaves of cucumber grown in potting medium amended with barley grain inocula (2%, w/w) of plant growth promoting fungal (PGPF) isolates for 21 days and challenge inoculated with three spore concentrations (10^4, 10^5, 10^6) of *Colletotrichum orbiculare*

PGPF isolates	Total lesion number[x,y]			Total lesion area (mm)[x,y]		
	10^4	10^5	10^6	10^4	10^5	10^6
GS6-1	11.8 a[z]	20.0 a	20.0 a	49.8 a	80.2 a	109.4 a
GS6-2	10.8 a	17.4 b	20.0 a	32.6 b	68.7 ab	106.0 a
GS6-4	8.2 b	20.0 a	20.0 a	23.9 c	78.7 a	101.3 a
GS7-4	11.0 a	17.1 b	20.0 a	32.6 b	61.4 a–c	110.3 a
GS8-1	1.2 d	11.9 d	14.5 d	2.8 e	38.7 cd	70.5 c
GS8-2	1.3 d	12.8 cd	15.4 c	3.0 e	46.7 b–d	91.4 b
GS8-3	2.0 d	14.2 c	16.7 b	6.4 e	49.9 b–d	110.2 a
GS10-1	6.5 c	17.0 b	20.0 a	19.1 d	69.9 ab	108.7 a
GU21-2	5.2 c	10.9 d	17.4 b	14.5 e	31.2 d	74.8 c
GU23-3	2.3 d	14.1 c	15.3 c	6.4 e	52.8 b–d	76.1 c
GU24-3	6.1 c	11.5 d	20.0 a	19.5 d	47.2 b–d	105.0 a
Control	12.3 a	20.0 a	20.0 a	48.5 a	81.7 a	112.1 a

[x] Means of three trials each with four replicates.

[y] Experiment conducted in a factorial design. There were significant interactions ($P \leq 0.05$) between inducer treatment and spore concentration. For comparing means of lesion number in a row, LSD = 1.51 ($P = 0.05$) and 2.01 ($P = 0.01$). For comparing means of lesion area in a row, LSD = 12.32 mm ($P = 0.05$) and 16.44 mm ($P = 0.01$).

[z] Means carrying same letters in a column are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

10^5 spores per milliliter. GS8-2 and GS8-3, which were effective in reducing the total area of lesions at 10^4 spores per milliliter of the pathogen, were not effective at 10^5 spores per milliliter (Table 3).

When the pathogen was challenge inoculated at 10^6 spores per milliliter, the MI of GS6-1 and GU21-2 when incubated for 24 and 72 h were highly effective in reducing the total lesion number and area. The MI of GS7-4 and GU24-3 were less effective when incubated for 24 h, although their effectiveness increased after 72 h of incubation.

TABLE 2. Total number of lesions on leaves of cucumber induced with mycelial inocula of plant growth promoting fungal (PGPF) isolates for 24 and 72 h and challenge inoculated with three spore concentrations of *Colletotrichum orbiculare*

	Total lesion number[x]					
	Pathogen spore concentration ml^{-1}/Duration of induction (h)[y]					
	10^4		10^5		10^6	
PGPF isolates	24	72	24	72	24	72
GS6-1	4.2 f[z]	3.3 f	17.2 c	13.2 c	16.1 c	15.7 c
GS6-2	9.7 c–e	6.7 cd	17.3 c	14.8 bc	20.0 a	20.0 a
GS6-4	13.7 a	11.6 ab	20.0 a	17.9 a	20.0 a	20.0 a
GS7-4	9.4 de	7.9 c	17.4 c	14.0 bc	20.0 a	15.1 cd
GS8-1	12.1 a–c	11.4 ab	19.0 a–c	18.8 a	20.0 a	18.1 b
GS8-2	11.7 a–d	11.1 ab	17.5 c	16.0 b	20.0 a	17.5 b
GS8-3	12.6 ab	11.2 ab	19.6 ab	19.2 a	20.0 a	19.9 a
GS10-1	13.3 a	10.3 b	20.0 a	18.5 a	20.0 a	20.0 a
GU21-2	7.9 e	4.4 ef	13.5 d	10.0 d	17.4 b	12.5 e
GU23-3	10.7 b–d	6.0 de	14.4 d	14.6 bc	18.6 b	18.1 b
GU24-3	9.9 c–e	6.0 de	17.8 bc	12.6 c	20.0 a	14.6 d
Control	13.3 a	12.5 a	20.0 a	20.0 a	20.0 a	20.0 a

