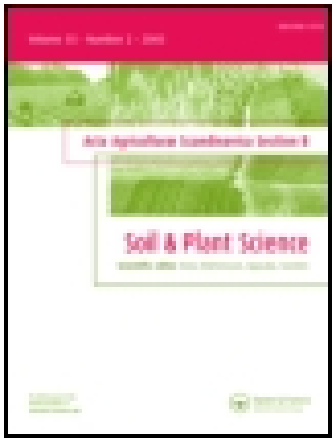

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Mechanism of induced systemic resistance against anthracnose disease in cucumber by plant growth-promoting fungi

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ORIGINAL ARTICLE

Mechanism of induced systemic resistance against anthracnose disease in cucumber by plant growth-promoting fungi

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Plant growth-promoting fungi (PGPF) such as *Phoma* sp. (isolates GS8-1, GS8-2 and GS8-3) and non-sporulating fungus (isolate GU21-2) were tested for their ability to induce systemic resistance against *Colletotrichum orbiculare* in cucumber. These isolates, used as colonized barley kernels to natural soils, induced systemic resistance in the greenhouse as well as in the field. Different elicitors from PGPF such as cell walls retaining or lacking protein and lipids, cell walls lipid fraction, and three fractions of culture filtrate (CF) with different molecular weight ranges were tested for their ability to elicit the defense response of cucumber plants under controlled conditions. Cell wall fraction lacking protein and lipids and the cell wall lipid fraction from root colonizing isolates GS8-1, GS8-2, and GS8-3 protected plants against *C. orbiculare*. While, only cell wall lipid fraction and CF fractions of different molecular weight ranges of the isolate GU21-2 protected plants against *C. orbiculare* infection. Methanol-soluble substances from CF fractions with molecular weight greater than 12,000 and less than 8000 consistently protected plants against the pathogen. Germination of *C. orbiculare* spores decreased significantly at 72 h of incubation on leaves of PGPF-protected plants. Isolate GU21-2 was the most effective in inhibiting spore germination. On the other hand, isolates GS8-1 and GU21-2 induced lignifications in the hypocotyls of seven-day-old cucumber seedlings after challenge inoculation with *C. orbiculare*. Three-week-old cucumber plants treated with isolate GU21-2 and challenged with *C. orbiculare* showed increased activities of exo- and endo-forms of glucanase and chitinase, as well as peroxidase and polyphenol oxidase in the second true leaves. Induction treatment with isolate GS8-1 also increased the activities of these enzymes with the exception of exo-glucanase. This study shows that the inoculation of PGPF or its CF resulted in additive effect on the suppression of anthracnose disease in cucumber.

Keywords: *Colletotrichum orbiculare*; defense-related enzymes; *Phoma* sp.; culture filtrate

Introduction

Induced systemic resistance (ISR) has been studied in great detail in cucumber (*Cucumis sativus* L.; Hammerschimdt & Kuc 1982; Irving & Kuc 1990; Chandanie et al. 2006, 2009; Saldajeno & Hyakumachi 2011; Elsharkawy et al. 2012b; Shimizu et al. 2013). The initiation of ISR in cucumber has been mainly attributed to infection caused by pathogens such as *Tobacco necrosis virus*, *Cucumber mosaic virus*, *Fusarium oxysporum* f. sp. *radicis-lycopersici*, *Colletotrichum orbiculare* (Berk. & Mont.), and *Cladosporium cucumerinum* (Ellis & Arth.; Staub & Kuc 1980; Bashan & Cohen 1983; Koike et al. 2001; Elsharkawy et al. 2012b). It has been shown that plants immunized with pathogen responded to further attack by increasing the activities of hydrolases (Ward et al. 1991) and producing lignin around the place of attempted entry (Hammerschimdt 1984) thus restricting the pathogen establishment. Glucanase and chitinase are reported to be the major produced enzymes in defense reactions of plants against pathogens (Maurhofer et al. 1994; Shimizu et al. 2013). The roles of chitinase, glucanase, peroxidase (PO), polyphenol oxidase, and phenylalanine ammonia

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lyase (PAL) in induced resistance against cucumber diseases have been previously reported (Tuzun et al. 1989; Irving & Kuc 1990; Schneider & Ullrich 1994; Elsharkawy et al. 2012b; Shimizu et al. 2013).

Certain beneficial microorganisms such as plant growth-promoting rhizobacteria (PGPR) and plant growth-promoting fungi (PGPF) have been shown to induce systemic resistance in cucumber (Van Peer & Schippers 1992; Liu et al. 1995; Meera et al. 1995a, 1995b; Koike et al. 2001). The efficacy of PGPF in plant-growth promotion as well as elicitation of systemic disease protection has been previously reported (Elsharkawy et al. 2012a, 2014). The factors that elicit resistance in cucumber plants might differ in their nature and mode of action depending on the PGPF isolate.

The present investigation reports the defense response of cucumber plants in response to induction of systemic resistance against *C. orbiculare* by non-pathogenic fungal isolates. The objectives of this work were to test the ability of certain PGPF isolates to induce systemic resistance in the field and also to test whether certain elicitors originating from PGPF could induce resistance under controlled conditions. The ability of *C. orbiculare* to germinate in PGPF-induced cucumber plants and the formation of lignin in PGPF-protected plants in response to pathogen infection were also studied. Lastly, the activities of glucanase, chitinase, PO, polyphenoloxidase (PPO), and PAL in plants systemically induced with PGPF were also elucidated.

Materials and methods

Plant and pathogen

C. orbiculare (= *Colletotrichum lagenarium* (Pass.) Ellis & Halst) isolate 104T, cultured on potato dextrose agar (PDA; 3% agar) was used in all experiments. Barley (*Hordeum vulgare* L.) kernel inocula of the PGPF were prepared as described in Elsharkawy et al. 2013. *Phoma* sp. isolates GS8-1, GS8-2, and GS8-3 and non-sporulating fungus GU21-2, originally isolated from zoysiagrass rhizosphere and maintained as part of the PGPF collection at the Laboratory of Plant Pathology, Gifu University, were used as PGPF isolates. Cucumber cv. Gibai was used in all experiments. Soil collected from the field (Gifu University Experimental Plot, Gifu, Japan) was used for greenhouse experiments. Analyses of soil samples showed that it was a brown loam soil (pH 6.8) and contained 0.71% total organic matter, 0.073% soluble carbon, 0.002% total nitrogen, 0.355% total phosphorus (P_2O_5), 0.046% soluble phosphorus (P_2O_5), and 0.30% potassium.

