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Effects of Fungicides on Fungal Development, Conidiophore Morphology, and Conidial Releases from Single Strawberry Powdery Mildew Colonies Assessed Using an Electrostatic Technique

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Abstract: We evaluated the effects of fungicides on strawberry powdery mildew (PM) fungus, *Podosphaera aphanis*, using an electrostatic technique. Thirty-six fungicides were sprayed on single colonies of *P. aphanis* on leaves of strawberry seedlings (*Fragaria* × *ananassa* Duchesne ex Rozier). Colony development varied depending on the tested fungicides. Particularly, pyraziflumid, triflumizole, triforine, polyoxin, sodium hydrogen carbonate + copper wettable powder, and flutianil + mepanipyrim were highly effective for reducing colony development. *P. aphanis* colonies were histochemically stained to observe the morphological characteristics of fungal cells forming normal and abnormal conidiophores. Abnormal conidiophores were classified into seven types based on their morphological and cytological characteristics. Finally, asexual conidia were collected from single *P. aphanis* colonies on the leaves spray-treated with fungicides using a dielectrically polarized insulator plate (electrostatic spore collector); conidia attracted to the insulator plates were counted using a high-fidelity digital microscope. Most tested fungicides highly inhibited the production and/or germination of asexual conidia. The germination of asexual conidia was observed only in thiophanate-methyl (methyl benzimidazole carbamates fungicides; MBC fungicides) and azoxystrobin (quinone outside inhibitors; QoI fungicides). Assessing with the electrostatic technique, we clarified that *P. aphanis* has developed resistance to both thiophanate-methyl and azoxystrobin. Thus, the methodological assessment analyzing the colony development and the number of conidia released from single colonies will be helpful information for screening effective fungicides.

Keywords: azoxystrobin; catenated conidia; conidiophores; electrostatic spore collectors; MBC fungicides; *Podosphaera aphanis*; QoI fungicides; thiophanate-methyl

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1. Introduction

Strawberry cultivation is very popular at our prefecture (Nara Prefecture) in Japan. Powdery mildew (PM) caused by *Podosphaera aphanis* (syn. *Sphaerotheca aphanis*, *S. humuli*, *S. macularis*, and *S. macularis* f. sp. *fragariae*) [1,2], an obligate biotrophic fungal pathogen, is one of the most important diseases of strawberry plants (*Fragaria* × *ananassa* Duchesne ex Rozier) of the Rosaceae family in many countries. PM occurs on the surfaces of epigeal organs, including the leaves, petioles, stolons, fruits, receptacles, runners, and flowers [3–7]. A severe outbreak of PM can significantly reduce strawberry fruit productivity [6–10]. Regarding the pathogenicity of *P. aphanis*, some have reported the sporulation of PM

isolates on the leaves of commercial strawberry cultivars (*Fragaria* × *ananassa* Duchesne) [3,4,11–13]. The ability of strawberry PM isolates to infect other plant species including *Fragaria vesca* L. (wild strawberry) and *Potentilla hebiichigo* (syn. *Duchesnea chrysantha* Miq.) (false strawberry) has been confirmed. Wild strawberry (*F. vesca* L.) is susceptible to the isolates described by Abiko [11] and Iwasaki et al. [14] but is resistant to the isolate studied by Peries [3], perhaps reflecting the existence of different pathotypes among strawberry PM isolates. Thus, the host range of strawberry PM fungi is very narrow, as described previously [11,13,14].

Chemical methods are commonly used in Japan and elsewhere to control strawberry PM diseases [15–18]. Strawberry PMs can be controlled using fungicides that target fungal respiration, nucleic acid synthesis, sterol biosynthesis, and signal transduction [19]. Notably, controlling PM in the fruit is more difficult than in the leaves. As such, fungicide application during early flowering period is important to efficiently control strawberry PM. During this period, protectant (sulfur) or systemic fungicides (myclobutanil and triflumizole) are mainly used [20,21]. However, previous studies have reported the resistance of strawberry PM to commercial fungicides, such as demethylation inhibitors (DMIs), including piperazines (triforine), pyrimidines (fenarimol and nuarimol), and triazoles (bitertanol, myclobutanil, penconazole, triadimefon, and triadimenol) [19,22–24]. In the current study, we examined the effects of commercial fungicides on the developments of *P. aphanis* Japanese isolate ex *Fragaria* × *ananassa* Duchesne ex Rozier, cv. Sagahonoka.

