European Journal of Plant Pathology **107:** 523–533, 2001.

Induction of systemic resistance in cucumber against several diseases by plant growth-promoting fungi: lignification and superoxide generation

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Accepted 2 April 2001

Key words: induced systemic resistance, plant growth-promoting fungi, cucumber, lignification, superoxide generation

Abstract

Five fungal isolates (*Trichoderma*, *Fusarium*, *Penicillium*, *Phoma* and a sterile fungus) from zoysiagrass rhizosphere that promote plant growth were tested for their ability to induce systemic resistance in cucumber plants against *Colletotrichum orbiculare*. Roots of cucumber plants were treated with these fungal isolates using barley grain inocula (BGI), mycelial inocula (MI) or culture filtrate (CF). Most isolate/inoculum form combinations significantly reduced the disease except BGI of *Trichoderma*. These fungal isolates were also evaluated for induction of systemic resistance against bacterial angular leaf spot and Fusarium wilt by treatment with BGI. *Penicillium*, *Phoma* and the sterile fungus significantly reduced the disease incidence of bacterial angular leaf spot. *Phoma* and sterile fungus protected plants significantly against Fusarium wilt. Roots treated with CFs of these fungal isolates induced lignification at *Colletotrichum* penetration points indicating the presence of an elicitor in the CFs. The elicitor activity of CFs was evaluated by the chemiluminescence assay using tobacco callus and cucumber fruit disks. The CFs of all isolates elicited conspicuous superoxide generation. The chemiluminescence activity of the CF of *Penicillium* was extremely high, and its intensity was almost 100-fold higher than that of other isolates. The chemiluminescence activity was not lost following treatment with protease or autoclaving or after removal of lipid. The MW 12,000 dialyzed CF fraction was highly effective in eliciting chemiluminescence activity. Chemiluminescence emission from cucumber fruit disks treated with *Penicillium* was the same as that obtained from tobacco callus, except that the lipid fraction also showed a high activity. Both the MW 12,000 fraction and the lipid fraction induced lignification in the epidermal tissues of cucumber hypocotyls.

Abbreviations: PGPF – Plant growth-promoting fungi; BGI – barley grain inocula; MI – mycelial inocula; CF – culture filtrate; CL – chemiluminescence.

Introduction

Induced systemic resistance in plants to fungal, bacterial and viral pathogens has been demonstrated after preinoculation with weakly aggressive, avirulent or incompatible forms of the disease-causing organisms. This response of plants after induction is known as systemic acquired resistance (SAR) and has been demonstrated in several plant–pathogen systems (Ross et al., 1961; Kuć, 1983; Ishiba et al., 1981). Most of our knowledge on the induction of disease resistance has been gained by using cucumber and the anthracnose pathogen, *Colletotrichum orbiculare*, as a model. Recently, however, some

beneficial microorganisms such as plant growth-promoting rhizobacteria (PGPR) were shown to induce systemic resistance (Wei et al., 1991; Van Loon et al., 1998). Previous research demonstrated that fungal isolates collected from the rhizospheres of zoysiagrass enhanced the growth of a variety of crop plants and thus these isolates were designated as plant growth-promoting fungi (PGPF) (Meera et al., 1994). The PGPF belonged to the genera *Fusarium*, *Penicillium*, *Phoma*, *Trichoderma* and sterile fungi. Meera et al. (1994) reported that systemic resistance was induced in cucumber using the *Phoma* sp. and the sterile fungus against anthracnose caused by *C. orbiculare*. Cucumber roots treated with culture filtrates (CFs) of PGPF isolates also induced resistance against anthracnose. CF-treated plants expressed resistance to pathogen infection by an alteration of various metabolisms, such as high increases in activities of chitinase, β-1,3-glucanase, peroxidase, polyphenol oxidase and phenylalanine ammonia lyase, indicating that an elicitor substance(s) existed in the CFs (Meera, 1994).

The objectives of this study were (i) to elucidate whether PGPF isolates belonging to several genera, besides *Phoma* and sterile fungi, could induce resistance in cucumber against not only anthracnose but also bacterial angular leaf spot and Fusarium wilt, and (ii) to identify elicitor substance(s) in the CF using a chemiluminescence (CL) assay.

Materials and methods

Host and pathogen culture

The cucumber cv. Gibai was used throughout this study. The anthracnose pathogen, *C. orbiculare* (= *C. lagenarium*) isolate 104T, was maintained on potato-dextrose agar (PDA). The bacterial angular leaf spot pathogen, *Pseudomonas syringae* pv. *lachrymans* isolate 201, was stored in King's B (KB) medium with 10% skim milk solution at −80 °C. The Fusarium wilt pathogen, *Fusarium oxysporum* f.sp. *cucumerinum* isolate 501, was stored at 4 °C on PDA. The pathogens were transferred to Petri plates containing appropriate media (PDA for *C. orbiculare* and *F.o. cucumerinum* and KB medium for *P.s. lachrymans*). Before use, *C. orbiculare*, *P.s. lachrymans* and *F.o. cucumerinum* were incubated for seven, three and 14 days, respectively.

