Detection of *Phomopsis sclerotioides* in Commercial Cucurbit Field Soil by Nested Time-Release PCR

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Abstract

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A polymerase chain reaction (PCR)-based molecular method to detect *Phomopsis sclerotioides* in soil was developed using a species-specific primer pair. To improve sensitivity of the detection, three PCR techniques were used; namely, nested PCR using the primer pair internal transcribed spacer (ITS)1 and ITS4, time-release PCR using two different DNA polymerases (recombinant Taq and AmpliTaq Gold), and fluorescent PCR to obtain fluorescent-labeled PCR products that can be analyzed by capillary electrophoresis. The latter two techniques were combined and termed nested time-release fluorescent (NTRF)-PCR. The minimum concentration of DNA required to obtain species-

specific PCR products successfully was 50 fg/ μ g. Using the NTRF-PCR method, the fungus could be detected in sandy soil that was artificially infested at a density of 10 CFU/g. The pathogen was detected in most soil samples collected from commercial cucumber fields in which visual disease symptoms had appeared, and even in samples collected from fields where visual disease symptoms had not appeared. To prevent the invasion and establishment of root-inhabiting pathogens such as *P. sclerotioides*, it is critical to detect the fungus in soil as soon as possible after its introduction into a cucumber-growing region.

Black root rot of cucurbit crops is caused by the soilborne fungus *Phomopsis sclerotioides* Kesteren (10). Infected plants lose the ability to obtain water through the root system (13), so that the stem and leaves wilt, resulting in severe loss of yields. It is unknown how *P. sclerotioides* survives in soil but it is assumed that pseudostromata and pseudomicrosclerotia play an important role as survival structures in soil.

The disease was first reported in The Netherlands and subsequently has been identified in several areas of Europe and South and East Asia (1,2). In Japan, this disease was initially found on cucumber (*Cucumis sativus* L.) grafted onto pumpkin (*Cucurbita maxima* Duch. and *C. moschata* Duch.) or figleaf gourd (*C. ficifolia* Bouche) in Saitama Prefecture, located in the central part of Honshu Island, in 1983 (7). Since then, the area of cucurbit crops infested by black root rot has increased, mainly in northern parts of Honshu Island. Because the pathogen seems to be neither native nor widespread throughout Japan at present, preventing invasion of the fungus into areas currently uninfested is of critical importance to avoid disease occurrence and yield loss. Techniques to detect and monitor the pathogen in the field are necessary to preclude the spread of the pathogen and increase in its spatial distribution. A selective medium to isolate this pathogen from soil has not been reported. Growing plants sensitive to *P. sclerotioides* in sample soil has been the only available and reliable method of detection. However, this method is time-consuming and takes more than 3 weeks; therefore, it is difficult to use for large numbers of soil samples.

Recently, Shishido et al. (17) reported a species-specific primer pair to detect *P. sclerotioides*, and successfully detected the fungus in root tissues with typical black rot symptoms and soil samples

collected from the vicinity of infected roots. However, detection of the pathogen by a conventional polymerase chain reaction (PCR) technique using the primer pair is not highly sensitive, as is demonstrated later in this article; therefore, it is desirable to develop a more sensitive and reliable technique to detect the pathogen in soil. Therefore, we conducted a study to develop a highly sensitive and reliable molecular technique to detect *P. sclerotioides* in soil collected from commercial fields, based on the primer pair reported by Shishido et al. (17).

