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FUNGAL DISEASES

First report of broccoli foot rot caused by *Rhizoctonia solani* AG-2-2 IV and pathogenicity comparison of the pathogen with related pathogens

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Abstract Foot rot and wilt symptoms were found on mature broccoli plants in Hokkaido, Japan, in 2007. The causal fungus was identified as *Rhizoctonia solani* anastomosis group (AG)-2-2 IV based on a hyphal fusion test, cultural characteristics and DNA analysis. We compared the isolate with two reference isolates, the pathogen of broccoli damping-off, belonging to AG-1 IC and AG-2-2 IIIB, in terms of differences in virulence to broccoli plants at different growth stages under various conditions. All the isolates caused damping-off on all three broccoli cultivars tested. However, only the present isolate incited foot rot with wilt symptoms on mature plants, when temperature was optimum for plant growth (20–25 °C), indicating that the foot rot pathogen may be discriminated from the damping-off pathogen in terms of virulence to mature broccoli. We also compared the isolate with 10 reference isolates from various crops, belonging to AG-2-2 IV, AG-2-2 IIIB and AG-1 IC, in terms of virulence to broccoli. All the isolates caused damping-off on broccoli seedlings with one exception; however, only the present isolate and one of four AG-2-2 IV isolates tested caused foot rot with wilt symptoms on mature broccoli in all the replicates. These results suggest that a group of isolates among AG-2-2 IV population is capable of causing foot rot on mature broccoli plants. This is the first report of broccoli foot rot caused by *R. solani* AG-2-2 IV.

Keywords AG-2-2 IV · Broccoli · Foot rot · *Rhizoctonia solani*

Broccoli (*Brassica oleracea* L. var. *italica*) is one of the most important vegetables in the world, including Japan. In the 2012 growing season, approximately 13,600 ha were planted with broccoli in Japan. Hokkaido, the northernmost island of Japan, is a major broccoli production area. Approximately 1-month-old seedlings, raised in the greenhouse, are transplanted in open fields, and the plants are harvested in about 2 months (Arai et al. 2010). In Hokkaido, broccoli is cultivated from April through October. Basal stems of the plants are sometimes covered with soil between 15 and 20 days after transplanting to prevent lodging.

Foot rot of mature broccoli plants was found in the field in Setana, Hokkaido in July 2007. *Rhizoctonia solani* Kühn was consistently isolated from damaged broccoli plants, which were characterized by constriction and soft rot on the basal stem. The only *Rhizoctonia* disease on broccoli so far reported in Japan was damping-off caused by *R. solani* anastomosis group (AG)-2-2 IIIB (Kubota and Abiko 1997) and AG-1 IC (Kubota et al. 2009). The disease occurs immediately after transplanting. Little information such as virulence of the damping-off pathogen to mature

The nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases as accessions AB911320–911323.

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plants is available on the pathogenicity of *R. solani* isolates from broccoli on broccoli plants. The objectives of this study, therefore, were to confirm that *R. solani* isolated from the damaged plants was the causal agent of the disease, to identify the isolate, and to compare the pathogenicity of the isolate with reference isolates belonging to various AGs, including the broccoli damping-off pathogens.

The wilt symptom on mature broccoli plants is an important character to determine the virulence of each pathogen. Here, we therefore defined any basal stem rot on mature broccoli with wilt symptoms as foot rot, and the representative isolate from broccoli foot rot is referred to as the BFR isolate.

Materials and methods

Disease occurrence and fungal isolation

The disease occurred in a broccoli field (ca. 1 ha; cv. Pixel) in Setana, southern Hokkaido in July 2007. We walked throughout the field and evaluated the incidence and distribution of the disease. Damaged broccoli plants were collected from the field, and stem sections (ca. 5 mm cube) were washed in running water, surface disinfested in sodium hypochlorite solution (1.0 % v/v active chlorine) for 1 min, blotted on sterilized filter paper, and then placed on 9-cm-diameter petri dishes containing potato-dextrose agar (PDA) amended with 20 mg/L streptomycin sulfate. After a 2-d incubation at 25 °C, an edge of the mycelium growing from each stem section was transferred to a petri dish containing water agar (WA) and grown for 3 days at 25 °C. Hyphal tips were then isolated from each colony, and a representative isolate was randomly selected as the BFR isolate and maintained on PDA slants at 4 °C.

