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Fusarium equiseti GF191 as an effective biocontrol agent against Fusarium crown and root rot of tomato in rock wool systems

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Abstract

Six isolates of plant growth-promoting fungi (PGPF), non-pathogenic *Fusarium oxysporum*, and five isolates of bacteria were tested in hydroponic rock wool systems as potential biocontrol agents of Fusarium crown and root rot (FCRR) of tomato caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL). PGPF *Fusarium equiseti* proved the most effective organism in controlling FCRR, and the reduction rate by *F. equiseti* against FCRR was consistently high and significant in four experiments. The numbers of colony-forming units of FORL per gram fresh weight of stems were significantly reduced ($P = 0.05$) in plants treated with *F. equiseti*. Stem extracts from *F. equiseti*-treated and pathogen-challenged plants significantly inhibited the germination and germ tube length of FORL microconidia. Moreover, stem extracts from *F. equiseti*-treated plants (not treated with pathogen) significantly inhibited the germination of FORL microconidia and production of FORL budding-cells.

Keywords: *Fusarium oxysporum* f. sp. *radicis-lycopersici*; *Lycopersicon esculentum*; PGPF *Fusarium equiseti*; Biological control

1. Introduction

Fusarium crown and root rot (FCRR) of tomato (*Lycopersicon esculentum* Mill.) caused by *Fusarium oxysporum* (Schlecht.) f. sp. *radicis-lycopersici* (FORL) (Jarvis and Shoemaker, 1978) is a major disease worldwide (Rowe and Farley, 1977). FCRR was first reported in Japan in 1969, when it caused 33% and 44% of the yield losses in Hokkaido and Kochi Prefectures, respectively (Ogura and Ban, 1971; Sato and Araki, 1974). FCRR has now spread to all tomato production areas in Japan, whether the plants are grown in fields or greenhouses. Although resistant cultivars are one of the methods used to control FCRR, their effectiveness has been unsatisfactory (Elgersma et al., 1972; Scott and Jones, 2000). Another method is the use of stock tomato cultivars. To control FCRR, tomato cultivars have been grafted to resistant stock cultivars. While this method is effective for controlling various soil-borne diseases including FCRR, tomato growers are reluctant to use it because of its high cost. The most effective method of controlling FCRR has been soil disinfection using methyl bromide, but because that causes severe environmental problems, its use was outlawed in 2005.

There are some reports that FCRR was effectively managed by application of the fungicide benomyl (Mihuta-Grimm et al., 1990) and metam sodium (McGovern et al., 1998). However, repeated applications of such broad-spectrum fungicides may also cause environmental problems and run the risk of encouraging the appearance of fungicide-resistant strains (Dekker, 1979). Thus, further alternative control measures need to be made available as soon as possible.

In recent years, the production of greenhouse tomatoes has begun to shift from soil cultivation to various

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hydroponic culture systems, including the rock wool system (Mihuta-Grimm et al., 1990). However, there is no fungicide registered for use in controlling FCRR in such hydroponic systems (Omori, 1998).

Recently, a severe outbreak of FCRR in a rock wool system in Gifu Prefecture, Japan has demonstrated its vulnerability. In the greenhouse, 63% of tomato plants were infected by FCRR (Kato et al., 1998). This showed that the pathogen could spread easily throughout entire system. Moreover, in those systems, fungicides such as benomyl cannot be used for controlling this disease because of the risk of water pollution. For these reasons, there is an urgent need to establish a biological control method for this disease.

Biological control shows promise in the management of FCRR, with several antagonists already known to control FCRR. *Trichoderma harzianum* (Marois et al., 1981; Sivan et al., 1987), non-pathogenic *Fusarium* (Komada, 1994; Louter and Edgington, 1990), *Bacillus subtilis* (Phae et al., 1992), and *Pseudomonas fluorescens* (Dekkers et al., 2000; Duffy and Defago, 1997; Piga et al., 1997) have been reported to reduce the incidence of FCRR. An application of *T. harzianum* in combination with methyl bromide resulted in the most effective control to date against FCRR (Sivan et al., 1987). In addition, Duffy and Defago (1997) reported that a zinc amendment improved the biocontrol activity of *P. fluorescens* against FCRR. Although the biological control of FCRR in tomatoes grown in soil has been well studied, few investigations have been done on rock wool hydroponic systems. Muslim et al. (2003) reported that hypovirulent binucleate *Rhizoctonia* (HBNR) isolates could control FCRR in hydroponic rock wool systems. They also found that stem extracts from HBNR-treated plants in the rock wool system inhibited germination and production of budding-cells of FORL. The observed inhibitory effect of stem extracts from HBNR-treated plants on germination and proliferation of FCRR suggested that HBNR causes changes in the composition of plant extracts.

Saprophytic plant growth-promoting fungi (PGPF) belonging to *Fusarium equiseti*, *Trichoderma* spp., *Penicillium* spp. and *Phoma* sp., all isolated from turfgrass rhizospheres, were reported to have promoted significant plant growth and suppressed several soil-borne diseases (Hyakumachi, 1994; Hyakumachi and Kubota, 2004). The mechanisms of disease suppression caused by PGPF have also been intensively studied, and induced systemic resistance is thought to be involved as one of the mechanisms (Koike et al., 2001; Meera et al., 1995a, b; Shivanna et al., 1996).