Preparation of PGPF inoculum

Five PGPF isolates (*Trichoderma* GT3-2, *Fusarium* GF18-3, *Penicillium* GP17-2, *Phoma* GS8-2 and sterile fungus GU23-3) from zoysiagrass rhizospheres were screened for their effectiveness as inducers of resistance against anthracnose. Cucumber roots were treated with three inoculum forms of PGPF: barley grain inocula (BGI), mycelial inocula (MI) or culture filtrate (CF). BGI of five isolates were also used to induce resistance in cucumber against bacterial angular leaf spot and Fusarium wilt disease.

BGI

Autoclaved barley grains were inoculated with mycelial disks of each PGPF isolate. After 10–12 days of incubation at 25 °C, the completely colonized barley grains were air dried at 22–25 °C. The dried BGI was ground to 1- to 2-mm particle size and mixed (2%, w/w) with autoclaved potting medium.

Mycelial inoculum (MI) and culture filtrates (CF)

Each of the PGPF isolates was cultured in potato-dextrose broth (pH 6.5) without shaking at 25 °C for 10–12 days in darkness. The fungal mat was separated from the CF by pouring through five layers of cheesecloth. The mycelia were thoroughly washed with sterile distilled water to remove the remaining CF from the mycelial mat and placed on filter paper to remove excess moisture. The mat was weighed and distilled water was added (1 g of mycelial mat/4 ml of water). Mycelia were blended in a blender at 5,000 rpm for 5 min. The crude CF was separated from mycelia and filtered three times through five layers of filter paper (Whatman No. 1). The CF was filtered (Milipore filter, pore size 0.45 μm). One milliliter of the CF was added to 4 ml of sterile distilled water and used for cucumber ISR assay.

Cucumber ISR assays

Barley grain inocula (BGI) experiments

One hundred and twenty grams of the potting medium/inoculum infested BGI mixture (2%, w/w) were placed in each sterilized plastic pot (autoclavable, ϕ6 cm × 7.5 cm). Cucumber seeds which had previously been surface sterilized with 0.2% NaOCl for 30 min, were sown in the mixture (one seed per pot). The plants were grown at 25 °C for 21 days in a growth

chamber with a 10 and 14 h light (24,000 lux)/dark period. The plants grown in potting medium with untreated BGI were used as a control. Each treatment consisted of four replicates, with three plants in each replicate.

Mycelial inoculam and culture filtrate experiments

Paper pots (Nippon Beet Sugar Co. Ltd., Japan) containing 162 pots (each pot ϕ3.8 cm × 5.0 cm) were autoclaved and filled with approximately 70 g of potting medium. In each pot, an individual surface-sterilized cucumber seed was sown. The pots were separated into individual pots, and the seeds were allowed to germinate and grow at 25 °C for 21 days in the greenhouse. The bottom portion of the pot was partially removed to expose tips of roots. After trimming the root rips, plant roots were dipped into either 4 ml of MI, 20 ml of CF, or 20 ml of water contained in sterile pots. After 72 h of incubation, the treated roots were covered with the autoclaved sterile potting medium. Plants were challenge inoculated after 72 h of incubation. Each treatment consisted of four replicates, with three plants in each replicate.

In the case of Fusarium wilt disease, a split-root system was used. Three-week-old cucumber seedlings were removed from the paper pots. The roots were washed with sterile distilled water and split into two halves. The plants were transferred to sterile plastic pots in which one half of the roots was covered with a sterile potting medium amended with BGI (2%, w/w) of PGPF and the other half with sterile potting medium. These split-root plants were grown in a greenhouse for seven days. This experiment consisted of three replicates, with five plants in each replicate.

Challenge inoculation

Plants were inoculated by placing 20 drops (10 µl) of the spore or bacterial suspension (10^5 spores/ml and 10^8 CFU/ml, respectively) on the second true leaf. The inoculated plants were incubated at 25 °C for 30 h in a dark, humid chamber and were transferred back to the greenhouse for another six days before evaluating the disease. The total number of lesions and total diameter of lesion per leaf caused by anthracnose and bacterial angular leaf spot in BGI treated and untreated plants were measured. For Fusarium wilt disease, the half of the cucumber roots that were covered with only potting medium were inoculated with 10^4 microconidia of the pathogen per g of dry soil. After 14 days in the greenhouse, the plants were evaluated for wilt symptoms.