Asexual conidia of *P. aphanis* on conidiophores are an infection source for the host plants; PM dissemination over a wide area from conidiophores occurs under natural conditions [25–27]. Matsuda et al. [28] previously described that asexual conidia that enter into an electric field are attracted to polarized dipole insulators and can become trapped by them. This electrostatic technique has been successfully applied to collect asexual progeny conidia from living single PM colonies (i.e., barley, melon, strawberry, or tomato PM Japanese isolates) without harming or damaging the PM conidia [29]. For example, using the electrostatic technique, one research group was able to clarify that the strawberry PM Japanese isolate releases an average of 6.7×10^4 conidia from each of the single colonies over approximately 816 h during the fungal lifetime [29]. In a second experiment, our group counted the number of asexual conidia released from single PM colonies on strawberry plants spray-treated with commercial fungicides by combining digital microscopic and electrostatic techniques, and then examined the morphological characteristics of the conidiophores in the single PM colonies. To the best of our knowledge, the current study is the first study to assess the fungicide effects of PM fungi by quantitatively analyzing the conidia released from single strawberry PM colonies, spray-treated with individual different fungicides, on the leaves of living host seedlings. This scientific information is important for better understanding the ecological mechanisms of conidial releases from conidiophores in the strawberry production field.

2. Materials and Methods

2.1. Plant Materials and Cultivation

The seeds of strawberry (S1 progeny from *Fragaria* × *ananassa* Duchesne cv. Sagahonoka) were stored in a refrigerator at 4 °C for 7 days. The dormant seeds were placed on wet filter paper inside Petri dishes. The seeds germinated for 14–16 days in an incubation room, under conditions controlled at 22 ± 1 °C, 60–70% relative humidity (RH), and continuous illumination of 59.5 μmoL m^{-2} s^{-1} under a 14 h photoperiod. Cotyledonal seedlings were placed on polyurethane cubic sponge supports (3 cm × 3 cm × 3 cm). The sponge supports with cotyledonal seedlings were inserted into 30 mL cylindrical plastic containers (diameter: 3 cm; length: 5 cm) containing 20 mL liquid fertilizer (Kyowa, Osaka, Japan). The seedlings were incubated for 60 days at 22 ± 1 °C, 60–70% RH, illumination of 210–260 μmoL m^{-2} s^{-1}, and photoperiods of 14 h light/10 h dark, as described by Iwasaki et al. [14]. Light intensity was measured using an LI-250A light meter (LI-COR, Tokyo,

Japan) fitted with a quantum sensor that measures photosynthetically active radiation (400–700 nm).

Sixty-day-old healthy seedlings were transferred to a polystyrene plate (61.5 cm × 60.5 cm × 3.0 cm) floating in hydroponic solution in a hydroponic culture trough (67.0 cm × 65.5 cm × 21.0 cm) (Home Hyponica 303; Kyowa) of a cultivating table (100 cm high) that was installed in a pathogen-free greenhouse (10.0 m × 6.0 m; 22 ± 1 °C) [28]. The seedlings were used for maintaining strawberry PM Japanese isolates and spray-treating single strawberry PM colonies on the leaves with fungicides.

2.2. Fungal Materials, Inoculation, and Incubation

After isolation of a single conidium from the PM-infected strawberry leaves (cv. Sagahonoka) and multiplication of the fungal mycelia on PM-free strawberry leaves, Iwasaki et al. [14] identified the PM Japanese isolate based on the morphological characteristics of domestic and foreign isolates described previously [2,30,31] and through genetic analysis of ribosomal DNA internal transcribed spacer (rDNA-ITS) sequences [32], and then designated the Japanese isolate *Podosphaera aphanis* (Wallroth) U. Braun & S. Takamatsu var. *aphanis* KSP-7N. Mature conidia were collected from conidiophores on KSP-7N-infected strawberry leaves using a pencil-type electrostatic insulator probe. The insulator probe, consisting of an ebonite rod with a pointed tip (length: 7 cm; diameter: 4 mm; pointed tip diameter: 5 μm), was mounted on a micromanipulator placed near a KH-2700 high-fidelity digital microscope (KH-2700 DM; Hirox, Tokyo, Japan) [14]. The insulator probe was negatively charged by a direct-current HVA 10K202NA electrostatic voltage generator (Logy Electric, Tokyo, Japan). The conidia were inoculated onto well-developed young true leaves of three 60-day-old healthy strawberry seedlings, as described previously [14]. The isolate was maintained on potted strawberry plants for 14 days after inoculation of conidia in a greenhouse (10.0 m × 6.0 m) or in an LH-240N growth chamber (Nippon Medical and Chemical Instruments, Osaka, Japan) at 22 ± 1 °C.