[x]Mean of three trials, each with four replicates.
[y]Experiment conducted in a factorial design. There were significant interactions ($P \leq 0.05$) among inducer treatment, spore concentration, and duration of induction. For comparing means of duration in a row, LSD = 1.65 ($P = 0.05$) and 2.20 ($P = 0.01$). For comparing spore concentration means within each inducer treatment duration in a row, LSD = 1.59 ($P = 0.05$) and 2.12 ($P = 0.01$).
[z]Means carrying same letters in a column are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

TABLE 3. Total lesion area on leaves of cucumber induced with mycelial inocula of plant growth promoting fungal (PGPF) isolates for 24 and 72 h and challenge inoculated with three spore concentrations of *Colletotrichum orbiculare*

	Total lesion area (mm)[x]					
	Pathogen spore concentration ml^{-1}/Duration of induction (h)[y]					
	10^4		10^5		10^6	
PGPF isolates	24	72	24	72	24	72
GS6-1	19.9 e[z]	16.8 h	56.7 cd	39.8 ef	63.6 c	52.8 e
GS6-2	44.1 cd	34.6 g	66.7 b–d	51.2 c–f	108.4 a	103.3 a
GS6-4	74.8 a	64.5 ab	100.0 a	80.9 ab	105.5 a	103.7 a
GS7-4	58.2 a–c	44.0 d–g	83.0 ab	53.6 c–e	102.5 a	79.1 c
GS8-1	65.9 a	51.4 cd	78.2 a–c	66.9 bc	104.7 a	92.9 b
GS8-2	66.4 a	52.9 b–d	82.5 a–c	83.6 ab	103.7 a	91.9 b
GS8-3	62.7 ab	59.6 bc	98.9 a	95.9 a	101.8 a	102.0 a
GS10-1	74.4 a	49.4 c–e	99.7 a	63.6 b–d	102.5 a	105.3 a
GU21-2	31.6 de	20.7 h	52.7 d	31.6 f	67.5 c	48.2 e
GU23-3	58.9 a–c	38.8 e–g	65.6 cd	53.5 c–e	82.0 b	62.2 d
GU24-3	46.9 b–d	37.8 fg	53.0 d	43.5 d–f	104.8 a	52.5 e
Control	75.4 a	74.6 a	93.6 a	95.3 a	107.8 a	105.0 a

[x]Mean of three trials, each with four replicates.
[y]Experiment conducted in a factorial design. There were significant interactions ($P \leq 0.05$) among inducer treatment, spore concentration, and duration of induction. For comparing means of duration in a row, LSD = 15.17 mm ($P = 0.05$) and 20.15 mm ($P = 0.01$). For comparing spore concentration means within each inducer treatment duration in a row, LSD = 13.42 mm ($P = 0.05$) and 18.13 mm ($P = 0.01$).
[z]Means carrying same letters in a column are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

Significant interactions ($P \leq 0.05$) among inducer treatment, duration for induction, and pathogen spore concentration obtained indicated that PGPF isolates, when used as MI, became more effective with an increase in duration for induction. However, with increase in pathogen spore concentration, the effectiveness of inducer isolates was reduced (Tables 2 and 3).

Culture filtrate. Isolates GS6-1 and GU21-2 that were highly effective in reducing total lesion number as MI were also effective as CF, after incubating for 24 and 72 h, and against a pathogen spore concentration of 10^4 spores per milliliter. The CF of GS6-2, GS8-3, and GU24-3 were next in their effectiveness after 24 and 72 h of incubation. On the other hand, isolates GS6-4, GS8-2, and GU23-3 reduced the total lesion number only after a 72-h incubation, compared with control (Table 4). The isolates that reduced the total lesion number also reduced the total lesion area. However, the effectiveness of these isolates increased with an increase in incubation period (Table 5). Reductions in the total lesion number and area ranged 78.3–87.9% in plants treated with CF of GS6-1 and GU21-2 for 72 h.