Disease assessment

The total number of lesions and the diameter of the lesions on leaf lamina of protected and unprotected plants grown under greenhouse conditions were measured. Disease severity (DS) of *C. orbiculare* and *P. syringae* pv. *lachrimans* was calculated using the disease index (DI), which is related to the total diameter of lesions ranging between 0 (no lesion) through 5 (lesion measuring 5 mm and above). The formula used is as follows:

$$\text{DS} = \Sigma\text{DI}/\text{Total number of inoculum drops applied (20)}$$

Disease severity of Fusarium wilt on each plant was calculated using the DI ranging between 0 (no wilt symptom) through 4 (completely wilted or dead plant). The formula used is as follows:

$$\text{DS} = \Sigma\text{DI}/\text{Total number of plants}$$

The level of protection induced by PGPF isolates against *C. orbiculare*, *P. syringae* pv. *lachrymans* and *F. oxysporum* f. sp. *cucumerinum* was calculated based on DS as follows:

$$\text{Protection} = (1 - \text{DS in induced plants}/\text{DS in control plants}) \times 100\%$$

Testing for lignin formation

Cucumber seeds were sown in moist sterilized filter paper and incubated for seven days in the dark at 25 °C. Roots of each seedling were individually dipped in 5.0 ml of CF contained in tubes and incubated for 24 h. After incubation the hypocotyls of the seedlings were inoculated with 10, 5 µl drops of spore suspension (1×10^5 spores/ml) of *C. orbiculare*. The incubated seedlings were further incubated for 20 h. The epidermal strips from hypocotyls of the seedlings were stained for the lignin using the toluidine blue O or phloroglucinol-HCl (Sherwood and Vance, 1976) and observed for the degree of lignification under the microscope.

Spores of *C. orbiculare* germinated 90% or more on cucumber hypocotyls. The degree of lignin deposition

was evaluated by determining the percentage of germinated spores together with appressoria around which lignin depositions were induced. For each treatment 100 germinated spores were evaluated. The percentage values of 0%, <30%; 31–70% and >70% were shown as −, +, ++ and +++, respectively.

Measuring of superoxide generation using a chemiluminescence assay

The elicitor activity of culture filtrates was evaluated in tobacco callus and cucumber fruit disks using a CL assay (Miura et al., 1995). Tobacco calli were incubated in MS medium for five days. After the incubation, the calli were suspended in MES buffer and stirred. The supernatant was discarded and the calli were collected. After aging 1.5 g calli for 30 min, the calli were treated with 3 ml of non-diluted CF and the amount of the CL emission was counted using a CL reader (Chemiluminescence Counter ELC-20, Tohoku Denshi Industrial Corporation, Japan). For cucumber assays, slices were placed in a humid chamber at 25 °C for 16 h for aging. A mixture of 0.05 ml of Tris-HCl buffer (pH 7.4) containing 1 mM luminol and 0.05 ml of non-diluted CF was painted on the surface of the cucumber slices and the emission of the CL pattern appearing from them was recorded. The amount of the CL emission was counted using ARGUS-50 (Hamamatsu Photonics K.K., Japan).

Fractionation of CF

The CF of isolate GP17-2, which was a good inducer of resistance against anthracnose, induced strong lignification in plants and showed extremely high CL activity, was used for fractionation studies. To determine whether the superoxide generating fractions of CF also function as elicitors and induce lignification in plants, the CF was fractionated as follows: (1) protein inactivated fraction; CF was treated with protease K at 37 °C for 1 h and after centrifugation at 12,000 rpm the supernatant was collected to remove the protein precipitate; (2) lipid fraction; CF was suspended in two volumes of diethyl ether and stirred for 15 min. The suspension was filtered through a sintered glass funnel. The ether fraction was concentrated by flash evaporation. The concentrated ether fraction was dissolved in a small amount of acetone and suspended in sterile distilled water; (3) >MW 12,000, MW 8,000–12,000 and <MW 8,000 fractions; to obtain different molecular weight fractions of CF, CF was dialyzed against sterile distilled water for 24 h at 4 °C, using a cellulose with a pore size capable of retaining substance with >MW 12,000 membrane (Cellulose tubing UC 36-32, mol wt, cut off 12,000–14,000, Viskase Sales Corporation, Japan). The dialyzed fraction containing substances <MW 12,000 were again concentrated as mentioned earlier and dialyzed over night in sterile distilled water at 4 °C using a membrane with a medium pore size, capable of retaining substances of >MW 8,000 (Spectra/Por molecularporous membrane 7, mol wt, cut off 8,000, Spectrum, USA).

Results

Systemic induced resistance in cucumber against anthracnose using different inocula of PGPF

To investigate whether the PGPF isolates induce resistance against *C. orbiculare*, roots of cucumber plants were treated with BGI, MI, or CF of the PGPF and the level of protection was evaluated.

Barley grain inoculum

Among the five isolates used, four excluding *Trichoderma* isolate GT3-2 significantly reduced the total number of lesions and the total lesion diameter ($P = 0.05$) in challenge inoculated plants, in comparison with challenge inoculated control plants (Table 1). The level of protection of plants treated with PGPF isolates of the sterile fungus, *Phoma*, *Fusarium* and *Penicillium* were 75.8%, 73.4%, 65.4% and 65.6%, respectively (Table 2).