Induction of resistance in the greenhouse

Cucumber seeds were sown in 8 × 9 cm pots containing natural soil amended with barley kernels colonized by individual PGPF isolate (2%, w/w) and un-amended or amended with autoclaved un-infested barley kernels (2%, w/w) were served as control treatments. Plants were grown for three weeks in the greenhouse under 12 h light regime. Artificial daylight fluorescent tubes were employed to supplement solar illumination. The greenhouse was maintained at an average of 85% RH and 25°C throughout the experiment. The spore suspension of *C. orbiculare* was adjusted to final concentration of 10^5 spores ml^{-1}. The study was conducted four times with three replicates in each treatment.

Induction of resistance in the field

Experiments were carried out in an experimental plot at Gifu University Experimental Plot, Gifu, Japan. Three replicate plots (5 × 7 m) were prepared in the randomized complete block design, each plot containing six rows. Each row (5 m long, 1 m apart) represented a treatment with four pits (each 15 cm diameter × 20 cm deep). The rows were raised 15 cm above the ground level. Soil (2500 g) removed from the pit was mixed with 2% (w/w) barley kernel inocula of each PGPF isolate and returned to the pits before sowing cucumber seeds (four cucumber seeds per pit). In each plot, pits of one row were not amended, while pits of another row were amended with the autoclaved un-infested barley kernels to serve as untreated and treated controls, respectively. The soil of other four pits received barley kernel inocula of isolates GS8-1, GS8-2, GS8-3, or GU21-2. Ten days after sowing seeds, two seedlings out of four were removed from each pit. Plants were grown for six weeks and trained on plant stakes. During the course of experiment, plants received a total of 25 cm rainfall, relative humidity of 75–90%, and temperature of 23–32°C. At six weeks, the top most fully expanded leaves of unprotected and protected plants were challenged with 20–30 µl drops of 10^6 spores ml^{-1} of *C. orbiculare*. The challenged plants were covered for 40 h with black polyethylene to keep humidity levels high. Sheets were held above the leaf surface at a distance of about 22–24 cm. Disease severity was assessed after 10 days of incubation. The experiment was conducted two times.

Disease assessment

The total number and diameter (mm) of lesions on leaves of protected or unprotected plants grown in

the greenhouse or field were measured (Meera et al. 1995a).

Root colonization ability of PGPF isolates

Roots from four plants per treatment grown in the field were removed intact from the soil and washed gently in the running tap water. The entire root system was surface disinfected (NaOCl, 1% for 3 min), excised into 200 segments (2–3 cm long), and placed on chloramphenicol-amended PDA under sterile conditions as reported (Meera et al. 1995b). The fungal colonies were investigated for the confirmation of identity (Meera et al. 1995b). Root colonization experiment was conducted two times with three replicates per treatment.

Effect of fungal elicitors on the suppression of anthracnose

Preparation of fungal cell wall fractions

Mycelia of PGPF isolates GS8-1, GS8-2, GS8-3, and GU21-2 and *C. orbiculare* were grown separately in potato dextrose broth (PDB, pH 6.5) by following the procedures described by Meera et al. (1994). Mycelia cell wall was extracted following the procedures of Woodward and Pegg (1986) with some modifications. A measure of 10 gm of mycelia mat were homogenized in 20 ml cold 10 mM sodium phosphate buffer pH 7.0 and sonicated for 10 min at 4°C to disrupt cell walls. The resultant mycelia homogenate was centrifuged to obtain the crude mycelia cell wall fraction. The crude preparation was washed three times with the buffer and two times with dionized water to remove cytoplasmic contaminants. The presence of cytoplasmic contaminants in the crude wall preparation was tested by observing under the light microscope. The crude mycelia cell wall preparation was divided into two portions. The first portion was freeze-dried and used as cell wall preparation that retained protein and lipids, while the second portion was processed further to obtain a lipid alone fraction and a cell wall fraction that lacked protein and lipids. The second portion of the crude wall preparation was homogenized in the saline (0.5 M NaCl) 10 mM sodium phosphate buffer two times before washing with 1% sodium dodecyl sulfate (SDS). Washing with saline buffer and SDS was repeated followed by washing with sterile deionized water for de-proteinization of the mycelia cell wall. Lipids were removed from the de-proteinized cell walls by standing in a mixture of 2:1 chloroform–methanol for 30 min. The chloroform–methanol fraction was centrifuged (1500 g for 5 min) and the supernatant was evaporated in a rotary evaporator at 30°C. The portions and lipids-removed cell wall fraction were finally washed with acetone. The acetone fraction was concentrated by vacuum evaporation at room temperature.

The cell wall fraction that lacked protein and lipids (Table 1) was suspended in 10 ml deionized water and autoclaved under reduced pressure at 121°C for 3 h to release heat-soluble elicitor fraction. The heat soluble fraction was filtered (0.22 μm Millipore filter) by using Bio-Rad protein dye reagent (Bio-Rad Laboratories Inc., USA). The elicitor preparations were stored at –30°C.

Preparation of culture filtrate (CF) fraction

Isolates GS6-1 and GU21-2 which gave high protection as CF were cultured in PDB. A measure of 1 l of CF was concentrated to 50 ml with a flash evaporator at 32–35°C. The concentrated CF was dialyzed serially against sterilized distilled water for 24–36 h at 5°C, using two membranes (Cellulose

Table 1. Amount of different cell wall fractions[a] obtained from 1 gm of mycelia mat[b] of plant growth promoting fungal (PGPF) isolate that was used for treating roots of a single plant.