At high spore density (10^5 and 10^6 spores per milliliter) of the pathogen, the CF of GS6-1 and GU21-2 when incubated for 72 h were highly effective and reduced the total number of lesions. Isolates GS6-2, GS6-4, GS8-2, GS8-3, and GU24-3 were less effective (Table 4). The CF of all the above isolates, along with that of GS8-1 on incubation for 72 h, reduced the total lesion area on plants challenge inoculated with 10^5 spores per milliliter of the pathogen. However, at 10^6 spores per milliliter of the pathogen, GS6-1 and GU21-2 were the only two isolates that reduced the total lesion number and area after 24 and 72 h of incubation, followed by isolate GS8-3 (Table 5). Significant interactions ($P \leq 0.05$) among inducer treatment, duration for induction, and pathogen spore concentration were obtained as in the case of MI treatment (Tables 4 and 5).

Comparison of induction abilities of PGPF isolates and *C. orbiculare*. Among the selected PGPF isolates, isolate GS6-1 as CF and isolate GU21-2 as BGI and MI were not significantly different ($P = 0.001$) from *C. orbiculare* in reducing the total lesion number and lesion area (Table 6). Induction of plants with the remainder of the PGPF isolates gave less protection than with *C. orbiculare*.

TABLE 4. Total number of lesions on leaves of cucumber induced with culture filtrates of plant growth promoting fungal (PGPF) isolates for 24 and 72 h and challenge inoculated with three spore concentrations of *Colletotrichum orbiculare*

	Total lesion number[x]					
	Pathogen spore concentration ml^{-1}/Duration of induction (h)[y]					
	10^4		10^5		10^6	
PGPF isolates	24	72	24	72	24	72
GS6-1	5.3 de[z]	2.6 f	14.4 e	9.2 f	15.6 d	13.2 d
GS6-2	6.8 cd	6.1 de	15.8 c–e	15.2 d	20.0 a	20.0 a
GS6-4	10.4 b	8.8 bc	19.0 ab	16.8 cd	20.0 a	20.0 a
GS7-4	13.0 a	11.9 a	19.3 ab	20.0 a	20.0 a	20.0 a
GS8-1	11.2 ab	10.6 ab	18.2 a–c	17.6 bc	20.0 a	18.6 b
GS8-2	11.3 ab	7.8 cd	19.7 ab	15.2 d	20.0 a	16.1 c
GS8-3	7.5 c	6.3 de	14.5 e	11.4 e	20.0 a	16.1 c
GS10-1	13.0 a	11.7 a	20.0 a	19.0 ab	20.0 a	20.0 a
GU21-2	3.4 e	1.9 f	15.0 de	12.7 e	18.2 c	14.2 d
GU23-3	11.2 ab	5.4 e	17.2 b–d	17.1 c	18.9 bc	18.5 b
GU24-3	8.0 c	6.1 de	19.4 ab	16.7 cd	19.1 b	18.7 b
Control	12.9 a	12.0 a	20.0 a	20.0 a	20.0 a	20.0 a

[x]Mean of three trials, each with four replicates.
[y]Experiment conducted in a factorial design. There were significant interactions ($P \leq 0.05$) among inducer treatment, spore concentration, and duration of induction. For comparing means of duration in a row, LSD = 1.17 ($P = 0.05$) and 1.56 ($P = 0.01$). For comparing spore concentration means within each inducer treatment duration in a row, LSD = 1.67 ($P = 0.05$) and 2.23 ($P = 0.01$).
[z]Means carrying same letters in a column are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

Root colonization ability. The isolation frequencies of PGPF isolates did not vary between surface-disinfected and nondisinfected roots. Among 16 isolates of PGPF tested, only three isolates—GS8-1, GS8-2, and GS8-3—had the ability to colonize roots. Analysis of results revealed significant ($P \leq 0.05$) interactions among the PGPF treatments, region of colonization, and days of isolation. Isolate GS8-1 colonized only after 7 days and the colonization increased significantly ($P = 0.01$) in the upper and lower regions with an increase in the age of the seedling. GS8-2 was isolated in high frequencies during the first 7 days of seedling growth, but with increased seedling age the isolation frequency did not increase significantly. GS8-3, on the other hand, was a rapid colonizer, whose isolation frequency increased to 100% in the upper root segments by 21 days. The colonization of roots by these isolates was usually more in the upper than in the middle and lower root parts (Fig. 1). The lower parts of the root were always less colonized except in the case of GS8-3. None of the PGPF isolates were reisolated from the aboveground parts.