Mycelial inoculum

All isolates reduced the total lesion number and diameter significantly (Table 1) and provided 50.7–85.3% protection (Table 2). Among the isolates, GP17-2, GS8-2 and GU23-3 were effective in reduction of both the total lesion number and diameter and above all GP17-2 showed the highest protection at 85.3%.

Culture filtrate

All isolates reduced the total lesion number and diameter significantly (Table 1). Isolates GU23-3 and GP17-2 were highly effective in reducing the total lesion number. Their protections were 80.4% and 82.1%, respectively (Table 2).

Table 1. Effect of plant growth promoting fungi on the total lesion number and diameter on leaves of cucumber plants challenge inoculated with *C. orbiculare*

PGPF isolates	Total lesion number[a]			Total lesion diameter (mm)[a]		
	BGI[b]	MI[c]	CF[c]	BGI	MI	CF
Trichoderma GT3-2	12.9 a	7.3 bc	8.2 b	67.3 b	19.3 bc	30.8 b
Fusarium GF19-2	6.1 bc	8.9 b	7.1 bc	17.5 c	38.3 b	29.5 b
Penicillium GP17-2	4.5 c	4.2 c	4.3 c	25.7 c	10.5 c	18.5 b
Phoma GS8-2	7.2 b	4.7 c	6.7 bc	23.1 c	15.3 c	23.5 b
sterile GU23-3	5.4 bc	5.9 bc	4.8 c	12.1 c	12.1 c	13.3 b
Control	15.1 a	15.3 a	15.3 a	85.4 a	81.5 a	81.5 a

[a]Mean of three trials each with four replicates. Means carrying different letters in a column are significantly different ($P = 0.05$) according to Fisher's LSD.
[b]Plants were grown in potting medium amended with BGI (barley grain inocula) of PGPF isolates (2%, w/w) for 21 days and challenge inoculated with 10 µl drops of 10^5 spores/ml of *C. orbiculare* at 20 locations on the second true leaves.
[c]Plants were grown in unamended potting medium for 21 days and their roots were treated with MI (mycelial inocula) or CF (culture filtrates) of PGPF isolates 1 for 24 h and challenge inoculated with 10 µl drops of 10^5 spores/ml of *C. orbiculare* at 20 locations on the second true leaves.

Table 2. Protection of cucumber induced by growing plants for 21 days in potting medium amended with plant growth-promoting fungal isolates, following challenge inoculation with *C. orbiculare* and *P. syringae* pv. *lachrymans*

PGPF isolates	Protection (%)[a]			
	C. orbiculare		*P.s. lachrymans*	
	BGI[b]	MI[c]	CF[c]	BGI[b]
Trichoderma GT3-2	13.7	59.4	52.2	10.2
Fusarium GF19-2	65.4	50.7	76.0	19.2
Penicillium GP17-2	65.6	85.3	82.1	52.0
Phoma GS8-2	73.4	82.6	75.5	31.6
sterile GU23-3	75.8	78.8	80.4	59.3

[a]The total number of lesions and the diameter of the lesions on leaf lamina of protected and unprotected plants grown under greenhouse conditions were measured. Disease severity (DS) of *Colletotrihum orbiculare* and *P. syringae* pv. *lachrimans* was calculated using the disease index (DI), assessing the total diameter of lesions with a range between 0 (no lesion) through 5 (lesion measuring 5 mm and above). The formula used is as follows, DS = ΣDI/Total number of inoculum drops applied (20). The level of protection induced by PGPF isolates was calculated based on disease severity (DS) as follows, Protection = (1 – DS in induced plants/DS in control plants)×100%.
[b]Plants were grown in potting medium amended with BGI (barley grain inocula) of PGPF isolates (2%, w/w) for 21 days and challenge inoculated with *C. orbiculare* and *P. syringae* pv. *lachrymans*.
[c]Plants were grown in unamended potting medium for 21 days and their roots were treated with MI (mycelial inocula) or CF (culture filtrates) of PGPF isolates for 24 h and challenge inoculated with *C. orbiculare*.

Table 3. Total lesion number and diameter on leaves of cucumber grown for 21 days in potting medium amended with barley grain inocula (2%, w/w) of PGPF isolates, after challenge inoculation with 10 µl drops of *P. syringae* pv. *lachrymans* at 20 locations

PGPF isolates	Total lesion number[a]	Total lesion diameter (mm)[a]
Trichoderma GT3-2	10.7 b	37.5 a
Fusarium GF19-2	12.8 ab	27.6 b
Penicillium GP17-2	5.6 c	18.7 bc
Phoma GS8-2	7.0 c	23.6 bc
sterile GU23-3	6.3 c	15.8 c
Control	15.0 a	39.8 a

[a]Mean of three trials each with four replicates. Means carrying different letters in a column are significantly different ($P = 0.05$) according to Fisher's LSD.

