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Digital microscopic analysis of conidiogenesis of powdery mildew pathogens isolated from melon leaves

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Abstract Melons (*Cucumis melo* L.) grown hydroponically in a greenhouse were heavily infested with powdery mildew. We isolated powdery mildew pathogens from the melon leaves and identified the isolate as *Podosphaera xanthii* KMP-6N, based on morphological characteristics and sequences of ribosomal DNA internal transcribed spacer (rDNA-ITS) regions. Host ranges of KMP-6N were determined by estimating the infectivity or pathogenicity after inoculating the conidia onto multiple plant species. The fungi caused severe powdery mildew symptoms on Cucurbitaceae plants, producing scattered conidia on conidiophores. The goal of this study was to observe KMP-6N conidiogenesis on melon leaves. The pathogen formed completely catenated conidiophores approximately 24 h from conidiophore erection to release of mature conidia. Six conidia were produced on the conidiophores and only the conidia at the apex reached maturity. The cycles of conidial release were repeated on melon leaves 14 to 18 times, at approximately 6-h intervals. In the final stage, conidia were released without causing growth and septation of generative cells. Conidiophores produced an average of 36 conidia during a 90-h period. In our study, the modes of conidiogenesis, lifetime of conidiophores and productivity of conidia on a conidiophore were described for powdery mildew fungi.

Keywords Conidiophores · Electrostatic insulator probe · Host ranges · rDNA-ITS sequence · Phylogenic tree · *Podosphaera xanthii*

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Introduction

A heavy occurrence of powdery mildew has been observed on leaves of melons (*Cucumis melo* L.) grown hydroponically in a greenhouse. Cucurbit powdery mildews, caused by multiple fungi including *Oidium* sp. of polygoni-type (*Oidium* subgenus *Pseudoidium*; Sato *et al*. 1996), *Sphaerotheca fusca* (=*Podosphaera xanthii*; anamorph: *Oidium* subgenus *Fibroidium*; Braun *et al*. 2001) and *Golovinomyces orontii* (=*Erysiphe cucurbitacearum*; anamorph: *Oidium* subgenus *Reticuloidium*; Uchida *et al*. 2009), have been reported in Japan. Powdery mildew caused by the *Sphaerotheca* (=*Podosphaera*) genus on melon plants (Reifschneider *et al*. 1985; Hosoya *et al*. 1999; Tomason & Gibson 2006), which is known to significantly reduce melon productivity, has been investigated extensively.

Physical (Munger 1979), chemical (Matheron & Porchas 2007) and biological (Krístková *et al*. 2002; McCreight 2003; Romero *et al*. 2004, 2007) methods

have been tested to control powdery mildew fungi on melons. In response, the fungi have become resistant to commercial fungicides applied to cucurbit plants (Huggenberger *et al*. 1984; Lebeda & Sedláková 2008; McGrath & Shishkoff 2001, 2003). As a result, new approaches to effectively control cucurbit powdery mildew are urgently needed. To this end, we observed the morphological characteristics of powdery mildew fungi isolated from infected melon plants, as the fungi infecting greenhouse-grown melon plants had not been fully characterized. In particular, we studied the fungal conidiophores, given the abundance of conidia and their broad, windblown distribution in the environment. We focused on the process of conidiogenesis.

In our laboratory, we carefully observed conidiophores of powdery mildew pathogens previously isolated in the field. Powdery mildew fungi have been observed on tomatoes (*Solanum lycopersicum* L.; Kashimoto *et al*. 2003; Seifi *et al*. 2012; Nonomura *et al*. 2014), red clover (*Trifolium pretense* L.; Takikawa *et al*. 2011), barley (*Hordeum vulgare* L.; Moriura *et al*. 2006a) and Japanese mallotus (*Mallotus japonicus* [L.f.] Muell.-Arg.; Nonomura *et al*. 2013). We were previously successful in describing conidiogenesis of tomato powdery mildew *O. neolycopersici* (non-catenated conidiophores, but pseudochain formation under no or low wind; Oichi *et al*. 2004, 2006) and barley powdery mildew *Blumeria graminis* f. sp. *hordei* race 1 (catenated conidiophores; Moriura *et al*. 2006a, 2006b), using a high-fidelity digital microscope. The digital microscope enabled us to observe powdery mildew fungi on plant leaves without fixation or staining.

We have been interested in determining when and how mature conidia are produced on conidiophores of powdery mildew pathogens found on melons. A dielectrically polarized insulator (ebonite) probe was used to collect the mature conidia as they were released from the conidiophores. The conidia were subsequently viewed with a digital microscope (Moriura *et al*. 2006b; Nonomura *et al*. 2009). This method enables collecting viable conidia on conidiophores, without harming the conidia (Nonomura *et al*. 2009).

In the present study, we first isolated a single conidium from melon leaves displaying powdery mildew symptoms and then multiplied the fungal mycelia on powdery mildew-free melon plants. Subsequently, we identified the isolate on the basis of morphological characteristics (Reifschneider *et al*. 1985; Braun 1987; Cosme *et al*. 2012) and sequence of the ribosomal DNA internal transcribed spacer (rDNA-ITS) region amplified by polymerase chain reaction (PCR) (Chen *et al*. 2008; Hirata *et al*. 2000). We attempted to observe, in detail, conidiophore formation of the powdery mildew isolate under the digital microscope, to better understand the mode of conidiation, longevity of the conidiophores and productivity of conidia per conidiophore. This is the first report of conidiogenesis of the *Sphaerotheca* genus. Deciphering the developmental process of conidiogenesis will facilitate taxonomical classification of the fungi causing powdery mildew.

Materials and methods

Plant materials Seeds of melon (cv. Earl's Favourite) were supplied by the Yuasa Experimental Farm of Kinki University (Wakayama, Japan). The seeds were placed on wet filter paper in petri dishes and germinated for 3-4 d in a growth chamber, under continuous illumination (4,000 lux) at 25 ± 2°C. Cotyledonal seedlings were placed in sponge cubes (3 × 3 × 3 cm^3). The sponge cubes and cotyledonal seedlings are inserted into a 30-ml cylindrical plastic case (diameter 3 cm, length 5 cm) containing fertilizer (see Fig. 4A) and incubated for 14 days in a controlled environment room at 25 ± 1°C, 60-70% relative humidity (RH) and continuous illumination of 4,000 lux.

Fungal materials Mature conidia were collected from conidiophores on powdery mildew-infected melon leaves using an electrostatic spore collector (Nonomura *et al*. 2009). The conidia were transferred onto leaves of 14-day-old healthy melon seedlings, with the aid of a high-fidelity digital microscope KH-2700 (Hirox, Tokyo, Japan; Matsuda *et al*. 2005). New conidia were re-isolated from the conidiophores (Kashimoto *et al*. 2003). Isolation was repeated three times. The melon powdery mildew isolate was subsequently designated KMP-6N. Mature conidia of KMP-6N were inoculated onto leaves of 14-day-old healthy melon seedlings, as described above. The isolate was maintained by incubating for 14 days in a growth chamber at 25 ± 1°C, 70-80% RH and continuous illumination of 4,000 lux.