Materials and Methods

Infestation of soil samples by *P. sclerotioides*. The isolate Ph-19 of *P. sclerotioides* used in the study was obtained in 2004 from diseased cucumber roots collected in the city of Nihonmatsu, Fukushima Prefecture, and is highly virulent to cucumber in inoculation tests. The isolate was grown for 4 to 5 weeks in 1,000-ml Erlenmeyer flasks containing 150 ml of potato dextrose broth at 25°C in the dark. A dark-colored, dense, and fairly hard mycelial mat developed during incubation, and was collected by filtration and washed with a large quantity of sterilized water. The mycelial mat was added to sterilized distilled water, and homogenized with a blender (Ace Homogenizer AM-8, Nihonseiki Kaisha, Japan) at 7,000 rpm for 1 min. Homogenization broke the mycelial mat into pieces of 60 to 450 by 100 to 780 μ m (mean 357.4 ± 26.4 by 226.2 ± 16.9 μ m), slightly larger than the pseudomicrosclerotia found in infected root tissues. Thus, the size of the mycelial mat fragments was less than 800 μ m. These small pieces of mat, including small quantities of coarse mycelial fragments, were suspended in sterilized distilled water. CFU in the suspension were counted using the dilution plate method. Potato dextrose agar (PDA) medium was used for incubation of the fragments after amendment with streptomycin sulfate at 300 mg/liter and adjustment to pH 4.0 with 20% lactic acid. The suspension (1 ml) was spread on the amended PDA plates and incubated for 2 weeks at 25°C, and the number of the developed colonies was counted. Ten PDA plates were used at each dilution level. The mean number of colonies was calculated for each dilution.

Sandy soil, two different volcanic ash soils with high or low organic contents, alluvial soil with heavy clay minerals, and a com-

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mercial potting medium, mainly composed of peat (Genki-kun Tokugo soil; Sakata Seed Co., Japan), were used. Cucumber is mostly grown in volcanic ash soil and alluvial soil in Japan. Alluvial soil was obtained from a paddy field where cucurbits have been grown as a rotational crop. Each soil sample was infested with the suspension of mycelial fragments and small stromata-like pieces of the pathogen at 3×10^3 CFU/g of dry soil and diluted to 1, 10, 10^2 , and 10^3 CFU/g.

DNA extraction from mycelia, plant tissues, and soil samples. Fungal DNA was extracted from mycelia grown in potato dextrose broth by the following method. Mycelia (50 mg) in a 2-ml tube was frozen using liquid nitrogen and powdered using a multibead shocker (model MB455AU; Yasui Kikai, Japan) at 1,500 rpm for 10 s. Powdered mycelia were mixed with 500 μ l of extraction buffer (50 mM EDTA and 0.2% sodium dodecyl sulfate, pH 7.5) and incubated for 10 min at 68°C. The crude homogenate was added to a 1/10 volume of 4 M potassium acetate (pH 5.8) and incubated for 5 min on ice. After centrifugation (15,000 rpm for 5 min), the supernatant was mixed with an equal volume of 2-propanol and centrifuged for 5 min at 25°C. The pellet was washed with 70% ethanol and dissolved in Tris-EDTA (pH 8.0) containing RNase A (1 μ g/ml). After incubation for 30 min at 37°C, DNA was purified by phenol/chloroform extraction followed by ethanol precipitation.

Total DNA was extracted from 100 mg of plant root tissue cut into 5- to 10-mm pieces. Plant roots were powdered using the method described above and DNA extraction was performed using the DNeasy Plant Mini Kit (Qiagen, USA).

Meta DNA in the soil sample was prepared as described by Ikeda et al. (8) or using the FastDNA SPIN Kit for Soil (MP Biochemicals, USA).

Time-release PCR. Time-release PCR was performed by modification of the conventional PCR technique reported by Shishido et al. (17) using Takara PCR Thermal Cycler Dice (Takara Bio Inc., Japan) to improve sensitivity. Time-release PCR was carried out in 20- μ l reaction volumes, each containing 0.1 μ l of recombinant *Taq* DNA polymerase (50 U/ml; TaKaRa, Japan) and 0.1 μ l of AmpliTaq Gold DNA polymerase (50 U/ml; Applied Biosystems, USA), 2 μ l of 10 $\times$ PCR buffer, 2 μ l of 20 mM dNTPs, 2 μ l of bovine serum albumin (10 mg/ml), and 0.5 μ l of 20 mM each primer (CPs-1 5'-GCCTCGGCGCAGGCCGGCCTCACC-3' and CPs-2 5'-GGGGCCTTCCAGAACGAAATATAATT-3'). As a control, a conventional PCR was also performed using 0.5 enzyme units in the reaction solution. The PCR conditions were one cycle of 94°C for 3 min; 30 or 50 cycles of 94°C for 30 s, 67°C for 30 s, 72°C for 20 s; and finally one cycle of 72°C for 10 min. Time-release PCR was also carried out using the CPs-1 primer (at the same concentration) labeled with the VIC fluorescent pigment (Applied Biosystems).