TABLE 5. Total lesion area on leaves of cucumber induced with culture filtrates of plant growth promoting fungal (PGPF) isolates for 24 and 72 h and challenge inoculated with three spore concentrations of *Colletotrichum orbiculare*

PGPF isolates	Total lesion area (mm)[x]					
	Pathogen spore concentration ml^{-1}/ Duration of induction (h)[y]					
	10^4		10^5		10^6	
	24	72	24	72	24	72
GS6-1	24.3 d[z]	12.6 f	41.3 c	30.9 ef	60.7 b	35.5 c
GS6-2	31.3 d	25.9 e	46.7 c	48.5 d	106.2 a	110.0 a
GS6-4	76.9 a	39.0 cd	80.5 ab	54.0 cd	106.2 a	102.8 a
GS7-4	73.0 ab	73.7 a	94.8 a	96.3 a	107.0 a	107.9 a
GS8-1	74.7 ab	48.1 bc	81.8 ab	61.0 c	103.3 a	103.5 a
GS8-2	76.4 a	46.5 bc	83.2 ab	59.7 c	103.7 a	108.6 a
GS8-3	32.1 d	24.1 e	43.7 c	43.4 e	104.4 a	59.7 b
GS10-1	77.6 a	55.8 b	94.5 a	77.1 b	107.0 a	108.3 a
GU21-2	14.8 e	8.8 f	48.3 c	37.7 f	69.2 b	58.9 b
GU23-3	67.0 b	30.2 de	75.2 b	83.7 b	96.1 a	97.6 a
GU24-3	43.1 c	33.2 de	92.7 a	63.8 c	101.1 a	101.8 a
Control	76.0 a	72.9 a	93.3 a	96.7 a	106.5 a	108.9 a

[x] Mean of three trials, each with four replicates.
[y] Experiment conducted in a factorial design. There were significant interactions ($P \leq 0.05$) among inducer treatment, spore concentration, and duration of induction. For comparing means of duration in a row, LSD = 19.65 mm ($P = 0.05$) and 26.1 mm ($P = 0.01$). For comparing spore concentration means within each inducer treatment duration in a row, LSD = 15.84 mm ($P = 0.05$) and 21.46 mm ($P = 0.01$).
[z] Means carrying same letters in a column are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

TABLE 6. Effect of plant growth promoting fungal (PGPF) isolates and *Colletotrichum orbiculare* on the total lesion number[w] and total lesion area[w] on leaves of cucumber plants challenge inoculated with *C. orbiculare*

Inducer treatment	Total lesion number			Total lesion area (mm)		
	BGI[x]	MI[y]	CF[y]	BGI	MI	CF
GS6-1	18.9	10.0***	7.0***	88.8	27.8***	22.1***
GS8-1	8.5***	18.0	16.0	41.7***	68.7***	61.0***
GS8-2	11.1***	15.2**	14.1**	47.2***	86.1*	56.3***
GS10-1	18.7	18.7	17.8	66.8***	61.5***	84.3
GU21-2	7.3***	7.1***	10.4***	21.7***	23.0***	34.4***
C. orbiculare[z]	3.1***	3.4***	3.0***	17.8***	19.2***	20.2***
Noninduced	18.8	18.9	18.0	89.5	90.7	89.1
LSD $P = 0.05$	2.37	2.04	2.58	10.05	4.74	6.90
$P = 0.01$	3.23	2.78	3.51	13.67	6.05	9.39
$P = 0.001$	4.36	3.75	4.74	18.45	8.16	12.67

[w] Values are the mean of two trials, each with four replicates.
[x] Plants were grown in potting medium amended with BGI (barley grain inocula) of PGPF isolates (2%, w/w) for 21 days in the growth chamber and challenge inoculated with *C. orbiculare* on the second true leaves.
[y] Plants were grown in unamended potting medium for 21 days and their roots were treated with MI (mycelia inocula) or CF (culture filtratres) of PGPF isolates for 72 h and challenge inoculated with *C. orbiculare* on the laminae of second true leaves.
[z] Plants were grown in unamended potting medium for 15 days and induced by inoculating with *C. orbiculare* (10^5 spores ml^{-1}, twenty 10 μl drops) on the first true leaves. After 6 days, the second true leaves of the pathogen-induced plants were challenge inoculated with the same spore density of *C. orbiculare*. *, **, and *** indicate significant decrease in disease over noninduced control at $P = 0.05$, $P = 0.01$, and $P = 0.001$, respectively.

In vitro interaction. Growth of the pathogen in the presence of PGPF isolates was not retarded or inhibited when compared with growth in the absence of PGPF. Some PGPF isolates produced pink pigments when the colony came in contact with that of the pathogen; however, no zone of inhibition was observed. On the other hand, some isolates were fast-growing and hence grew over the colony of the pathogen without restricting its growth rate.