Morphological observation of KMP-6N using microscopes KMP-6N conidia were transferred to glass slides and melon leaves to examine morphological characteristics of the isolate. The conidia were observed

under a light microscope BX-60 (Olympus, Tokyo, Japan) and a digital microscope. Experiments were conducted using 100 mature conidia.

Leaves of potted melon plants were inoculated with KMP-6N conidia in September 2013 and the inoculated plants were placed outside the greenhouse. Three months later, the leaves forming chasmothecia were collected for microscopic observation. Chasmothecia were gently scraped from the leaves with a glass needle, transferred to a glass slide and pressed with a cover glass to push asci and ascospores out of the chasmothecia. The chasmothecia on melon leaves and the broken chasmothecia were observed under a stereomicroscope SZ-PT (Olympus, Tokyo, Japan) and a light microscope BX-60, respectively.

rDNA-ITS sequence and phylogenic analysis To sequence the rDNA-ITS of KMP-6N, conidia were collected from KMP-6N-infected leaves. Chromosomal DNA was extracted from the conidia (Chen *et al*. 2008). PCR was performed in a PCR Thermal Cycler Dice TP600 (Takara Bio Inc., Shiga, Japan) using S1 (5'-GGA TCA TTA CTG AGC GCG AGG CCC CG-3') and S2 (5'-CGC CGC CCT GGC GCG AGA TAC A-3') fungal-specific primers designed on the basis of the ITS sequence of rDNA obtained from GenBank at the National Centre for Biotechnology Information (Hirata *et al*. 2000). PCR was performed according to the protocol described by Chen *et al*. (2008), involving denaturation at 94°C and 30 cycles for 5 min, 40 sec of denaturation at 94°C, 1 min of annealing at 62°C and 1.5 min of extension at 72°C, followed by a final extension cycle of 5 min at 72°C. The nucleotide sequence of the amplified region was determined using the BigDye Terminator Cycle Sequencing Ready Reaction Kit from Applied Biosystems (Tokyo, Japan) on an ABI Prism 3130 Genetic Analyzer (Applied Biosystems) in the Pharmaceutical Research and Technology Institute, Kinki University, Japan. For phylogenetic analysis, the rDNA-ITS sequences of different powdery mildew isolates (accession numbers, EU367960.1 *P. xanthii*, EU294368.1 *P. fuliginea*, AB040336.1 *P. fusca*, AB040309.1 *P. fusca*, AB040306.1 *P. euphorbiae*, AF011319.1 *P. fusca*, EU327327.1 *P. xanthii*, AB040347.1 *Podosphaera* sp., AY450961.1 *P. xanthii*, D84387.1 *P. xanthii*, EU424056.1 *P. fusca*, AB040316.1 *P. xanthii*, EF442023.1 *P. fusca*, AB046989.1 *P. fusca*, FJ625796.1 *P. balsaminae*, EF010913.1 *P. xanthii*, EF137856.1 *P. fusca* and HQ316143 *P. xanthii*), including our KMP-6N isolate, were obtained from the DNA Data Bank of Japan (DDBJ) database and aligned using neighbor-joining analysis with the ClustalW software (version 2.1). The Dendroscope 3 software was used to create distance matrices and to infer dendrograms (Huson *et al*. 2007; Huson & Scornavacca 2012), with the sequence (EF010914) of *G. cichoracearum* as the reference species.

Host range tests Eleven plant families, containing 25 species (totally 42 cultivars), were inoculated with KMP-6N conidia to determine host range of the isolate (Table 1). Seeds of all test plants were purchased from the originators or were obtained from our university seed collection. Seeds were placed on wet filter paper in petri dishes and germinated for 3-4 days in a growth chamber under continuous illumination (4,000 lux) at 25 ± 2°C. Cotyledonal seedlings were placed in sponge cubes (3 × 3 × 3 cm^3) and grown for 14-21 days in a growth chamber under the conditions stated above. Each inoculation involved three test plants. We obtained one or two leaves from large-leaf plants, such as Amaranthaceae, Apiaceae, Asteraceae, Brassicaceae, Cucurbitaceae, Lamiaceae, Polygonaceae and Solanaceae, four to six true leaves from small-leaf plants such as Fabaceae and Pedaliaceae, and primary leaves from Gramineae plants and three melon plants (14-day-old seedlings of Earl's Favourite) as controls. The leaves were placed in a cubic frame covered with electrostatic spore precipitators (Matsuda *et al*. 2006). Conidia were sprayed onto the leaves (Nonomura *et al*. 2009). Successful inoculation was confirmed by observing pustule expansion on leaf surfaces of the control plants. Fungal development on the inoculated leaves was observed 14 days after inoculation.

To observe cytological responses caused by invasion of KMP-6N into the inoculated leaves, samples were prepared as follows. Leaf segments (approximately 1 × 1 cm^2 in size) were cut from KMP-6N-inoculated plants. The leaf segments were fixed and color was removed by boiling in an alcoholic lactophenol solution for 1-2 min, and stained with aniline blue (Nacalai Tesque, Tokyo, Japan; Sameshima *et al*. 2004). The samples were observed under a light and fluorescent microscope BX-60 (Olympus, Tokyo, Japan) with B excitation, a B absorption filter and an O-515 barrier filter.

Developmental process of conidiogenesis Mature KMP-6N conidia were collected from conidiophores