Amplified PCR products were electrophoresed in a 2.0% agarose gel (1 $\times$ Tris-acetate-EDTA buffer) and existence of the ex-

pected band was confirmed by staining with ethidium bromide at 0.5 μ g/ml and visualization with ultraviolet light. Fluorescent-labeled PCR products were analyzed by capillary electrophoresis using a genetic analyzer (model 3100; Applied Biosystems). The genetic analyzer provided fluorescence intensity as peak height and the peak position corresponded to the length of the DNA; both are shown as numerical data.

Nested time-release PCR. Nested PCR was performed to obtain a higher sensitivity than time-release PCR. As a single-step PCR, the internal transcribed spacer (ITS) region, including the 5.8S rDNA region, was amplified using the fungal universal primer pair ITS1 and ITS4 described by White et al. (18), because the primers CPs-1 and CPs-2 were designed within this region. PCR was carried out using AmpliTaq Gold DNA polymerase with the following conditions: one cycle of 95°C for 10 min; 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min; and finally one cycle of 72°C for 7 min. The PCR product (1 μ l) obtained by the single-step PCR was used as template for nested PCR with the above-mentioned time-release PCR technique using the fluorescent-labeled primer. Nested PCR products were also analyzed by agarose or capillary electrophoresis. Hereafter, the PCR technique using the fluorescent-labeled primer is termed fluorescent PCR.

Detection of *P. sclerotoides* in field soil. Detection of *P. sclerotoides* in field soil was attempted in two trials, designated A and B.

Soil samples for trial A were collected from 42 commercial cucumber fields in Iwate prefecture. The sampling was conducted on 19 November 2007, immediately after cucumber harvest was completed. The disease incidence in each field was evaluated first by observation of wilted plants. When wilted plants were found, production of pseudomicrosclerotia or pseudostromata on the roots of the plants was recorded. In all, 50 to 100 g of soil was collected from the uppermost 10-cm layer at five sites in each field. These were placed in one plastic bag and mixed thoroughly by hand.

Soil samples for trial B were collected from 49 locations in the melon-growing regions of the towns Wakami and Hachiryu, Akita Prefecture. Black root rot of melon (*Cucumis melo*) was first recognized in these neighboring towns in June 2008. Sampling was carried out 3 to 4 months after the first detection of the disease (i.e., 6 to 16 weeks after melons were harvested). Of the 49 samples, 40 were collected from commercial melon fields, comprising 28 from Hachiryu and 12 from Wakami. The other nine samples were collected from four asparagus fields, one Welsh onion field, one potato field, one pine nursery, and two miscellaneous vegetable fields neighboring the melon fields in Hachiryu. Sandy soil was present at every sampling location in Hachiryu and seven fields of Wakami, while volcanic ash soil with high organic content was present in the other five fields in Wakami. Soil was obtained from five different sites in a field as described above.

Approximately 20 g of each soil sample was dried at 60°C for 12 h in a constant-temperature oven (DK810; Yamato Scientific Co., Japan), passed through a 2 mm mesh sieve, and crushed with a cell disruptor (multi-beads shocker MB455AU(S), Yasui Kikai) at 2,500 rpm for 300 s.

Results

Sensitivity of fungus detection in soil by conventional PCR. A product slightly less than 400 bp in length was obtained with single-step PCR using the primer pair CPs-1 and CPs-2 and DNA extracted from mycelia of *P. sclerotoides* as the template. A PCR product with almost the same length was successfully amplified by the same primer pair using DNA extracted from soil samples containing *P. sclerotoides* by both the technique of Ikeda et al. (8) and using the FastDNA SPIN Kit for Soil. The minimum concentrations of *P. sclerotoides* in soil samples that were successfully detected were 1,000 CFU/g using the method of Ikeda et al. (8) and 100 CFU/g using the FastDNA SPIN Kit for Soil (Fig. 1). Thus, the latter technique was adopted to extract DNA from soil samples.