Plant growth promotion ability. Among 16 isolates tested, 14 promoted the plant height and biomass significantly ($P = 0.05$), compared with plants grown in potting medium amended with uninfested barley grains and in unamended medium (Fig. 2). GS6-4 and GS8-6 induced maximal increases in plant height and biomass. Isolates GS7-3 and GU23-3, and isolates GS7-3 and GS7-4, did not induce a significant increase in plant height and biomass, respectively.

Only the MI of GS6-2 and GU21-2, among the seven isolates tested, enhanced plant height and biomass significantly ($P = 0.05$). However, the CF of GS6-1, GS6-2, GS8-2, and GU21-2 increased plant height and biomass significantly ($P = 0.05$). The MI and CF of GS6-2 increased both parameters to the maximum extent, compared with other isolates (Fig. 3). Plants grown in soil unamended or amended with uninfested barley grains did not show enhanced growth or protection.

DISCUSSION

The present study revealed that disease caused by *C. orbiculare* on leaves can be suppressed by using PGPF isolates. Suppression of disease appeared to be systemic, as roots were treated with PGPF isolates and the pathogen was challenge inoculated on leaves, thereby separating the two spatially, although biochemical studies are needed for further confirmation of induced resistance. However, no effective PGPF isolates could be recovered from the aboveground parts. This is in agreement with the findings of van Peer et al (40) and Wei et al (42).

The present study indicated that induction of systemic resistance in cucumber plants not only depended on the type of inducer

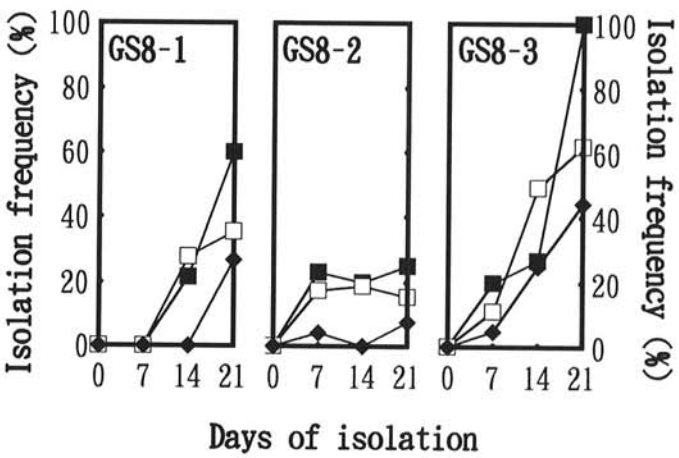

Fig. 1. Isolation frequencies of plant growth promoting fungal (PGPF) isolates GS8-1, GS8-2, and GS8-3 from the upper (toward stem) (■), middle (□), and lower (toward root tip) (■) root segments of 7-, 14-, and 21-day-old cucumber seedlings grown in potting medium amended with barley grain inocula (2%, w/w). For comparing treatment means of isolation frequency within plant age, LSD = 2.6% ($P = 0.05$) and 3.5% ($P = 0.01$).