Table 1 Host range tests for the melon powdery mildew isolate KMP-6N

Family, genus and species	Common names	Cultivars	Producers [a]	Cytological responses [b]	Pathogenicity [c]
Amaranthaceae					
Gomphrena globosa	Globe amaranth	Sennichiko	TA	-	R
Apiaceae					
Daucus carota subsp. sativus	Carrot	Kinkouyonsun	SA	-	R
Asteraceae					
Arctium lappa	Edible burdock	Salada musume	TA	-	R
Glebionis coronaria	Edible chrysanthemum	Kikujirou	TA	HR	R
Helianthus annuus	Sunflower	Sunrich lemon 50	TA	-	R
Lactuca sativa	Lettuce	Summer green	SA	-	R
Brassicaceae					
Brassica oleracea var. capitata	Cabbage	Shikimaki cabbage	SA	-	R
Brassica oleracea var. italica	Broccoli	Ryokurei	SA	-	R
Cucurbitaceae					
Benincasa cerifera	Winter melon	Himetougan	TA	-	S
Citrullus lanatus	Watermelon	Kabuki	TA	-	S
		Kansen	MA	-	S
Cucumis melo	Melon	Earl's favourite	KU	-	S
		Earl's nabio	MA	-	S
		Happiness	MA	-	S
		Harmonika	MA	-	S
		Ichibakouji	MA	-	S
		Ichibakouji red	MA	-	S
		Kurarineto	MA	-	S
		Mondo	MA	-	S
		Newmelon	MA	-	S
		Reimi	MA	-	S
Cucumis sativus	Cucumber	Hokushin	TA	-	S
		Kyoshizuku	TA	-	S
		Natsunokagayaki	AT	-	S
		Natsususzumi	TA	-	S
		Satsukimidori	SA	-	S
		Senba	AT	-	S
Cucurbita moschata	Squash	Ebisu	TA	-	S
Cucurbita pepo	Zucchini	Daina	TA	-	S
Luffa cylindrica	Sponge gourd	Futohethima	KA	-	S
Momordica charantia var. pavel	Bitter melon	Goya	SA	-	S
Fabaceae (Leguminosae)					
Trifolium pratense	Red clover	Meziumu	TA	-	R
Gramineae (Poaceae)					
Hordeum vulgare	Barley	Goseshikoku	KU	Pa	R
		Kobinkatagi	KU	Pa	R
Lamiaceae					
Ocimum basilicum	Basil	Sweet basil	TA	-	R

Table 1 (continued)

Family, genus and species	Common names	Cultivars	Producers [a]	Cytological responses [b]	Pathogenicity [c]
Pedaliaceae					
Sesamum indicum	Sesame	Shiro Goma	KU	-	R
		Kuro Goma	KU	-	R
Polygonaceae					
Fagopyrum esculentum	Buckwheat	Shinshu Oosoba	TA	-	R
Solanaceae					
Capsicum annuum	Bell pepper	Kyoumidori	TA	HR	R
Capsicum annuum var. angulosum	Sweet pepper	Kairyou shishitou	SA	HR	R
Solanum lycopersicum	Tomato	Moneymaker	KU	HR	R
Solanum melongena	Eggplant	Senryou Ni-gou	TA	HR	R

[a] TA, Takii seeds, Kyoto, Japan; SA, Sakata seeds, Yokohama, Japan; AT, Atariya Noen, Chiba, Japan; MA, Marutane, Kyoto, Japan; KA, Kaneko seeds, Tochigi, Japan; KU, Kinki University seed collection

[b] By 14 days after inoculation. HR, hypersensitive cell death; Pa, papilla formation; -, no necrosis

[c] By 14 days after inoculation. R, non-infection; S, infection with sporulation and extensive spread of the colony

formed in the fungal colonies and then transferred onto well-developed, young leaves of 14-day-old melon seedlings with an electrostatic insulator probe (Nonomura *et al*. 2009). A cylindrical plastic case containing the KMP-6N-inoculated seedling was placed on the stage of a digital microscope in a temperature-controlled room (at 25 ± 1°C, 45-55% RH; Fig. 4A). Wind at 0.5 m s^{-1} was blown continuously onto the inoculated seedling, using an electric fan placed 1 m from the seedlings. At 4-5 days after inoculation, immature conidiophores were selected and observed under the digital microscope. Conidiophores selected as the targets for microscopic observation were photographed at 30 min intervals using a 1/2" Interline transfer charge-coupled device (CCD) camera of the digital microscope. Photographs were treated using image-processing software (Adobe Photoshop ver. 5; Adobe Systems, CA, USA). More than 30 conidiophores were observed to trace their development.

A fluorescent brightener, Calcofluor white (CFW; Sigma, MO, USA), was used to stain conidiophores on melon leaves (Oichi *et al*. 2004). A few drops of 10% potassium hydroxide containing 0.05% CFW were placed onto the KMP-6N-inoculated melon leaves and incubated at room temperature for 5 min. Fluorescence-stained conidiophores were observed with a fluorescence microscope BX-60 (U excitation with BP330-385 excitation filter and BA420 absorption filter).

Electrostatic collection of mature conidia on conidiophores An electrostatic insulator probe used to collect mature conidia was a pencil-type, ebonite rod with a pointed tip (7-cm length; 4-mm diameter; tip 5-μm diameter; Moriura *et al*. 2006b; Nonomura *et al*. 2009, 2014). The insulator probe was negatively charged by a direct current (DC) voltage generator HVA 10K202PA (Max Electronics, Tokyo, Japan) and was attached to the micromanipulator on the digital microscope as shown in Fig. 4A. Conidiophores located at the edge of the colony were selected as a target for consecutive collection of the conidia. The insulator probe with static electricity of 5.2×10^{-1} nC was placed approximately 80-100 μm from the conidiophore apex to collect the released conidia (Nonomura *et al*. 2009). When the septum of the apical conidial cell in a full-length conidial chain was fully constricted, the surface of the insulator probe was negatively polarized by the current provided by the generator (Moriura *et al*. 2006b; Nonomura *et al*. 2009, 2014). The conidia trapped on the probe were inoculated onto a healthy melon leaf to test infectivity. The collection procedure was repeated and the intervals between each release of a conidium were timed until conidia were no longer released. Finally, all conidia from a given conidiophore were counted.

Results

Morphological observation and molecular analysis of KMP-6N We isolated conidia from powdery mildew-

infected melon leaves and then multiplied the fungus on leaves of healthy melon plants. Subsequently, KMP-6N conidia were inoculated onto glass slides and leaves of melon plants to observe morphological characteristics. The focus was on conidial shapes and sizes, fibrosin bodies, appressorial shapes, conidiophores and chasmothecia. The conidia with sizes of 23-33 x 15-22 μm were hyaline and ellipsoid-ovoid to doliiform, and contained fibrosin bodies (Fig. 1A). All conidia germinated 3-4 h after inoculation and then formed indistinct appressoria at the tip of the germ tubes (Fig. 1B). Conidiophores were catenated, with conidia formed in straight chains approximately 45-86 × 10-18 μm in size (Fig. 1C). Numerous chasmothecia formed within three months of conidia inoculation (Fig. 1D). Each chasmothecium possessed only one ascus, with each ascus having eight ascospores and rod-like appendages (Fig. 1E).

The data obtained in this study were similar to those on *P. xanthii* (sexual stage) morphological characteristics reported previously by Braun (1987) and Cosme *et al*. (2012). We conducted gene amplification of the rDNA-ITS region of KMP-6N to determine the nucleotide sequences of the PCR products (455 bp). The rDNA-ITS sequence of KMP-6N was compared with sequences of other cucurbit powdery mildew isolates registered in GenBank. In addition, on the basis of these results, we established the phylogenic tree of their powdery mildew isolates (Fig. 2). The nucleotide sequence of the KMP-6N rDNA-ITS region was highly homologous to that of *P. xanthii* (HQ316143). KMP-6N was assigned to the cluster containing isolates of *P. xanthii*. The rDNA-ITS sequence of KMP-6N was registered as accession number LC012888 in DDBJ.