Identification and sequencing of rDNA-ITS region

The number of nuclei per cell, cultural appearance, and growth temperature relations of the BFR isolate were investigated as described previously (Misawa and Kuninaga 2010, 2013). Hyphal fusion tests were made with each of seven tester isolates of *R. solani* AGs 1-5: isolates CS-Gi (AG-1 IA), RPS-9 (AG-2-1), C-96 (AG-2-2 IIIB), RI-64 (AG-2-2 IV), ST9710HK (AG-3 PT), AH-1 (AG-4 HG-I), and ST9710HN (AG-5). Hyphal interactions at the contact point were observed microscopically, and fusion frequency was determined as follows: low < 30 %, moderate $= 30$–50 %, high > 50 % (Sneh et al. 1991), and the interactions were scored as C0–C3 reactions based on the scale developed by Carling (1996).

Subgroups within AG-2-2 were identified by PCR using subgroup-specific primer pairs, i.e., P22-IIIB for AG-2-2 IIIB, P22-IV for AG-2-2 IV, and P22-LP for AG-2-2 LP (Carling et al. 2002). Fungal DNA of the BFR isolate was extracted using a DNeasy Plant Mini Kit (Qiagen, Chatsworth, CA, USA) from mycelium grown on a PDA plate for 10 days at 25 °C. The PCR amplification was performed in 25 μL of mixture containing 0.5 μL of template DNA, 200 μM of each primer, and 12.5 μL of HotStarTaq Master Mix Kit (Qiagen) in a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA). Cycling conditions for amplification consisted of 95 °C for 15 min; followed by 30 cycles of denaturation at 94 °C for 40 s, annealing at 62 °C for 1 min, and extension at 72 °C for 1 min; then a final extension for 10 min at 72 °C. PCR products were separated on 2 % agarose gels, stained with ethidium bromide, and viewed using a UV transilluminator.

Nucleotide sequences of the internal transcribed spacer region, including the 5.8S ribosomal gene (rDNA-ITS region) were analyzed for the BFR isolate and three reference isolates of AG-2-2 IV (Table 1). DNA was extracted from the four fungal isolates as described already. The rDNA-ITS region was amplified using primers ITS1 and ITS4 (White et al. 1990). The PCR reactions were performed as described, except for annealing at 55 °C for 1 min. PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, USA) were cloned in pT7Blue T-Vector (Novagen, Madison, WI, USA) and transferred into *Escherichia coli* JM109 competent cells (Takara Bio, Ohtsu, Japan) according to the manufacturer's instructions. The sequencing was performed using a BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Carlsbad, CA, USA) with the primers designed for the vector plasmid and analyzed with a 3500 Genetic Analyzer (Applied Biosystems).

The rDNA-ITS sequences of AG-2-2 IIIB isolate of C-96 and AG-1 IC isolate of BV-7 were obtained from the DDBJ/EMBL/GenBank databases. Similarity of rDNA-ITS region among the BFR isolate, three AG-2-2 IV isolates and two isolates of C-96 (AG-2-2 IIIB) and BV-7 (AG-1 IC) were calculated by the program GENETYX ver. 11(GENETYX, Tokyo, Japan).

Inoculation tests

Pathogenicity of the BFR isolate on broccoli plants was compared with that of damping-off pathogens belonging to AG-2-2 IIIB (isolate RS-B) and AG-1 IC (isolate BR1) (Table 1) in terms of growth stage of plants, temperature, and culture condition.

For pathogenicity tests, greenhouse-grown broccoli plants of three major cultivars (Pixel, Heights-SP and

Table 1 Isolates of *Rhizoctonia solani* used in this study

Isolate	AG/subgroup	Host	Portion	Source	Geographic origin	MAFF[b]
SBFl[a]	AG-2-2 IV	Broccoli	Basal stem	This study	Setana, Hokkaido, Japan	241951
SD1	AG-2-2 IV	Soybean	Hypocotyl	T. Komatsu	Kami-furano, Hokkaido, Japan	242303
JRN1	AG-2-2 IV	Japanese radish	Root	T. Misawa	Nanae, Hokkaido, Japan	242980
RI-64	AG-2-2 IV	Sugar beet	Root	S. Kuninaga	Ibaraki, Japan	(76125)[c]
RS-B	AG-2-2 IIIB	Broccoli	Hypocotyl	M. Kubota	Tsu, Mie, Japan	726525
WLS81	AG-2-2 IIIB	Welsh onion	Leaf sheath	T. Misawa	Hokuto, Hokkaido, Japan	242301
JHS2	AG-2-2 IIIB	Japanese honeywort	Hypocotyl	T. Misawa	Shiriuchi, Hokkaido, Japan	242305
C-96	AG-2-2 IIIB	Rush	Stem	S. Kuninaga	Chikugo, Fukuoka, Japan	237429
BR1	AG-1 IC	Broccoli	Hypocotyl	K. Tomioka	Mitoyo, Kagawa, Japan	726726
BV-7	AG-1 IC	Sugar beet	Root	S. Kuninaga	Hakozaki, Fukuoka, Japan	305910
RD1	AG-1 IC	Radish	Hypocotyl	T. Komatsu	Naka-furano, Hokkaido, Japan	243448