2.3. Fungicide Testing

Thirty-six commercial fungicides were used to examine the effects of the fungicides on developments and conidial releases of *P. aphanis* (Table 1). All test fungicides were purchased from agrochemical manufacturers in Japan. The fungicides were diluted based on the manufacturer's instructions. The leaves of 60-day-old strawberry seedlings were inoculated with single *P. aphanis* conidia using the insulator probe described. Single 7-day-old *P. aphanis* colonies (fungal fleck) on the leaves, as shown in Figure 1A, were spray-treated with the solution of adjusted fungicides, and then incubated for 3 days in a greenhouse at 22 ± 1 °C, 50–70% RH, under illumination of 190.6 to 400.4 μmol m^{-2} s^{-1} during the daytime hours. Each spray treatment with a fungicide involved five test plants. Fungal development on strawberry leaves and conidiophores, as shown in Figure 1B, was observed with a digital microscope. In addition, five fungal colonies on strawberry seedlings spray-treated with tap water served as controls.

Table 1. Mode of action of commercial fungicides used in this study to assess fungicide sensitivity against *Podosphaera aphanis* KSP-7N.

Mode of Action	Code	Target Site	Group Name	Active Ingredient	FRAC Code [19]
Cytoskeleton and motor protein	B1	β-tubulin assembly in mitosis	Methyl-benzimidazole carbamates (MBC fungicides)	Thiophanate-methyl	1
	B6	Actin/myosin/fimbrin function	Aryl-phenyl-ketones	Pyriofenone	50
Respiration	C2	Complex II: succinate dehydrogenase	Succinate dehydrogenase inhibitors (SDHIs)	Fluopyram	7

		(SdhB, SdhC, and SdhD)			
				Isopyrazam	7
				Penthiopyrad	7
				Pyraziflumid	7
	C3	Complex III: cytochrome bc1 at Qo site (Cytb)	Quinone outside inhibitors (QoI fungicides)	Azoxystrobin	11
				Kresoxim-methyl	11
Amino acids and protein synthesis	D1	Methionine biosynthesis (proposed *cgs* gene)	Anilinopyrimidines (AP fungicides)	Mepanipyrim	9
Sterol biosynthesis in membranes	G1	C14-demethylase in sterol biosynthesis (*erg11*/*cyp51*)	Demethylation inhibitors (DMI fungicides)	Difenoconazole	3
				Fenarimol	3
				Myclobutanil	3
				Simeconazole	3
				Triflumizole	3
				Triforine	3
Cell wall biosynthesis	H4	Chitin synthase	Polyoxins	Polyoxin	19
Not specified	–	Unknown	Diverse	Pottasium bicarbonate	NC
				Sodium bicarbonate	NC
Chemicals with multi-site activity	M	Multi-site contact activity	Inorganic	Dodecylbenzenesulfonic acid bisethylenediamine copper complex salt (II) (DBEDC)	M01
				Sulfur	M02
			Bis-guanidines	Iminoctadine albesilate	M07
			Quinoxalines	Chinomethionate	M10
Biologicals with multiple modes of action: microbial	BM	Multiple effects described	Microbial	*Bacillus subtilis*	BM02
Respiration	C2, C3	Complex II: succinate dehydrogenase; Complex III: cytochrome bc1 at Qo site	SDHIs, QoI fungicides	Boscalid, kresoxim-methyl	7, 11
Chemicals with multi-site activity	M	Multi-site contact activity	Inorganic	Copper, sulfur	M01, M02
Chemicals with multi-site activity, respiration	M, C3	Multi-site contact activity, complex III: cytochrome bc1 at Qo site	Bis-guanidines, QoI fungicides	Iminoctadine albesilate, pyribencarb	M07, 11

