Biological Characterization of *Marssonina coronaria* Associated with Apple Blotch Disease

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Marssonina coronaria associated with apple blotch disease causes severe premature defoliation, and is widely distributed in Korea. Thirteen isolates were collected from orchards located in Gyeongbuk Province from 2005–2007. All isolates displayed over 99.6% and 99.2% sequence similarity to each other in internal transcribed spacer regions and partial sequences of 28S rDNA, respectively. The isolates were phylogenetically closely related to Chinese isolates. Selected isolates did not differ in their pathogenicity. The optimum conditions for fungal growth were 20°C and pH 6 on peptone potato dextrose agar (PPDA). Peptone and mannose were the best nitrogen and carbon source, respectively. Fungal growth was better on PPDA than on common potato dextrose agar. This study provides valuable information for integrated disease management program and facilitates the routine culturing of *M. coronaria*.

KEYWORDS : Apple blotch, Fungal growth, *Marssonina coronaria*, Pathogenicity, Phylogeny

Apple blotch is one of the most severe apple diseases known. The disease is widely-distributed, being reported in North America, Oceania, and Asia [1-3]. This disease is caused by the fungus *Diplocarpon mali* (Y. Harada & K. Sawamura [anamorph *Marssonina coronaria* (Ellis & J. J. Davis) J.J. Davis, syn. *M. mali* (Henn.) S. Ito]) [1]. The fungus primarily infects apple leaves, and conidia formed in acervuli causes infection of the leaves and fruits during the growing season. The apothecia produced on overwintered diseased leaves are sources of the inoculum. The disease first appears as dark green circular patches on the upper surface of the mature leaves in mid-summer. As the disease progresses, the leaf spots coalesce and black pinhead-like asexual fruiting bodies (acervuli) develop on the affected surfaces. Severe infections of leaves result in premature defoliation, reducing the quantity and quality of apples produced [4, 5].

The occurrence of apple blotch in Korea was first reported on 1988 and the first disease outbreak happened in 1993 [6]. In 2006, leave defoliation reached 87.7% in an experimental field in mid-September, with nearly all leaves being infected. The disease has continued and remained serious to the present day. Most pertinent research has focused on the intensive management of this disease

through the development of a fungicidal spraying program [7]. However, thiophanate-methyl-resistant strains of *D. mali* were found in Japan in 1997 [8]. The occurrence of this disease throughout the country prompted the gathering of information on the phylogenetic distribution of *Marssonina* species. Basic studies on causal fungus and its disease often involve culturing the fungus in media and production of a large quantity of conidia for inoculation of plant tissues [3]. However, *Marssonina* sp. are particularly difficult to culture *in vitro* because it grows very slowly on commonly used potato dextrose agar (PDA) medium. Suitable cultural conditions for this fungus have been unclear. This study was conducted to determine the phylogenetic relationship of *Marssonina* isolates based on the sequences of internal transcribed spacer (ITS) region and the partial sequences of 28S rDNA, to determine their pathogenic behavior, and to facilitate the establishment of a suitable culturing method by determining suitable nutrients sources, optimum temperature, and pH for growth.

Materials and Methods

Collection of isolates. Infected apple leaves were collected from three different regions of Gyeongbuk Province from

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2005~2007: three orchards in Cheongsong in 2005, three orchards in Yeongju in 2006, two orchards in Cheongsong in 2007, and five orchards of Gunwi in 2007. The fungi were isolated separately from collected leaves. Infected lesions of leaves including acervuli were cut into small pieces (3~5 mm) and surface sterilized in 1% (v/v) sodium hypochloride for 2 min followed by three rinses with sterilized distilled water (SDW). The pieces were then placed on water agar (WA) and incubated at 25°C in darkness for 2 wk. Conidia produced on the pieces were dislodged in SDW and 1 mL of the suspension was spread on WA. After incubation at 25°C for 18 hr, a germinating single spore was transferred to PDA including peptone (PPDA; 20 g peptone, 200 g potato, 20 g dextrose, 15 g agar and 1.0 L of distilled water [DW], pH 6.0). Single-spore isolates were kept at 25°C for further use. The isolates from Cheongsong were coded as CS01, 02, and 03 (2005) and CS06 and 08 (2007). Isolates from Yeongju were coded as YJ01, 02, and 04. Isolates from Gunwi were coded as GW01, 04, 07, 08, and 10.