	Amount (mg)			
		Cell wall lipids		
PGPF isolates	Cell wall retaining protein and lipids	Acetone	Chloroform–Methanol	Cell wall lacking protein and lipids
GS6-1	110	–[c]	8.0	102.0
GS8-1	25	3.0	6.0	12.9
GS8-2	30	0.3	2.0	28.9
GS8-3	28	0.8	4.0	24.1
GU21-2	80	0.7	7.4	74.4

[a]The different cell wall fractions were obtained from the mycelia mat by following the procedures modified from Woodward and Pegg (1986).
[b]A measure of 1 gm of mycelia mat of PGPF was sufficient to induce systemic resistance to *C. orbiculare*.
[c]Not obtained.

tubing UC 36–32, molecular weight cut-off 12,000–14,000, viskase sales corp, Tokyo, Japan and spectra/Por molecular porous membrane 7, molecular weight cut-off 8000, spectrum, CA, USA) with pore sizes capable of retaining substances having molecular weight more than 12,000 and 8000, respectively. The dialyzed fractions were collected separately and again concentrated to 50 ml with flash evaporator to provide stock solutions. This process yielded CF fractions of three different molecular weight ranges. The stock solutions of different fractions were diluted with sterile deionized water and used for treatment. In another set of experiment, the stock solutions were freeze-dried and were extracted with cold methanol. The methanol fraction was evaporated in vacuo at room temperature.

In a separate experiment, proteins present in the CF were fractionated. A measure of 1 l of CF was added with ammonium sulfate and the final concentration was brought to 65% and incubated overnight at 4°C. The precipitate was removed by centrifugation at 1500*g* for 15 min. The supernatant was centrifuged again to collect the remaining precipitate. The pellets were pooled and dissolved in a little water and dialyzed (cellulose tubing, molecular cut-off 6000) extensively against sterile deionized water at 4°C. Retained proteins were freeze-dried and stored at –30°C. Total protein content was measured.

Treatment of elicitors from mycelia cell wall and CF fractions

The amount of elicitor fraction of fungal cell wall treated per plant is given in Table 1. The mycelia cell wall fraction (retained protein and lipids) was suspended in 5 ml of sterile deionized water, while the volume of soluble fraction after autoclaving of cell wall fraction (lacked proteins and lipids) was used without further dilution. Fungal cell wall lipids were dissolved in 0.5 ml of methanol and suspended in 2.5 ml sterile deionized water. Methanol was vacuum evaporated for 24 h and lipid-water suspension was treated to roots.

Roots of three-week-old plants were immersed in the elicitor fraction in small tubes (2.5 × 5.5 cm). A 10 µl solution of antibiotic mixture (200 mg L^{-1} each of chloramphenicol and penicillin G) was added to the tubes to prevent microbial contamination of the roots. Sterile deionized water was added when required to prevent drying of the roots. Control plants received 2.5 ml of sterile deionized water and 10 µl of antibiotic solution. After 72 h of incubation in diffused light at 25°C, plants were transferred to sterile plastic pots and their roots were completely covered with autoclaved potting medium.

CF fraction of different molecular weight ranges was diluted with sterile deionized water to give different concentration (100%, 50%, 25%, 12.5%, 6.25%, and 0%). A measure of 2.5 ml of the CF stock solution was taken in a small tube and treated to the roots as described above. Since 2.5 ml of undiluted stock solution of CF fractions of GS6-1 or GU21-2 induced 40–60% reduction in total leaf diameter, methanol soluble substances from 2.5 ml of the stock were extracted and used for treatment. Measures of 2.4 mg, 0.85 mg, or 15.5 mg of methanol soluble substances from high, intermediate, or low molecular weight stocks, respectively, of GS6-1 CF were treated to the roots. On the other hand, 2.4 mg, 2.2 mg, or 16.1 mg of methanol soluble substances from high, intermediate, or low molecular weight stocks, respectively, of GU21-2 CF were treated to the roots. The methanol soluble substance was dissolved in methanol and then in 2.5 ml of sterile deionized water as described previously.

The ammonium sulfate precipitation of CF of GS6-1 and GU21-2 yielded 20 and 15 mg proteins 10^{-1} of CF, respectively. Measures of 1, 2.5, 5.0, or 10.0 mg of protein fraction were taken separately in plastic pot and were dissolved in 4 ml of sterile deionized water. Roots of plants were then immersed in the protein solution for 72 h and incubated as described previously.

Plants after treatment with elicitor fractions were transferred to a growth chamber for a further incubation of 24 h. The second true leaves of treated and untreated plants were challenge incubated with 10–20 µl drops of 10^5 spores ml^{-1} of *C. orbiculare* and incubated (Meera et al. 1994). The reductions in the total lesion number and diameter due to different cell wall elicitors were determined. Experiment was performed three times in a randomized complete block design with three replicates in each treatment.

Effect of protecting plants with PGPF on spore germination of C. orbiculare

This experiment was conducted to test the germination of *C. orbiculare* spores on plants systemically protected with PGPF. Cucumber plants were grown for three weeks in autoclaved potting medium unamended and amended with 2% (w/w) of PGPF isolate GS8-1 or GU21-2. Twenty pieces (1 × 1 cm) of polycarbonate nucleopore membrane (Nucleopore, USA) were placed on the lamina of the second true leaf. A measure of 10 µl drop containing ca. 300 spores of the pathogen was placed on the membrane

and plants were incubated at 25°C, in dark humid champer for 72 h. The nucleopore membrane was removed from the leaf after incubation, placed on a glass slide, and stained with cotton blue (0.5%, w/v) in lactophenol. The numbers of spores which germinated and produced appressoria and infection hyphae were counted by observing under light microscope. The experiment was conducted three times with three replicates each time.

Formation of lignin in seedlings systemically protected with PGPF

C. orbiculare was cultured on PDA and spore suspension was prepared in distilled water. Two PGPF isolates GS8-1 and GU21-2 were cultured in PDB for seven days. The CF was separated and mycelia mat was washed in sterile water. A measure of 5.0 g of mycelial mat was homogenized in sterile distilled water at 5000 rpm for 5 min and the final homogenate volume was made up to 20 ml and was used as mycelia inoculums (MI).