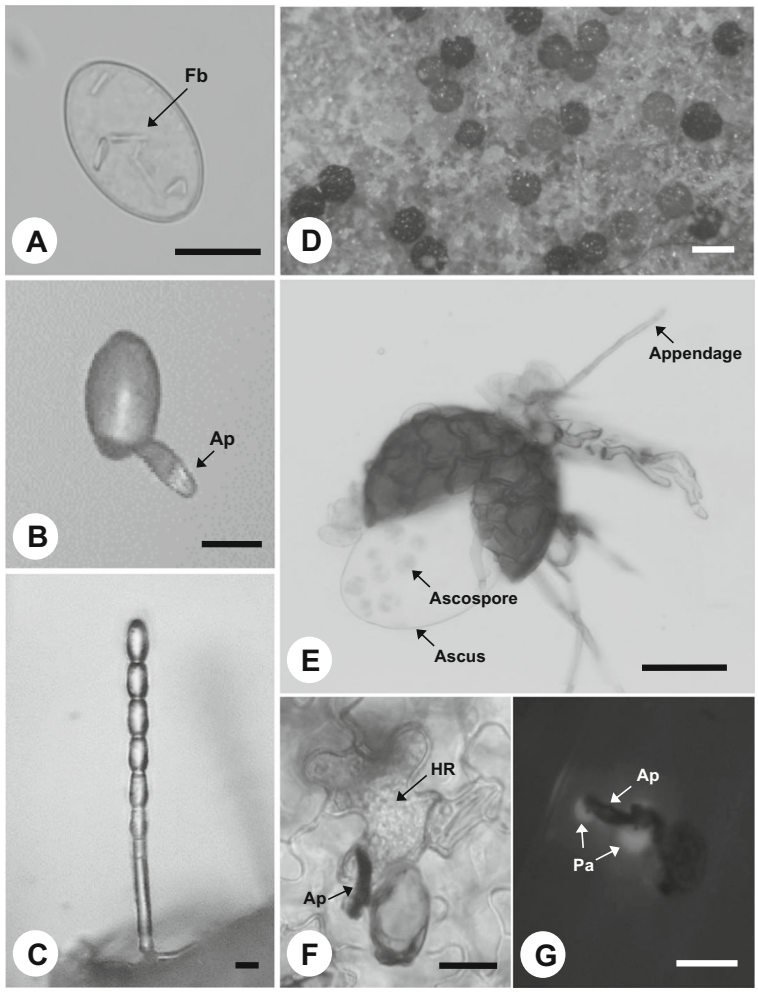

Fig. 1 Light (**A, E** and **F**), digital (**B** and **C**), stereo (**D**) and fluorescence **(G)** micrographs showing morphological characteristics of KMP-6N. **A,** Fibrosin bodies (Fb) in a conidium. **B,** Appressorial (Ap) shape at tip of a germ tube. **C,** Catenated conidiophore possessing six immature conidia. **D,** Chasmothecia on melon leaves. **E,** Ascus in a broken chasmothecium, eight ascospores in ascus appendages. **F,** Hypersensitive cell death (HR) induced by insertion of KMP-6N into tomato leaves. **G,** Papillae (Pa) formed by insertion of KMP-6N into barley leaves. Bars represent 10 μm (**A** and **B**), 20 μm (**C, F** and **G**), 50 μm (**E**) and 100 μm (**D**)

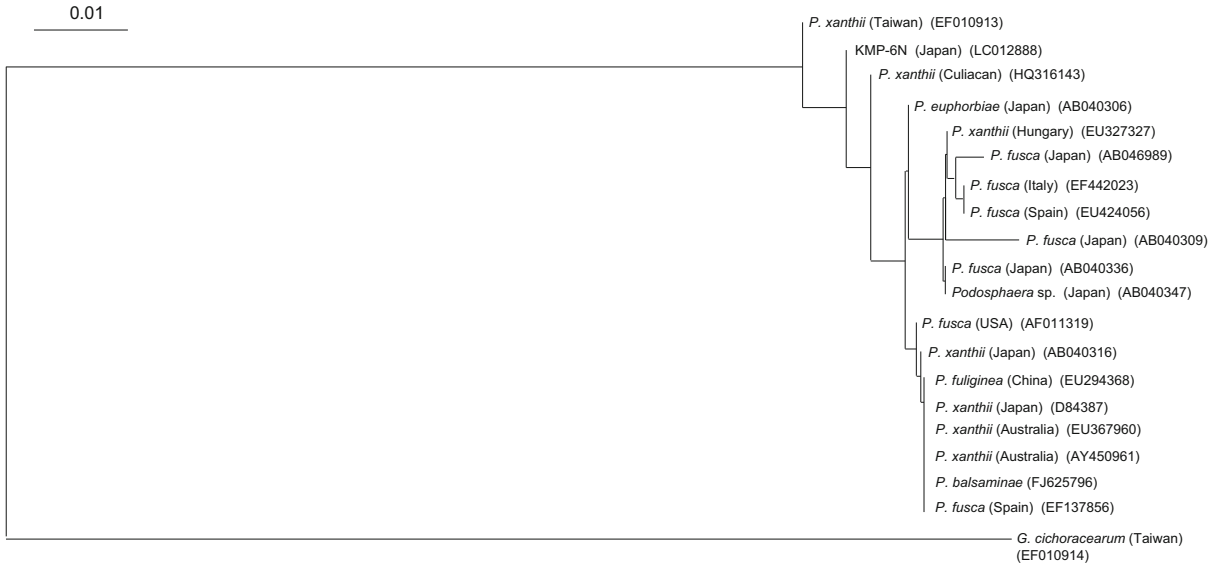

Fig. 2 Phylogenetic tree of various cucurbit powdery mildew isolates based on rDNA internal transcribed spacer sequences. Newly discovered melon powdery mildew isolate KMP-6N belonged to the *Podosphaera xanthii* clade. The dendrogram was constructed using the Dendroscope 3 software, with the *Golovinomyces cichoracearum* sequence (EF010914) as the outgroup species sequence

Host range tests Table 1 shows the host range tests for melon powdery mildew isolate KMP-6N. In the tests, KMP-6N heavily infected and sporulated on the inoculated leaves of Cucurbitaceae plants, which included winter melon, watermelon, melon, cucumber, squash, zucchini, sponge gourd and bitter melon, without causing hypersensitive cell death (HR), necrosis or papillae in the leaf epidermal cells. The HR reaction was induced in epidermal cells of the inoculated leaves of Asteraceae (edible chrysanthemum) and Solanaceae (bell pepper, sweet pepper, tomato and eggplant) plants (Fig. 1F). Additionally, papillae were induced in the epidermal cells by insertion of KMP-6N into barley leaves (Fig. 1G). Hyphal elongation of KMP-6N was completely suppressed by the resistant plant species. Among the remaining plant species tested, KMP-6N did not infect their leaves without causing resistance responses in their epidermal cells.