[a] Broccoli foot rot (BFR) isolate (present isolate)

[b] Ten isolates, except isolate RI-64, were deposited in Genebank, National Institute of Agrobiological Sciences

[c] Deposited in American Type Culture Collection with the Accession Number ATCC76125

Ryokurei): (1) 2-week-old seedlings with an expanded primary leaf, grown in 7-cm-diameter plastic pots; (2) 8-week-old young plants with 6–8 expanded leaves, grown in 15-cm-diameter plastic pots; (3) 3- to 3.5-month-old mature plants that were ready for harvest, grown in 1/5,000-a (15.9 cm diameter × 19.3 cm depth) plastic pots. All plants were grown in a commercial potting soil (Pottace, Katakura Chikkarin Co., Tokyo, Japan), which had been autoclaved at 121 °C for 60 min.

Wheat bran culture incubated at 25 °C for 2 weeks was used as inoculum. The three sizes of pots (7-cm-diameter, 15-cm-diameter and 1/5,000-a) planted with single broccoli plants of different sizes received additional soil, each containing 1, 10 and 15 g of inoculum on the soil surface and to 5 and 10 cm deep, respectively. Seedling, young plants, and mature plants were inoculated and incubated in greenhouses with average temperatures of 26.4°,26.3° and 21.7 °C, respectively, and plant damage was evaluated 17, 15 and 19 days after inoculation, respectively. Stem damage was categorized as follows: 0, no symptom; 1, basal rot on part of stem; 2, basal rot girdled stem superficially, but plants did not wilt; 3, basal rot deeply girdled stem, stem base was constricted, roots were sometimes detached from stem, and plants wilted (Fig. 1a). Sterilized wheat bran served as the control. Each inoculation test consisted of 5 replicate pots.

To reveal the relationship between the extent of plant damage and temperature, the BFR isolate and broccoli damping-off pathogens were used to inoculate mature broccoli plants (cv. Pixel) at various temperatures in a growth chamber controlled at 15°, 20°, 25°, 30° and 35 °C with 16-h photoperiod. Plant damage was evaluated 14–15 days after inoculation. Each inoculation test was made using 3 pots.

To investigate the effect of hilling on the extent of foot rot, we inoculated mature plants of cv. Pixel with the BFR isolate and broccoli damping-off pathogens and simulated hilling by adding the soil-inoculum mixture to 10 cm deep or the lack of hilling with soil to 1 cm deep in pots and then grew the plants in a greenhouse with an average temperature of 26.9 °C. Plant damage was evaluated 13 days after inoculation. Each inoculation treatment consisted of 3 pots.

Pathogenicity of the BFR isolate on broccoli was compared with that of 10 reference isolates (three isolates of AG-2-2 IV, four isolates of AG-2-2 IIIB and three isolates of AG-1 IC). Reference isolates were obtained from various crops such as sugar beet, soybean, carrot, Welsh onion in addition to broccoli (Table 1). The reference isolates had already been identified by PCR using the subgroup-specific primers developed by Carling et al. (2002) and Kuninaga (2003), i.e., P22-IIIB for AG-2-2 IIIB, P22IV for AG-2-2 IV and AG-1 IC-F (GAGTTGTTGCTGGCCTCTGG) and AG-1 IC-R (CCAAGTCAATGGACTATTG) for AG-1 IC. The PCR reactions for identification of AG-1 IC isolates were performed as described above, except for annealing at 58 °C for 1 min. Seedlings and mature broccoli plants of cv. Pixel were inoculated, then grown in a greenhouse with average temperatures of 26.4 °C and 27.0 °C, respectively. Plant damage was evaluated 11–14 days after inoculation. Five pots were used for each inoculation test.