DNA extraction. Genomic DNA was extracted from a fungal colony as described previously [9]. Fresh mycelia were collected from 30-day-old cultures grown on PPDA and ground with a sterile mortar and pestle in 1.5 mL microtubes containing 500 µL of lysis buffer (400 mM Tris-HCl [pH 8.0], 60 mM EDTA [pH 8.0], 150 mM NaCl, 1% sodium dodecyl sulfate). The tubes were left at room temperature for 10 min. After adding 150 µL of potassium acetate (11.5 mL of glacial acetic acid, and 28.5 mL of DW), the tube was shaken using vortex briefly and spun at $>10,000 \times g$ for 1 min. The supernatant was transferred to another tube and an equal volume of 2-propanol was added. After mixing the tube by inversion, the tube was spun at $>10,000 \times g$ for 2 min and the supernatant was discarded. The resultant DNA pellet was washed in 300 µL of 70% ethanol. After the pellet was spun at 10,000 rpm for 1 min, the supernatant was discarded. The DNA pellet was dried by using evaporator for 5~10 min and dissolved in 50 µL of 1× Tris-EDTA.

PCR amplification, sequencing and phylogenetic analyses. The ITS ribosomal DNA regions and the partial of 28S rDNA were amplified by PCR using the universal primer pairs, ITS1 (5'-TCC GTA GGT GAA CCT GCG-3')/ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') [10] and NL1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3')/NL4 (5'-GGT CCG TGT TTC AAG ACG G-3') [11]. Amplification reactions were performed in a total volume of 20 µL containing 2 µL of 10× PCR buffer, 0.4 µL of dNTP, 2 µL of each primer, 0.2 µL of Taq polymerase, 2 µL of genomic DNA, and 13.4 µL of SDW. PCR was performed using a thermal cycle 9700 (Applied Biosystems, Foster City, CA, USA) with the following

program: an initial denaturation stage of 5 min at 94°C, followed by 35 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 50°C, extension for 2 min at 72°C, and a final 10 min extension at 72°C for ITS gene. For the partial of 28S rDNA amplification, PCR conditions were an initial denaturation stage of 2 min at 94°C, followed by 35 cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at 52°C, extension for 1 min at 72°C, and a final 10 min extension at 72°C. Amplified products were analyzed by gel electrophoreses on a 1% agarose gel with 100 bp Plus DNA ladder as a marker.

The amplified PCR products were then purified and sequenced ABI 3730 XL DNA sequencer (SolGent, Daejeon, Korea) using the same primers. Sequences were edited by DNASTAR genetic information processing software (Madison, WI, USA) and aligned with reference sequences of related taxa obtained from the NCBI database. Phylogenetic analyses based on ITS regions and the partial of 28S rDNA sequences were constructed by neighbor-joining method using Tree View (Win32 ver. 1.6.1). Bootstrap values were generated with 100 replicates to examine the reliability of the interior branches and the validity of the trees obtained. *Diplocarpon mespili* and *M. rosae* were as the outgroups.

Pathogenicity tests. The inoculums were prepared by using 50 mL of 30-day-old fungal cultures in potato dextrose broth (PDB). Conidia were collected by centrifugation at 3,000 rpm for 5 min and washed the pellets twice with 40 mL of DW. The final pellets were ground in sterilized mortar and pestle with DW and adjusted concentration to 10^5 conidia/mL. Fourteen leaves of potted apple seedlings cultivar (cv.) “Fuji” (4-year-old) were sprayed with 100 mL aliquots of conidial suspension on upper surface of each leaf. The inoculated plants were kept in a greenhouse under controlled condition (100% relative humidity, 20°C) for 3 days prior to transferring them in the field (24~28°C). The experiment was conducted on June when environmental conditions were the most favorable for pathogen infection and natural incidence was prevalent.