Cucumber seeds were germinated in sterilized moistened Whatman filter papers in darkness at 25°C. The roots of seven-day-old seedlings were treated individually by immersing in 5.0 ml of MI contained tubes and seedlings incubated for 72 h in the presence of red light to avoid chlorophyll development. The treated roots were washed in sterile water and seedlings were placed in petri dishes lined with moistened sterile blotters and their hypocotyls were inoculated with 5–10 µl drops of water. The inoculated seedlings were further incubated for 72 h. The epidermal strips from hypocotyls of seedlings were prepared (Hammerschimdt & Kuc 1982) and tested for lignin by employing the histochemical techniques using phloroglucinol-HCl (Sherwood & Vance 1976) and toluidine blue (O'Brien et al. 1964) tests and by enzyme maceration (Ride 1975). The experiment was performed three times with three replicates each time.

Activity of defense-related enzymes in plants systemically induced with PGPF isolates

Plants were grown for three weeks in autoclaved potting medium un-amended or amended with barley kernels colonized with isolate GS8-1 or GU21-2. The second true leaves of three-week-old plants were challenged with 10–20 µl drops of 10^5 spores ml^{-1} of *C. orbiculare* or with the same amount of distilled water and incubated as suggested Meera et al. (1994). The disease incidence was assessed. The second true leaves were excised after challenge inoculation and frozen. To understand whether the defense response is initiated earlier in the PGPF-protected plants, disease was assessed at three, six, or nine days after challenge inoculation and second true leaves at these intervals were collected, frozen and assayed for the activities of endoglucanase, endochitinase, and phenylalanine ammonia lyase. The frozen leaves were ground in liquid nitrogen and stored at –80°C until used. The experiment was repeated three times. Various dilutions of leaf extract were prepared in 100 mM sodium acetate buffer (pH 5.0) for assaying activities of glucanase and chitinase, while dilutions made with 10 mM sodium phosphate buffer were used for assaying PO and PPO activities. Samples extracted with 10 mM sodium borate-HCl buffer (pH 8.8) were tested for PAL activity. Protein content of enzyme samples was measured by the micro assay method using Bio-Rad protein assay reagent according to the directions of the manufacturer. Bovine serum albumin was used as the standard.

1, 3-Glucanase

The activity was assayed by measuring the rate of release of reducing sugar from the substrate laminarin (1.0%; Sigma, USA). The exoglucanase and endoglucanase activities were determined according to the method described by Brown (1987) and Keen and Yoshikawa (1983), respectively. The reaction mixture for exoglucanase assay consisted of 0.6 ml of enzyme solution and 0.6 ml of laminaring and incubated at 50°C for 1h. The reducing sugar released was assayed by the dinitro salicylic acid method of Miller (1972). For assaying endoglucanase activity, 0.6 ml of enzyme solution and 0.6 ml of laminarium were included in the reaction mixture which was incubated at 37°C for 30 min. The release of reducing sugar was assayed by the Nelson-Somogyi's method (Marais et al. 1966) and used as control. The enzyme activity was expressed as nano katals (nkat) g^{-1} protein where 1 nkat is the enzyme activity catalyzing the formation of 1 nano mole of glucose in 1 sec.

Chitinase

The exochitinase and endochitinase assays were done by determining the release of N-acetyl-D-glucosamine from 10 mg ml^{-1} colloidal chitin. The assay mixture for exochitinase contained 0.5 ml of crude enzyme solution and 0.5 ml of colloidal chitin that was incubated at 37°C for 2 h in a shaking water bath. The assay mixture for endochitinase assay was similar to exochitinase, however, after incubation, supernatant of the reaction mixture was

incubated with desalted snail gut enzyme as described by Boller and Mauch (1988). The resulting N-acetylglucosamine after exochitinase and endochitinase was used as the initial standard. Enzyme alone and substrate alone were used as controls. Enzyme activity was expressed as nkat g^{-1} protein. Formation of reaction products in glucanase and chitinase assays was not proportional to enzyme concentration. Therefore, standard curve relating the amount of enzyme to the rate of product formation was prepared and enzyme activities were calculated as described by Boller and Mauch (1988).

Peroxidase

The PO activity of the enzyme solution phosphate buffer (pH 7.0) was measured by using 20 mM guaiacol (O methoxy phenol) as the hydrogen donor in the presence of 12.3 mM hydrogen peroxide at 25°C (Putter 1974). One enzyme unit corresponded to the amount of enzyme that caused a variation of 0.1– 0.2 absorbance units per minute at 470 nm. Specific activity of the enzyme was calculated by using the linear part of the curve and expressed as unit mg^{-1} protein min^{-1}.

Polyphenoloxidase

Assay of PPO was carried out using 0.5 M pyrocatechol as the substrate. The reaction mixture consisted of 2.5 ml of phosphate buffer (pH 6.5), 0.3 ml pyrocatechol, and 0.2 ml of enzyme extract. The increase in absorbance was recorded at 495 nm (Vance et al. 1980). The change in absorbance of 0.01–0.02 units per minute was used to calculate the enzyme activity. Specific enzyme activity was expressed as units g^{-1} protein min^{-1}.

Phenylalanine ammonia lyase

The activity of PAL was tested as described by Brueske (1980) with some modification. Reaction mixture contains 0.5 ml of enzyme extract in 100 mM sodium borate-HCl buffer (pH 8.8), 1.5 ml of borate buffer, and 1 ml of 100 mM L-phenylalanine in water (pH 8.7). After incubating the reaction mixture at 32°C for 60 min, the reaction was stopped by adding 0.5 ml of 1M trichloroacetic acid and the incubation was continued further for 5 min at 37°C. The amount of t-cinnamate produced (nM mg^{-1} protein h^{-1}) was measured at 290 nm. An internal control without phenylalanine was included to correct for the presence of endogenous substrate (Hughes & Dickerson 1989).

Experimental setup and statistical analysis

The greenhouse and field experiments were performed in the randomized complete block design with six treatments and each treatment contained three replicates. The experimental data from trials were tested for homogeneity of variance and replicate data from trials were combined for analysis of variance. Other experiments of root colonization, fungal elicitors, spore germination, and enzyme activity also were performed with a completely randomized design. Means were separated by Duncan's multiple range test (DMRT, $P = 0.05$) or by Fisher's least significant difference test (LSD, $P = 0.05, 0.01$).

