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SHORT COMMUNICATION

BIOCONTROL OF FUSARIUM WILT OF SPINACH BY THE PLANT GROWTH PROMOTING FUNGUS *FUSARIUM EQUISETI* GF183

H. Horinouchi[1], A. Muslim[2] and M. Hyakumachi[3]

[1] Gifu Prefectural Agricultural Technology Center, 729 Matamaru, Gifu 501-1152, Japan
[2] Department of Plant Pests and Diseases, Faculty of Agriculture, Sriwijaya University, Indralaya Campus, Palembang 30662, Indonesia
[3] Laboratory of Plant Pathology, Faculty of Applied Biological Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan

SUMMARY

The plant growth promoting fungus *Fusarium equiseti* GF183 effectively controlled Fusarium wilt of spinach caused by *Fusarium oxysporum* f. sp. *spinaciae* in transplanting systems using paper pots. Reduction in disease severity ranged from 43.5 to 91.8%. Double application of *F. equiseti* GF183 increased the protective effects. The number of colony-forming units of *F. oxysporum* f. sp. *spinaciae* per gram fresh weight of roots was significantly reduced ($P<0.05$) in plants treated with *F. equiseti*. Root extracts from both *F. equiseti*-treated plants and *F. equiseti* and pathogen-treated plants significantly inhibited new production of budding-cells of *F. oxysporum* f. sp. *spinaciae*.

Key words: *Fusarium oxysporum* f. sp. *spinaciae,* PGPF *Fusarium equiseti,* biocontrol, spinach.

Fusarium wilt of spinach (*Spinacia oleracea* L.) (FWS) caused by *Fusarium oxysporum* Schlechtend: Fr. f. sp. *spinaciae* (Sherb) W.C. Snyder & H.N. Hansen (FOS) is a serious constraint to spinach production worldwide (Correl *et al*., 1994; Larsson and Gerhardson, 1992). It causes damping-off, wilting, root rot and discoloration of the vascular system of seedlings and mature plants. Although the use of resistant cultivars is considered an effective means to control Fusarium wilt (Beckman, 1987), FWS-resistant cultivars have been reported against FWS, including non-pathogenic *F. oxysporum* (Katsube and Akasaka, 1997), hypovirulent binucleate *Rhizoctonia* (Muslim *et al*., 2003) and *Enterobacter cloacae* (Tsuda *et al*., 2001). A paper pot transplanting system is another method used for controlling soil-borne diseases such as FWS (Katsube and Akasaka, 1997) and common potato scab (Naito *et al*., 1998). Katsube and Akasaka (1997) reported that a high suppression effect against FWS was obtained by transplanting spinach seedlings grown in paper pots containing non-pathogenic *F. oxysporum*.

The saprophyte plant growth promoting fungus (PGPF) *Fusarium equiseti*, obtained from turfgrass rhizospheres, has been reported to enhance plant growth significantly and suppress several soil-borne diseases caused by *Pythium* spp., *Rhizoctonia solani*, *Sclerotium rolfsii*, *Gaeumannomyces graminis* var. *tritici* and *Cochliobolus sativus* (Hyakumachi, 1994; Hyakumachi and Kubota, 2004). We have previously shown that *F. equiseti* can also control Fusarium crown and root rot of tomato caused by *F. oxysporum* f. sp. *radicis-lycopersici* in both a hydroponic rock-wool system and soil system (Horinouchi *et al*., 2007, 2008). From the results of our preliminary experiments, we found that among several bacteria and fungi used as biocontrol agents, the isolate of PGPF *F. equiseti* effectively controls FWS.

The present study was conducted to investigate the effect of *F. equiseti* in controlling FWS. We used *F. equiseti* isolate GF183. The FOS isolate, S1HI-4, obtained

ported to have only some resistance to the disease (O'Brien and Winter, 1977). Although soil fumigation with chloropicrin or methyl bromide has been used to control the disease in Japan, its use has caused severe environmental problems. Thus, alternative control methods must be made available as soon as possible.