The objective of this study was to select from among known biocontrol agents including PGPF those, which could control FCRR throughout the tomato-growing period in a rock wool hydroponic system. The effect of stem extracts from tomatoes treated with selected biocontrol agents on the germination and proliferation of pathogens was also determined.

2. Materials and methods

2.1. Organisms

PGPF isolates of *Trichoderma harzianum* GT31, *Penicillium simplicissimum* GP172, *Phoma* sp. GS81, and GS122, *Fusarium equiseti* GF191 (Hyakumachi, 1994; Hyakumachi and Kubota, 2004; Meera et al., 1995a, b; Shivanna et al., 1996) and an isolate of non-pathogenic *Fusarium oxysporum* F13 (Komada, 1994), were screen tested as biocontrol agents. Bacterial antagonists, i.e., *Pseudomonas fluorescens* F-2, *Xanthomonas campestris* K-3, *Bacillus subtilis* B-1, and B-2, *Bacillus amyloliquefaciens* B-3, obtained from the collection of the Plant Pathology Laboratory, Gifu University, were also used for comparison. The *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL) isolate, RJNI, obtained from a tomato plant with FCRR was used as a pathogen.

2.2. Plant

Tomato cv. "House Momotaro," (Takii seed Co., Ltd., Japan) which is a popular cultivar used mainly in greenhouses in Japan and is susceptible to FCRR, was used throughout the experiments. All seeds were surface-disinfected with 1% sodium hypochlorite for 5 min and rinsed three times in sterile distilled water prior to sowing.

2.3. Inoculum preparation

(1) *Fungal organisms*: Isolates of *T. harzianum* and *P. simplicissimum* were cultured on potato dextrose agar (PDA) in 9-cm Petri dishes for 7 d in the dark at 25 °C. Sterile distilled water was added to each Petri dish, and conidia of these isolates were harvested. Conidial suspensions were then adjusted to 10^7 spores/ml and used as inocula sources. Isolates of *F. equiseti*, non-pathogenic *Fusarium* and two isolates of *Phoma* sp. were cultured on PDA for 5 d in the dark at 25 °C. Five mycelial disks (5 mm in diameter) of these isolates taken from the edges of 5-d-old cultures were transferred to 100 ml potato dextrose broth (PDB) in 300-ml Erlenmeyer flasks and incubated for 7 d at 25 °C in a rotary shaker (NR-150, Taitec Co., Ltd., Japan) at 120 rpm. To obtain budding-cells of *F. equiseti* and non-pathogenic *Fusarium*, the fungal cultures were filtered through three layers of sterile gauze cloth. The resulting fungal suspensions were then diluted to 10^7 budding-cells/ml with sterile distilled water and used as inoculum sources. To obtain inoculum of two isolates of *Phoma* sp., the mycelial mass together with a culture filtrate was then homogenized using a blender (Model AM, Ace Homogenizer, Nihonseiki Kaisha Ltd., Japan) at 8000 rpm for 5 min and diluted with 200 ml sterile distilled water. The mycelial suspensions were used as inoculum sources.

(2) *Bacterial organisms*: Each isolate of *B. subtilis*, *B. amiloliquefaciens*, *P. fluorescens*, *X. campestris* was cultured in 100 ml King's B media (liquid type) in 300-ml Erlenmeyer flasks and incubated for 2 d at 25 °C with shaking (120 rpm). The bacterial-cell suspensions were then diluted to 10^8 cells/ml with sterile distilled water and used as inoculum sources.
(3) *Inoculum of FORL*: An isolate of FORL RJNI was also cultured in the same way as *F. equiseti* and non-pathogenic *Fusarium* as previously described. The collected budding-cell suspensions were adjusted with sterile distilled water to 10^5 budding-cells/ml and used as a pathogen inoculum.