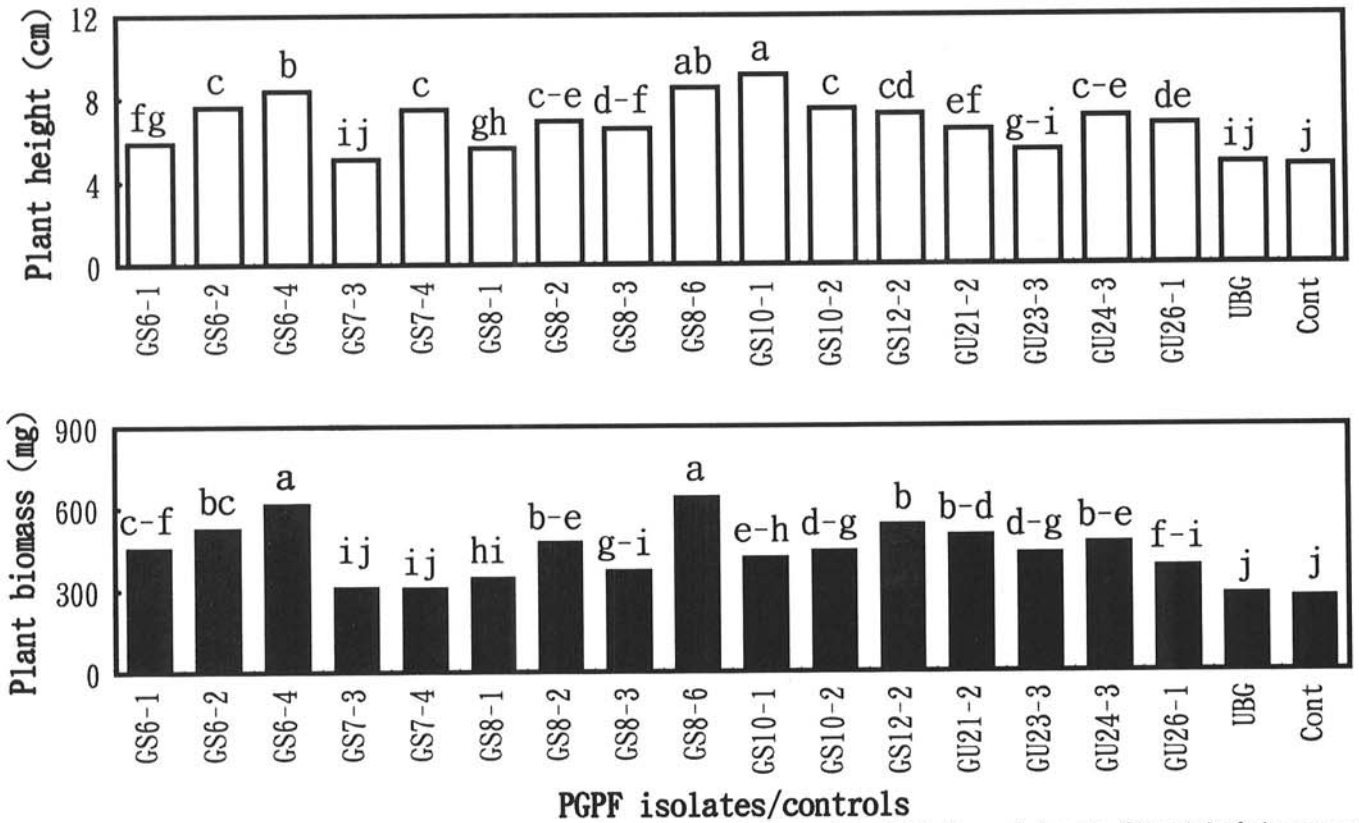

Fig. 2. Plant height and dry biomass of cucumber plants grown in potting medium amended with barley grain inocula (2%, w/w) of plant growth promoting fungal (PGPF) isolates for 21 days. UBG = Plants grown in potting medium amended with uninfested barley grains. Cont = Plants grown in potting medium not amended with barley grains. Bars with same letters are not significantly different ($P = 0.05$), according to Duncan's multiple range test. Histograms represent the mean of three trials, each with four replicates per treatment.