Suitable nutrient sources, optimum temperature, and optimum pH. In the study of suitable nutrient for fungal growth, the experiments were conducted on basal medium, Czapek-Dox agar medium (0.05 g MgSO₄, 0.46 g KH₂PO₄, 1.0 g K₂HPO₄, 120 µg thiamine-HCl, 20 g agar, and 1 L of DW) amended with one of seven carbon sources (glucose, sucrose, maltose, mannose, glycerin, dextrin, and galactose), and seven nitrogen sources [bacto peptone, yeast extract, tryptone, malt extract, (NH₄)₂SO₄, Ca(NO₃)₂, and NaNO₃]. Each carbon and nitrogen source was added to the basal medium separately at the concentration of 0.1 M. For evaluation of vitamin and minerals, malt-extract-glucose agar broth was cooperated with one of five vitamin sources

(pyridoxine, biotin, thiamine, riboflavin, and folic acid) and four mineral sources (FeSO_4 , FeCl_3 , nicotinic acid, and MgSO_4). The final concentrations of vitamins and mineral salts were 0.5 mg/L and 0.2 mg/L, respectively. The diameters of colonies cultured on these media were measured on 35 days after incubation at 25°C. The fungal cultures on PPDA were incubated for 45 days at -4, 0, 10, 15, 20, 25, 30, and 40°C. The PPDA media were adjusted to the range of pH 3~9 with 1.0 N HCl or NaOH, and incubated for 45 days at 25°C.

Effect of media on fungal growth. The fungus was first cultured on PPDA for 30 days in darkness at 20°C. Then, a small amount of fungal colony was scraped and ground in 1.5 mL microtube containing 1 mL of SDW with a sterile plastic pestle. The suspension was adjusted to 10^6 ~ 10^7 conidia. One hundred microliters of fungal suspension was put into 50 mL of PDB while 100 μL was also spread on PPDA for comparison. After incubation at 20°C for 30 days, the culture on PDB and the suspended colony from PPDA in DW were filtered and dehydrated at 80°C for 5~6 hr. Dry weights of fungi grown on these media were determined.

Results and Discussion

Morphological characteristic of isolates. All 13 isolates had almost the same morphological and cultural characteristics. The colonies of all isolates were dark brown to black without aerial mycelia, formed a wrinkled surface, and ranged from 5~7 mm in diameter on PPDA after incubation for 30 days in darkness at 20°C (Fig. 1A). Conidia were hyaline, straight to slightly curved, obovoid, unequally two-celled and ranged from $12\sim 20 \times 4\sim 6 \mu\text{m}$ in size (Fig. 1B). The size and shape of conidia from all

Fig. 1. Morphological characteristics of *Marssonina coronaria* isolated from apple leaves. A, Dark brown to black wrinkled surfaced colony without aerial mycelia after 30 days on peptone potato dextrose agar; B, Hyaline, ampule-shaped conidia with two cells, constricted at the septum.

Fig. 2. Phylogenetic relationship of *Marssonina coronaria* isolates based on internal transcribed spacer regions. The tree was constructed by neighbor-joining analysis. Numbers on the branches are confidence values obtained from 100 replicated (only values above 80% are shown). The bar represents a phylogenetic distance of 1%. EF, AY, FJ, EU, GQ numbers are DNA sequence accession number from GenBank. *Diplocarpon mespili* and *M. rosae* were used as the outgroups.

isolates were consistent with previous descriptions of *M. coronaria* [1, 2]. Little difference in the appearance of colonies (color and size) and conidial characters were observed among isolates collected from three locales of Gyeongbuk Province from 2005~2009.

ITS and partial 28S rDNA sequence analyses. The ITS regions amplified by ITS1/ITS4 primers ranged from

Fig. 3. Symptoms of *Marssonina* blotch appeared on inoculated leaf by CS1 isolate. Initial yellowish patch after 21 days (A) and dark green spots surrounded by chlorotic area after 45 days of inoculation (B).