Amplification by single-step time-release PCR. Minimum concentrations of DNA extracted from mycelia of *P. sclerotoides*

Fig. 1. Detection of *Phomopsis sclerotoides* by a single-step polymerase chain reaction-based technique using specific primers in artificially infested sandy soil. Lane 1: sterile distilled water, lanes 2 and 8: 0 CFU/g, lanes 3 and 9: 1 CFU/g, lanes 4 and 10: 10 CFU/g, lanes 5 and 11: 100 CFU/g, lanes 6 and 12: 1,000 CFU/g, lanes 7 and 13: 10,000 CFU/g, lane 14: *P. sclerotoides* DNA. Soil DNA was extracted using the FastDNA SPIN Kit for Soil (MP Biochemicals) (lanes 2–7) and the method of Ikeda et al. (8) (lanes 8–13).

were 500 and 50 pg/μl when 30 or 50 PCR cycles of denaturation, annealing, and extension were programmed (Fig. 2). Under these PCR conditions, either rTaq or AmpliTaq Gold DNA polymerase was added to the PCR mixture, and no differences in the minimum concentration of DNA required were observed between the polymerases. On the other hand, PCR solutions containing both rTaq and AmpliTaq Gold yielded PCR products from DNA at 50 pg/μl as a minimum concentration with a PCR program of 30 cycles (Fig. 3). When 50 cycles were performed, sensitivity of the detection was further improved, indicating that a minimum DNA concentration of 50 fg/μl was required for successful PCR amplification.

Detection of *P. sclerotioide*s in soil by time-release fluorescent PCR. Detection of the fungus in artificially infested soil samples by time-release PCR with fluorescent-labeled primers CPs-1 and CPs-2 was conducted. As mentioned above, analysis of the PCR products with a genetic analyzer provided both fluorescence intensity and the peak position of the amplified PCR product. We tentatively considered that a fluorescent intensity ≥ 400 indicated detection of the fungus, values from 100 to 399 indicated detection with caution, and values less than 100 were negative. The peak position of the PCR product amplified from DNA of *P. sclerotioide*s using the genetic analyzer was 383 to 390, depending on the experimental run.

*P. sclerotioide*s was detected in sandy soil and commercial potting medium whenever these contained *P. sclerotioide*s at more than 10 CFU/g (Table 1). In addition, the fungus was detected in volcanic ash soil with low organic content, alluvial heavy clay soil,

and volcanic ash soil with high organic content containing *P. sclerotioide*s at more than 100, 100 to 1,000, and 1,000 to 3,000 CFU/g, respectively.

Detection of *P. sclerotioide*s in soil by nested time-release fluorescent PCR. The ITS region of rDNA from three soil samples (sandy soil, alluvial heavy clay soil, and commercial potting medium) with a low concentration of *P. sclerotioide*s (10 CFU/g) was amplified by nested time-release fluorescent (NTRF)-PCR (Table 2; Fig. 4). In the case of volcanic ash soil with low or high organic content, PCR products were obtained from DNA extracted from soil samples with *P. sclerotioide*s at 100 CFU/g.

Detection of *P. sclerotioide*s in commercial field soil. In trial A (Table 3), the fluorescence intensity (i.e., peak height) of PCR products of 10 soil samples from the fields where disease incidence had been observed was very high (between 3,358 and 6,096), clearly indicating that *P. sclerotioide*s was detected in the samples. The values for 31 of the 32 other soil samples where disease incidence had not been observed were less than 100, indicating that the pathogen was not detected in the samples. The sample obtained from field number 16 showed a peak height of 295, although black rot had not been observed in the field. The disease was recognized 2 years after soil was collected from the field and *P. sclerotioide*s was isolated from symptomatic cucurbit roots.

In trial B, *P. sclerotioide*s was detected in 15 of 40 soil samples collected from melon fields, and detected with caution in 9 samples. These included 17 of 28 samples collected from Hachiryu and 7 of 12 samples from Wakami. The fungus was also detected in two samples collected from asparagus and potato fields showing fluorescence intensity values of 132 and 7,106, respectively; these were next to melon fields. Fields where the pathogen was detected were widely distributed in the melon-growing area of the towns (Table 4, Fig. 5).

