Mycoscience (2002) 43:447–451

FULL PAPER

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Screening of fungal antagonists against yellows of cabbage caused by *Fusarium oxysporum* f. sp. *conglutinans*

Received: August 8, 2001 / Accepted: August 28, 2002

Abstract Screening of fungal antagonists against yellows of cabbage caused by *Fusarium oxysporum* f. sp. *conglutinans* was carried out. We obtained 78 seed-borne fungal isolates from 20 kinds of vegetable roots. Fifty-five soilborne fungal isolates were obtained from the surface-sterilized roots of seven vegetables. Twelve isolates were from field soil using a baiting method. By in vitro and in vivo screening, two seedborne species of *Penicillium* (S-34) and *P. citrinum* (S-59), and four soilborne *Epicoccum nigrum* (TC-33), *Fusarium solani* (SS-6, CM02), and *F. oxysporum* f. sp. *lactucae* (F-9501) suppressed yellows of cabbage effectively. Reductions in disease incidence ranged from 28% to 63%.

Key words Biological control · *Fusarium oxysporum* f. sp. *conglutinans* · Yellows disease

Introduction

The yellows of cabbage, caused by the soilborne fungus *Fusarium oxysporum* f. sp. *conglutinans* (Wollenweb.) W.C. Snyder & H.N. Hansen, is one of the most serious soilborne diseases in Japan. It first broke out in 1952 (Nomura et al. 1976), spread rapidly, and became serious in most of the cabbage fields in Japan. Although *Fusarium*-resistant cultivars of cabbage can be used for controlling the disease (Nomura et al. 1976), the appearance of new pathogenic races has been a continuing problem (Ramirez-villupadua et al. 1985). Soil fumigation with methyl bromide (MBr) has been carried out to control soilborne diseases, but MBr is an ozone-depleting compound (Yagi et al. 1993). Because of its potential effect on human health, the Ministry of Agriculture, Forestry and Fisheries of Japan has scheduled removing MBr completely from the market by 2005 (Suzui et al. 2000). Therefore, alternative measures are needed for controlling the disease.

As one of the tools suppressing soilborne diseases, plant pathologists have been interested in the effect of microorganisms. A variety of soilborne fungi have demonstrated potential activity for controlling various soilborne plant pathogens (Aberra et al. 1998; Jackson et al. 1994; Larkin and Fravel 1998). Some mycorrhizal fungi have been known to be effective in inhibiting the development of pathogens and reducing incidence of the disease (Caron 1989; Perrin 1990). Cabbage is a member of the Cruciferae, which is an exceptional family consisting of few or no mycorrhizas (Ocampo et al. 1980), although most plant species including a large number of crops develop mycorrhizas. Recently, an attempt for screening useful fungal endophytes has been made for cruciferous plants. *Heteroconium chaetospira* (Grove) M.B. Ellis suppressed clubroot disease in Chinese cabbage (Narisawa et al. 1998). Thus, it is necessary to screen useful fungal strains from endophytic or soilborne fungi for suppressing soilborne diseases of cruciferous vegetables.

Generally, biological control is more variable and less effective than chemical control because of the poor ecological competence of the biocontrol microorganism, i.e., the ability of the control organism to survive and compete in nature (Harman and Lumsden 1990). However, in some cases, biocontrol agents used as microbial seed treatments have been shown to be as effective as manufactured chemical agents in achieving soilborne disease control (McQuilken et al. 1990; Parke 1990). Seed treatment applications have been shown to be effective against the patho-

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genic fungus for considerable periods in naturally infested soil in some situations (Ahmed and Tribe 1977; Utkhede and Rahe 1980), whereas the pathogens are limited in time (Paulitz 1992). We postulate that seed treatments with seedborne fungi may be effective for controlling plant pathogenic fungus. The activities of seedborne fungi for controlling soilborne plant pathogens have never been reported, although a variety of soilborne fungi have been tested and their activities against soilborne or seedborne plant pathogens demonstrated (Aberra et al. 1998; Jackson et al. 1994; Larkin and Fravel 1998; Teperi et al. 1998). In this study, we screened fungal antagonists against yellows of cabbage from seedborne as well as soilborne fungal isolates with microbial seed treatments.