572~600 bp in the isolates. High homologies among the sequences of isolates were found (99.6~100%). Among them, 100% sequence similarity was evident for six of the 13 isolates (GW01, GW04, GW10, YJ01, YJ02, and YJ04). Similarly, 100% sequence similarity resulted from three of the Cheongsong isolates (CS01, CS02, and CS03), two of the Cheongsong isolates (CS06 and CS08) and two of the Gunwi isolates (GW07 and GW08). The sequences of representative isolates CS01, CS06, GW01, and GW07 were deposited in the DNA database of Japan (DDBJ) under accession numbers were AB494960, AB494961, AB494962, and AB494963, respectively. Phylogenetic relationship based on ITS sequences indicated that all Korean isolates were closely related to Chinese isolates (EU329732, FJ606800, and EU329735) obtained from NCBI GenBank (Fig. 2).

Similarly, the homologies of the partial sequences of 28S rDNA of all isolates were also compared after sequencing with NL1/NL4 primers. The sequences ranged in length from 569~600 bp and homologies among them were 99.2~100%. In case of the partial sequences of 28S rDNA, nine isolates displayed 100% sequence similarity (CS01, CS02, CS03, GW01, GW04, GW10, YJ01, YJ02, and CS06), CS08 and YJ04 remained as one, and GW07 was the same as GW08. The sequences of CS01 and GW07 were deposited at DDBJ under accession numbers AB494964 and AB494965. The Korean isolates and Chinese isolates were highly similar based on their partial sequences of 28S rDNA. Among the Korean isolates, the partial 28S rDNA sequences of two isolates (GW07 and GW08) displayed high similarity with Chinese isolates FJ618560 and FJ618562.

In this study, all isolates showed high similarities, with > 99.6% of their ITS sequences and > 99.2% of their partial sequences of 28S rDNA. All 13 isolates possessing high similarity were obtained from the same geographical area around Gyeongbuk Province and the same apple species, cv. Fuji. However, four isolates (CS06, CS08, GW7, and

GW08) displayed little variation in sequences of the ITS regions of rDNA. White *et al.* [10] reported that ITS sequences are genetically constant or show little variation within species, but vary between species in a genus. Therefore, little genetic diversity among isolates of the apple blotch fungus was indicated by the combined results of ITS and the partial sequences of 28S rDNA. Analyses of only the ITS gene sequences were more useful than the combined sequences of ITS and 28S rDNA to clarify genetic relationships among *M. coronaria* isolates.

Pathogenicity tests. Tiny yellow spots appeared on upper surface of leaves after 21 days of inoculation (Fig. 3A). Typical *Marssonina* blotch symptoms as natural occurrence in field were observed after 40~45 days of inoculation (Fig. 3B). The tiny yellow spots enlarged to form grayish brown circular lesions (5~10 mm diameter) that were tinged purple at the periphery. Small, black pinhead-like asexual fruiting bodies (acervuli) developed on the lesions. Re-isolation of the identical fungus from the inoculated leaves completed Koch's postulates. The pathogenicities of isolates were not visually different in their symptoms developed on inoculated leaves until 5~6 wk after inoculation.

Successful fungal infection depended on moist incubation under controlled condition (100% relative humidity, 20°C) prior to transfer outside. An incubation period of 3 days incubation resulted in the highest disease severity (72.5%), with much diminished severities of 20.3% obtained on 2-day moist incubated plants and 0% on 1-day moist incubated plants. This result suggests that this fungus needs an extended moist period to produce a successful infection. This partially explains why this disease usually starts after rain and occurs mostly in June and July. This knowledge will be valuable in disease control programs.