Results

Disease occurrence

The field survey revealed foot rot on approximately 1 % of broccoli plants in the field just before harvest, i.e.,

Fig. 1 Broccoli foot rot caused by *Rhizoctonia solani* AG-2-2 IV. **a** Disease index from 0 to 3. The numbers within each image indicate the disease index: *0* no symptom, *1* basal rot on part of stem, *2* basal rot girdled stem superficially, but plants did not wilt, *3* basal rot deeply girdled stem, stem base was constricted, roots were sometimes detached from stem, and plants had wilted. **b** Wilted mature plants in field. **c** Foot rot with browning and constriction of basal stem. **d** Cultural morphology of isolate SBF1 after 3 weeks on PDA at 25 °C. **e** Wilt and foot rot of mature broccoli plant (cv. Pixel) 14 days after inoculation with isolate SBF1 (*right*). *Left*: uninoculated control

2 months after transplanting. Diseased plants were severely wilted. Three to seven plants adjacent to each other were diseased along the row (Fig. 1b). The stem base of diseased plants turned brown and became soft and constricted between 0 cm and 15 cm below the soil line (Fig. 1c). The basal stems in the field were covered with soil to make ridges as was the case with potato cultivation. Microscopic observations revealed the presence of *Rhizoctonia*-like hyphae in the lesion.

Identification of the isolate

Rhizoctonia-like fungi were obtained frequently from diseased stems. Isolate SBF1, the BFR isolate, was randomly selected for further detailed study. Its hyphae lacked clamp connections and were 6.1–11.1 µm (mean 7.9 µm) wide and found to have 5–13 nuclei (average 7.1 nuclei) per cell by the DAPI staining method (Martin 1987). Young hyphae branched near the distal septum of cells and formed a constriction near the point of hyphal branching. These features indicated that the isolate was *R. solani* Kühn (Ogoshi 1987).

Colonies of the isolate on PDA were white at first, then gradually turned brown. Brown zonation was formed on the surface of a 3-week-old culture. Sclerotial formation was not apparent (Fig. 1d). When incubated at 5–40 °C on PDA, the isolate grew at 10–35 °C with an optimum growth at 25 °C; growth rates at 25°, 30° and 35 °C were 18.5, 16.8 and 0.3 mm/24 h, respectively. Cultural appearance and growth temperature relations of the isolate were similar to those of culture type IV within AG-2-2 described by Watanabe and Matsuda (1966) and Ogoshi (1976).

The isolate showed the C2 reaction both with the AG-2-2 IV and AG-2-2 IIIB tester isolates with high fusion frequencies of more than 50 %, the C1 reaction with the AG-2-1 tester isolate with a frequency of less than 30 %, and the C0 reaction with tester isolates of AGs 1 and 3-5.

AG-2-2 IV subgroup-specific primer pair amplified the expected PCR product of 500 bp long from DNA of the isolate (Fig. 2). From the results of morphology, anastomosis reactions and the specific PCR, we identified the isolate as *R. solani* AG-2-2 IV.

Sequence similarities of the rDNA-ITS region between the isolate and AG-2-2 IV reference isolates ranged from 97.6 to 98.9 % (Table 2). While, AG-2-2 IIIB isolates of C-96 and AG-1 IC isolate of BV-7 had 95.5–96.3 % and 91.1–92.4 % similarity, respectively, with four AG-2-2 IV isolates in the region. Sequence analysis fortified the identification of the isolate as AG-2-2 IV.

Inoculation tests

Effect of growth stages and cultivars

The BFR isolate, as well as the reference, damping-off isolates, caused damping-off (disease index = 3) on all the seedlings and on 2–5 of 5 young broccoli plants (Table 3). Two of five mature plants inoculated exclusively with the BFR isolate showed foot rot symptoms (disease index = 3) to reproduce field symptoms (Fig. 1e). The 2 damping-off isolates showed basal stem rot (rated as disease index 1 or

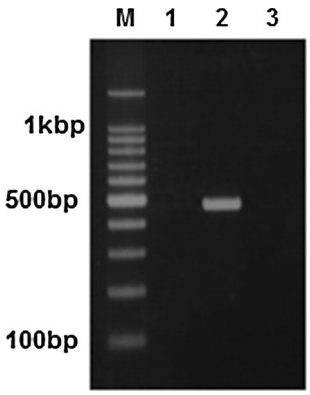

Fig. 2 Agarose gel electrophoresis of PCR product amplified from DNA of *Rhizoctonia solani* isolate SBF1 from broccoli. Amplifications were done using primer pairs designed for specific detection of *R. solani* AG-2-2 IIIB (*lane 1*), IV (*lane 2*) and LP (*lane 3*). Lane M 100-bp ladder