Discussion

*P. sclerotioide*s can be isolated from infected root tissues by a standard isolation method using agar media, such as water agar or acidic PDA plates, combined with surface sterilization with ethyl alcohol or NaOCl. We used a semiselective medium for isolation of the fungus from root tissues, such as PDA plates with streptomycin sulfate (300 mg/ml) and pentachloronitrobenzene (1,000 mg/ml; Hokkai Sankyo Co., Kitahiroshima, Japan), adjusted to pH 4.2 with 20% lactic acid. With such media, isolation from infected tissues is much easier but is not selective enough to isolate the fungus from soil. We are not aware of any previous report using a

Fig. 2. Amplification of the rDNA-specific region in *Phomopsis sclerotioide*s by polymerase chain reaction using primers CPs1 and CPs2 with either rTaq or AmpliTaq Gold DNA polymerase. Lane 1: 500 pg/μl, lane 2: 50 pg/μl, lane 3: 5 pg/μl, lane 4: 500 fg/μl, lane 5: 50 fg/μl, lane 6: 5 fg/μl, lane 7: 500 ag/μl, lane 8: 50 ag/μl, lane 9: 5 ag/μl, lane 10: sterilized distilled water.

Fig. 3. Amplification of the rDNA-specific region in *Phomopsis sclerotioide*s by time-release polymerase chain reaction using primers CPs1 and CPs2 with both rTaq or AmpliTaq Gold DNA polymerase. Lane 1: 500 pg/μl, lane 2: 50 pg/μl, lane 3: 5 pg/μl, lane 4: 500 fg/μl, lane 5: 50 fg/μl, lane 6: 5 fg/μl, lane 7: 500 ag/μl, lane 8: 50 ag/μl, lane 9: 5 ag/μl, lane 10: sterilized distilled water.

conventional technique to isolate or detect the fungus directly in soil.

Reports describing PCR-based methods to detect soilborne pathogens in soil have accumulated over the last two decades (4,5,9,11,14,16). Shishido et al. (17) reported a PCR-based method to detect *P. sclerotoides* using a species-specific primer pair and successfully detected *P. sclerotoides* in root tissues and some of the soil samples collected adjacent to infected roots. The NTRF-PCR technique developed in the present study by combining three PCR techniques (namely, time-release PCR, nested PCR, and fluorescent PCR) is more sensitive for detection of the fungus in soil

than the technique based on conventional PCR (17). Shishido et al. (17) also reported a method based on real-time PCR technique using SYBR Green I dye to quantify the DNA of *P. sclerotoides*. The method was highly sensitive, and able to detect as little as 1 fg of fungal DNA. However, this real-time PCR technique using dye is able to quantify every product amplified with the primer sets used, including products of other sizes originating from micro-organisms other than *P. sclerotoides* in soils (17). The method we have developed provides peak position data that are strictly equivalent to the base pair numbers of PCR products in each analysis. Therefore, we consider that the method developed here is more

Table 1. Detection of *Phomopsis sclerotoides* using time-release fluorescent polymerase chain reaction in artificially infested soil samples

Population (CFU/g) ^b	Soil sample ^a				
	Sandy soil	Volcanic soil (low OM)	Potting medium	Alluvial soil	Volcanic soil (high MO)
Test 1					
0	50>	50>	50>	50>	50>
1	50>	50>	50>	50>	50>
10	2,000	78	300	50>	50>
10 ²	4,000	560	3,400	50>	50>
10 ³	7,400	7,400	7,400<	780	7,400
3 × 10 ³	7,200	5,600	2,600	1,400	420
Test 2					
0	50>	50>	50>	50>	50>
1	50>	58	340	50>	50>
10	7,600	50>	200	50>	50>
10 ²	5,800	880	720	260	50>
10 ³	7,200	4,800	6,600<	260	56
3 × 10 ³	6,200	7,600	7,400	2,200	2,800

^a OM = organic material. Values indicate fluorescence intensity.

^b Population density of the soil sample in CFU per 1 g of soil sample.