Materials and methods

Isolation of seedborne fungi

The seeds from 20 different vegetables belonging to the families Alliaceae, Asteraceae, Chenopodiaceae, Cruciferae, Cucurbitaceae, Solanaceae, and Umbelliferae were used for isolating seedborne fungi (Table 1). One hundred seeds for each vegetable (Takii Seeds, Kyoto, Japan) were surface-sterilized by immersion in 70% ethanol for 2 min and then in sodium hypochlorite solution (1% available chlorine) for 5 min. The seeds were then rinsed five times in sterile distilled water. The surface-sterilized seeds were placed on 1% water agar plates (9 cm in diameter) and incubated at 25°C for germination. Two weeks later, only germinating seeds with no microorganism were transferred to a 100-ml test tube that contained 50 ml 0.5% water agar. The numbers of germinated seeds are shown in Table 1. The axenic seedlings were incubated at 25°C (6L:8D) in sterilized conditions for 2 weeks. The roots were separated from the 2-week-old seedlings by forceps and cut into 1-cm segments. The root segments were transferred on potato dextrose agar (PDA) or malt extract agar (MEA) plates (9 cm in diameter), and then incubated at 25°C under black light (Toshiba FL20S BLB-A 20W; with the light source at a distance of 30 cm from the Petri dishes) for 2 months to induce sporulation. Fungal conidia or mycelia grown from the root pieces were transferred and cultured on LCA medium (glucose 1 g, KH_2PO_4 1 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, KCl 0.2 g, $NaNO_3$ 2 g, yeast extract 0.2 g, agar 15 g l^{-1}) (Miura and Kudo 1970). Identification of the fungal isolates was carried out under microscopic observation according to appropriate taxonomic keys (Ellis 1971, 1976; Domsch et al. 1993; Watanabe 1993).

Isolation of soilborne fungi

Method 1

Eight kinds of cultivated vegetables including cabbage, Chinese cabbage, corn, soybean, strawberry, sweet potato, taro, and tomato were sampled from five farms in Japan (Wako, Saitama; Miura, Kanagawa; and Bunkyo, Tokyo). Twenty pieces of root from each plant were surface-sterilized by ethanol and sodium hypochlorite solutions using the aforementioned method.

Table 1. Frequency of seedborne fungi isolated from 20 kinds of vegetables

Host	Germination rate of seeds (%)	Number of isolates											
		Alt.	Asp.	Cla.	Cur.	Cyl.	Glio.	Fus.	Nig.	Pae.	Pen.	Ste	Total
Bell pepper	83						1		1	1			3
Broccoli	100						1					1	2
Cabbage	100			1			1	1		1	3		7
Carrot	75	1			1			1			1		4
Cauliflower	88		1	1							1	1	4
Celery	49	1											1
Chinese cabbage	100		1	1						1	3		6
Cocklebur	75	1						1					2
Cucumber	100										3		3
Eggplant	83			1			1				2		4
Green onion	83		1	1			1			1	2		6
Hudanna	25										1		1
Komatuna	100			1						1			3
Lettuce	100	1	2	1						1	3		7
Rape	100											1	1
Taisai	100			1			1				1		3
Tomato	100		2	1							6	1	10
Turnip	100		1	1			1						3
Spinach	17					1	2						3
Raddish	58		1	1				1			2		5
Total number of isolates		4	9	11	1	1	9	4	1	6	28	4	78

Alt., *Alternaria* spp.; Asp., *Aspergillus* spp.; Cla., *Cladosporium* spp.; Cur., *Curvularia* sp.; Cyl., *Cylindrocarpon* sp.; Glio., *Gliocladium* spp.; Fus., *Fusarium* spp.; Nig., *Nigrospora* sp.; Pae., *Paecilomyces* spp.; Pen., *Penicillium* spp.; Ste, unidentified sterile fungi; Total, total number of isolates

Method 2

Four soil samples were collected from the three fields of lettuce (Goka, Kitaooi, and Seba; Nagano) and a field of Chinese cabbage (Kiso; Nagano). In Goka, no soilborne disease has been reported even under continuous cropping for more than 35 years. In the other three fields, serious soilborne *Fusarium* disease broke out recently. For isolating soilborne fungi, all roots of axenic seedlings of cabbage (*Brassica oleracea*) cultivar "Shikitori" (Takii) were used as a bait (Narisawa et al. 1998). The isolation and identification procedures are the same as already mentioned.