Chemicals with multi-site activity, cell wall biosynthesis	M, H4	Multi-site contact activity, chitin synthase	Bis-guanidines, polyoxins	Iminoctadine albesilate, polyoxin complex	M07, 19
Chemicals with multi-site activity, cytoskeleton and motor protein	M, B6	Multi-site contact activity, actin/myosin/fimbrin function	Bis-guanidines, aryl-phenyl-ketones	Iminoctadine albesilate, pyriofenone	M07, 50
Not specified, chemicals with multi-site activity	U, M	Unknown, multi-site contact activity	Diverse, inorganic	Sodium hydrogen carbonate and copper wettable powder	NC, M01
Unknown, sterol biosynthesis in membranes	U, G1	Unknown, C14-demethylase in sterol biosynthesis	Phenyl-acetamide, DMI fungicides	Cyflufenamid, triflumizole	U06, 3
Unknown, amino acids and protein synthesis	U, D1	Unknown, methionine biosynthesis	Thiazolidine, AP fungicides	Flutianil, mepanipyrim	U13, 9
	–			Sorbitan fatty acid ester emulsion	–
	–			Polyglycerol fatty acid ester	–
	–			Propylene glycol fatty acid monoester	–
	–			Sodium oleate	–
	–			Decanoyl octanoyl glycerol	–

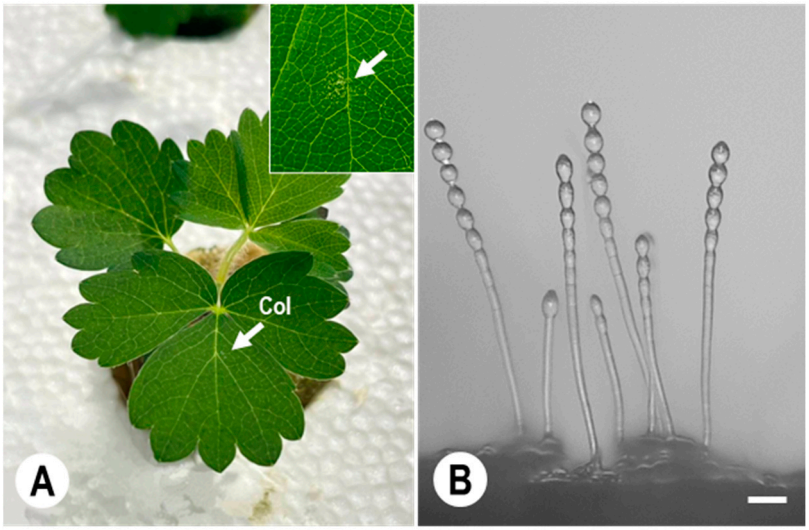

Figure 1. Single PM colonies (Col) that developed on true leaves of 60-day-old strawberry seedlings used for fungicide testing and for collecting asexual conidia with an electrostatic spore collector 3 days after fungicide spray treatment (**A**), and digital micrograph of conidiophores formed in 7-day-old *P. aphanis* colonies (**B**). Inserted photo shows enlargement of a 7-day-old *P. aphanis* colony. The scale bars are 20 μm. PM: powdery mildew. White arrows show single 7-day-old *P. aphanis* colonies.

2.4. Electrostatic Spore Collector

An electrostatic spore collector, a conductor plate (aluminum, 60 mm × 30 mm) between two insulator plates (polyethylene terephthalate, 80 mm × 50 mm), was used to collect all asexual conidia released from a single colony (Figure 2). The conductor (aluminum) plate was connected to an electric line from an HVA 10K202NA electrostatic voltage

generator (Logy Electric). Electric current was supplied from the voltage generator to the aluminum plate. The outer surface of the insulator plate was negatively polarized by the current from the voltage generator. The transparent insulator plate (collector plate), charged with static electricity of 5.2×10^{-1} nC (nanocoulombs), was placed approximately 2 cm from the single *P. aphanis* colony on a strawberry leaf to collect the asexual conidia released, as mentioned previously [33]. An electrostatic field was produced by the negative charge on the outer surface of the electrified insulator plate (negatively charged on the conidium-collection side), thus creating an attractive force for trapping asexual conidia that entered into the field. Asexual conidia from normal *P. aphanis* conidiophores were collected on the collector plate by the electrostatic attraction.