Table 2 Sequence similarity of rDNA-ITS regions including 5.8S gene among the broccoli foot rot (BFR) isolate and reference isolates belonging to AG-2-2 IV, AG-2-2 IIIB and AG-1 IC

AG/ subgroup	Isolate[a]	AG-2-2 IV				AG-2-2 IIIB	AG-1 IC
		SBF1	SD1	JRN1	RI-64	C-96	BV-7
AG-2-2 IV	SBF1[b]	–	–	–	–	–	–
AG-2-2 IV	SDl[b]	98.3[d]	–	–	–	–	–
AG-2-2 IV	JRN1[b]	98.9	98.9	–	–	–	–
AG-2-2 IV	RI-64[b]	97.6	98.9	98.2	–	–	–
AG-2-2 IIIB	C-96[c]	96.0	96.1	96.3	95.5	–	–
AG-1 IC	BV-7[c]	92.2	92.1	92.2	91.1	92.4	–

[a] For details, see Table 1. Isolate SBF1: broccoli foot rot (BFR) isolate (the present isolate)

[b] The rDNA-ITS sequence for isolates SBF1, SD1, JRN1 and RI-64 were deposited in the DDBJ/EMBL/GenBank databases with Accession Numbers AB911320, 911321, 911322 and 911323, respectively

[c] The rDNA-ITS sequence of isolates C-96 (AB054854) and BV-7 (FJ492101) were obtained from the DDBJ/EMBL/GenBank databases

[d] Percentage similarity was calculated for different nucleotide sites (transition, transversion, or insertion/deletion) using the Lipman and Pearson (1985) algorithm

2) on mature plants; however, they failed to cause foot rot. No varietal differences were evident in any experiment. No symptoms appeared on the control plants. The inoculated fungus was consistently reisolated from diseased stem base, but not from the control plants.

Effect of temperature

The BFR isolate reproduced foot rot symptoms on all the mature broccoli plants at between 20° and 25 °C but not at 15°, 30°, or 35 °C (Table 4). Other reference isolates failed to cause foot rot symptoms with the exception of isolate RS-B at 30° and 35 °C. No symptoms developed on the control plants.

Effect of hilling

No effect of hilling was obvious for the BFR isolate; foot rot symptoms developed on plants in pots filled with soil-inoculum mixture to 1 cm deep as well as 10 cm deep (Table 5). The two reference isolates caused basal stem rot on plants in all the pots with or without hilling.

Pathogenicity of isolates belonging to AG-2-2 IV, AG-2-2 IIIB and AG-1 IC to broccoli

All the isolates listed in Table 1 caused damping-off on broccoli seedlings with one exception (isolate RI-64 of AG-2-2 IV). The BFR isolate caused foot rot symptoms on all mature plants inoculated (Table 6). The broccoli damping-off pathogen, isolate BR1 of AG-1 IC caused foot rot symptoms on 1 of 5 inoculated mature plants. While, the other broccoli damping-off pathogen, isolate RS-B of AG-2-2 IIIB, did not incite foot rot symptoms on mature plants. Thus, the BFR isolate was more virulent on mature plants than broccoli damping-off pathogens.

Of four AG-2-2 IV isolates, only the BFR isolate and isolate SD1, causing soybean damping off, caused foot rot symptoms on mature broccoli plants in all 5 replicates. Isolate JRN1 from Japanese radish incited foot rot symptoms on 2 of 5 mature plants. Differences in virulence to mature broccoli plants were evident also among isolates belonging to the same subgroups; only isolate WLS81, Welsh onion leaf sheath blight pathogen, caused foot rot symptoms on mature broccoli plants among the isolates belonging to AG-2-2 IIIB, and in the case of AG-1 IC, all 3 isolates induced foot rot symptoms on 1 of 5 mature plants. No symptoms appeared on the control plants.