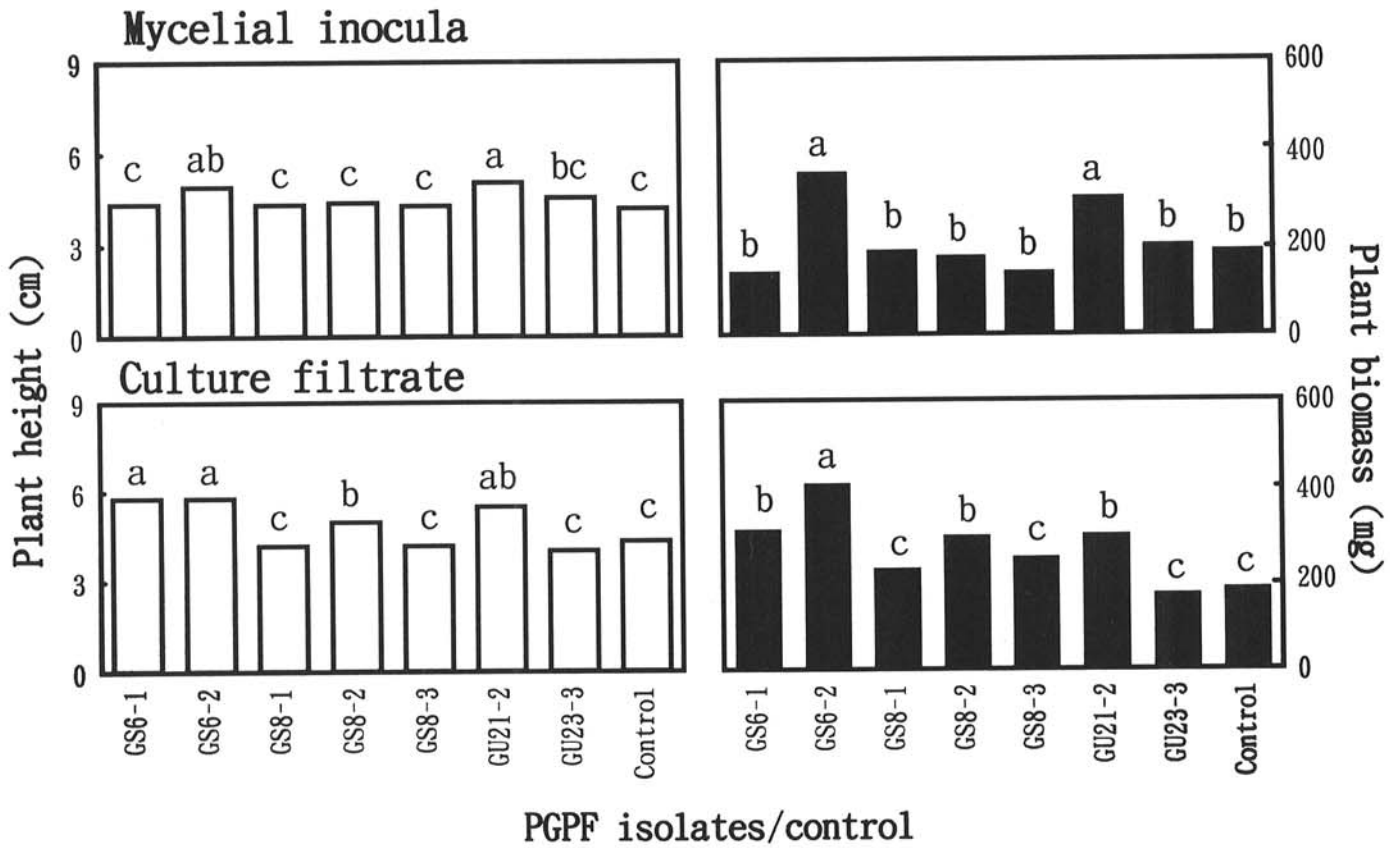

Fig. 3. Effect of treatment of cucumber seeds with mycelial inocula (MI) and culture filtrates (CF) of plant growth promoting fungal (PGPF) isolates on height and dry biomass of plants grown for 21 days in potting medium. Control = Seeds not treated with MI or CF. Bars with same letters are not significantly different ($P = 0.05$), according to Duncan's multiple range test. Histograms represent the mean of three trials, each with four replicates per treatment.

isolate, type of inocula, and duration of induction, but also on the density of the challenger inoculum. PGPF isolates induced resistance when provided to plants as BGI, MI, or CF. Millet grain inocula, homogenized mycelia, and culture filtrates of inducer agents have been shown to reduce disease in plants (11,12,38). Among the different PGPF isolates tested, GS8-1, GS8-2, and GS8-3, when used as BGI rather than as MI, suppressed the disease to the maximum extent compared with other isolates, and these isolates were capable of colonizing roots. This suggested that a longer period of their association with roots was necessary for the induction of resistance. Previous reports on foliar-induced resistance also suggested that root colonization by the biocontrol agent was necessary before resistance was realized (3,27,42,44). On the other hand, certain isolates (such as GU21-2, GU23-3, and GU24-3) that lacked root colonization ability induced resistance when roots were treated with their MI or CF for as little as 24 or 72 h, indicating that some factor(s) other than root colonization ability might be responsible for induction. These isolates also induced resistance when provided as BGI after 21 days of plant growth.

Certain isolates (GS6-1 and GU21-2) were effective as MI or CF in inducing resistance. The in vitro interaction experiment revealed that they did not produce any zone of inhibition, suggesting that they were not antagonistic, thus ruling out the possible involvement of toxins or antifungal compounds in disease suppression. Our results confirm those of Wei et al (42), who reported that antifungal compounds were not involved in the induction of resistance in cucumber while using plant growth promoting rhizobacteria (PGPR) as an inducer. CF of pathogens and nonpathogens have also been shown to initiate host defense responses in cucumber and carnations (1,20,26,37).