Suitable nutrient sources, optimum temperature, and optimum pH. Several carbon, nitrogen, vitamin, and mineral nutrient sources were tested. The results were

Table 1. Effect of different types of four nutrients sources on mycelial growth (mm)/dry mycelial weight (g/L) of *Marssonina coronaria* after 35 days of incubation

Mycelial growth (mm) ^a				Dry mycelial weight (g/L) ^a			
Carbon ^b		Nitrogen ^b		Vitamin ^c		Mineral salt ^c	
Glucose	4.8 c	Bacto peptone	18.5 a	Pyridoxine	5.9 a	FeSO ₄	2.4 a
Sucrose	3.9 cd	Yeast extract	15.6 ab	Biotin	5.8 a	FeCl ₃	2.2 a
Maltose	3.5 d	Tryptone	13.2 b	Thiamine	4.9 ab	Nicotinic acid	1.9 ab
Mannose	9.0 a	Malt extract	13.1 b	Riboflavin	4.6 b	MgSO ₄	1.7 b
Glycerin	4.9 c	(NH ₄) ₂ SO ₄	2.6 c	Folic acid	1.2 c		
Dextrin	6.5 b	Ca(NO ₃) ₂	2.5 c				
Galactose	0.5 e	NaNO ₃	1.2 c				

^aMeans in a column followed by a different letter differ significantly at $p = 0.05$ by Duncan multiple ranged test.

^bEach carbon and nitrogen source was supplemented in the Czapek-Dox agar medium (0.05 g MgSO₄, 0.46 g KH₂PO₄, 1.0 g K₂HPO₄, 120 µg thiamine-HCL, 5 g peptone, 20 g agar, and 1,000 mL distilled water) at the concentration of 0.1 M.

^cMalt extract-glucose broth (pH 6.0) was used to determine vitamin and mineral sources at final concentration of 0.5 mg/L and 0.2 mg/L, respectively.

shown in Table 1. The highest mycelial growth was observed on mannose and bacto-peptone amended media with colonies 9.0 and 18.5 mm in diameter resulting using the carbon and nitrogen sources. Pyridoxine and biotin were the best vitamins, and FeSO₄ and FeCl₃ were the best mineral salt sources for mycelial growth, producing the highest dry mycelial weight. The optimum temperature for mycelial growth was 20°C among the eight different temperatures and the optimum pH was 6 within the pH range of 4~9 (data not shown). The optimum temperature for growth of *M. coronaria* varied from 18~22°C according to their geographical origin [1, 3, 4, 8].

Effective media for fungal growth. *M. coronaria* grew slowly on PDA media and took 1 mon to produce a small colony about 3 mm in diameter in our preliminary study. In this study, quite rapid fungal growth was obtained on PPDA and resulted in the same colony diameter within 3 wk, and a colony diameter of 5~7 mm after 30 days. Based on this result, the study was extended by culturing of fungus grown on PPDA and transferring those to PDB at 20°C for 30 days with constant shaking (120 rpm) and using PPDA for comparison. The fungus grown on PPDA to PDB resulted in 0.23 g/mL, while those on PPDA to PDA resulted in 0.12 g/mL. Therefore, broth culturing (PDB) promoted the growth of fungus rather than continuous growth on PPDA. On the other hand, potato and carrot sucrose broth (PCSB) has been recommended for mycelial growth and conidial production of *D. mali* [12]. Broth culturing experiments were further conducted by using PDB, peptone and potato dextrose broth (PPDB), and PCSB. PDB and PCSB resulted in better growth of the fungus than PPDB (data not shown). The peptone efficacy for enhancing fungal growth might be reduced, while it is incorporated in broth culture.

Generally, growth of the fungi could be enhanced by using special media prepared by parts of their host.

However, *M. coronaria* did produce colonies only 5~7 mm in diameter on apple leaf agar after 3 mon incubation at 20~22°C [3]. Some authors grew *M. rosae* on PDA media for 3 mon to complete their genetic study [13]. Thus, the fungus culturing on PPDA to PDB provided as an efficient method to overcome time consuming. Presently, supplementation of bacto-peptone as the nitrogen source could be used to enhance the growth of this fungus in PDA. Peptone-supplementation might have stimulated further growth to some extent, although the fungi generally do not exhibit good or normal growth on the media that are commonly used to culture bacteria [14].

This study provides valuable information concerning facilitating the growth of *M. coronaria*. More information is needed to understand biology and genetics of the fungus.

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