Discussion

In Japan, *R. solani* isolates belonging to different AGs are known to incite different symptoms on the same crop, e.g., radish (Nagai and Fukatsu 1971; Yonemoto et al. 2008), Japanese radish (Eimori et al. 2005; Homma and Ishii 1984; Takano and Toyota 1985) and Chinese chive (Misawa and Kuninaga 2013; Nakayama et al. 1992). For example, different plant parts of Japanese radish are attacked by many *R. solani* AGs, i.e., root rot caused by AG-1, AG-2-1, AG-2-2, AG-4, AG-5 and AG-7 (Homma and Ishii 1984), leaf blight (Takano and Toyota 1985) and damping-off (Eimori et al. 2005) by AG-2-1. However,

Table 3 Pathogenicity of the broccoli foot rot (BFR) isolate and broccoli damping-off pathogens to three broccoli cultivars at three growth stages[a]

Isolate		Seedlings[b]				Young plants[b]				Mature plants[b]			
		Disease index[c]				Disease index				Disease index			
(AG/Subgroup)	Cultivar	0	1	2	**3**	0	1	2	**3**	0	1	2	**3**
SBFl[d]	Pixel	0[f]	0	0	**5**	0	0	3	**2**	0	0	3	**2**
(AG-2-2 IV)	Heights-SP	0	0	0	**5**	0	0	0	**5**	0	0	3	**2**
	Ryokurei	0	0	0	**5**	0	0	0	**5**	0	0	3	**2**
RS-B[e]	Pixel	0	0	0	**5**	0	0	3	**2**	0	5	0	**0**
(AG-2-2 IIIB)	Heights-SP	0	0	0	**5**	0	0	3	**2**	0	5	0	**0**
	Ryokurei	0	0	0	**5**	0	0	2	**3**	0	5	0	**0**
BRI[e]	Pixel	0	0	0	**5**	0	0	2	**3**	0	4	1	**0**
(AG-1 IC)	Heights-SP	0	0	0	**5**	0	0	0	**5**	0	5	0	**0**
	Ryokurei	0	0	0	**5**	0	0	1	**4**	0	4	1	**0**
Control	Pixel	5	0	0	**0**	5	0	0	**0**	5	0	0	**0**
	Heights-SP	5	0	0	**0**	5	0	0	**0**	5	0	0	**0**
	Ryokurei	5	0	0	**0**	5	0	0	**0**	5	0	0	**0**

[a] Each inoculation test on seedlings, young plants, and mature plants was conducted at average temperatures of 26.4, 26.3 and 21.7 °C, respectively

[b] Seedlings: 2-week-old plants, Young plants: 8-week-old plants, Mature plants: 3 to 3.5-month-old plants

[c] Disease index: *0* no symptom, *1* basal rot on part of stem, *2* basal rot girdled stem superficially, but plants did not wilt, *3* basal rot deeply girdled stem, stem base was constricted, roots were sometimes detached from stem, and plants wilted (see Fig. 1a)

[d] Broccoli foot rot (BFR) isolate (present isolate)

[e] Isolates causing broccoli damping-off (detailed in Table 1)

[f] Number of plants categorized based on the disease indices

Table 4 Pathogenicity of the broccoli foot rot (BFR) isolate and broccoli damping-off pathogens on mature broccoli plants at various temperatures

Isolate	15 °C				20 °C				25 °C				30 °C				35 °C			
	Disease index[c]				Disease index				Disease index				Disease index				Disease index			
	0	1	2	**3**	0	1	2	**3**	0	1	2	**3**	0	1	2	**3**	0	1	2	**3**
SBFl[a]	0[d]	0	3	**0**	0	0	0	**3**	0	0	0	**3**	0	1	1	**1**	3	0	0	**0**
RS-B[b]	0	3	0	**0**	0	3	0	**0**	0	2	1	**0**	0	2	0	**1**	0	1	0	**2**
BR1[b]	0	3	0	**0**	0	2	1	**0**	0	3	0	**0**	0	1	2	**0**	2	1	0	**0**
Control	3	0	0	**0**	3	0	0	**0**	3	0	0	**0**	3	0	0	**0**	3	0	0	**0**

[a] Broccoli foot rot (BFR) isolate (present isolate)

[b] Isolates causing broccoli damping-off (for details, see Table 1)

[c] Disease index: *0* no symptom, *1* basal rot on part of stem, *2* basal rot girdled stem superficially, but plants did not wilt, *3* basal rot deeply girdled stem, stem base was constricted, roots were sometimes detached from stem, and plants wilted (See Fig. 1a)

[d] Number of plants categorized based on the disease indices

little information has been available on differences in virulence among the different AGs. Therefore, in this study, we compared virulence under various conditions of the broccoli foot rot pathogen with the broccoli damping-off pathogen, each belonging to different AGs.