In vitro screening of antagonists

All isolated fungal strains were used for in vitro screening. Dual cultures of *Fusarium oxysporum* f. sp. *conglutinans* (J.-Y. Park no. R-56, supplied by T. Arie) and a candidate of antagonists were conducted on 20% PDA plates (9 cm in diameter) at 25°C. Growth inhibition zones of *F. oxysporum* f. sp. *conglutinans* were assessed after 2-week incubation. The antagonists showing a clear inhibition zone were selected as candidates for the in vivo screening test.

In vivo screening of antagonists

The selected antagonists, all the isolates of non-pathogenic species of *Fusarium*, and unidentified hyaline or dark sterile fungi were chosen as candidates for the in vivo screening test. The seed treatments with the chosen fungal isolates were carried out using the method of Teperi et al. (1998) with slight modifications. The isolates were incubated on PDA for 1–2 weeks at room temperature, 25°C. The spores and mycelia (in nonsporulating sterile fungi, only mycelia) from the surface of one PDA plate were scraped into 25 ml sterile distilled water to make fungal suspensions that were adjusted to 10^6 conidia or hyphal segments per milliliter. After homogenization, the suspensions were used for treatment of the cabbage seeds, cultivar "Shikitori" (Takii). The seeds were soaked in the fungal suspensions at room temperature for 2 h, and then sowed in six plastic pots (four seeds per 30-ml pot) containing autoclaved (121°C, 1 h) soil (Kureha Chemical, Tokyo, Japan). Seeds treated with autoclaved distilled water were used as a control. All treated and untreated seeds with fungal isolates were cultivated at 27°C (16L:8D). Two weeks later, 5 ml 10^6 conidia/ml of *F. oxysporum* f. sp. *conglutinans* (prepared from potato dextrose broth shaken in 25°C for 48 h) were inoculated directly into the pot using a pipette. Disease assessment was conducted 11 days after the inoculation using the disease index (Matuo et al. 1980; Ohata et al. 1995) with slight modifications. Disease severity was visually categorized into six classes: 0 (no disease), 1 (slight yellowing on vein), 2 (slight yellowing and wilt on leave), 3 (moderate yellowing and wilt on about half of leaves), 4 (severe yellowing and wilt on all leaves), and 5 (complete wilt and dead leaves). Twelve plants in each treatment were recovered from three pots to assess the disease symptoms. The experiments were conducted twice.

All isolated antagonists were deposited to the Japan Collection of Microorganisms (JCM), RIKEN, and three strains of *Fusarium* to the National Institute of Agrobiological Sciences, the Ministry of Agriculture, Forestry, and Fisheries of Japan (MAFF). JCM and MAFF strain numbers are shown in Table 2.

Statistical analysis

The proportion of plant disease symptoms was analyzed using Fisher's protected least significant difference (PLSD) test with the Abacus Concept StatView procedure (Abacus Concept, Berkeley, CA, USA).

Table 2. Disease reduction rates of yellows of cabbage by fungal antagonists

Antagonists	Strain number[a]	Host	Disease reduction[b] (%)	Disease severity[c] (control)
Seedborne fungi				
Penicillium sp.	S-34	Hudanna	61*	0.88 (2.26)
Penicillium citrinum	S-59	Green onion	28*	1.63 (2.26)
Soilborne fungi				
Epicoccum nigrum	TC-33	Corn	34*	1.50 (2.26)
Fusarium solani	SS-6	Cabbage	63*	1.25 (3.42)
Fusarium solani	CM02	Cabbage	43*	2.28 (4.00)
Fusarium oxysporum	F-9501	Lettuce	48*	1.79 (3.42)

[a] *Penicillium* sp. S-34 (= JCM 11377), *P. citrinum* S-59 (= JCM 11378), *E. nigrum* TC-33 (= JCM 11380), *F. solani* SS-6 (= JCM 11383, = MAFF 238326), *F. solani* CM02 (= JCM 11384, = MAFF 238327)

[b] Disease reduction (%) = (1 − disease severity of treated plant ÷ disease severity of untreated plant) × 100

[c] Described in Materials and methods

* Values marked by an asterisk denote significant reduction of disease relative to the only pathogen-infected control treatment according to Fisher's protected least significant difference (PLSD) at $P = 0.05$