2.4. Assay of biocontrol agents for control of FCRR in a hydroponic rock wool system

Two sizes of cube and rock wool substrate, mini-cube (3.6 cm × 3.6 cm × 4 cm) and large-cube (7.5 cm × 7.5 cm × 6.5 cm) (Grodan; Grodania A/S, Hedehusene, Denmark), were used. Tomato seeds were individually sown in rock wool mini-cubes into which a 20-ml suspension of the appropriate biocontrol inoculum was poured. The seedlings were grown for about 25 d in a greenhouse. Each mini-cube with its tomato seedling was then inserted into a large cube. A 200-ml suspension of the appropriate biocontrol inoculum was then poured into the large cube. Four experiments were conducted from 1999 to 2002 using this system. In experiments 1, 2, and 4, four large-cube transplants were placed on rock wool slabs (15 cm × 45 cm × 7.5 cm) (Grodan; Grodania A/S, Hedehusene, Denmark) in polypropylene boxes (50 cm × 50 cm × 50 cm). Each plant was grown in a sub-irrigation culture system. Plastic baskets (38 cm × 29 cm × 13 cm) were placed in the bottom of the boxes to create a space underneath the slabs. Tap water was added to the boxes up to 3 cm below the surface of the slabs. Plant fertilizers "Nutricote 12-0-0-23 (NPKCa)" and "Nutricote 13-11-13 (NPK)" (Asahikasei Co., Ltd., Japan) were used at 4.3 g per plant and 19.2 g per plant, respectively. Each box contained two rock wool slabs in which two tomato plants were inserted about 20 cm apart. After 7 d, a spore suspension of FORL (5.4×10^4 budding-cells/cm^3) was inoculated into each rock wool cube. As controls, tomato plants in rock wool cubes untreated with biocontrol agents and either challenged or unchallenged with FORL were prepared. The experiments were conducted in the greenhouse for 71 d (experiment 1), 117 d (experiment 2), and 140 d (experiment 4), respectively, at 15–30 °C. A short-term experiment (experiment 1) was conducted to select and narrow down more effective biocontrol agents against FCRR, and long-term experiments (experiment 2 and 4) were conducted to confirm the efficacy of the selected biocontrol agents. Furthermore, in a commercial production system, we conducted an experiment (experiment 3) to confirm the efficacy of biocontrol agents that were selected in experiments 1 and 2. Experiment 3 was carried out in a manner similar to that used in a standard commercial production facility. The transplants were placed onto rock wool slabs (20 cm × 91 cm × 7.5 cm) (Grodan; Grodania A/S, Hedehusene, Denmark) in plastic sleeves. Each slab contained four tomato plants about 20 cm apart. In this system, the slabs were placed on the greenhouse floor. Tomato transplants grown in large cubes of rock wool were placed on the surface of the slabs over holes (10 cm × 10 cm) cut in the plastic sleeve. Nutrient solutions, "High-Tempo Ar" and "High-Tempo Cu" (Taiyou Kogyou Co., Ltd., Japan), were provided at all times through drip irrigation emitters placed at the base of each transplant. Excess nutrient solution drained out through slits cut in the bottom of the plastic sleeves. The rock wool slabs were maintained at saturation with nutrient solution at all times. After 7 d, a spore suspension of FORL (5.4×10^4 budding-cells/cm^3) was inoculated into each rock wool cube. Rock wool cubes untreated with biocontrol agents and either challenged or unchallenged with FORL were set up as controls. The experiments were conducted for 97 d with temperatures between 14 (minimum) and 27 °C (maximum) (experiment 3) after inoculation with the pathogen. All treatments had four replicates, with four plants per replication. For experiments 1, 2, 3, and 4, disease severity based on foliar symptoms of wilting was monitored for 71, 98–117, 76–96 and 77–140 d after pathogen inoculation (DAPI), respectively. Discoloration severity of vascular tissue/cortex/xylem was assayed at the end of the experiments. Disease severity based on foliar symptoms was assessed on a scale of 0–4: 0 = healthy; 1 = yellowing; 2 = slight wilting; 3 = severe wilting; 4 = dead plant. Discoloration severity of vascular tissue/cortex/xylem was assessed subjectively after cutting the basal stem or crown of each plant as the midpoint percentage on the following scale: 0 = healthy (no vascular discoloration); 1 ⩽ 33% (midpoint = 16.5%); 2 ⩾ 33–67% (midpoint 50%); and 3 ⩾ 67–100% (midpoint = 83.5%).

2.5. Monitoring of FORL in stems

Populations of FORL in the stems were estimated at the end of experiments 1–3. In experiment 1, 20-cm long stems from all plants in each treatment were cut into 5-cm-long segments of 0–5, 5–10, 10–15, and 15–20 cm above the soil surface and the stem segments in each position were combined. In experiments 2 and 3, the 20-cm long stems from all plants in each disease score based on discoloration severity within each treatment were combined. The stems were washed separately in tap water to remove adhering rock wool, then homogenized in sterile distilled water (1:10 w/v) using a blender (Model AM, Ace Homogenizer, Nihonseiki Kaisha Ltd., Japan) at 8000 rpm for 10 min. The homogenized stems were filtered through two layers of gauze, diluted 10- to 100-fold and plated on Komada's selective medium (Komada, 1975) in six plates per replication. In experiment 1, we recorded the number of

colony-forming units of FORL per gram fresh weight of each of the stems in the 5-cm long segments within each treatment. The average populations of FORL in each replication within the treatment were calculated using the formula $(P_{0-5}A+P_{5-10}B+P_{10-15}C+P_{15-20}D)/N\times 100$, where $P_{0-5,\ 5-10,\ 10-15,\ \text{and}\ 15-20}$ is the pathogen population in stems 0–5, 5–10, 10–15, and 15–20 cm above soil surface, A the fresh weight of stems 0–5 cm above soil surface, B the fresh weight 5–10 cm above, C the fresh weight 10–15 cm above, D the fresh weight of stems 15–20 cm above, N the total weight of stems. Each treatment for four plants was replicated four times. In experiments 2 and 3, we recorded the number of colony-forming units of FORL per gram fresh weight of the stems in each score, based on discoloration severity within each treatment. The average populations of FORL in each replication within each treatment were calculated using the formula $(P_0A+P_1B+P_2C+P_3D)/N\times 100$, where $P_{0,\ 1,\ 2,\ \text{and}\ 3}$ is the population of pathogens in scales 0, 1, 2, and 3, respectively, A the fresh weight of stems on scale 0, B the fresh weight on scale 1, C the fresh weight on scale 2, D the fresh weight on scale 3, N the total weight of stems. Each treatment for four plants was replicated four times.