Table 2. Detection of *Phomopsis sclerotoides* using nested time-release fluorescent polymerase chain reaction in artificially infested soil samples

Population density (CFU/g) ^b	Soil sample ^a				
	Sandy soil	Volcanic soil (low OM)	Potting medium	Alluvial soil	Volcanic soil (high OM)
Test 1					
0	50>	50>	50>	50>	50>
1	50>	50>	50>	50>	50>
10	6,390	50>	2,300	4,130	50>
10 ²	4,560	5,320	6,300	5,400	346
10 ³	4,590	4,680	4,810	5,050	5,840
3 × 10 ³	4,920	5,140	...	4,890	5,100
Test 2					
0	50>	50>	50>	50>	50>
1	50>	50>	50>	50>	50>
10	2,400	340	780	168	50>
10 ²	3,800	960	620	220	180
10 ³	7,200	7,200	5,000	...	800
3 × 10 ³	5,400	7,400	3,800	2,400	2,920

^a OM = organic material. Values indicate fluorescence intensity.

^b CFU per 1 g of soil sample.

Fig. 4. Detection of *Phomopsis sclerotoides* by nested time-release polymerase chain reaction using specific primers in artificially infested soils. Lanes 1–4: 0 CFU/g, lanes 5–9: 1 CFU/g, lanes 10–14: 10 CFU/g, lanes 15–19: 100 CFU/g, lanes 20–24: 1,000 CFU/g, lanes 25–29: 3,000 CFU/g, lane Ph: *P. sclerotoides* DNA. Lanes 5, 10, 15, 20, and 25: sandy soil; lanes 1, 6, 11, 16, 21, and 26: potting medium; lanes 2, 7, 12, 17, 22, and 27: volcanic ash soil with high organic content; lanes 3, 8, 13, 18, 23, and 28: volcanic ash soil with low organic content; lanes 4, 9, 14, 19, 24, and 29: alluvial heavy clay soil.

reliable than the real-time PCR technique for detection of the fungus in environmental DNA, such as that extracted from soil, plant debris, or compost. Using our technique, detection of the pathogen may be practicable in soil samples collected from fields where visual symptoms caused by the pathogen have not been observed, as suggested in trial A (Fig. 3).

We used small pieces of dark-colored, dense, hard mycelial mat obtained in pure culture as a substitute for the presumed survival structures of the pathogen (i.e., pseudomicrosclerotia and pseudostromata). The pieces were almost as large as pseudomicrosclerotia in size; therefore, quantities of DNA would be the same or similar. In addition, these pieces were pigmented like the pseudomicrosclerotia produced in cucurbit roots. It can be expected that these mycelial mat pieces were an adequate substitute but further studies are necessary to decide the lowest population density of the pathogen in soil that can be detected using this technique.

PCR-based diagnostic techniques require much less time and labor than conventional techniques, such as soil dilution methods using selective media and baiting methods using host plants. The NTRF-PCR technique is even less time- and labor-consuming than standard PCR-based diagnostic techniques, because the post-PCR analysis is automated by capillary electrophoresis in the genetic analyzer, which utilizes a fluorescent signal for detection. Capillary electrophoresis provides information on the amplicon amount and also on the nucleotide number of the amplicon. Electrophoresis can discriminate even a single nucleotide difference and exhibits much higher resolution than conventional electrophoresis as a post-

PCR analysis. Therefore, NTRF-PCR is more reliable than standard PCR-based techniques. In addition, because the post-PCR analysis is automated, large-scale analysis of samples, which is critical for ecological or epidemiological studies and extension services (16), can be accomplished.