We first confirmed that the BFR isolate, but not the damping-off isolates, causes foot rot symptoms on mature broccoli plants of various cultivars.

Second, we further investigated disease-temperature relations for these isolates using mature plants; the BFR isolate of AG-2-2 IV caused foot rot at 20 °C and 25 °C, while isolate RS-B of AG-2-2 IIIB, the damping-off pathogen, incited the symptoms at 30 °C and 35 °C. These results agree with the findings that different subgroups within AG-2-2 differ in ecological characteristics and that AG-2-2 IV prefers moderate temperatures near 25 °C,

Table 5 Pathogenicity of the broccoli foot rot (BFR) isolate and broccoli damping-off pathogens to mature broccoli plants grown in pots with or without hilling[a]

Isolate	Depth of hilling[d] (cm)	Disease index[e]			
(AG/Subgroup)		0	1	2	**3**
SBFl[b]	10 (with hilling)	0[f]	0	0	**3**
(AG-2-2 IV)	1 (without hilling)	0	0	0	**3**
RS-B[c]	10 (with hilling)	0	3	0	**0**
(AG-2-2 IIIB)	1 (without hilling)	0	3	0	**0**
BR1[c]	10 (with hilling)	0	0	3	**0**
(AG-1 IC)	1 (without hilling)	0	1	2	**0**
Control	10 (with hilling)	3	0	0	**0**
	1 (without hilling)	3	0	0	**0**

[a] Inoculation test was conducted with average temperature at 26.9 °C

[b] Broccoli foot rot (BFR) isolate (present isolate)

[c] Isolates causing broccoli damping-off (for details, see Table 1)

[d] Plants received soil-inoculum mixture to 10 cm deep (with hilling) or 1 cm deep (without hilling) in pots

[e] Disease index: *0* no symptom, *1* basal rot on part of stem, *2* basal rot girdled stem superficially, but plants did not wilt, *3* basal rot deeply girdled stem, stem base was constricted, roots were sometimes detached from stem, and plants wilted (See Fig. 1a)

[f] Number of plants categorized based on the disease indices

while AG-2-2 IIIB grows better at high temperatures near 30 °C (Watanabe and Matsuda 1966). Broccoli grows best at 15–20 °C and normally at 25 °C (Fujime 2010). However, its floral development is disrupted over 30 °C (Björkman and Pearson 1998). The optimum temperature for infection of the BFR isolate was consistent with that for plant growth, while, the optimum for the damping-off isolate differed from that for plant growth. These results indicate that only the BFR isolate causes foot rot symptoms on mature broccoli plants under field conditions.

In some broccoli fields including the field where the foot rot was found, basal stems are covered with soil to make ridges. We hypothesized that hilling might be important factors to promote the occurrence of foot rot symptoms; however, the number of plants with foot rot symptoms was not correlated with the depth of hilling, indicating that hilling to cover stem base does not promote foot rot and that this disease could occur in fields where stem bases are not covered with soil.

Inoculation experiments by Kubota and Abiko (1997) revealed that *R. solani* isolates belonging to culture types IB, II, IIIB and IV isolated from various crops, had the potential to cause damping-off on broccoli seedlings. Subsequently, AG-4 HG-I (Yamauchi et al. 2009) and AG-2-1 (Misawa et al. 2013) were reported as broccoli damping-off pathogens. In this study, 10 of 11 isolates belonging to AG-2-2 IV, AG-2-2 IIIB and AG-1 IC caused damping-off on broccoli seedlings. However, only AG-2-2 IV isolates of SBF1 and SD1 caused foot rot symptoms on mature plants in all 5 replicates, indicating that mature plants are more resistant than seedlings to *R. solani*, as was the case with tomato (McCarter 1991). The isolate WLS81 of AG-2-2 IIIB obtained from Welsh onion was as virulent as the BFR isolate in the greenhouse maintained at relatively high temperatures. These results predict the potential of AG-2-2 IIIB isolates to incite disease on mature plants in warm regions.