The use of biocontrol agents to control Fusarium wilt has been reported for many crops including tomato, cucumber, melon, strawberry, banana and carnation. There are also some reports on the use of biocontrol

Corresponding author: M. Hyakumachi
Fax: +81.58.2932847
E-mail: hyakumac@gifu-u.ac.jp

from the Iwata Prefectual Agricultural Experiment Station, was used as a pathogen. Spinach cv. Samalto (Sakata Seed, Japan), a popular cultivar susceptible to FWS, was used throughout the experiment. All seeds were surface-disinfected with 1% sodium hypochlorite solution for 5 min and rinsed three times in sterile distilled water prior to sowing.

GF183 and S1HI-4 were separately cultured on potato dextrose agar (PDA) in 9-cm Petri dishes for 5 days in the dark at 25°C. Five mycelial disks (5 mm in diameter), taken from the edges of 5-day-old cultures, were transferred into 100 ml of potato dextrose broth (PDB) in 300 ml Erlenmeyer flasks and incubated for 7 days at 25°C on a rotary shaker (NR-150, Taitec Co., Japan) at 120 rpm. To obtain budding-cells of *Fusarium* isolates,

the fungal cultures were filtered through three layers of sterile gauze. Fungal suspensions were then diluted to 10^7 budding-cells per ml with sterile distilled water and each was used as an inoculum source.

In all experiments, a paper pot set (28.0×58.0 cm) containing 162 pots (5.0 cm depth × 1.5 cm diameter per pot), manufactured by Nippon Beet Sugar Co., Japan, was filled with potting soil "Star Bed" (Zen-Noh, Tokyo, Japan). The potting soil contains clay, peat, zeolite, and composted plant material. The nitrogen, phosphorus and potassium contents of the potting soil were 200:1500:200 mg per liter, and the pH ranged from 6.0 to 7.0. A 10 ml suspension of GF183 was poured into each pot, and one spinach seed was sown. The seedlings were grown for 14 days in a greenhouse. The spinach seedlings grown in paper pots were transplanted approximately 15 cm apart into concrete blocks (50×100×80 cm) filled with pathogen-infested soil (FOS concentration was 1.0×10^4 CFU g^{-1} soil) in polytunnels. Seedlings not treated with GF183 and challenged or not challenged with FOS were set up as controls.

Experiments 4 and 5 were conducted to compare the effectiveness of three applications of GF183 against FWS. The three applications were: GF183 single application at seeding, single application one day before transplanting, and GF183 double application at both seeding and one day before transplanting. In experiment 5, an additional test was conducted for comparison, in which the spinach seeds were sown directly in pathogen-infested soil. Experiments 1, 2, 3, 4 and 5 were conducted for 22, 24, 27, 22 and 26 days after transplanting to pathogen-infested soil between May 18 and June 9, 1999, June 4 and June 28, 2000, June 28 and July 28, 2003, May 26 and June 18, 2004 and June 6 and July 5, 2006, respectively.

Disease severity based on the foliar symptoms of wilt-

in which A = number of plants on scale 4; B: = number of plants on scale 3; C = number of plants on scale 2; D = number of plants on scale 1; N = total number of plants.

The experiments were arranged in a randomized complete block design of four replications, with nine plants per replication. First, analysis of variance was performed for the treatment means obtained for disease severity. 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 disease severity were analyzed after transformation to arc sine $\sqrt{x}$.

Spinach plants treated with GF183 had less disease than pathogen-control plants in experiments 1, 2, and 3 (Table 1). The protective effects based on disease severity were 91.8%, 51.5% and 79.9%, respectively, at the end of experiments 1, 2, and 3.

In experiment 4, the GF183 single application at seeding and GF183 double application at both seeding and one day before transplanting significantly ($P<0.05$) reduced disease severity by 52.5% and 58.1%, respectively, compared to the pathogen control (Table 2). The GF183 single application one day before transplanting was not effective. In experiment 5, the protective effect of the GF183 double application was higher than that of the GF183 single application at seeding, though the difference was not significant. The GF183 double application significantly ($P<0.05$) reduced disease severity by 87.3% compared to the pathogen control. The protective effect of the GF183 double application was further enhanced, reaching 94.8% when compared to direct sowing.