2.6. Re-isolation of GF191 from tomato roots and stems

Tomato seeds were sown individually in rock wool mini-cubes after which a 20 ml suspension of *F. equiseti* GF191 was poured into each mini-cube. The seedlings were grown for 45 d in a greenhouse. Tomato plants treated with GF191 and unchallenged with FORL were used for the experiments in re-isolation and populations of GF191 from tomato roots and stems. The roots and stems (0–10 cm above the soil surface) were collected 25 and 45 d after seeding, respectively. The roots and stems from four plants were washed separately in tap water to remove adhering rock wool and surface-sterilized (0.5% NaOCl, for 3 min). The roots were cut into segments 1 cm long, and the stems were sliced into segments around 3 mm in thickness. Ten such segments of each root and stem segment in six plates per replication were placed on peptone-PCNB medium. The isolation frequency was determined by counting the number of colonized root and stem segments. Roots and stems that were surface-disinfected were homogenized in sterile distilled water (1:10 w/v) using a blender (Model AM, Ace homogenizer, Nihonseiki Kaisha Ltd., Japan) at 8000 rpm for 10 min. The homogenized roots and stems were filtered through two layers of gauze, diluted 100- to 1000-fold, and plated on peptone-PCNB medium at six plates per replication. The numbers of colony-forming units of GF191 per gram fresh weight of roots and stems were recorded.

2.7. Effect of stem extracts on germination and proliferation of FORL

The tomato plants treated with *F. equiseti* GF191 challenged with FORL, and showing discoloration scale 1, were used. The stems were collected at the end of experiment 3 (96 DAPI). The filtrates of homogenized experiment-3 stems were centrifuged at 3000 rpm for 10 min. The supernatant solutions were collected and filtered with 0.45 μm Millipore ("Millex-HV", Millipore Co., Bedford, MA). Two ml of stem extracts was placed in small Petri dishes (40 mm × 20 mm). Cellophane was then positioned on the surface of the extract, and 40 μl of spore suspension FORL (5×10^5 budding-cells/ml) was immediately placed on the cellophane. Germination percentage was determined by randomly counting 400 spores per Petri dish. Germ tube lengths for 40 spores per Petri dish were also measured under the microscope. The tomato plants treated with *F. equiseti* GF191 and unchallenged with FORL were also used in experiments for the germination and proliferation of FORL. The stems were collected 75 d after seeding. The effect of stem extracts on pathogen proliferation was evaluated by counting newly formed budding-cells in the stem extracts. Nine ml of stem extracts and 1 ml of budding-cells of FORL (1.0×10^6 budding-cells/ml) were mixed in a 100-ml Erlenmeyer flask. The flask was then incubated at 25 °C on a reciprocal shaker at 120 rpm for 24 d. The numbers of budding-cells formed were determined with a haemacytometer. Each Petri dish and Erlenmeyer flask constituted one replicate, and each experiment was conducted four times.

2.8. Data analysis

The experiments in rock wool systems were arranged in a randomized complete block design of four replications with four plants per replication. First, we did a variance analysis of treatment means obtained for disease severity, discoloration severity, pathogen population, and effect of stem extracts on pathogen germination and proliferation; and when the F test results were significant, these means were compared using Fisher's protected least significant difference test at $P=0.05$. Data on the severity of disease and discoloration were analyzed after transformation to Arc Sine $\sqrt{x}$.

3. Results

3.1. Assay of biocontrol agents for control of FCRR of tomato

The disease severity of FCRR was 59.3% in pathogen-infested controls 71 DAPI of FORL in experiment 1 (Table 1). Six biocontrol agents, including *F. equiseti* GF191, *Phoma* sp. GS81, GS122, *B. subtilis* B-2, *B. amyloliquefaciens* B-3 and non-pathogenic *Fusarium* F13, significantly reduced FCRR by 100%, 94%, 94%, 87%, 87%, and 87%, respectively. On the other hand, *P. simplicissimum* GP172, *B. subtilis* B1, *P. fluorescens* F-2, and *X. campestris* K-3 reduced FCRR disease severity at lower levels of 31%, 50%, 38%, and 50%, respectively. Isolate GF191 also significantly reduced the discoloration severity of vascular

Table 1
Effect of biocontrol agents on disease and discoloration severity of crown and root rot of tomato caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici* in greenhouse rock wool system (experiment 1)

Treatment		Disease severity of foliar symptom[1]	Vascular discoloration severity[2]
Trichoderma harzianum	GT31	14.8 ab[3]	81.5 c
Phoma sp.	GS81	3.7 a	40.7 ab
Phoma sp.	GS122	3.7 a	70.4 bc
Penicillium simplicissimun	GP172	40.7 c	81.5 bc
Fusarium equiseti	GF191	0.0 a	3.7 a
non-P *Fusarium*	F13	7.4 a	37.0 ab
Bacillus subtilis	B-1	29.6 bc	77.8 bc
Bacillus subtilis	B-2	7.4 a	70.4 bc
Bacillus amyloliquefaciens	B-3	7.4 a	81.5 c
Pseudomonas fluorescens	F-2	37.0 c	85.2 c
Xanthomonas campestris	K-3	29.6 bc	74.1 bc
Pathogen		59.3 d	92.6 c
Control		0.0 a	0.0 a

[1]Disease severity $= (4A+3B+2C+D)/4N \times 100$. A, number of plants on index 4; B, number of plants on index 3; C, number of plants on index 2; D, number of plants on index 1; N, total number of plants. Disease index: 0, no disease; 1, yellowing; 2, slight wilt; 3, heavy wilt; and 4, dead.

[2]Discoloration severity $= (3A+2B+C)/3N \times 100$. A, number of plants on scale 3; B, number of plants on scale 2; C, number of plants on scale 1; and N, total number of plants. Discoloration scale: 0, no vascular discoloration; 1, <33%; 2, >33–67%; 3, >67–100% discoloration of vascular tissue/cortex/xylem.