In the inoculation test shown in Table 6, we investigated the disease index of inoculated plant 7 and 14 days after inoculation. Of 5 plants inoculated with the BFR isolate and broccoli damping-off isolates of RS-B and BR1, 5, 0 and 1 plants had wilt symptoms, respectively, 7 days after inoculation (data not shown). The number of plants with wilt did not increase between 7 and 14 days after inoculation. Considering that mature plants are relatively resistant to the pathogen, if the inoculated plants were grown for more than 14 days, damping-off pathogens should not cause wilt on mature plants.

Watanabe and Matsuda (1966) inoculated seedlings of various crops such as sugar beet, cucumber, cabbage, tomato and eggplant with many isolates of *R. solani*

Table 6 Pathogenicity comparison of the broccoli foot rot (BFR) isolate with 10 reference isolates belonging to AG-2-2 IV, AG-2-2 IIIB and AG-1 IC obtained from various crops[a]

AG/Subgroup	Isolate[b]	Seedlings[c]				Mature plants[c]			
		Disease index[d]				Disease index			
		0	1	2	**3**	0	1	2	**3**
AG-2-2 IV	SBF1[e]	0[f]	0	0	**5**	0	0	0	**5**
	SD1	0	0	0	**5**	0	0	0	**5**
	JRN1	0	0	1	**4**	0	0	3	**2**
	RI-64	5	0	0	**0**	0	1	4	**0**
AG-2-2 IIIB	RS-B	0	0	0	**5**	0	5	0	**0**
	WLS81	0	0	0	**5**	0	0	1	**4**
	JHS2	0	0	0	**5**	0	0	5	**0**
	C-96	0	0	0	**5**	0	0	5	**0**
AG-1 IC	BR1	0	0	0	**5**	0	0	4	**1**
	BV-7	0	0	0	**5**	0	0	4	**1**
	RD1	0	0	0	**5**	0	0	4	**1**
Control		5	0	0	**0**	5	0	0	**0**

[a] Each inoculation test of seedlings and mature plants was done at average temperature of 26.4 °C and 27.0 °C, respectively

[b] Origins of all isolates are described in Table 1

[c] Seedlings: 2-week-old plants, mature plants: 3- to 3.5-month-old mature plants

[d] Disease index was categorized as follows: *0* no symptom, *1* basal rot on part of stem, *2* basal rot girdled stem superficially, but plants did not wilt, *3* basal rot deeply girdled stem, stem base was constricted, roots were sometimes detached from stem, and plants wilted (See Fig. 1a)

[e] Broccoli foot rot (BFR) isolate (present isolate)

[f] Number of plants categorized based on the disease indices

belonging to culture types IA, IB, IIIA, IIIB, and IV. They found that a root rot pathogen of sugar beet belonging to culture type IV was weakly virulent. Our inoculation tests obtained similar results that isolate RI-64 of AG-2-2 IV, root rot pathogen of sugar beet, failed to cause damping-off on broccoli seedlings.

In our inoculation tests, we tested only one isolate (the BFR isolate) as a pathogen of foot rot of broccoli. To clarify the characters of this pathogen in more detail, further research using additional broccoli foot rot isolates is required.

Our inoculation tests revealed that 2 of 4 isolates of AG-2-2 IV were strongly virulent on mature broccoli plants (Table 6). These results suggest that a group of isolates among AG-2-2 IV population is capable of causing foot rot on mature broccoli plants; however, these isolates were indistinguishable from other isolates in rDNA-ITS sequences (Table 2). AG-2-2 IV isolates are distributed throughout Japan (Ogoshi 1976; Watanabe and Matsuda 1966). Further research is required to differentiate these two groups within AG-2-2 IV in terms of foot rot on mature broccoli plants so that an outbreak of the disease may be predicted.

Kuramae et al. (2003) reported damping-off of broccoli caused by AG-4 HG-III in Brazil. Budge et al. (2009) examined AG-2-1 and AG-4 HG-II isolates from symptomless stem base of broccoli in UK to find that they were pathogenic to cauliflower seedlings in inoculation tests. To our knowledge, no previous reports are available on foot rot of mature broccoli plants caused by *R. solani* or with a detailed comparison of the virulence of *R. solani* isolates on broccoli.

In conclusion, we identified that broccoli foot rot is caused by a different pathogen than that causing broccoli damping-off; both pathogens affect different growth stages of plants in the field, and different levels of virulence can be ascribed to each pathogen on mature broccoli plants. We refer to this disease as "foot rot of broccoli (kabugusarebyo in Japanese)". Isolate SBF1 was deposited in the NIAS Genebank, National Institute of Agrobiological Sciences as MAFF 241951.

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