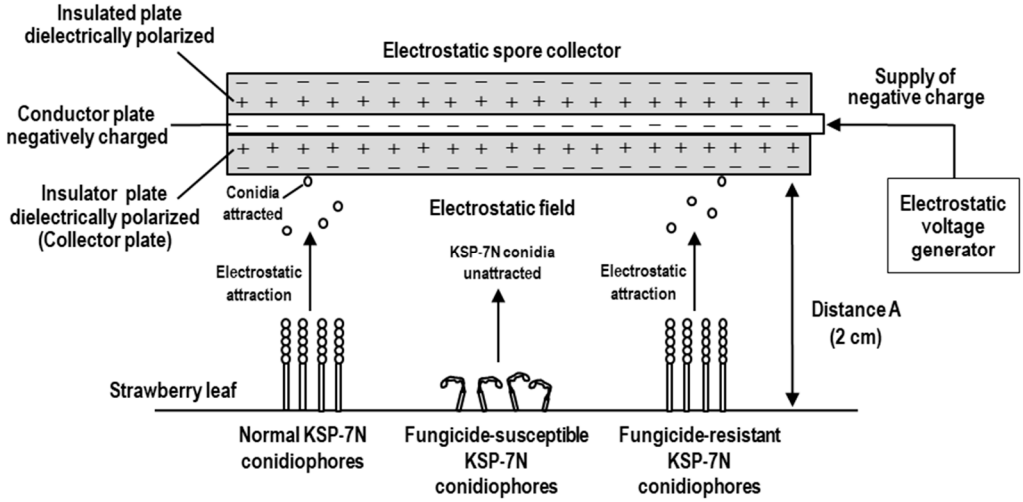

Figure 2. Electrostatic spore collector system for collecting asexual conidia released from single *Podosphaera aphanis* colonies with and without spray treatment with commercial fungicides (cross-sectional view). The collector plate (5.2×10^{-1} nC) was placed < 2 cm from a *P. aphanis* colony on a strawberry leaf (distance A). 'nC' means a unit of charge quantity.

2.5. Microscopic Observation of Single Conidiophores and Colonies Spray-Treated with Fungicides

Conidia of *P. aphanis* from the greenhouse were inoculated onto true leaves of 60-day-old strawberry seedlings and then incubated for 7 days under greenhouse conditions. The 7-day-old *P. aphanis* colonies were spray-treated with each fungicide. Three days later, conidiophores in colonies were observed using a KH-2700 DM and a BX-60 light microscope (BX-60 LM; Olympus, Tokyo, Japan). At first, the conidiophores were photographed using the ½-inch Interline transfer charge-coupled device (CCD) camera of the KH-2700 DM. Photographs were processed using an image-processing software of Adobe Photoshop version 5 (Adobe Systems, Mountain View, CA, USA). After the asexual conidia from single colonies spray-treated with each fungicide were electrostatically collected as mentioned above, leaf segments (size: approximately 2 cm × 2 cm) were cut from the KSP-7N-infected strawberry seedlings. The leaf segments were fixed, and then, their color was removed by boiling in an alcoholic lactophenol solution including 10 mL glycerol, 10 mL phenol, 10 mL lactic acid, 10 mL distilled water, and 40 mL 99.8% ethanol for 1 to 2 min. Then, the leaves were stained histochemically using 0.1% Aniline Blue (Nacalai Tesque, Tokyo, Japan) dissolved in distilled water, as described previously [29]. Subsequently, the stained *P. aphanis* colonies were observed under a BX-60 LM and then photographed with a digital camera (EOS KISSX6i; Canon, Tokyo, Japan) mounted on the light microscope. The total number of normal and abnormal conidiophores in the five individual 10-day-old colonies from each leaf was calculated. Mycelial areas were calculated using the

ImageJ software (National Institutes of Health, Bethesda, MD, USA). Data are presented as the mean ± the standard deviation (SD) of five replicates.