[3]Values with same letter in each column are not significantly different ($P = 0.05$) according to Fisher's protected least significant difference test.

tissue/cortex/xylem by 96% 71 DAPI. Isolates of GS81 and F13 also reduced discoloration severity by 56% and 60%, respectively, while isolates of GS122, B-2, and B-3, though reducing disease severity, did not significantly reduce discoloration severity. For these reasons, isolates of GF191, GS81, and F13 were selected as the more effective biocontrol agents based on reductions in the severity of both disease and discoloration. Isolate B-2 was also selected based on its reduction of disease severity. These four isolates were primarily used in the subsequent experiments designed to compare their biocontrol efficacy.

In experiment 2, GF191 and B2 significantly reduced FCRR disease severity by 85% and 78%, respectively (Table 2). Isolate GS81 significantly reduced FCRR until 103 DAPI, but it did not suppress it from 110 to 117 d (Fig. 1). At 117 DAPI, its suppressive effect on disease severity was only 22%. Isolate F13 significantly reduced FCRR disease severity by 52%, which was markedly lower than those of GF191 and B-2. The reductions in FCRR discoloration severity by treatment with GF191, B-2, and F13 were low, and they reduced discoloration severity by 34%, 30%, and 20%, respectively.

Table 2
Effect of biocontrol agents on severity of discoloration inside stems of crown and root rot of tomato caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici* in rock wool system

Treatment		Discoloration severity[1]		
		Experiment 2 (117 d)[2]	Experiment 3 (97 d)	Experiment 4 (140 d)
Fusarium equiseti	GF191	60.4 b[3]	41.7 b	81.3 b
non-P *Fusarium*	F13	72.9 b	33.3 ab	93.8 c
Phoma sp.	GS81	93.8 c	ND[4]	ND
Bacillus subtilis	B-2	64.6 b	79.2 c	85.4 b
Pathogen		91.7 c	100.0 d	95.8 c
Control		0.0 a	0.0 a	0.0 a

[1]Discoloration severity $= (3A+2B+C)/3N \times 100$. A, number of plants on scale 3; B, number of plants on scale 2; C, number of plants on scale 1; and N, total number of plants. Discoloration scale: 0, no vascular discoloration; 1, ≤33%; 2, >33–67%; 3, >67–100% discoloration of vascular tissue/cortex/xylem.

[2]Days after inoculation of pathogen.

[3]Means of four replications with four plants per replication; values with same letter in each column are not significantly ($P = 0.05$) different according to Fisher's protected least significant difference test.

[4]Not done.

In experiment 3, which was conducted in the field, GF191, B-2, and F13 significantly reduced FCRR disease severity by 88%, 92%, and 81%, respectively (Fig. 1). Isolates of GF191 and F13 also significantly reduced discoloration severity 97 DAPI by 58% and 67%, respectively.

Experiment 4 was conducted over a long period. Isolate GF191 significantly reduced disease severity by 63% 140 DAPI (Fig. 1). Isolate F13 significantly reduced disease severity by 67% up to 120% DAPI, but did not reduce it until the end of the experiment because disease severity rapidly increase afterward thereafter. Isolate B-2 did not significantly reduce disease severity during the experiment. Isolates of GF191, F13, and B-2 were unable to significantly reduce discoloration severity 140 DAPI.

3.2. Monitoring of FORL in tomato stems

Tomato plants treated with both isolates of *F. equiseti* GF191 and non-pathogenic *Fusarium* F13 in experiment 1 showed significantly ($P = 0.05$) lower FORL population densities in all stem segments except the 10–15 cm segment for F13. Their average of FORL populations in stems from 0 to 20 cm above the soil surface were significantly reduced by 94% and 91%, respectively (Table 3). In experiment 2, FORL population densities in stems at scales 0, 2, and 3 of discoloration severity were significantly ($P = 0.05$) reduced by 98%, 100%, and 97%, respectively, compared to pathogen-infested control in the GF191 treatment (Table 4). Furthermore, the average of FORL populations in stems treated with GF191 was reduced by 99.9% relative to the pathogen-infested control. The average FORL populations in stems treated with F13, *Phoma* sp. GS81,