Induced resistance by PGPF against bacterial angular leaf spot and Fusarium wilt

Cucumber roots treated with BGI of PGPF isolates GP17-2, GS8-2 and GU23-3 decreased the total lesion number and diameter of angular leaf spot significantly when compared to the untreated control plants (Table 3). The level of disease protection induced by these isolates was 52.0%, 31.6% and 59.3%, respectively (Table 2). A reduction in total lesion diameter was observed with isolate GF19-2 though total lesion number was unchanged. Contrastingly, GT3-2 reduced the total lesion number without affecting the total lesion diameter.

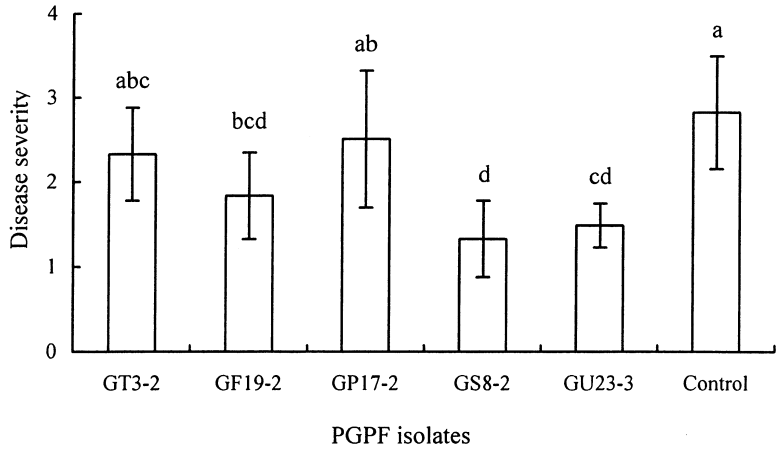

Figure 1. Reduction of Fusarium wilt in cucumber caused by *Fusarium oxysporum* f.sp. *cucumerinum* in plant grown in potting medium amended with barley grain inocula (2%, w/w) of plant growth-promoting fungi isolates *Trichoderma* GT3-2 (GT3-2), *Fusarium* GF19-2 (GF19-2), *Penicillium* GP17-2 (GP17-2), *Phoma* GS8-2 (GS8-2) and sterile fungus GU23-3 (GU23-3). Disease severity (DS) of Fusarium wilt on each plant was calculated using the disease index (DI) ranging between 0 (no wilt symptom) through 4 (completely wilted or dead plant). The formula used is as follows: DS = ΣDI/Total number of plants. Histograms represent the mean of three trials, each with five replicates per treatment. Bars represent standard deviation of means. Different letters representing the histograms indicate statistical significance among treatment by Fisher's LSD ($P = 0.05$).

After treatment with GS8-2, GU23-3 and GF19-2, the disease severity of Fusarium wilt was 1.33, 1.49 and 1.84, respectively, on a scale of 0–4 (Figure 1). The values were significantly lower than that of untreated control plants (2.8). Isolates GT3-2 and GP17-2 did not cause a significant reduction in Fusarium wilt symptoms.

In case of the aerial pathogens *C. orbiculare* and *P.s. lachrymans*, the treated PGPF were not isolated from the stems and leaves of cucumbers which were grown in the pots inoculated with the PGPF. Also, in case of the Fusarium wilt, the treated PGPF were not isolated from the cucumber roots and soils of the other pots in which pathogen was inoculated (data not shown). Therefore, the observed protection cannot be attributed to direct antagonism but must result from a PGPF-mediated induced resistance response.

Induction of lignin deposition in the epidermal tissues of cucumber hypocotyls by CFs of PGPF

Lignin deposition is known as one of the mechanisms of systemic resistance induced resistance (Hammerschmidt and Kuć, 1982). Therefore the level of lignin deposition at the point of pathogen infection in the epidermal tissues of cucumber hypocotyls was examined after treatment with CFs of the five PGPF isolates. To this end, cucumber hypocotyls were pretreated with CFs of the five PGPF isolates and inoculated with spores of *C. orbiculare*. Subsequently, the extent of lignin deposition was examined around 100 germinated spores for each treatment. Lignification was apparent as an intense blue-green or red color of the lignified cell walls (toluidine blue O and phloroglucinol–HCl test, respectively). Cucumber hypocotyls pretreated with any of the CFs of the five PGPF isolates showed an enhanced lignin deposition after infection by *C. orbiculare* compared to control treated hypocotyls with isolates GP17-2 and GU23-3 performing best (Table 4).

Emission of chemiluminescence from plant tissues treated with CFs of PGPF

The oxidative burst is characterized by a rapid and transient generation of active oxygen species immediately following fungal elicitor treatment. Miura et al. (1995) demonstrated that the oxidative burst from potato tuber slices treated with the fungal cell elicitor of incompatible race of *Phytophthora infestans* occurred within several minutes, depending on the elicitor concentrations. In this study, the elicitor activity of CFs of PGPF was evaluated by the CL assay to measure emission of active oxygen species from tobacco callus and cucumber

fruit disks. The CFs of the five PGPF isolates elicited a conspicuous superoxide generation from tobacco callus when compared with the control (Table 5). The emission from the callus started shortly after CFs treatment of all PGPF isolates and peak emission was seen at 20 min followed by a decrease until 180 min. Maximum CL activity was shown by the CF of GP17-2 with intensity almost 100-fold higher than that of other isolates.