Developmental process of conidiophores Conidiophore formation by KMP-6N on melon leaves was observed under a digital microscope. Figures 3A and B show digital and fluorescent micrographs of conidiophores. Septation and conidial development in the conidiophores were traced using a digital microscope (Fig. 3A, 1-11). Septation was confirmed by staining conidiophores with CFW stains at designated stages (Fig. 3B). The sites of conidiophore septation observed with the digital microscope coincided with those we observed in CFW-stained conidiophores under a fluorescence microscope.

Collection of mature conidia on conidiophores using an electrostatic spore collection technique We attempted to collect mature conidia released from conidiophores, utilizing an electrostatic spore collection technique (Fig. 4A). The non-polarized insulator probe did not attract conidia (Fig. 4B). However, conidiophores were attracted to the probe when the insulator probe was negatively polarized (Fig. 4C) and then the mature conidial cell (C1-1) produced on top of the conidiophore was collected with the probe (Fig. 4D). The probe was then depolarized, and the conidiophore returned to its original position. The conidiophore was attracted to the probe when the insulator probe was, once again, negatively polarized. The next apical conidial cell (C1-2) was similarly attracted to the probe 2 h later (Fig. 4E). We repeatedly collected mature conidia on conidiophores using the same technique. Consequently, the apical cell C(n)-1 was attracted to the probe and the C(n)-2 to the probe approximately 2 h later. The conidiophores became progressively shorter. By collecting all the released conidia, we determined the entire production of conidia by individual conidiophores and the

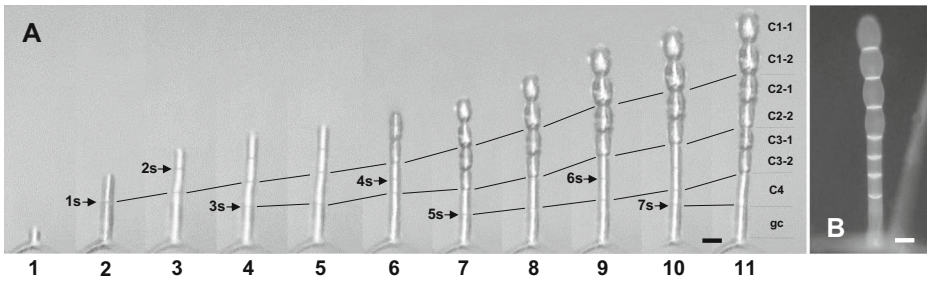

Fig. 3 Digital **(A)** and fluorescence (**B**) micrographs of conidiophores formed by *Podosphaera xanthii* KMP-6N on melon leaves. **A-1 to -11,** The same conidiophore was consecutively observed until it formed full-length chains of six conidial cells (C1-1 to C3-2), an undivided conidial cell (C4) and generative cell (gc) as shown in Fig. 5A. Arrows represent the first to seventh septation (1 s to 7 s). **B,** Stage of Calcofluor white (CFW)-stained conidiophore corresponds to that in **A-10**. Septation of the conidiophores was confirmed by CFW staining. Bars represent 10 μm

timing of conidial secession (Table 2). Individual conidiophores released an average of 36 conidia at approximately 90 h during conidial secession.

Analysis of conidiogenesis process on conidiophores Figure 5 shows the developmental process of conidiogenesis based on actual data. The developmental process occurred in three phases. The first phase involved the completion of a set of developed cells (Fig. 5A), the second phase involved repetition of conidia formation by generative cells (Fig. 5B), and the third phase consisted of the release of conidia, without division and growth of generative cells (Fig. 5C). Under the conditions we induced for conidiogenesis of KMP-6N, the hyphae initiated to form erect conidiophores on hyphae 4-5 days after inoculation (Figs. 3A-1, 5A-1). Initially, the slender apical portion of the generative cell elongated on hyphae and a septum (1 s) formed to divide a given cell into two cells after 5 h (Figs. 3A-2, 5A-2). Within 7 h, the upper **C1** cell was divided by a second septation (2 s) into two cells (Figs. 3A-3, 5A-3). The resulting two cells developed into primary (C1-1) and secondary (C1-2) conidia during the subsequent stage. A third septation (3 s) formed in a generative cell for subsequent conidia formation (Figs. 3A-4, 5A-4). The generative cells elongated (Figs. 3A-5, 5A-5) and the upper **C2** cell was divided by a fourth septation (4 s) into two cells within 13 h of formation (Figs. 3A-6, 5A-6). These two cells developed into third (C2-1) and fourth (C2-2) conidia during the subsequent stage. Thus, the generative cells repeatedly septated, elongated and produced two immature conidia by splitting into two cells (Figs 3A-7 to -11, 5A-7 to -11).

At this stage, approximately 24 h after the formation of primordial cells, the conidiophores produced full-length chains of six conidial cells (C1-1 to C3-2), one undivided conidial cell **(C4)** and one generative cell (gc). During the second phase (Fig. 5B), the first mature conidium (C1-1; Fig. 5B-12) and subsequent conidia (C1-2; Fig. 5B-13) were released from the conidiophores by division (8 s) of the upper **C4** cell and septation (9 s) in the generative cell. After release of their conidia, two mature conidia were repeatedly produced with elongation of the upper **C5** cell (Fig. 5B-14), division of the **C5** cell by septation (10s) into two cells (Fig. 5B-15), and elongation of the split upper cells and septation (11 s) in generative cells (Fig. 5B-16). The upper cells that were split formed two immature conidia (C5-1 and C5-2) (Fig. 5B-17).

During the repeated cycles, two mature conidia were released at approximately 6 h intervals. This cycle was repeated on melon leaves 14 to 18 times (Table 2). At the final stage of conidial secession, as shown in Fig. 5C, the generative cells ceased division and growth, although four conidia were released (Fig. 5C-18 to -21). Secession of the conidial cells (two immature conidia) remaining on the conidiophores was not detected, even when the observation period was extended by 12 h. Consequently, lifetime of the conidiophores was determined to be 120-140 h, from the primordial formation until final conidial release on conidiophores (i.e., first to third phases).