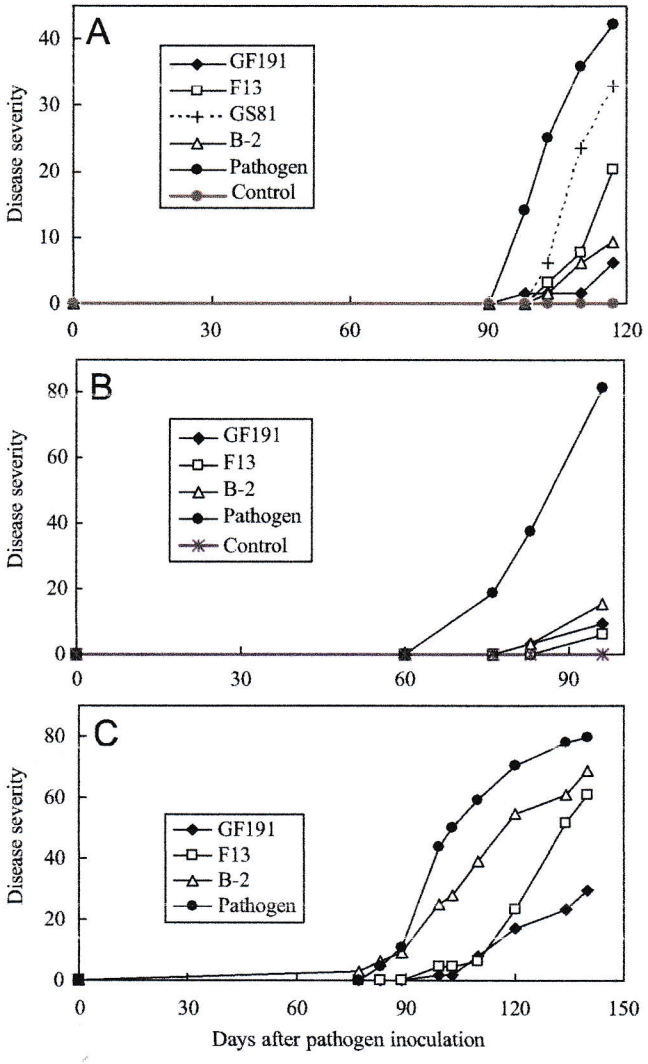

Fig. 1. Progress of disease severity of foliar symptoms caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici* on tomato treated with biocontrol agents in (A) experiment 2, (B) experiment 3 and (C) experiment 4.

and *B. subtilis* B-2 were significantly reduced by 26%, 75%, and 68%, respectively, compared to the pathogen-infested control. In experiment 3, the averages of FORL populations in stems treated with GF191 and F13 were reduced by 93% and 41%, respectively, compared to the pathogen-infested control (Table 5), whereas in stems treated with B-2, averages increased by 267% relative to the control.

3.3. Re-isolation of GF191 from tomato roots and stems

GF191 was re-isolated from 94.3% of the root segments 25 d after seeding (Table 6). Such a high isolation frequency of GF191 from root segments was also found 45 d after seeding. The population of GF191 increased from 25 to 45 d, ranging from 6.7×10^3 to 1.6×10^4 cfu/g. However, GF191 was not re-isolated nor detected from stem segments.

Table 3
Population density of *Fusarium oxysporum* f. sp. *radicis-lycopersici* in tomato stems collected from 0 to 20 cm above soil surface in experiment 1

Treatment	Population of FORL ($\times 10^2$ cfu/g) in different portion of tomato stems[1]				Average FORL population ($\times 10^2$ cfu/g)[2]
	0–5 cm	5–10 cm	10–15 cm	15–20 cm	
GF191	0.1 a[3]	0.4 ab	0.2 ab	0.1 a	0.2 a
F13	0.3 a	0.0 a	0.6 b	0.1 a	0.3 a
Pathogen	12.0 b	2.6 b	0.3 ab	0.3 a	3.5 b
Control	0.0 a	0.0 a	0.0 a	0.0	0.0 a

[1]Stems taken from 0 to 20 cm above soil surface, and cut into segments 5 cm long 0–5, 5–10, 10–15, and 15–20 cm above soil surface.
[2]Average population = Σ (population of different portion of tomato stem × weight)/total weight.
[3]Values with same letter in each column are not significantly different ($P = 0.05$) according to Fisher's protected least significant difference test.

Table 4
Population density of *Fusarium oxysporum* f. sp. *radicis-lycopersici* in tomato stems collected from 0–20 cm above soil surface (experiment 2)

Treatment	FORL population ($\times 10^2$ cfu/g) in discoloration scale[1]				Average FORL population ($\times 10^2$ cfu/g)[2]
	0	1	2	3	
GF191	0.5 a[3]	0.7 a	0.0 a	16.7 a	0.8 a
F13	0.2 a	31.3 b	833.3 c	370.0 c	433.7 d
GS81	n.s.[4]	n.s.	0.0 a	186.7 b	146.6 b
B-2	0.4 a	8.0 a	298.3 b	915.0 e	188.7 c
Pathogen	25.0 b	n.s.	58.4 a	653.3 d	582.2 e
Control	0.0 a	n.s.	n.s.	n.s.	0.0 a

[1]Discoloration scale: 0, no vascular discoloration; 1: ≦33%; 2, >33–67%; 3, >67–100% discoloration vascular tissue/cortex/xylem.
[2]Average population = Σ (population of different discoloration scale × weight)/total weight.
[3]Values with same letter in each column are not significantly different ($P = 0.05$) according to Fisher's protected least significant difference test.
[4]No sample.

3.4. Effect of stem extracts on FORL germination and proliferation

Stem extracts from tomato plants treated with *F. equiseti* GF191 and challenged with pathogen significantly ($P = 0.05$) inhibited the spore germination and germ tube length of FORL (Table 7). Germination and germ tube length in stem extracts of *F. equiseti*-treated plants were inhibited by 18% and 23%, respectively, compared with that of untreated and unchallenged plants. The stem extracts taken from plants inoculated only with *F. equiseti* significantly ($P = 0.05$) inhibited spore germination from 8 to 12 h after inoculation (Fig. 2). Spore germination was inhibited between 13% and 68% by stem extracts of *F. equiseti*-treated plants compared with those of untreated plants. The rate of budding-cell formation increased rapidly during the first 7 d of incubation and then declined

Table 5
Population density of *Fusarium oxysporum* f. sp. *radicis-lycopersici* in tomato stems collected from 0 to 20 cm above soil surface (experiment 3)