Table 4. Lignification of hypocotyls of cucumber seedlings induced by culture filtrates of plant growth promoting fungi, following challenge inoculation with *C. orbiculare*[a]

Treatment	Challenge inoculation with *C. orbiculare*	
	Before[b]	After[c]
Trichoderma GT3-2	−	++
Fusarium GF19-2	−	++
Penicillium GP17-2	−	+++
Phoma GS8-2	−	++
sterile GU23-3	−	+++
Sterile distilled water	−	+

[a]The degree of lignin deposition was evaluated by determining the percentage of germinated spores together with appressoria around which lignin depositions were induced. Presence of 100 germinated spores were evaluated for each treatment. The percentage values of 0%, <30%; 31–70% and >70% were shown as −, +, ++ and +++, respectively.
[b]Roots of cucumber plants grown in moist sterilized filter paper for seven days were treated with culture filtrates of PGPF and not challenged.
[c]Cucumber plants were treated as earlier and the hypocotyls were challenged with 5 µl drops of 10^5 spores/ml of *C. orbiculare* at 10 locations.

Thus, CF of GP17-2 was chosen to determine the substance(s) generating active oxygen species. Among the six fractions of this CF, the MW >12,000 fraction elicited highest generation of superoxide from tobacco callus (Figure 2). The responses shown by fraction devoid of lipid and protease treatment fraction were similar to that of non-fractionated CF. Contrastingly, MW 8,000–12,000, MW <8,000 and lipid fractions elicited only small amount of CL as sterile distilled water and potato dextrose broth. In cucumber fruit disks used in this study, the MW >12,000 fraction and the lipid fractions of GP17-2 generated remarkably high amount of CL emission with peaks at 15 min and 40 min after treatment, respectively (Figure 3). In addition, three other fractions (fraction devoid of lipid, protease treatment fraction, and MW 8,000–12,000 fraction) generated almost equal amount of CL to non-fractionated CF. However, the fraction of MW <8,000 scarcely generated CL as similar to sterile distilled water and potato dextrose broth. Whether the superoxide generating fractions of CF could induce lignification or not was subsequently determined. A high correlation between superoxide generation ability and lignification ability was detected. Extensive lignification was induced in the epidermal tissues of cucumber hypocotyls by the treatment with MW >12,000 and the lipid fractions (Table 6).

Discussion

The disease caused by *C. orbiculare* on leaves was suppressed by different PGPF isolates. Suppression of the

Table 5. Emission of chemiluminescence from tobacco callus incubated in MS medium for five days treated with culture filtrates of PGPF isolates[a] as evaluated by chemiluminescence reader[b]

PGPF isolates	Time (min)									
	0	20	40	60	80	100	120	140	160	180
GT3-2	200	3374	3268	3745	3880	3954	4216	4410	4290	4177
GF19-2	2257	3694	2845	2657	2242	2067	2000	1888	1742	1603
GP17-2	245513	340432	152024	83651	48609	29748	18489	11399	6098	5576
GS8-2	751	3710	2805	2416	2469	2234	2077	1907	1826	1689
GU23-3	2579	5074	3962	3153	2727	2499	2234	2197	2107	2029
SDW[c]	142	151	242	316	312	407	387	426	402	400
PDB[d]	1211	791	742	892	1041	1113	1188	1108	1178	1127

[a]PGPF isolates were used as follows, *Trichoderma* GT3-2 (GT3-2), *Fusarium* GF19-2 (GF19-2), *Penicillium* GP17-2 (GP17-2), *Phoma* GS8-2 (GS8-2) and sterile fungus GU23-3 (GU23-3).
[b]Chemiluminescence activity was quantified from the photons emitted in 1 min by the tobacco callus aging for 30 min.
[c]Sterile distilled water.
[d]Potato dextrose broth.