In this study, GF183 effectively controlled FWS, based on the results of five different experiments. Protective effects of GF183 were 43.5-91.8% and 58.1-87.3% by single application at seeding and double ap-

ing was assayed at the end of the experiments using a scale of 0 to 4: 0 = healthy; 1 = yellowing; 2 = slight wilting; 3 = severe wilting; and 4 = dead plant, was calculated with the following formula:

$$(4A+3B+2C+D)/4N\times100$$

plication at seeding and one day before transplanting, respectively. These effects were almost the same as with non-pathogenic *F. oxysporum* (Katsube and Akasaka, 1997), although the treatment methods were slightly different. In the latter case, non-pathogenic *F. oxysporum* was tested singly at 1 or 5 days after sowing (protective

Table 1. Effects of *Fusarium equiseti* GF183 on disease severity of Fusarium wilt of spinach caused by *F. oxysporum* f. sp. *spinaciae* under polytunnel conditions.

Treatment	Disease severity		
	Exp. 1	Exp. 2	Exp. 3
Fusarium equiseti (GF183) + Pathogen	3.4 b	22.9 b	6.3 b
Pathogen only	41.7 c	47.2 c	31.3 c
Control	0.0 a	0.0 a	0.0 a

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

Table 2. Effects of single and double application of *Fusarium equiseti* GF183 on disease severity of Fusarium wilt of spinach caused by *F. oxysporum* f. sp. *spinaciae* under polytunnel conditions.

Treatment	Disease severity	
	Exp. 4	Exp. 5
GF183 single application at seeding + Pathogen	41.6 b	20.4 bc
GF183 single application one day before transplanting + Pathogen	71.3 bc	ND
GF183 double application at both seeding and one day before transplanting + Pathogen	36.7 ab	4.6 b
Pathogen only	87.5 c	36.1 c
Direct sowing + Pathogen	ND	89.8 d
Control	0.0 a	0.0 a

ND: Not done.
Values with the same letter in each column are not significantly different ($P<0.05$) according to Fisher's protected least significant difference test.

effects 38.2-48.1%), or 5 days before transplanting (87.1%).

A different strain, PGPF GF191 of *F. equiseti*, could also effectively decrease Fusarium crown and root rot of tomato both in rock wool and soil systems (Horinouchi

FOS populations in roots were estimated at the end of experiment 4. The roots in the paper pots were collected from all plants in each disease category within each treatment, were pooled, washed in tap water and homogenized in sterile distilled water (1:10 w/v) using a

et al., 2007; 2008). The protective effects were 63-100% and 53-62% in rock wool and soil systems, respectively. GF191 could also decrease Fusarium wilt of tomato (unpublished data). Like them, *F. equiseti* could be a potential candidate as a biocontrol agent. GF183 was frequently re-isolated from spinach roots (data not shown), suggesting that the high ability of GF183 to colonize spinach roots might be related to the mechanism of disease suppression. Reports indicate that PGPF isolates, including *F. equiseti,* 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 responses (Meera *et al*., 1995). Recently, Maciá-Vicente *et al*. (2009) demonstrated endophytic colonization by *F. equiseti* and indicated that root colonization by the biocontrol agent is an important factor for the control of root pathogens.

Although a single application of GF183 one day before transplanting to pathogen-infested soil did not significantly reduce disease severity, a single application of GF183 at seeding and a double application at both seeding and one day before transplanting to pathogen-infested soil served to control FWS (Table 2). These results also suggest that a high degree of colonization of GF183 is necessary for disease suppression.

blender (Ace Homogenizer, Model AM, Nihonseiki Kaisha, Japan) at 8,000 rpm for 5 min. The homogenate was filtered through two layers of gauze, diluted 10- to 1000-fold and plated on Komada's selective medium (Komada, 1975) with six plates per dilution. The experiment included four replicates.