Discussion

Cucurbit powdery mildew fungi were previously known as *S. fuliginea* or two species of *Golovinomyces* (*G. orontii* and *G. cucurbitacearum*; Braun *et al.* 2001,

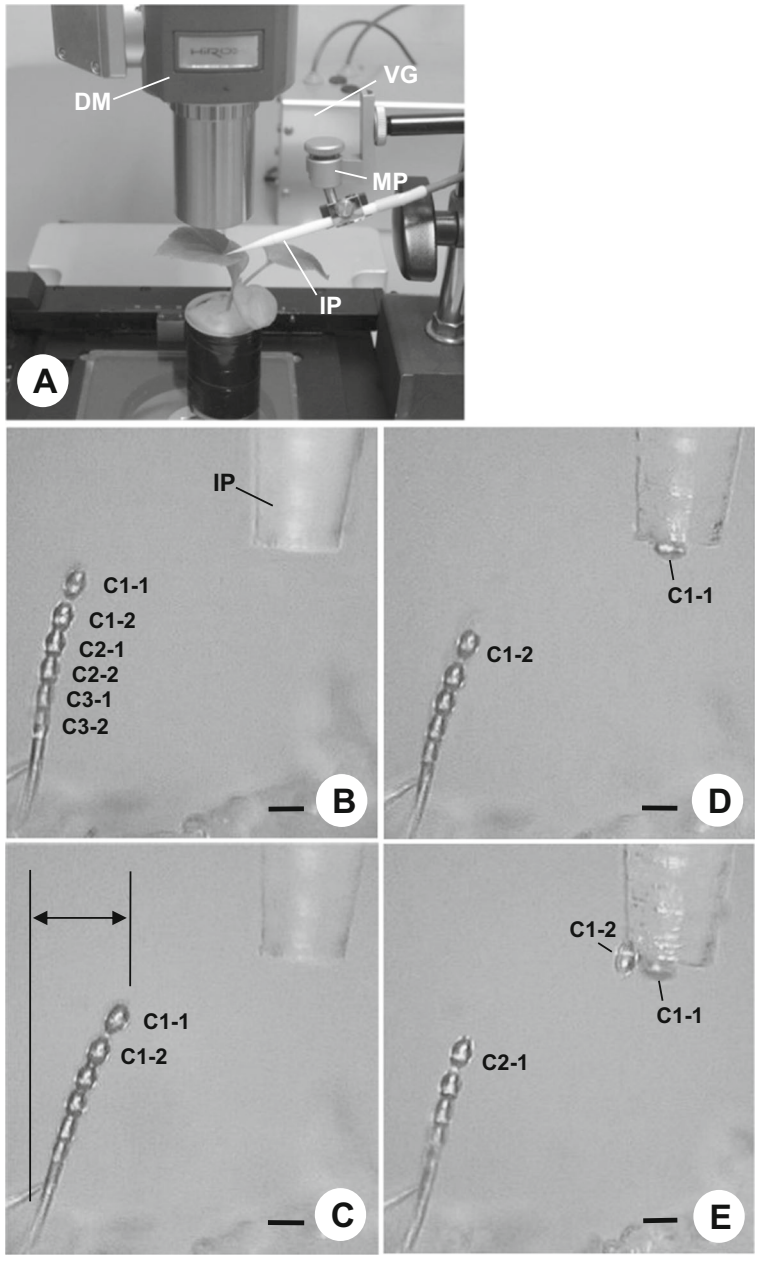

Fig. 4 Collection of mature conidia released from conidiophores of KMP-6N on melon leaves used an electrostatic spore collection technique. **A,** Electrostatic spore collection method used to collect conidia from a conidiophore. A KMP-6N-inoculated melon seedling grown in a culture case containing fertilizer was placed on a stage of the digital microscope (DM) for direct observation of conidiophores on the leaf. A pointed insulator probe (IP) connected from a direct current voltage generator (VG) was installed on a manipulator (MP) apparatus. **B** and **C,** A conidiophore before (**B**) and after (**C**) negatively electrifying the insulator probe. **D** and **E,** C1-1 (**D**) and C1-2 (**E**) mature conidia captured on an insulator probe. Note successful attraction of the erect conidiophore to the activated insulator probe placed at 100 μm from the apex of the conidiophore and collection of C1-1 and C1-2 conidia. Bar represents 20 μm (**B** to **E**)

Vakalounakis & Klironomou 2001). Recently, based on morphological characteristics of cucurbit powdery mildew fungi and molecular phylogenetic analyses of their isolates, the genus *Sphaerotheca* was merged with the genus *Podosphaera* (Braun & Takamatsu 2000). The scientific name of cucurbit powdery mildew pathogens was then changed to *P. xanthii* (Braun *et al.* 2001).

In Japan, infestation of *P. xanthii* and *G. orontii* on melon plants has been reported (Hosoya *et al.* 1999). These fungi are morphologically distinguished by the presence of Fibrosin bodies in conidial cells and appressorial shapes, as described by Braun (1987) and Uchida *et al.* (2009). Our data strongly suggested that KMP-6N was identical to *P. xanthii*, based on morphological characteristics including shape of chasmothecia formed in sexual stages, sequences of rDNA-ITS regions and phylogenetic analysis. Furthermore, as a result of experiments designed to understand the pathogenicity of KMP-6N to various plant species, we confirmed its host range. The isolate KMP-6N heavily

Table 2 Time intervals of conidial secession by individual conidiophores of melon powdery mildew KMP-6N on host leaves