Results and discussion

Isolation of seedborne and soilborne fungi

Seventy eight strains of seedborne fungi were obtained from 20 kinds of vegetables (see Table 1). The seeds of hudanna, spinach, and celery were severely contaminated with microorganisms, so their seed germination rates were very low (25%, 17%, and 49%, respectively). Species of *Alternaria*, *Aspergillus*, *Cladosporium*, *Curvularia*, *Cylindrocarpon*, *Fusarium*, *Gliocladium*, *Nigrospora*, *Paecilomyces*, *Penicillium*, and some unidentified sterile fungi were isolated from the germinated seeds. Species of *Aspergillus*, *Cladosporium*, *Gliocladium*, and *Penicillium* were common, and species of *Penicillium* were the most prevalent among seedborne fungal isolates (36%). There are some reports on a trial for investigating the incidence of seedborne fungi (El-Nagerabi and Elshafie 2001; Sinclair 1991; Valkonen and Koponen 1990). El-Nagerabi and Elshafie (2001) investigated the incidence of seedborne fungi in *Sudanese lentil* seeds: *Aspergillus* was the most prevalent genus, followed by the *Rhizopus*, *Penicillium*, *Fusarium*, *Chaetomium*, and *Cladosporium*. Sinclair (1991) isolated the following fungal genera from surface-sterilized soybean seeds: *Acremonium*, *Alternaria*, *Aspergillus*, *Botrytis*, *Cercospora*, *Chaetomium*, *Choanephora*, *Cladosporium*, *Diplodia*, *Fusarium*, *Penicllium*, *Pestalotia*, *Pythium*, *Rhizopus*, *Sclerotinia*, and *Thielavia*. Valkonen and Koponen (1990) investigated the incidence of fungal contamination in 44 commercial seed lots of *Brassica pekinensis*.

Sixty-seven strains of soilborne fungi, i.e., species of *Acremonium*, *Actinomucor*, *Alternaria*, *Arthrobotrys*, *Choaneophora*, *Cylindrocarpon*, *Drechslera*, *Epicoccum*, *Fusarium*, *Gliocladium*, *Humicola*, *Monacrosporium*, *Mucor*, *Paecilomyces*, *Penicillium*, *Rhizoctonia*, *Rhizopus*, *Scedosporium*, and some unidentified sterile fungi, were obtained from the roots of vegetables. Species of *Fusarium* were the most prevalent among soilborne fungal isolates (27%), and as had been also shown by Teperi et al. (1998) (30%). In contrast, species of *Fusarium* were not prevalent in seedborne fungi (Table 1).

In vitro and in vivo screening of antagonists

Six isolates, including *Aspergillus ochraceus* Wilhelm (S-61), *Epicoccum nigrum* Link (TC-33), species of *Gliocladium*, and three strains belonging to the genus of *Penicillium*, showed clear inhibition zones. *Aspergillus ochraceus* and three strains of species of *Penicillium* were isolated from seeds, and *E. nigrum* and species of *Gliocladium* were isolated from soil, respectively.

In vivo screening was conducted using these selected six isolates, all isolates of the non-pathogenic species of *Fusarium* and unidentified sterile fungi. Among six selected isolates from in vitro screening, the treatments of *E. nigrum* TC-33, *Penicillium* sp. S-34, and *P. citrinum* Thom S-59 effectively reduced the disease symptoms (see Table 2). Reductions in disease incidence ranged from 28% to 61%. *E. nigrum* (formerly treated as *E. purpurascens* Ehrenberg) has been reported as a potential biological control agent for white mold of bean caused by *Sclerotinia sclerotiorum* (Libert) de Bary (Boland and Inglis 1989; Zhou and Reeleder 1991), and the species of *Penicillium* have been reported as a potential biological control agent against various plant pathogenic fungi (De Cal et al. 2000; Jackson et al. 1994). Among 29 strains of nonpathogenic species of *Fusarium*, *F. solani* (Mart.) Sacc. CM02 and SS-6 reduced 43% and 63% of the disease, respectively. *F. oxysporum* f. sp. *lactucae* Matuo & Motohashi F-9501 reduced 48% of the disease. Many nonpathogenic species of *Fusarium* have been reported as effective biological control agents against *Fusarium* diseases (Larkin et al. 1996; Larkin and Fravel 1998). The disease symptoms of the plants treated with seedborne sterile fungi S-23, S-25, S-38, and soilborne sterile fungus TT-51 were more severe compared to the control (only pathogen-infected plot).

Acknowledgments We are grateful to Dr. T. Aoki (National Institute of Agrobiological Sciences, MAFF) for identifying *Fusarium* species, and Dr. Kyung-min Kim (Kyungbuk University, Korea) for advising with the statistical analysis.

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