2.6. Number and Germination Rates of Asexual Conidia Collected Electrostatically from Single Colonies Spray-Treated with Fungicides

Asexual conidia on each strawberry leaf, which was attached to the plants, were electrostatically collected from individual single colonies 3 days after the application of fungicides with the insulator plate, as described above. They were counted with the KH-2700 DM system and calculated at one replication for a single colony. Data are presented as the mean and SD of five replicates (for each fungicide). After collection, the insulator plates were placed in a humid box (RH, 95–99%) and incubated at 22 ± 1 °C for 24 h. The number of conidia germinated was counted with the KH-2700 DM, and the germination rates were determined. Data are presented as the mean ± SD of five replicates (for each fungicide). In addition, to test the infectivity of conidia into host leaves, the asexual conidia trapped on the electrostatic insulator plate from single thiophanate-methyl-spray-treated colonies were inoculated onto leaf epidermal cells of healthy 60-day-old strawberry seedlings, spray-treated with the same fungicides in advance, and then incubated under growth chamber conditions. The development of the colonies was observed using the KH-2700 DM and BX-60 LM systems.

2.7. Statistical Analysis with Tukey's Test

Tukey's test was performed using the EZR software version 1.54 (Jichi Medical University, Saitama, Japan) to identify significant differences among (1) colony areas, (2) the number of normal conidiophores in 10-day-old single colonies, (3) the number of asexual conidia collected from single colonies spray-treated with each fungicide, and (4) the conidial germination rates.

3. Results

3.1. Effects of Fungicides on the Development of P. aphanis Colonies

Colony areas of *P. aphanis* isolate were measured 3 days after spray treatments with tap water or individual fungicides (Table 2). The areas of colonies spray-treated with pyraziflumid (succinate dehydrogenase inhibitors; SDHIs) were much smaller, whereas those spray-treated with thiophanate-methyl (methyl benzimidazole carbamates fungicides; MBC fungicides) were only a little smaller compared to those treated with the tap water (Table 2). Statistically, the six fungicides that included the active ingredients of pyraziflumid, triflumizole, triforine, polyoxin, sodium hydrogen carbonate and copper wettable powder and both flutianil and mepanipyrim highly inhibited *P. aphanis* colony development. On the other hand, polyglycerol fatty acid ester did not completely inhibit colony development, and kresoxim-methyl and decanoyl octanoyl glycerol provided only moderate inhibition (Table 2).

Table 2. Colony development of *Podosphaera aphanis* after spray treatment with individual fungicides, as assessed by counting asexual conidia trapped on electrostatically activated insulator plates, and the rates of conidial germination.

Active Ingredients	Concentrations (μg mL^{-1})	Colony Areas (mm^2) *	The Number of Conidia Released from Single Colonies *	Germination Rates (%)
Thiophanate-methyl	466.6	31.6 ± 3.5 cd	72.0 ± 5.0 b	79.0 ± 3.0 b
Pyriofenone	89.3	34.2 ± 3.0 c	0 i	–
Fluopyram	208.5	18.4 ± 3.0 fg	0 i	–
Isopyrazam	187	33.5 ± 2.3 c	0 i	–
Penthiopyrad	100	22.6 ± 2.0 ef	10.0 ± 1.0 e	0 d