The number was recorded of CFU of FOS per gram of fresh weight of the roots in each scale within each treatment. Discoloration of the vascular tissue was assessed after transversely cutting the basal part of the taproot of each plant on the following scale: 0 = no vascular discoloration; 1 = < 33%; 2 = > 34 to 67%; and 3 = > 67 to 100% discoloration. The average FOS populations in each replication within the treatment were calculated using the formula:

$$(P_0A+P_1B+P_2C+P_3D)/N \times 100,$$

where $P_{0, 1, 2, \text{and } 3}$ = populations of the pathogen in scales 0, 1, 2 and 3, respectively; A = fresh weight of roots on scale 0; B = fresh weight on scale 1; C = fresh weight on scale 2; D = fresh weight on scale 3; and N = total weight of roots. Statistical analysis was performed as previously described.

The FOS population densities in roots treated with the GF183 single application at seeding and double application at seeding and one day before transplanting

Table 3. Effects of *Fusarium equiseti* GF183 treatments on population density of *Fusarium oxysporum* f. sp. *spinaciae* in spinach roots in experiment 4.

Treatment	FOS populations in different discoloration scores (x10_2 CFU g_1 fresh weight)				Average population of FOS (x10_2 CFU g_1 fresh weight)
	0	1	2	3	
GF183 single application at seeding + Pathogen	7.9 b	21.3	43.3 a	353.3 a	98.9 b
GF183 double application + Pathogen	7.3 b	55.0	73.3 ab	264.0 a	96.5 b
Pathogen only	n.s.	n.s.	95.3 b	1453.3 b	673.0 c
Control	0.0 a	n.s.	n.s.	n.s.	0.0 a

n.s.: No sample.
Values with the same letter in each column are not significantly different (P<0.05) according to Fisher's protected least significant difference test.

were significantly (P<0.05) reduced by 75.7% and 81.8%, respectively, at scale 3 of vascular discoloration, compared to the pathogen control (Table 3). Furthermore, the average FOS populations in roots treated with the GF183 single and double applications were significantly (P<0.05) reduced by 85.3% and 85.6%, respectively, relative to the pathogen control.

Pre-inoculation of spinach seedlings with GF183 not

which was then incubated at 25°C on a reciprocal shaker at 120 rpm. The numbers of budding-cells formed were determined with a haemocytometer. Each Petri dish and Erlenmeyer flask constituted one replicate, and each experiment was conducted four times. Statistical analysis was performed as previously described.

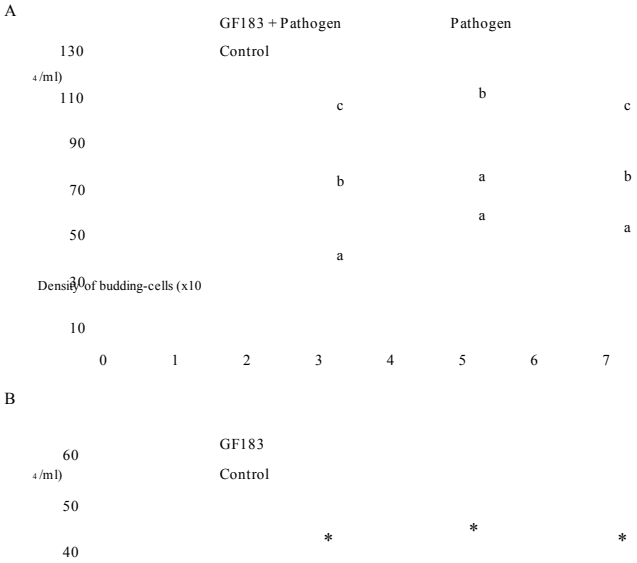
Root extracts from spinach plants treated with GF183 and challenged with pathogen inhibited signifi-

only suppressed the disease but also reduced pathogen populations in the roots. This result is similar to that of Nelson *et al*. (1992), who showed that pre-inoculation of tomato and cucumber with non-pathogenic *Fusaria* reduced the multiplication of *F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *cucumerinum*. In the present study, pathogen populations in the roots treated with GF183 were significantly lower (reduced by about 85%) than those of the pathogen control. These results suggest that, although FOS can enter spinach roots treated with *F. equiseti*, it cannot multiply there. This further suggests that, in this system, the observed disease reduction is attributable to indirect antagonism mediated through the host plant in response to root colonization by *F. equiseti*, and that induced resistance may be one of the mechanisms of FWS biocontrol.