Results

Induction of resistance in the greenhouse

Plants treated with PGPF isolates and challenged with *C. orbiculare* had significantly less total number and diameter of lesions compared with non-treated plants (Table 2). Isolates GS8-2 and GU21-2 were highly effective ($P = 0.01$) in reducing the lesion number and diameter caused by *C. orbiculare* followed by isolates GS8-1 and GS8-3.

Table 2. Effect of plant growth-promoting fungi (PGPF) isolates[w] on the total lesion number and total lesion diameter (mm/leaf) caused by inoculating *C. orbiculare* on leaves of cucumber cv. Gibai plants in the greenhouse and field[x].

	Greenhouse assay		Field assay	
	Total lesion		Total lesion	
PGPF isolates	Number	Diameter	Number	Diameter
GS8-1	10.7 b[y]	35.2 b	23.5 a	82.9 b
GS8-2	7.5 c	28.0 c	15.6 b	67.0 c
GS8-3	9.5 b	32.2 b	16.7 b	66.7 c
GU21-2	9.2 b	26.5 c	13.4 c	71.7 c
C. orbiculare[z]	5.0 d	20.2 d	5.0 d	50.5 d
Control	15.2 a	55.7 a	22.8 a	122.4 a

[w]Barley kernels colonized with PGPF were added to natural soil at the rate of 2% (w/w).
[x]The second true leaves of three-week-old plants in the greenhouse were challenged inoculated with 10–20 µl drops of 10^5 spores ml^{-1} of spores, while in the field, the top most fully expanded leaves of six-week-old plants were challenged with 10–30 µl drops of 10^6 spores ml^{-1} of *C. orbiculare.* Numbers represent mean values ($n = 9$).
[y]Values with the same letters in a column are not significantly different at $P = 0.05$, analyzed by DMRT.
[z]The first true leaf was induced with 10–20 µl drops of 10^5 spores ml^{-1} or 10–30 µl drops of 10^6 spores ml^{-1} of *C. orbiculare* in the greenhouse and field, respectively, and second true leaf was challenged with the same amount of pathogen inoculums after six days.

Induction of resistance in the field

C. orbiculare was effectively reduced the disease compared with PGPF isolates (Table 2). Isolates GS8-2, GS8-3, and GU21-2 reduced ($P = 0.05$) the total lesion number at six weeks compared with un-induced control plants followed by isolate GS8-1.

Root colonization ability of PGPF isolates

PGPF isolates varied in root colonization ability in the field. At six weeks, isolates GS8-3 and GS8-1 were isolated from 51% and 41% of root segments, respectively. Isolate GS8-2 was a poor colonizer and was isolated only from 15% of root segments. Isolate GU21-2 failed to colonize roots even after six weeks of plant growth.

Effect of fungal elicitors on disease suppression

Fungal cell wall fraction

The cell wall fraction that retained protein and lipids of all tested PGPF significantly ($P = 0.05$) reduced the total lesion number and diameter. The lipid fractions of all isolates, except GS8-3, also caused reduction ($P = 0.05$) in the total lesion number and diameter. The soluble cell wall fractions without proteins and lipids of isolates GS8-1, GS8-2, and GS8-3 reduced the disease significantly compared with the non-treated control plants. Acetone soluble fraction from PGPF cell wall elicited a weak and inconsistent defense response against the pathogen (Data not shown). Cell wall fraction of the *C. orbiculare* was more effective in comparison to that of PGPF isolates (Table 3).

CF fraction

The CF fractions of molecular weight more than 12,000 and less than 8000 from the isolates GS6-1 and GU21-2 showed concentration dependent abilities to elicit host defense responses. Substances with molecular weight ranging between 8000 and 12,000 were effective only at the highest concentration (100%; Tables 4 and 5). The methanol soluble substances obtained from high and low molecular weight CF fractions of isolates GS6-1 and GU21-2 caused a significant ($P = 0.05$) decrease in disease. However, the methanol soluble substance from the intermediate molecular weight fraction caused less disease reduction (Table 6).

Effect of protecting plants with PGPF on spore germination of C. orbiculare

Plants protected with isolates GS8-1 and GU21-2 showed reduced appressoria formation from *C. orbiculare* as early as 24 h compared with the un-induced control plants. However, 72 h after challenge inoculation, the percentages of germinated spores in plants protected with isolates GS8-1 and GU21-2 ranged between 46% and 47%, while in un-induced control plants was 82% (Table 7).

Formation of lignin in systemically protected seedlings

Lignin formation was observed in seedlings induced with isolates GS8-1 or GU21-2 at pathogen infection positions when tested by staining with phloroglucinol-HCl, or toluidine blue, or by maceration. Isolate GU21-2 induced more lignin formation compared

Table 3. Effect of cell wall fractions[u] of the plant growth-promoting fungi (PGPF) isolates on total number (TLN) and total lesion diameter (TLD mm/leaf) due to 10–20 µl drops of 10^5 spores ml^{-1} of *C. orbiculare* in three-week-old cucumber cv. Gibai[v].

	Cell wall retaining protein and lipids		Cell wall lipids		Cell wall lacking protein and lipids	
PGPF isolate	TLN	TLD	TLN	TLD	TLN	TLD
GS6-1	14.0 b[w]	60.6 b	11.0 b	50.5 c	16.5 a	80.8 a
GS8-1	11.7 bc	42.6 d	11.5 b	55.7 c	12.7 b	49.2 c
GS8-2	13.2 bc	50.2 c	12.5 b	50.6 c	12.5 b	58.0 b
GS8-3	11.0 c	47.7 c	17.0 a	85.9 b	12.2 b	37.5 d
GU21-2	13.0 bc	52.3 c	10.5 b	45.6 d	17.0 a	76.7 a
C. orbiculare[x]	8.0 d	27.5 e	–[y]	–	8.0 c	30.2 e
Control[z]	17.6 a	85.6 a	18.5 a	92.6 a	17.0 a	79.4 a

[u]The cell wall fraction retaining protein and lipids was suspended in 5 ml of sterile water. The cell wall fraction lacking protein and lipids in 10 ml water was autoclaved and filtered. The entire volume of filtrate was used for treating a plant. The cell wall lipid fraction was dissolved in a 0.5 ml of methanol and 2.5 ml water. The methanol in the suspension was vacuum evaporated. The fractions were treated to the roots for 72 h and the roots were then covered with autoclaved potting medium. After 12 h incubation, second true leaves were challenged.
[v]Values are means of three trials, each with three replicates.
[w]Values with the same letters in a column are not significantly different at $P = 0.05$, analyzed by Duncan's multiple range test (DMRT).
[x]The first true leaf was induced with 10–20 µl drops of 105 spores ml^{-1} *of C. orbiculare* and second true leaf was challenged with the same amount of pathogen inoculums after six days.
[y]Not tested.
[z]Roots of plants were treated with sterile dionized water.