Treatment	FORL population ($\times 10^2$ cfu/g) in discoloration scale[1]				Average FORL population ($\times 10^2$ cfu/g)[2]
	0	1	2	3	
GF191	0.3 a[3]	36.7 a	14.0 a	13.3 a	14.4 a
F13	137.0 b	129.0 b	1.7 a	155.3 ab	124.0 b
B-2	n.s.[4]	165.0 c	343.7 b	1326.7 c	776.8 d
Pathogen	n.s.	n.s.	n.s.	211.7 b	211.7 c
Control	0.0 a	n.s.	n.s.	n.s.	0.0 a

[1]Discoloration scale: 0, no vascular discoloration; 1, ≦33%; 2, >33–67%; 3, >67–100% discoloration of vascular tissue/cortex/xylem.
[2]Average population = Σ (population of different discoloration scale × weight)/total weight.
[3]Values with same letter in each column are not significantly different ($P = 0.05$) according to Fisher's protected least significant difference test.
[4]No sample.

Table 6
Re-isolation and population of *Fusarium equseti* from roots and stems of tomato after seeding

Treatment		% re-isolation of GF191		Population of GF191 ($\times 10^3$ cfu/g)	
		25 d	45 d	25 d	45 d
GF191	Roots	94.3	97.1	6.7	16.7
	Stems	0	0	0	0

Table 7
Effect of stem extracts from plants treated with *Fusarium equiseti* on spore germination and germtube length of *Fusarium oxysporum* f. sp. *radicis-lycopersici*[1]

Treatments	Discoloration scale	Germination (%)	Germ tube length (μm)
GF191 + pathogen	1	70.0 a[2]	11.4 a
Pathogen	1	85.5 b	14.8 b
Control	0	85.0 b	14.8 b

[1]Stem and root samples were collected 117 d after inoculation of FORL from experiment 2. Germination and germ tube length determined 12 h after incubation. Data are means of four replications with 200 and 40 spores per replication for germination and germ tube length, respectively.
[2]Values with same letter in each column are not significantly different ($P = 0.05$) according to Fisher's protected least significant difference test.

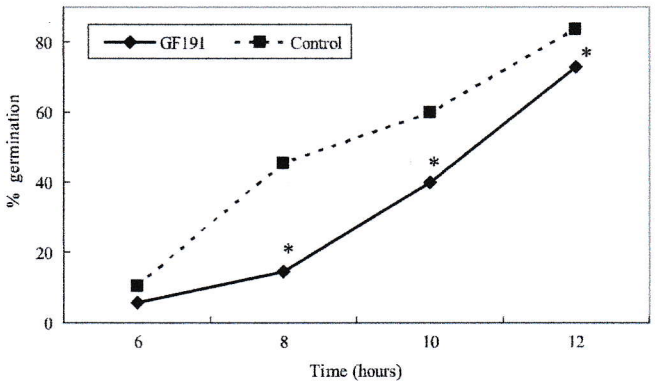

Fig. 2. Suppression of spore germination of *Fusarium oxysporum* f. sp. *radicis-lycopersici* in stem extracts of tomato treated with GF191. Asterisk denotes significant difference according to Student *t*-test at $P = 0.05$.

thereafter when stem extracts of those not treated and challenged or unchallenged with pathogen were used (Fig. 3). In contrast, when stem extracts from plants treated with *F. equiseti* and challenged with the pathogen were used, the rates of budding-cell formation were reduced by 33% throughout the experiment.

4. Discussion

In this study, *F. equiseti* effectively inhibited the development of FCRR under four different experimental conditions. The protection values based on disease severity of foliar symptoms against the disease were 63–100%, higher than those of other reports.

In this study, effective biocontrol agents were selected from the results of the four experiments. Experiment 1 was conducted 71 DAPI (short-term). The disease severity of foliar symptoms and vascular discoloration severity of FCRR were 59.3% and 92.6%, respectively, in pathogen-infested control at the end of the experiment. Although seven isolates of biocontrol agents significantly reduced FCRR based on disease severity of the foliar symptoms (protection values of 75–100%), only three among them, *F. equiseti* GF191, non-pathogenic *Fusarium* F13, and *Phoma* sp. GS81, significantly reduced FCRR based on vascular discoloration severity (protection values of 96%, 60%, and 56%, respectively). Discoloration severity is correlated with disease severity of foliar symptoms in many cases including those in the rock wool system

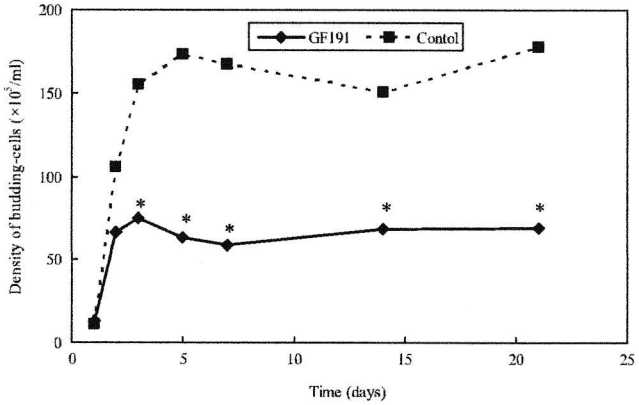

Fig. 3. Suppression of production of new budding-cells of *Fusarium oxysporum* f. sp. *radicis-lycopersici* in extracts of stem of tomato treated with *F. equiseti* GF191. Asterisk denotes significant difference according to Student *t*-test at $P = 0.05$.