We found that *P. sclerotioides* was widely distributed in the melon-growing region of the towns Wakami and Hachiryu, even in 2008, the year that the disease was first recognized here (Table 4). It is assumed that the pathogen was not transmitted to the region that year. This pathogen is likely to have been transported there at least several years previously and, subsequently, the population density had increased, because continuous cropping of melon for several years in the same fields is a common practice in the region. Several diseases of melon showing wilt symptoms have occurred in the area over many years, including root knot induced by *Meloidogyne hapla* Chitwood, root rot caused by *Monosporascus cannonballus* Pollack & Uecker and *Pyrenochaeta terrestris* (H.N. Hansen) Gorenz, J.C. Walker & Larson, and *Fusarium wilt* induced by *Fusarium oxysporum* f. sp. *melonis* W.C. Snyder & H.N. Hansen. Black root rot of melon might have been masked by symptoms of these diseases, so that it has been overlooked for several years. This phenomenon can happen with cucurbits other than melon, such as cucumber (2). It is critical to detect and monitor the pathogen in soil to avoid its spread to other cucurbit-growing regions before the appearance of typical symptoms on crops. The diagnostic technique developed here will be useful for this purpose.

Table 3. Detection of *Phomopsis sclerotioides* in soil samples collected from cucumber field plots in Iwate Prefecture

Field plot number	Location	Disease incidence ^a	Fluorescence intensity value	Soil pH (H ₂ O)
1	Hanamaki-shi 1	+	5,655	5.4
2	Hanamaki-shi 2	+	5,266	5.6
3	Morioka-shi 1	+	4,377	6.4
4	Sizukuishi-cho 1	+	4,504	5.7
5	Tono-shi 1	+	4,936	4.5
6	Tono-shi 2	+	6,096	5.2
7	Tono-shi 3	+	5,879	5.5
8	Tono-shi 4	+	3,392	6.4
9	Tono-shi 5	+	4,600	6.5
10	Tono-shi 6	+	3,358	5.7
11	Miyako-shi 1	—	<100	4.1
12	Miyako-shi 2	—	<100	6.6
13	Miyako-shi 3	—	<100	5.3
14	Yamada-machi 1	—	<100	5.7
15	Yamada-machi 2	—	<100	6.0
16	Yamada-machi 3	—	295	6.5
17	Yamada-machi 4	—	<100	6.2
18	Yamada-machi 5	—	<100	5.2
19	Yamada-machi 6	—	<100	5.6
20	Hachimantai-shi 1	—	<100	4.7
21	Hachimantai-shi 2	—	<100	5.4
22	Hachimantai-shi 3	—	<100	4.9
23	Hachimantai-shi 4	—	<100	5.0
24	Hachimantai-shi 5	—	<100	5.4
25	Hachimantai-shi 6	—	<100	6.1
26	Hachimantai-shi 7	—	<100	5.7
27	Hachimantai-shi 8	—	<100	5.6
28	Hachimantai-shi 9	—	<100	5.6
29	Hachimantai-shi 10	—	<100	5.4
30	Hachimantai-shi 11	—	<100	5.7
31	Ninohe-shi 1	—	<100	4.9
32	Ninohe-shi 2	—	<100	5.1
33	Morioka-shi 2	—	<100	6.4
34	Morioka-shi 3	—	<100	6.4
35	Shizukuishi-cho 2	—	<100	5.7
36	Shizukuishi-cho 3	—	<100	5.8
37	Shizukuishi-cho 4	—	<100	5.7
38	Shizukuishi-cho 5	—	<100	5.7
39	Shizukuishi-cho 6	—	<100	5.2
40	Shizukuishi-cho 7	—	<100	5.7
41	Shizukuishi-cho 8	—	<100	5.7
42	Shizukuishi-cho 9	—	<100	4.8

^a Disease was confirmed by observation of the presence of pseudostromata and microsclerotia.

Root-infecting fungi can be divided into root inhabitants and soil inhabitants based on their competitive saprophytic ability (CSA) (i.e., their competitive ability to colonize plant debris or litter in or on soil; 6). Production of conidia by *Phomopsis sclerotioides* in the field has never been reported (10) and hyphal growth is much slower than in most soil-inhabiting fungi, such as *Pythium* spp. and *Rhizoctonia solani*. In addition, *P. sclerotioides* is very sensitive to common antibiotics compared with the saprophytic *F. oxysporum* (12), and does not produce fungistatic or bacteriostatic substances on nutrient agar media such as PDA or cornmeal agar. These characteristics suggest that *P. sclerotioides* has a low CSA and, hence, is a root-inhabiting fungus (3,6).