Table 4. Effect of dialyzed fractions of culture filtrates (CFs) of plant growth-promoting fungi (PGPF) isolate GS6-1 on the total lesion number (TLN) and total lesion diameter (TLD nm/leaf) caused by *C. orbiculare* in three-week-old cucumber cv. Gibai plants[w].

	Mol. wt. CF fractions[x]					
	More than 12,000		8000–12,000		Less than 8000	
Concentration of CF (% v/v)	TLN[y]	TLD	TLN	TLD	TLN	TLD
100.0	9.5 b[z]	36.9 d	15.4 b	59.6 d	9.2 c	37.5 d
50.0	10.5 b	39.6 d	18.2 a	85.0 c	9.4 c	39.3 d
25.0	12.0 b	47.3 c	18.8 a	88.8 ab	9.7 c	39.8 d
12.5	17.5 a	63.0 b	18.7 a	88.0 b	15.1 b	49.1 c
6.2	18.1 a	65.0 b	19.5 a	89.9 ab	15.0 b	60.6 b
0.0	19.0 a	86.7 a	18.5 a	90.6 a	17.8 a	85.6 a

[w]Values are means of three trials, each with three replicates.
[x]The concentrated CF (50 ml) was serially dialyzed using two membranes to obtain three fractions. The first fraction contained substances of mol. wt. more than 12000, the second fraction contained substances of mol. wt. ranging between 8000 and 12,000, and the third fraction with mol. wt. less than 8000. The final volume (50 ml) of each fraction was considered as stock solution, from which various dilutions were derived with sterile distilled water. Roots, treated with 2.5 ml stock solution for 72 h, were later covered with autoclaved potting medium.
[y]The second true leaves of the plants were challenge inoculated with 10–20 µl drops of 10^5 spores ml^{-1} of *C. orbiculare* and incubated.
[z]Values with same letters in a column are not significantly different at $P = 0.05$, analyzed by DMRT.

Table 5. Effect of dialyzed fractions culture filtrate (CF) of plant growth-promoting fungi (PGPF) isolate GU21-2 on the total lesion number (TLN) and total lesion diameter (TLD mm/leaf) caused by *C. orbiculare* in three-week-old cucumber cv. Gibai plants[w].

	Mol. wt. CF fractions[x]					
	More than 12,000		8000–12,000		Less than 8000	
Concentration of CF (% v/v)	TLN[y]	TLD	TLN	TLD	TLN	TLD
100.0	10.0 c[z]	38.9 c	14.2 b	54.2 c	8.3 d	32.7 d
50.0	12.5 b	41.2 c	17.5 a	84.2 b	9.1 d	34.0 d
25.0	13.0 b	49.7 b	18.9 a	84.2 b	9.8 d	34.9 d
12.5	13.8 b	60.1 b	18.2 a	86.8 ab	12.0 c	41.1 c
6.2	12.3 b	52.8 b	18.7 a	85.0 ab	16.0 b	70.7 b
0.0	18.2 a	86.8 a	18.2 a	88.6 a	18.9 a	90.9 a

[w]Values are means of three trial, each with three replicates.
[x]The concentrated CF (50 ml) was serially dialyzed using two membranes to obtain three fractions: mol. wt. more than 12,000, mol. wt. ranging between 8000 and 12,000, and mol. wt. less than 8000. The final volume (50 ml) of each fraction was considered as stock solution, from which various dilutions were derived with sterile deionized water and treated to roots as in Table 4.
[y]The second true leaves of plants were challenge inoculated with 10–20 µl drops of 10^5 spores mL^{-1} of *C. orbiculare* and incubated.
[z]Values with the same letters in a column are not significantly different at $P = 0.05$, analyzed by DMRT.

to that of GS8-1. Un-induced but challenged control plants showed very weak reactions.

Activity of defense-related enzymes in plants systemically induced with PGPF isolates

The activities of exochitinase, PO, and PPO in plants treated with GS8-1 and GU21-2 were significantly increased ($P = 0.01$) compared with untreated control plants. Isolate GU21-2 increased exoglucanase activity more than isolate GS8-1 (Table 8). Time course activities of endoglucanase, endochitinase, and PAL were different for isolates GS8-1 and GU21-2. The activities of endoglucanase and endochitinase were remarkably high at six days. Isolate GS8-1 did not cause much increase compared with unprotected plants, however, increased activities of these enzymes were noticeable at nine days after challenge inoculation (Figure 1). In contrast to the activities of the above two enzymes, the activity of PAL increased due to the induction by PGPF at three days after challenge inoculation and the activity decreased with increase in incubation after challenge inoculation. Lesion development was observed 5–6 days after challenge inoculation in PGPF-protected plans, while in unprotected plants, lesion developed as early as 3–4 days. Isolate GU21-2 increased ($P = 0.05$) more PAL activity than isolate GS8-1. At nine days after challenge inoculation with *C. orbiculare*, PAL activity was lower ($P \leq 0.01$) in PGPF-induced plants than in un-induced challenged control plants (Figure 1).

Table 6. Effect of methanol soluble substances extracted from culture filtrate (CF) of different molecular weight ranges on total lesion number (TLN) and total lesion diameter (TLD mm/leaf) due to 10–20 µl drops of 10^5 spores ml^{-1} of *C. orbiculare* in three-week-old cucumber cv. Gibai plants[x].