Number of conidia	Conidia released	Hours required for conidial secession [a]								
		1	2	3	4	5	6	7	8 [b]	Means ± S.D.
1	C1-1	-	-	-	-	-	-	-	-	-
2	C1-2	1.9	2.0	2.2	1.8	1.9	2.4	1.5	2.3	2.0 ± 0.3
3	C2-1	3.8	4.1	3.7	4.3	3.8	4.0	3.7	3.8	3.9 ± 0.2
4	C2-2	2.2	2.3	2.2	2.1	2.0	1.7	2.0	1.7	2.0 ± 0.2
5	C3-1	4.3	3.6	4.1	3.6	4.3	3.9	3.7	4.3	4.0 ± 0.3
6	C3-2	2.1	2.3	2.3	1.8	2.2	2.4	2.2	2.3	2.2 ± 0.2
7	C4-1	4.4	3.4	3.8	4.3	3.9	4.2	4.1	4.1	4.0 ± 0.3
8	C4-2	1.7	1.8	2.0	1.9	2.2	2.4	1.6	2.1	2.0 ± 0.3
9	C5-1	4.2	4.0	4.1	4.4	3.9	3.5	4.5	4.2	4.1 ± 0.3
10	C5-2	2.1	2.1	1.9	1.8	2.4	2.1	2.0	2.0	2.1 ± 0.2
11	C6-1	3.7	4.1	3.8	4.0	3.8	4.3	4.4	4.3	4.1 ± 0.3
12	C6-2	2.3	2.4	2.3	2.3	2.0	2.4	1.8	2.2	2.2 ± 0.2
13	C7-1	4.3	3.7	4.3	4.3	3.9	4.2	4.1	3.8	4.1 ± 0.2
14	C7-2	1.9	1.8	2.0	2.4	2.0	1.9	2.3	2.2	2.1 ± 0.2
15	C8-1	4.0	4.3	3.8	4.3	3.8	4.3	3.7	4.1	4.0 ± 0.3
16	C8-2	2.2	2.3	2.0	2.2	2.0	1.8	1.8	1.6	2.0 ± 0.2
17	C9-1	3.7	3.5	3.8	4.1	4.3	4.0	4.4	4.2	4.0 ± 0.3
18	C9-2	2.1	2.4	2.3	1.9	2.0	1.9	2.0	2.4	2.1 ± 0.2
19	C10-1	3.9	3.7	4.0	3.7	4.1	3.8	4.1	3.7	3.9 ± 0.2
20	C10-2	2.3	2.3	1.8	2.2	2.0	2.1	1.7	1.8	2.0 ± 0.2
21	C11-1	4.3	3.7	3.8	3.8	4.1	4.4	4.0	4.4	4.1 ± 0.3
22	C11-2	1.8	2.2	2.3	2.0	2.2	2.2	2.2	1.7	2.1 ± 0.2
23	C12-1	4.3	4.2	3.6	4.2	3.7	4.4	4.2	4.2	4.1 ± 0.3
24	C12-2	2.0	1.9	2.3	1.7	2.1	1.9	1.9	2.1	2.0 ± 0.2
25	C13-1	3.6	4.2	3.8	4.3	4.2	3.9	4.2	4.3	4.1 ± 0.3
26	C13-2	1.9	2.0	2.3	2.2	1.4	2.0	1.8	2.0	2.0 ± 0.3
27	C14-1	3.8	4.2	3.7	3.8	3.9	4.2	3.8	4.3	4.0 ± 0.2
28	C14-2	2.2[c]	1.8	2.0	2.4	2.0	2.2	1.9	1.9	2.0 ± 0.2
29	C15-1	3.4	3.8	4.0	3.7	3.8	3.6	3.8	4.1	3.8 ± 0.2
30	C15-2	1.0	1.8	2.0	1.9	2.0	2.3	1.0	2.2	1.8 ± 0.5
31	C16-1	0.9	4.0	4.1	4.4	4.3	3.8	0.8	3.7	3.3 ± 1.5
32	C16-2	1.2	2.1	2.0	1.8	1.3	2.4	1.0	0.9	1.6 ± 0.6
33	C17-1		4.1	3.8	3.9	1.0	4.3		0.7	3.0 ± 1.7
34	C17-2		2.4	0.8	2.3	1.2	2.0		0.8	1.6 ± 0.7
35	C18-1		3.6	1.0	4.2		4.3			3.3 ± 1.5
36	C18-2		1.0	1.1	1.0		1.9			1.3 ± 0.4
37	C19-1		0.9		1.1		4.3			2.1 ± 1.9
38	C19-2		1.2		1.1		0.8			1.0 ± 0.2
39	C20-1						0.9			0.9
40	C20-2						0.8			0.8
Total number of conidia released		**32**	**38**	**36**	**38**	**34**	**40**	**32**	**34**	**35.5 ± 3.0**
Continuance (h) of conidial secession		**81.0**	**98.5**	**92.1**	**99.9**	**85.9**	**88.4**	**84.4**	**88.3**	**89.8 ± 6.6**

[a] The conidium-release time was mesured immediately after C1-1 conidia were abstricted.

[b] Number of conidiophores used in the present study

[c] Boundary between conidial secessions with and without growth and division of generative cells

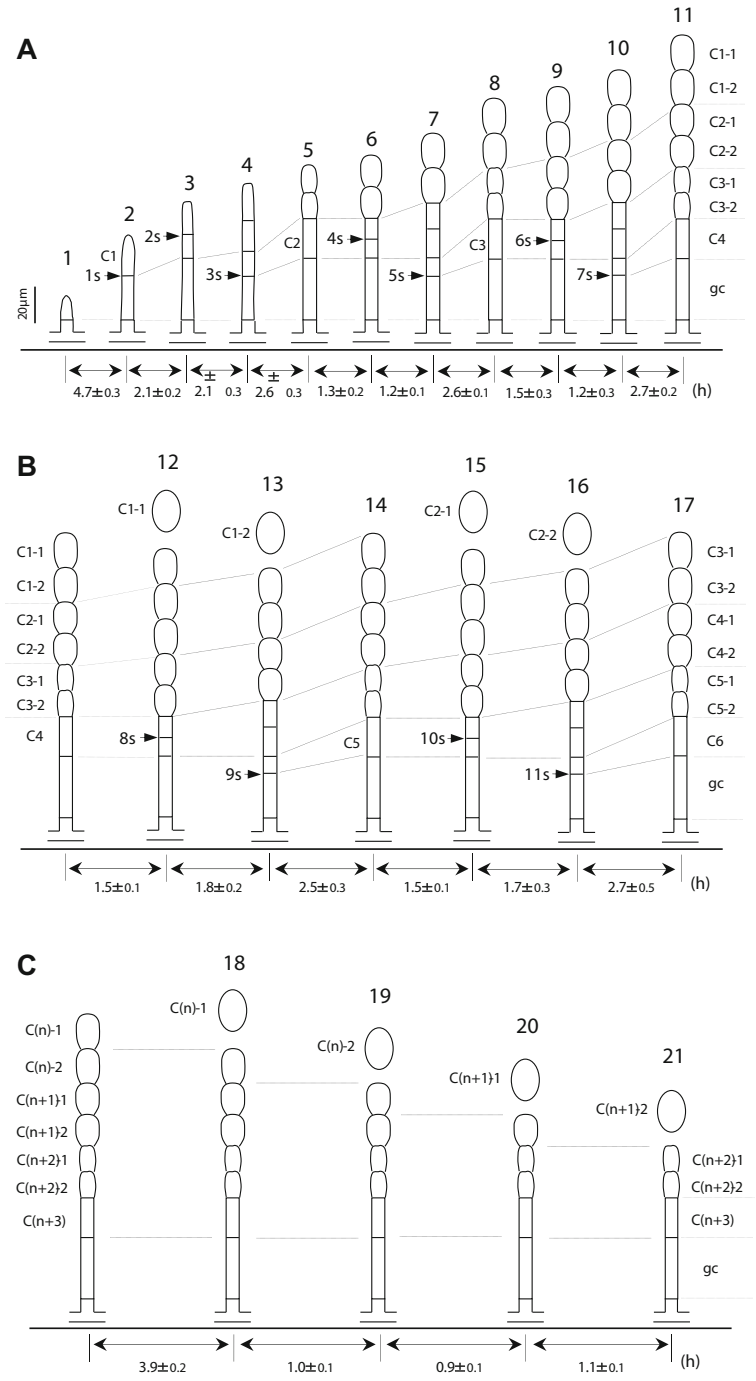

Fig. 5 Diagrams of the developmental process of full-length conidial chain formation (**A**) and successive secession of mature conidia (**B** and **C**) in conidiogenesis of *Podosphaera xanthii* KMP-6N. **A,** Process of conidiophore formation with elongation of the conidial chain, and the growth and septation of the generative cell (gc) at the first phase (1-11). **B,** Process of conidial secession repeated with elongation of the conidial chain, and growth and septation of the generative cell at the second phase (12-14 and 15-17). **C,** Process of conidial secession without elongation of the conidial chain, and growth and septation of the generative cell at the third phase (18-21). C(n+2)-1 and C(n+2)-2 were immature conidial cells that remained in a conidiophore. C(n+3) was the undivided cell without growth and septation. Time intervals (h) for elongation of the conidial chain, growth and septation of the generative cell, and conidial secession are shown at the bottom of the figure. Thirty conidiophores were observed, and the data are presented as means and standard deviation. Bar represents 20 μm