To avoid damage caused by root-inhabiting pathogens, preventing their invasion into areas where host crop plants are grown is the first step in managing the disease. Prevention may have been successful in British Columbia, Canada. The first record of *P. sclerotioides* in North America was in 1971, in a greenhouse near Vancouver, and it has been suggested that the fungus was brought from Europe on human footwear (15). However, since then, black rot has not been reported in North America, suggesting that further transmission of the fungus may have been successfully prevented. This may indicate that containment of the pathogen in the greenhouses where the fungus was first found can be accomplished if the pathogen is identified before it spreads further within a region.

Table 4. Detection of *Phomopsis sclerotioides* in soil samples collected from melon growing region in Akita prefecture

Town, field plot number	Location	Fluorescence intensity value ^a
Hachiryu-machi		
1	Ashizaki 1	5,281
2	Ashizaki 2	—
3	Ashizaki 3	432
4	Ashizaki 4	112
5	Ashizaki 5	92
6	Demizu	159
7	Hamada 1	—
8	Hamada 2	164
9	Hamada 3	2,687
10	Hamada 4	5,152
11	Hamada 5	142
12	Hamada 6	5,025
13	Hamada 7	4,103
14	Iwaisako 1	108
15	Iwaisako 2	214
16	Iwaisako 3	5,305
17	Iwaisako 4	—
18	Iwaisako 5	213
19	Iwaisako 6	—
20	Iwaisako 7	—
21	Iwaisako 8	—
22	Kamaya	—
23	Ooyachi 1	137
24	Ooyachi 2	69
25	Ooyachi 3	67
26	Ooyachi 4	71
27	Ooyachi 5	318
28	Ooyachi 6	5,322
Wakami-machi		
29	Fukumezawa 1	20>
30	Fukumezawa 2	5,179
31	Gomyouko 1	7,106
32	Gomyouko 2	49
33	Kamayachi	86
34	Michimura	4,381
35	Noishi 1	5,095
36	Noishi 2	4,718
37	Noishi 3	95
38	Tamano-ike	—
39	Wakami 1	6,199
40	Wakami 2	5,724

^a Values at the vicinity position as; — indicates that no peak appeared in the vicinity of the position for *P. sclerotioides* DNA.

Black root rot was not reported from Japan until the 1980s (7), even though cucurbit crops such as cucumber, watermelon (*Citrus lanatus*), several varieties of melon (*Cucumis melo*, *C. melo* var. *conomon*, and *C. melo* var. *makuwa*), pumpkin (*Cucurbita moschata*), and bottle gourd (*Lagenaria siceraria* var. *hispida*) have been widely grown for hundreds of years throughout the country. Therefore, it is presumed that this fungus is not native to Japan. It seems that the disease is currently spreading northward year by year since this disease was first recorded in central Japan. Detection of the pathogen and monitoring of its spread using NTRF-PCR has been carried out in several cucumber-growing regions in Akita Prefecture. The incidence of cucumber black root rot was first recognized in two commercial plots in 2009. The pathogen had been successfully detected in soil samples collected from several plots in two regions where the disease had not been noticed (*unpublished data*). Plots where the pathogen had been detected remain rather limited in number in the region. Local agronomists and farmers have started a practice to prevent further spread of the pathogen. This mainly consists of not taking soil out of the plots on footwear, tools, or machinery or in seedling pots.

The molecular-based, highly sensitive technique we have developed and described is able to detect the pathogen's DNA rather than the viable organism in the soil. It has been demonstrated that it is effective in detecting the pathogen before typical symptoms appear. Therefore, it may not be valuable to use the technique to evaluate the implementation of control measures that would result in loss of viability of the pathogen. In addition, it is obvious that negative soil samples do not necessarily indicate the absence of the pathogen. To better interpret the data and to describe the spatial patterns of fungal distribution, further research into the relationships between sample data and population parameters is necessary.

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Fig. 5. Detection of *Phomopsis sclerotioides* by nested time-release fluorescent polymerase chain reaction using specific primers in soil samples collected from commercial melon fields in the towns of Wakami and Hachiryu, Akita Prefecture, in 2008.

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