	Mol. wt. CF fractions[y]					
	More than 12,000		8000–12,000		Less than 8000	
Fungal isolates	TLN	TLD	TLN	TLD	TLN	TLD
GS6-1	13.0 b[z]	54.1 b	18.2 a	80.2 b	13.1 b	53.2 b
GU21-2	14.2 b	32.7 c	17.0 a	76.9 c	12.2 b	53.2 b
Control	18.0 a	86.2 a	17.2 a	88.1 a	18.1 a	90.2 a

[x]Values are the mean of three trials, each with three replicates.
[y]The concentrated CF (50 ml) of fungal isolates was serially dialyzed using two membranes to obtain three fractions: mol. wt. more than 12000, mol. wt. ranging 8000–12,000, and mol. wt. less than 8000 as in Table 4. The final volume of each fraction was freeze-dried and extracted with methanol and concentrated under vacuum. The volume of methanol soluble substances extracted from 2.5 ml of freeze-dried fraction was dissolved in 0.5 ml of methanol and in 2.5 ml sterile water. Methanol was completely evaporated before the fraction was treated to the roots. Roots treated with the fraction for 72 h were later covered with autoclaved potting medium.
[z]Values with the same letters in a column are not significantly different at $P = 0.05$, analyzed by DMRT. Roots of plants were treated with sterile deionized water.

Table 7. Effect of protecting cucumber plants systemically with barley kernels (2%, w/w) colonized with selected plant growth-promoting fungi (PGPF) isolate on the germination (%) of *C. orbiculare* spores, 24 h and 72 h after challenge inoculation[v].

	Inoculation after challenge[u]		
	24 h	72 h	
PGPF isolates	Only appressoria[w]	Only appressoria	Appressoria and infection hyphae[y]
GS8-1	81.5 b[y]	40.0 a	46.0 b
GU21-2	74.4 c	27.0 b	47.0 b
Control[z]	96.0 a	11.9 c	82.0 a

[u]The second true leaves of three-week-old plants were challenge inoculated with 10–20 µl drops containing ca. 300 spores ml^{-1} of *C orbiculare* and incubated.
[v]Values are the mean of three trials, each with three replicates.
[w]Spores germinating to produce appressoria.
[y]Values with the same letters in a column are not significantly different at $P = 0.05$, analyzed by DMRT.
[z]Control plants grown in un-amended autoclaved potting medium.

Discussion

PGPF are rhizoshere fungi with beneficial effects on plant growth and health. PGPF activate plant defense response against different pathogens through ISR (Elsharkawy et al. 2012a, 2012b, 2013; Sudisha et al. 2013; Hassan et al. 2014). In this study, all PGPF isolates protected cucumber plants against *C. orbiculare* in the field when tested at six weeks (Table 2). Fractions of cell wall and CF were tested for the induction of systemic resistance using colonized and non-colonized PGPF (Table 3). Our results showed that isolates GS6-1 and GU21-2 could not elicit defense response of plants when proteins and lipids were removed from the cell wall suggesting that cell wall saccharides of these isolates might not be the elicitor of defense response. However, the effectiveness of cell wall lipid fractions of these isolates indicates the involvement of free lipids in the elicitation. On the other hand, elicitation of defense by cell wall lipid fraction and cell wall fraction lacking protein and lipids (soluble sugar) of isolates GS8-1, GS8-2, and GS8-3 indicates that lipids and saccharides of cell wall might be responsible for induced resistance. Lipopolysaccharides and soluble cell wall components and non-pathogenic microorganisms like PGPR and a hypovirulent *Fusarium* species have been shown to elicit defense in carnation (*Dianthus caryophyllus* L.) and cucumber, respectively (Ishiba et al. 1981; Van Peer & Schippers 1992). Woodward and Pegg 1986 observed that the de-proteinized cell wall fractions *Verticillium albo-atrum* (Reinke & Berthier) elicited host defense response. However, the role of cell wall proteins of PGPF isolates in the mechanism of elicitation cannot be ruled out, since we did not fractionate and test cell wall protein fraction. In other studies, lipids, glycoproteins, and polysaccharides including chitin and glucans of fungal cell walls are documented to elicit plant defense response (Anderson 1980; Ricci et al. 1986; Heinkel et al. 1992). The present investigation indicates that CF fractions of PGPF could also be used as inducers of systemic resistance against anthracnose in cucumber plants. Both high and low molecular weight CF fractions of isolates GS6-1 and GU21-2 protected cucumber plants from anthracnose disease (Tables 4 and 5). On the other hand, Tjamos (1979) showed a major elicitors activity in high molecular weight fraction of CF of *V. albo-atrum* while the low molecular weight fraction processed low activity. Further work is necessary to understand the nature

Table 8. Activities of exoglucanase, exochitinase, peroxidase (PO), and polyphenoloxidase (PPO) in cucumber cv. Gibai systemically induced by growing plants in autoclaved potting medium amended with barley kernels (2%, w/w) colonized with individual plant growth promoting fungal isolates for three weeks and challenge inoculated with 10–20 µl drops of 10^5 $ml^{-1}$of *C. orbiculare*[w].

Fungal used for protection	TLD (mm)	Specific activity[x]			
		Exoglucanase	Exochitinase	PO	PPO
GS8-1	60.0**	25.31 NS	31.77**	2.32**	0.726**
GU21-2	43.5**	45.08**	39.57**	2.91**	1.014**
C. orbiculare	35.8**	41.16**	42.80**	2.36**	0.800**
Uninduced	–	–	–	–	–
Challenged control	99.5	28.96	20.08	1.71	0.688
LSD $P = 0.05$	6.6	4.03	4.23	0.33	0.005
LSD $P = 0.01$	11.0	4.81	5.05	0.39	0.006

Note: Values carrying double asterisks are significantly different at $P = 0.01$, compared to un-induced challenge control, analyzed by Fisher's LSD test.
TLD, Total lesion diameter (mm/leaf); NS, Nonsignificant.
[w]Values are means from three separate experiments.

of function of methanol soluble substances of high and low molecular weight CF fraction of isolates GS6-1 and GU21-2.