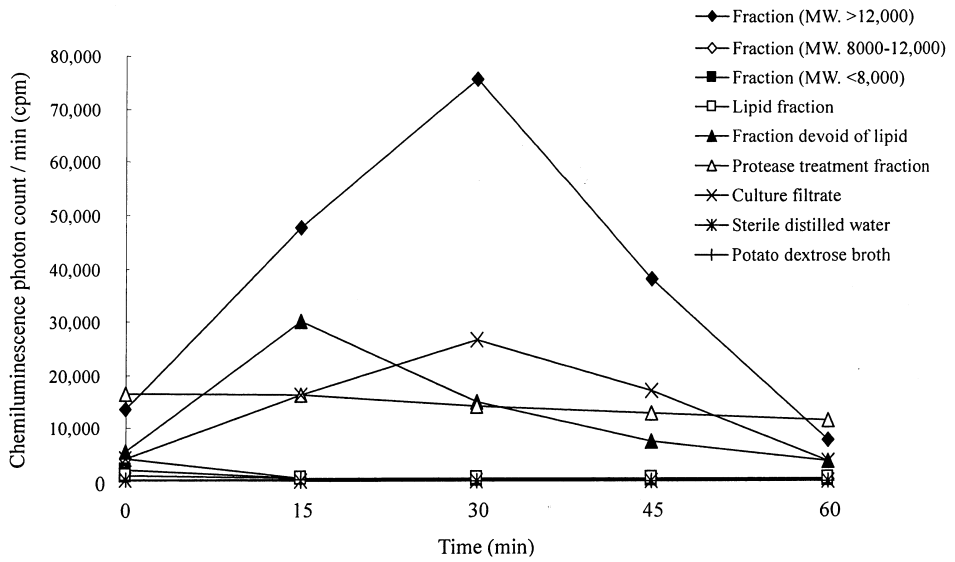

Figure 2. Emission of chemiluminescence from tobacco calli aging for 30 min following treatment with various fractions of culture filtrate of *Penicillium* GP17-2 as evaluated by chemiluminescence reader. The emission of chemiluminescence from the calli was recorded for 1 min.

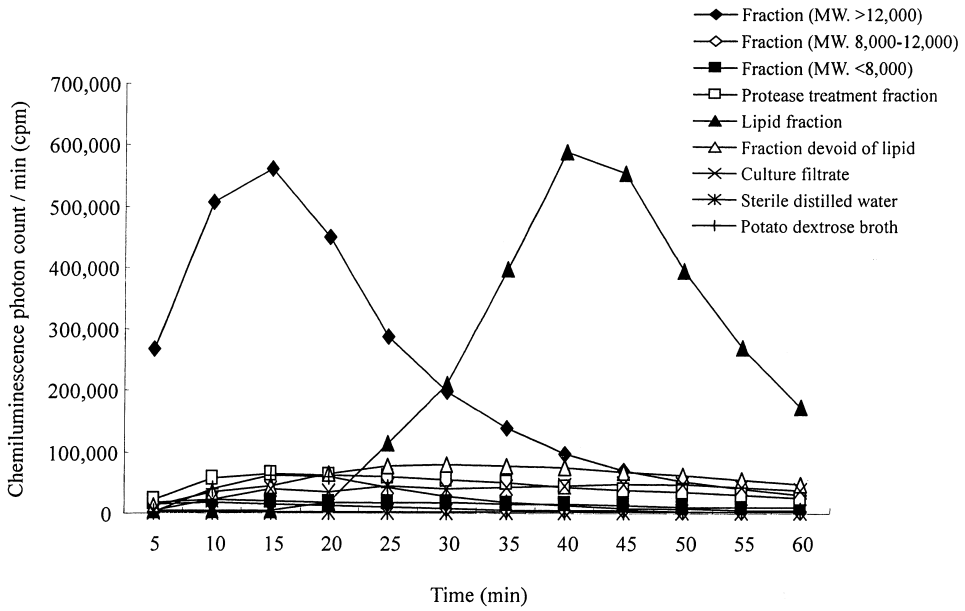

Figure 3. Emission of chemiluminescence with luminol from cucumber fruits disks aging for 16 h following treatment with various fractions of culture filtrate of *Penicillium* GP17-2 as evaluated by ARGUS-50. A mixture of 0.05 ml of Tris–HCl buffer (pH 7.4) containing 1 mM luminol and 0.05 ml of CF was painted on the surface of cucumber slices and the emission of chemiluminescence from the disk was recorded for 1 min.

Table 6. Lignification of hypocotyls of cucumber seedlings induced by culture filtrate of *Penicillium* GP17-2, following challenge inoculation with *C. orbiculare*[a]

Treated fraction	Challenge inoculation with *C. orbiculare*	
	Before[b]	After[c]
Fraction (MW. >12,000)	−	+++
Fraction (MW. 8,000–12,000)	−	+
Fraction (MW. <8,000)	−	+
Protease treatment fraction	−	++
Lipid fraction	−	+++
Fraction devoid of lipid	−	+
Culture filtrate	−	++
Potato dextrose broth	−	+
Sterile distilled water	−	+

[a]The degree of lignin deposition was evaluated by determining the percentage of germinated spores together with appressoria around which lignin depositions were induced. Presence of 100 germinated spores were evaluated for each treatment. The percentage values of 0%, <30%; 31–70% and >70% were shown as −, +, ++ and +++, respectively.
[b]Roots of cucumber plants grown in moist sterilized filter paper for seven days were treated with fraction of culture filtrates of *Penicillium* GP17-2 and not challenged.
[c]Cucumber plants were treated as earlier and the hypocotyls were challenged with 5 µl drops of 10^5 spores/ml of *C. orbiculare* at 10 locations.