An increase in the inducer treatment duration provided higher protection. The MI and CF of certain isolates afforded better protection when incubated for 72 rather than 24 h. On the other hand, some other isolates as BGI protected plants when the treatment duration was further prolonged to 21 days. However, isolate GS6-1, which was highly effective as MI and CF after 72 h, was not effective as BGI even after 21 days. This suggested that if the period between the induction and challenge was prolonged the protection effect gained in the short duration was lost. This observation corroborates those of Wymore and Baker (43).

Spore concentration of the pathogen appeared to play a significant role in inducing systemic resistance since the ability to suppress disease by different PGPF isolates decreased with increase in pathogen spore concentration. Similar observations were also made by Dean and Kuć (7), Gessler and Kuć (14) and Ishiba et al (20). Certain isolates—GS6-1 and GU21-2 (MI and CF) and GU24-3 (MI)—were capable of restricting the disease even against a high pathogen spore concentration. However, some PGPF isolates became ineffective when a high inoculum load was challenge inoculated on plants. GS8-3, which is a strong root colonizer, failed to restrict the disease at high pathogen spore concentration, suggesting that the reduction in disease depended not only on the root colonization but also on the inherent ability of the isolate.

The reduction in lesion number and diameter induced by *C. orbiculare* was more than that of certain PGPF treatments. However, the lesion diameter reduction caused by GS6-1 (CF) and GU21-2 (BGI and MI) was not significantly different from that of *C. orbiculare*–induced control. Maurhofer et al (28) also obtained nonsignificant differences between a tobacco necrosis virus (TNV)–induced and a PGPR-induced resistance against TNV infection. On the other hand, Wei et al (42) obtained high protection with *C. orbiculare* compared with PGPR. The high protection induced by *C. orbiculare* is expected since the inducer involved is a necrosis-causing pathogen.

Plant growth promotion by fungi has been demonstrated in a few crops (9,15,31) compared with a large number of reports on growth promotion by rhizobacteria (5,16,21). In our study, PGPF isolates caused growth promotion of plants when applied as BGI, MI, or CF, revealing that growth promotion might be independent of the root colonization ability. Seed treatment with MI or CF of certain PGPF isolates for 72 h resulted in a significant enhancement of plant growth compared with those of untreated ones and these isolates lacked colonization ability. These results suggest that such isolates might produce certain metabolites that induce growth promotion. Dewan and Sivasithamparam (10) demonstrated that some factors in exudates produced by a sterile red fungus were responsible for the growth enhancement of wheat and other crops. The growth promotion ability of microbes has also been largely attributed to the production of growth-regulating substances (32,36). However, certain other isolates failed to induce growth as MI or CF, but promoted growth as BGI and colonized roots. When wheat was grown in nutrient-depleted sterile soil amended with mycelial inocula of GS8-3, the plant length and biomass increased considerably compared with unamended soil, which was attributed to its root colonization (35). One mechanism of growth promotion could be the ability of certain isolates to colonize roots and provide minerals to plants in a more available form. Cowan (6) showed that isolates of *Phialophora graminicola* promoted growth of wheat plants when amended to soil and suggested that the promotion of growth was due to the increased uptake of mineral nutrients by plants. Similar observations were also made by Barber and Lynch (2). The fact that seed treatment with MI or CF of certain PGPF isolates resulted in growth promotion indicates that nutrition from the barley grain inoculum is not a possible factor in the growth promotion mechanism, at least for such type of isolates. However, the ability of PGPF isolates to degrade barley grains and release nutrients, thus resulting in the stimulation of plant growth, cannot be ruled out. Shivanna et al (35) showed that the ammonium-N content of barley grains colonized by certain PGPF isolates was increased due to colonization by these isolates and the ammonium form of N could be more readily utilized by roots leading to the enhancement of plant growth.

In the present study, isolates that induced systemic resistance promoted the growth of cucumber plants and some also colonized roots. The results suggest that these growth-promoting fungi are capable of inducing systemic protection against *C. orbiculare* by root colonization or by other means of triggering the host defense mechanism. This study sheds light on the potential of some saprophytic, sterile fungi as plant growth promoters as well as biocontrol agents. A detailed investigation is under way to understand the exact mechanism of growth promotion and systemic resistance in cucumber using PGPF isolates.

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