The elicitation of plant defense by cell wall lipid fractions alone and methanol soluble CF fractions of isolates GS6-1 and GU21-2 might suggest that these non-colonizing isolates induced resistance by their lipids (Table 6). Additionally, the ammonium sulfate precipitating fractions of CF and autoclaved CF did not induce resistance (data not shown) suggesting that proteins in CF might not have any role in induced resistance. The fact that liposaccharides present in the cell wall could elicit plant defense response suggests that lipids and saccharides of the root colonizing isolates might be recognized by plants during the process of root colonization resulting in induced systemic resistance.

A clear reduction in the number of germinated spores of *C. orbiculare* suggests that PGPF-protected plants produced some inhibitory biochemical substances (Table 7). Baker et al. (1978), using nucleopore filters, also observed impaired pathogen development in carnation after treatment with a non-pathogenic isolate of *F. roseum*.

Lignin deposition has been considered as an important step to suppress pathogen infection in systemically immunized plants (Kuc 1990; Shimizu et al. 2013). Hammerschimdt and Kuc (1982) suggested that lignin formation was responsible for the reduction of lesion area in *C. orbiculare*-induced plants. In the current study, increased lignin formation in response to pathogen infection was observed in plants treated with non-pathogenic PGPF.

Increased activity of hydrolytic enzyme is associated with induced resistance against necrosis pathogen (Maurhofer et al. 1994). Induction of systemic resistance in cucumber plants by GU21-2 resulted in increased activities of exo- and endo-forms of glucanase and chitinase, while, in case of isolate GS8-1, all of the previous enzymes were increased except exoglucanase (Table 8 and Figure 1). This might explain the result that isolate GU21-2 was more effective than isolate GS8-1 in ISR in cucumber plants. Maurhofer et al. (1994) also indicated variability in the induction of glucanase and chitinase activities using PGPR in tobacco plants. The delayed and reduced formation of lesions in PGPF-protected plants appeared to be related to the early and increased synthesis of endoglucanase and endochitinase than in unprotected plants. Chitinase started to accumulate at three days after challenge and reached the maximum level at six days in PGPF-treated plants compared with un-induced challenged plants. Tuzun et al. (1989) observed that chitinase started accumulation three days after challenge inoculation in tobacco plants with *Peronospora tabacina*. The role of increased PO activity in plant defense has been studied and discussed extensively (Hammerschimdt 1984; Irving & Kuc 1990). Our study showed that PGPF isolates enhanced the PO and PPO activities. Increased activities of PO and PPO due to protection with isolate GU21-2 might be related to the increased production of lignin that we observed in the protected seedling tissues. Increased activity of PAL was related to the increased necrotic area and lignin formation due to pathogen infection (Vegetti et al. 1975). The results of the present study showed increased activities of PAL at three days after challenge inoculation even before observing necrotic areas but the activity was reduced considerably at nine days. Our results indicate a slight increase in the activity of PPO at nine days after challenge inoculation in PGPF-induced plants. This might

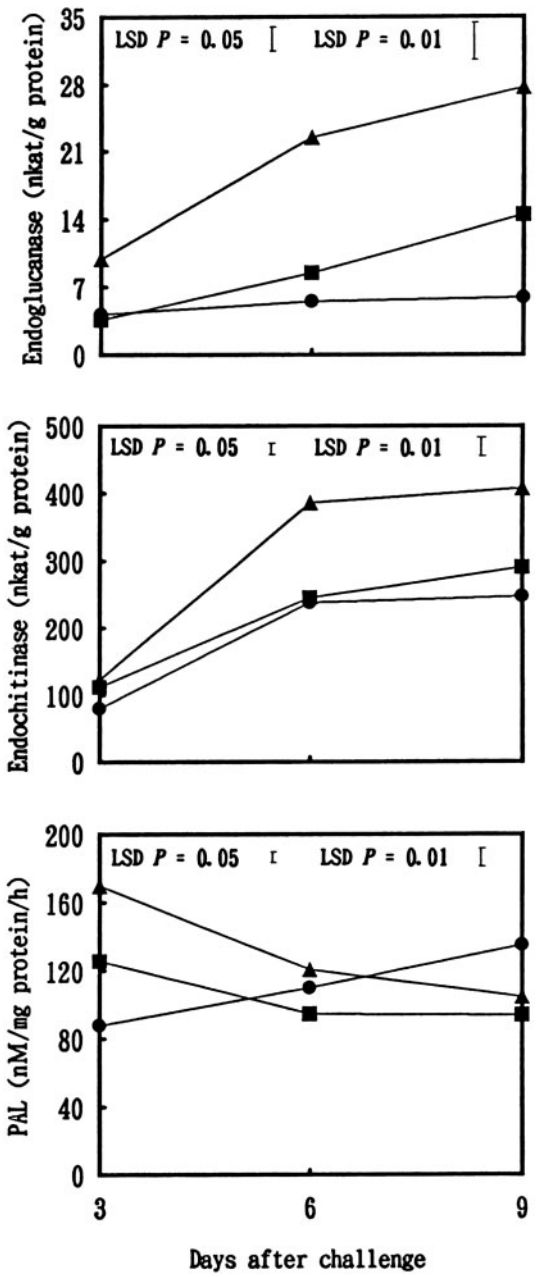

Figure 1. Activities of endoglucanase, endochitinase, and phenylalanine ammonia lyase (PAL) in three-week-old cucumber cv. Gibai plants unprotected (●) or protected systemically with plant growth-promoting fungi (PGPF) isolates GS8-1 (■) or GU21-2 (▲) at three, six, and nine days after challenge inoculation with 10–20 µl drops of 10^5 spores ml^{-1} of *C. orbiculare*.
Note: LSD bars compare means of two treatments at the same interval. Values are the means from three separate experiments and a repetition experiment with pooled samples from mix second true leaves per treatment.

show the role of PPO in induced resistance. Results of assays conducted with PGPF isolates in the present investigation provide support for the hypothesis that the inhibition of spore germination, formation of lignin, production of lytic and oxidative enzymes on protection with PGPF isolates are implicated in cucumber plant defense against attack by anthracnose disease.

Disclosure statement

No potential conflict of interest was reported by the authors.

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