(Mihuta-Grimm et al., 1990). These results indicated that, in a short-term experiment, it would be suitable to select and narrow down better biocontrol agents by investigating the vascular discoloration severity rather than the disease severity of foliar symptoms. Experiments 2 and 4 were conducted 117 and 140 DAPI (long-term). The disease severity of foliar symptoms and vascular discoloration severity of FCRR were 42.2–79.7% and 91.7–95.8%, respectively, in the pathogen-infested control at the end of the experiment. Although four isolates of biocontrol agents (GF191, F13, GS81, and B-2) did not reduce FCRR based on vascular discoloration severity, three isolates (GF191, F13, and B-2) significantly reduced FCRR based on disease severity of the foliar symptoms (protection values of 52–85%) in experiment 2. In experiment 4, however, only GF191 could show a high protection value (63%) based on disease severity of foliar symptom, but not on vascular discoloration severity (protection value, 15%). These results indicated that, in a long-term experiment it would be better to select better biocontrol agents by investigating the disease severity of foliar symptoms rather than the vascular discoloration severity.

Three fungal isolates (GF191, GS81, and F13) were more effective against FCRR than bacterial and other fungal isolates. Larkin and Fravel (1998) reported that non-pathogenic isolates of *F. oxysporum* and *Fusarium solani* were the most effective antagonists of Fusarium wilt of tomato among numerous fungi and bacteria that were tested using identical evaluation methods. They provided significant and consistent control. On the other hand, bacterial isolates generally failed to provide enough control levels.

Reports indicate that PGPF isolates colonize the epidermal and outer cortical cell layer of roots (Hyakumachi, 1994; Shivanna et al., 1996). The continuous presence of PGPF isolates on/in roots may trigger the plants to produce defence alarms (Meera et al., 1995b). Isolate GF191 was re-isolated very frequently from tomato roots, and its root population increased between 25 and 45 d after seeding (Table 6). This result suggests that the high colonization ability of GF191 in tomato roots might be related to the mechanisms of disease suppression.

The complete mechanism of the disease suppression in the tomato plants, i.e., *F. equiseti* interaction, remains unclear. The mechanisms of action associated with many biocontrol agents can be divided into two broad categories: direct antagonism of biocontrol agents to the pathogen and indirect antagonism mediated through the host plant (Mandeel and Baker, 1991, Postma and Rattink, 1992). In this study, pre-inoculation of tomato seedlings with *F. equiseti* GF191 not only suppressed the disease but also reduced pathogen populations in the stems. This result is similar to that of Nelson et al. (1992), who showed that pre-inoculation of tomato and cucumber with non-pathogenic *Fusarium* reduced the multiplication of pathogens in the plants. Similar results were obtained using resistant cultivars against wilt or crown and root rot disease of tomato where the pathogenic *Fusarium* population in stems and roots was significantly lower than that in susceptible cultivars (Elgersma et al., 1972; Stromberg and Corden, 1977). In this study, pathogen populations in the stems treated with GF191 were significantly lower (reduced by 93–100%) (Tables 3–5) than those of control, and were also lower in all discoloration scores (Tables 4 and 5) in both short- (71 d) and long-term (117 d) experiments. These results suggest that, although FORL can infect and even grow in tomato roots treated with GF191, it cannot multiply there.

Benhamou et al. (1997) revealed that *Pythium oligandrum* induced an accumulation of phenolic compounds creating a fungitoxic environment to FORL. In addition, Stromberg and Corden (1977) reported that the xylem extract from resistant tomato cultivars inoculated with *F. oxysporum* f. sp. *radicis-lycopersici* was highly fungitoxic to the growth of pathogen. Previous studies reported that the inhibition of pathogens in the roots and stems of resistant tomato cultivars after inoculation with Fusarium wilt pathogen was related to accumulations of tomatine and rishitin (Furui et al., 1998; Hammerchlag and Mace, 1975; Langcake et al., 1972; MacCane and Drysdale, 1975). We also observed such inhibitory effects of stem extracts from *F. equiseti*-treated plants on pathogen germination and proliferation (Table 7, Figs. 2 and 3), suggesting that *F. equiseti* might induce physiological changes in the composition of plant extracts. Such changes may be related to the production and release of fungitoxic materials by biocontrol agents themselves or by plants that have developed resistance through these agents. These changes allow for reduced inoculum levels of pathogen in the plants. Furthermore, *F. equiseti* GF191 was re-isolated from tomato roots but not from the stems, indicating that the hyphae of *F. equiseti* do not move between the two sites. In other words, in the stems, there is no direct interaction (parasitism and antibiosis) between GF191 and FORL. These results suggest that, in this system, the

observed disease reduction could be attributed to indirect antagonism mediated through the host plant in response to root colonization by GF191, and induced-resistance may be one of the mechanisms of the biocontrol of FCRR.

In this study, we have shown that *F. equiseti* GF191 was able to control FCRR of tomato in a hydroponic rock wool system under both sub-irrigation and drip irrigation methods during a term of 71–140 DAPI. This is the first report of the biocontrol of FCRR by *F. equiseti*. Further research is needed to determine the complete mechanisms of disease suppression involved in the *F. equiseti* system, and to develop methods for its practical use as a biocontrol agent.

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