The effect of root extracts on the proliferation of FOS was studied further using spinach plants treated or not with GF183 at seeding, challenged with FOS, and showing a discoloration scale of 3. Roots were collected at the end of experiment 4. Spinach plants treated with GF183 and unchallenged with FOS were also used. Roots were examined 30 days after seeding. Homogenized root filtrates were centrifuged at 3,000 rpm for 10 min, supernatants were collected and filtered through 0.45 µm Millipore ("Millex-HV," Millipore USA). The effect of these extracts on pathogen proliferation was evaluated by counting newly formed budding-cells in the root extracts. Nine milliliters of root extract and 1 ml of a FOS budding-cell suspension (1.0x10^6 budding-cells per ml) were mixed in a 100 ml Erlenmeyer flask

cantly ($P<0.05$) the production of new budding-cells of FOS compared with extracts of untreated and unchallenged plants 3, 5 and 7 days after inoculation (Fig. 1A), and the rates of budding-cell formation were reduced by 67.9%, 48.8% and 53.8%, respectively. Root extracts from spinach plants untreated with GF183 and challenged with pathogen also significantly ($P<0.05$) inhibited FOS proliferation compared with extracts of untreated and unchallenged plants, and the rates of budding-cell formation were reduced by 33.3%, 32.9% and 31.3%, respectively. Furthermore, root extracts from spinach plants treated with GF183 and unchallenged with pathogen also significantly ($P<0.05$) inhibited the production of new FOS budding-cells compared with extracts of untreated and unchallenged plants (Fig. 1B), and the rates of budding-cell formation were reduced by 19.7%, 24.1% and 30.9%, respectively.

In the present study, we found an inhibitory effect of spinach root extracts from pathogen-treated plants on pathogen proliferation (inhibitory effect was 31.3-33.3% for 3-7 days) but the effect was stronger (48.8-67.9%) in *F. equiseti*-pathogen-treated plants (Fig. 1A). An inhibitory effect was also given by root extracts from *F. equiseti*-treated plants (19.7-30.9%) (Fig. 1B), but it was not as strong as that from *F. equiseti*-pathogen-treated plants. These results suggest that *F. equiseti* might induce physiological changes in the composition of plant extracts and enhance defence mechanisms that are further triggered when the plant senses a potential pathogen in a phenomenon known as potentiation or conditioning (Kauss *et al*., 1993; Kessmann *et al*., 1994).



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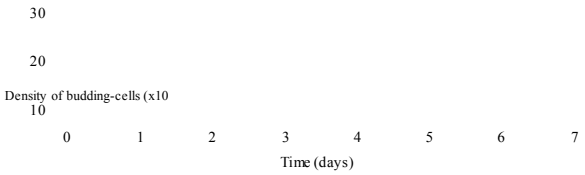

Fig. 1. Suppression of production of new budding-cells of *Fusarium oxysporum* f. sp. *spinaciae* in extracts of root of spinach (A) treated with *F. equiseti*-GF183 and pathogen, (B) treated with *F. equiseti*-GF183 and unchallenged with pathogen. Values with the same letter in each column are not significantly different (P<0.05) according to Fisher's protected least significant difference test. An asterisk denotes a significant difference according to Student's *t*-test at P<0.05. Bars indicate standard error of means.

Although *F. equiseti* is implicated in a few diseases, such as rotting of cucurbit fruits in contact with soil (Burgess *et al*., 1988), it is considered an unimportant or opportunistic pathogen. This fungus, however, has been characterized as a natural root endophyte able to colonize plant roots and endowed with properties that could make it a promising candidate for the biological control of root pathogens and nematodes (Maciá-Vicente *et al*., 2008; Nitao *et al*., 2001).

We have now shown that *F. equiseti* GF183 is able to control FWS, which, to our knowledge, is the first report of such an activity. Further research is nevertheless warranted to determine the complete mechanisms of disease suppression involved in the *F. equiseti* system, and to develop methods for its practical use in biocontrol.

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