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. Furthermore, PGPF isolates could not be recovered from the regions growing above the ground. This conclusion is in agreement with the findings of van Peer et al. (1991) and Wei et al. (1991). Induction of systemic resistance in cucumber plants depended on the type of inducer isolates and type of inocula which were provided to plants either as BGI, MI or CF.

Among the different PGPF isolates tested, GF19-2 suppressed the disease when used as BGI rather than as MI; BGI treatment was given to roots for more than 21 days but MI was only for three days. Longer period of their contact with roots may be necessary for the induction for resistance. Also, a need for a lag period was evident. This is in agreement with results obtained with bacterial and fungi that induce systemic resistance (Kuć, 1983; van Peer et al., 1991). Previous reports also suggested that root colonization by the inducer microorganisms was necessary before resistance was realized (Meera et al., 1995; Liu et al., 1995). However, GT3-2, GP17-2, GS8-2 and GU23-3 induced resistance when roots were treated either with their MI or CF just for 72 h, indicating that some factors other than root colonization might be responsible for induction. Hyakumachi (1997) revealed that the lipid fraction of mycelial cell walls of noncolonizing PGPF was effective in eliciting a resistance response, while the cell wall lipid fractions as well as polysaccharides of root colonizing PGPF were effective. Van Peer and Schippers (1992) reported that the membrane lipopolysaccharides of *Pseudomonas fluorescens* induced resistance in carnation to Fusarium wilt suggesting that the lipid/polysaccharide component may be involved with these PGPF isolates.

Besides anthracnose, PGPF isolates also suppressed bacterial angular leaf spot and Fusarium wilt. However, an induction of systemic resistance depended on the pathogen and PGPF combination. For example, although isolates GS8-2 and GU23-3 effectively induced systemic resistance in cucumber against anthracnose, bacterial angular leaf spot and Fusarium wilt diseases, GP17-2 did so only against anthracnose and bacterial angular leaf spot. It was shown previously that pretreatment with *P. fluorescens* protected radish through induction of systemic resistance not only against the fungal root pathogen *Fusarium oxysporum* f. sp. *raphani*, but also against the avirulent bacterial leaf pathogen *P. syringae* pv. *tomato* and the fungal leaf pathogen *Alternaria brassicicola* (Hoffland et al., 1996). The mechanism(s) of this specificity of ISR against diseases by PGPF isolates are not known.

When cucumber roots were treated with CFs of PGPF, lignin was formed at the point of attempted penetration by the pathogen in the epidermal tissues of cucumber hypocotyls. This suggested that the elicitor(s) was contained in CFs of PGPF. Lignin deposition has been considered as an important step to suppress pathogen infection in systemically immunized plants (Hammerschmidt et al., 1984; Dean and Kuć, 1987). The final enzymatic step of lignification utilizes peroxidase, an enzyme that can generate lignin polymers by catalyzing the formation of free radicals of the lignin monomer precursors (Hammerschmidt and Yang-Cashman, 1995). Peroxidase has several functions which could have an effect on the resistance of a plant. The possible role for peroxidase in plant resistance may be attributed to its ability to oxidize important metabolites including phenolic compounds of the host plant. Also, the findings of Apstol et al. (1989) as well as Peng and Kuć (1992) implicate peroxidase in an oxidative defense mechanism in the elicitor treated

or the infected plants, and peroxidase-generated hydrogen peroxidase may also function directly as an antimicrobial agent. Doke (1983) indicated that systemic oxidative burst is characterized by a rapid and transitory generation of active oxygen and this generation of active oxygen could be a key reaction triggering the active defense reaction in plants. Also, Doke et al. (1994) suggested that the metabolic changes in plant tissues developing systemic acquired resistance may be triggered initially by the systemically activated oxidative burst through some unknown translocated signal originating from tissues undergoing initial, localized oxidative burst. A rapid generation of superoxide was found from CF-treated tobacco callus and cucumber fruit disks. Although, both the >MW 12,000 and the lipid fractions of CF of GP17-2 elicited a high amount of CL emission from the cucumber fruit disks, only the >MW 12,000 fraction did so from the tobacco callus. The emission of CL by the protease-treated fraction was equal to the control. This suggests that polysaccharide(s) existing in CF of GP17-2, may be the common elicitor(s) that triggers resistance in different crops including tobacco and cucumber. However, lipids in the CF could also act as an elicitor of specific plant species. The fraction which induced high CL activity also induced lignin formation in plants. This suggested that CL activity is a good indicator of elicitor activity and ISR. Additional studies are needed to comprehend the relationship between the biocontrol activity of certain microorganisms and the CL activity in their CFs. This will also help in elucidating the mechanisms governing the induced systemic resistance by PGPF.

Acknowledgement

We thank Sachi. Sri Kantha for critical reading of the manuscript.

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