infected all tested melon cultivars and other Cucurbitaceae plants (Abiko 1978; Tomason & Gibson 2006; Křístková *et al*. 2009; Cosme *et al*. 2012). However, the ability of KMP-6N to infect other plant species has not been confirmed. Solanaceae plants were susceptible to the isolates described by Abiko (1978) and Braun *et al*. (2001), but not to KMP-6N. Sponge gourd (*Luffa cylindrica*) was resistant to the isolate studied by Tomason and Gibson (2006), but it was not resistant to KMP-6N. Thus, the results of host range tests for KMP-6N have been inconsistent over the range of isolates tested. This may reflect the existence of

different pathotypes among melon powdery mildew isolates.

Cucurbit powdery mildew pathogens have been reported as tolerant to commercial fungicides (Huggenberger *et al*. 1984; McGrath & Shishkoff 2001, 2003; Lebeda & Sedláková 2008). Fortunately, KMP-6N did not show tolerance to the main commercial fungicides in the present study **(data not shown)**. However, we must continuously screen fungicide-resistant cucurbit powdery mildews on host plants.

Powdery mildew widely expands the infection source by releasing progeny conidia from conidiophores. Therefore, the main objective of this study was to observe the process of conidiogenesis of the melon powdery mildew KMP-6N, as the morphological characteristics of conidiophores of powdery mildew fungi have not been analyzed in detail. Urquhart and Punja (1997) described conidia that appeared to be turgid and were formed in long chains on conidiophores of *Sphaerotheca fuliginea*. Cosme *et al*. (2012) presented conidiophores that were of the euoidium type (i.e., conidia in chains), with crenated margins. Pérez-García *et al*. (2001) and Rivera *et al*. (2002) described 5-10 ovoid-shaped conidia produced in chains at the tip of each conidiophore. Thus, the reported characteristics of conidiophores have been variable. To our knowledge, the developmental process of living conidiophores of cucurbit powdery mildew pathogens has not been presented.

From our digital microscopic observation, the melon powdery mildew isolate KMP-6N produced six conidia on conidiophores in chains (Fig. 1C). We assert that the number of conidia piled up and produced on conidiophores ought to be naturally determined among powdery mildew pathogens. In our previous reports, we demonstrated the developmental process of conidiogenesis of tomato powdery mildew *O. neolycopersici* (Oichi *et al*. 2004, 2006) and barley powdery mildew *Blumeria graminis* f. sp. *hordei* race1 on host leaves (Moriura *et al*. 2006a, 2006b). Tomato powdery mildew Japanese isolates (KTP-01 to -04) formed the non-catenated conidiophores with foot-cells (conidia singly produced on conidiophores) during windy conditions (1.0 m s^{-1}; Oichi *et al*. 2004, 2006; Seifi *et al*. 2012; Nonomura *et al*. 2014), while barley powdery mildew isolate produced catenated conidiophores and accumulated eight conidia on a conidiophore with a globularly bulky generative cell (Moriura *et al*. 2006b).

From our microscopic observation, KMP-6N also produced catenated conidiophores and accumulated six conidia on a conidiophore, with a non-bulky generative cell, under windy conditions (1.0 m s^{-1}). Among the conidiophore types, powdery mildew fungi possess conidia that form singly or catenescent in genuine chains, following cells composing of generative cells, and straight or sinuous-helicoid foot-cells (Braun & Cook 2012). However, KMP-6N formed straight generative cells on basal septa because the cell was divided by a septation into two cells during conidiogenesis. This was clearly different from the *O. neolycopersici* isolate KTP-01, as the *Pseudoidium* type possessed foot-cells on basal septa (Oichi *et al*. 2004). We could not determine if the straight hyphae on basal septa were foot-cells or generative cells, unless we consecutively observed the developmental process of conidiophores under a digital microscope. Boesewinkel (1980) showed that *S. fuliginea* forms conidiophores with foot-cells frequently slightly swollen at the base. The results obtained in the present study may help to understand the characteristics of conidiophores. On the other hand, the divided aspects by septation in generative cells of KMP-6N were clearly similar to those of barley powdery mildew fungi, except for initial formation of conidiophores (See Fig. 5A-4, -5 and -6) as reported previously by Moriura *et al*. (2006b). The divided aspects by septation in generative cells may be the main characteristics of catenated powdery mildew fungi with conidiophores piling up conidia in genuine chains.

Next, to confirm the longevity of conidiophores and lifelong production of conidia by living conidiophores on leaves, we used an electrostatically activated insulator probe (Nonomura *et al*. 2009). This technique enabled us to analyze the release process of mature conidia from conidiophores and count the total number of conidia produced by individual conidiophores throughout their lifetime. Consequently, we clarified the precise cycle of the conidial formation and release from conidiophores of KMP-6N. The release process of mature conidia from the apex of the conidiophores (at second phase) was similar for barley and melon powdery mildew pathogens; a succession of two conidia on the apex of conidiophores was repeatedly released at approximately 3-5 h (Moriura *et al*. 2006b). In addition, the conidia release process of KMP-6N was identical to that of barley powdery mildew pathogen at the third phase. Four conidia on the apex of conidiophores were continuously released and the conidiophores became shorter,

without elongating and dividing the generative cells. However, the release times varied among the fungi. KMP-6N released a conidium every 0.5-1 h, while barley powdery mildew isolates released conidia every 3 h (Moriura *et al*. 2006b). Finally, the longevity of conidiophores and lifelong production of conidia by KMP-6N were similar to those of barley powdery mildew isolates (Moriura *et al*. 2006b). Thus, we demonstrated conidiogenesis and the longevity of KMP-6N conidiophores. The data obtained in the present study provide important information describing conidiophores and conidiogenesis of powdery mildew fungi. Moreover, their characteristics will facilitate accurate taxonomical classification of powdery mildew fungi. In future, we will focus on the developmental process of conidiophores of other powdery mildew fungi.

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