Pyraziflumid	100	12.5 ± 3.2 g	0 i	–
Azoxystrobin	133	28.4 ± 2.6 de	36.5 ± 5.5 c	50.0 ± 10.0 c
Kresoxim-methyl	147	43.3 ± 3.4 b	0 i	–
Mepanipyrim	200	25.6 ± 1.4 e	1.0 ± 0.5 h	0 d
Difenoconazole	50	31.7 ± 3.1 cd	0 i	–
Fenarimol	30	17.2 ± 2.2 fg	0 i	–
Myclobutanil	50	16.0 ± 3.0 fg	0 i	–
Simeconazole	100	25.7 ± 2.8 def	0 i	–
Triflumizole	100	13.5 ± 3.8 g	0 i	–
Triforine	90	12.6 ± 2.2 g	0 i	–
Polyoxin	100	13.8 ± 1.7 g	0 i	–
Pottasium bicarbonate	1000	31.3 ± 3.4 cd	0 i	–
Sodium bicarbonate	1000	35.3 ± 2.8 c	19.0 ± 2.0 d	0 d
DBEDC	400	29.2 ± 1.8 d	7.0 ± 1.0 f	0 d
Sulfur	260	18.5 ± 2.4 fg	2.5 ± 1.0 h	0 d
Iminoctadine albesilate	150	20.8 ± 3.3 f	0 i	–
Chinomethionate	83.3	23.1 ± 3.6 ef	1.0 ± 0.5 h	0 d
Bacillus subtilis	1×10^8 CFU/g	17.9 ± 2.9 fg	0 i	–
Boscalid, kresoxim-methyl	133.5, 33.5	20.4 ± 3.1 f	0 i	–
Copper, sulfur	437.5, 312.5	22.1 ± 3.2 ef	0 i	–
Iminoctadine albesilate, pyribencarb	150, 100	23.8 ± 3.5 ef	0 i	–
Iminoctadine albesilate, polyoxin complex	62.5, 75	23.4 ± 3.1 ef	0 i	–
Iminoctadine albesilate, pyriofenone	150, 40	18.3 ± 2.3 fg	0 i	–
Sodium hydrogen carbonate and copper wettable powder	613.3, 400	14.2 ± 2.7 g	3.7 ± 2.3 gh	0 d
Cyflufenamid, triflumizole	17, 75	22.7 ± 3.2 ef	3.0 ± 2.0 gh	0 d
Flutianil, mepanipyrim	9, 100	13.9 ± 3.2 g	0 i	–
Sorbitan fatty acid ester emulsion	1400	22.4 ± 3.7 ef	0 i	–
Polyglycerol fatty acid ester	825	58.9 ± 3.5 a	0 i	–
Propylene glycol fatty acid monoester	350	24.7 ± 3.7 de	0 i	–
Sodium oleate	2000	18.6 ± 2.2 fg	4.0 ± 3.0 gh	0 d
Decanoyl octanoyl glycerol	3000	38.2 ± 2.8 bc	5.0 ± 1.0 g	0 d
Control (water)	–	59.5 ± 2.6 a	188.5 ± 21.5 a	85.5 ± 9.0 a

* Data show colony areas spray-treated with fungicides, and the number of asexual conidia collected using the electrostatic spore collector from individual 10-day-old colonies 3 days after spray treatment with fungicides. Single colonies were spray-treated with tap water, as a control. Data represent the mean and standard deviation of five replicates. Different letters in each column indicate significant differences ($p < 0.05$, Tukey's test).

3.2. Effects of Fungicides on Morphology of P. aphanis Conidiophores

Figure 3 shows the development of single *P. aphanis* colonies (7-day-old) spray-treated with tap water as a control and with pyraziflumid (SDHI) and thiophanate-methyl (MBC fungicides). Untreated *P. aphanis* conidiophores that formed in the colonies were concatenated. The morphology of *P. aphanis* colonies spray-treated with thiophanate-methyl (C) was clearly different from that spray-treated with pyraziflumid (B), while similar to that spray-treated with tap water (A). In addition, almost all *P. aphanis* conidiophores treated with tap water (D) and most of those treated with thiophanate-methyl (F) were normal, while almost all those treated with pyraziflumid (E) were abnormal (disruptive). The number of normal conidiophores in a single colony spray-treated with fungicides that included active ingredients such as pyraziflumid significantly decreased compared to those treated with tap water (Table 3). Those spray-treated with fungicides containing thiophanate-methyl showed higher numbers than those treated with the other tested

fungicides (Table 3). In the next experiments, *P. aphanis* colonies were histochemically stained to observe the morphologies and cytological characteristics of fungal cells forming normal (Figure 4A) and abnormal (Figure 4B–H) conidiophores. The seven abnormal (i.e., collapsed) conidiophores were classified into seven types (Figure 4B–H) based on their morphological and cytological characteristics. Table 3 shows the distributions of normal and abnormal morphologies of conidiophores in single colonies spray-treated with fungicides, based on the cytological characteristics of the histochemically stained conidiophores mentioned as footnotes to Figure 4. Particularly, the number of abnormal conidiophores in *P. aphanis* colonies spray-treated with thiophanate-methyl was ca. 18%, while that with nine fungicides including pyraziflumid was higher by >50% compared to those treated with the other tested fungicides (G in Table 3). Consequently, the conidiophores had different morphologies and cytological characteristics, even when spray-treated with the related fungicides. The six effective fungicides mentioned under paragraph 3.1 all reduced colony sizes to less than 14 mm^2, but all showed comparatively high percentages of category G damage (above 33%). The distribution was clearly different among the fungicides tested after three days.

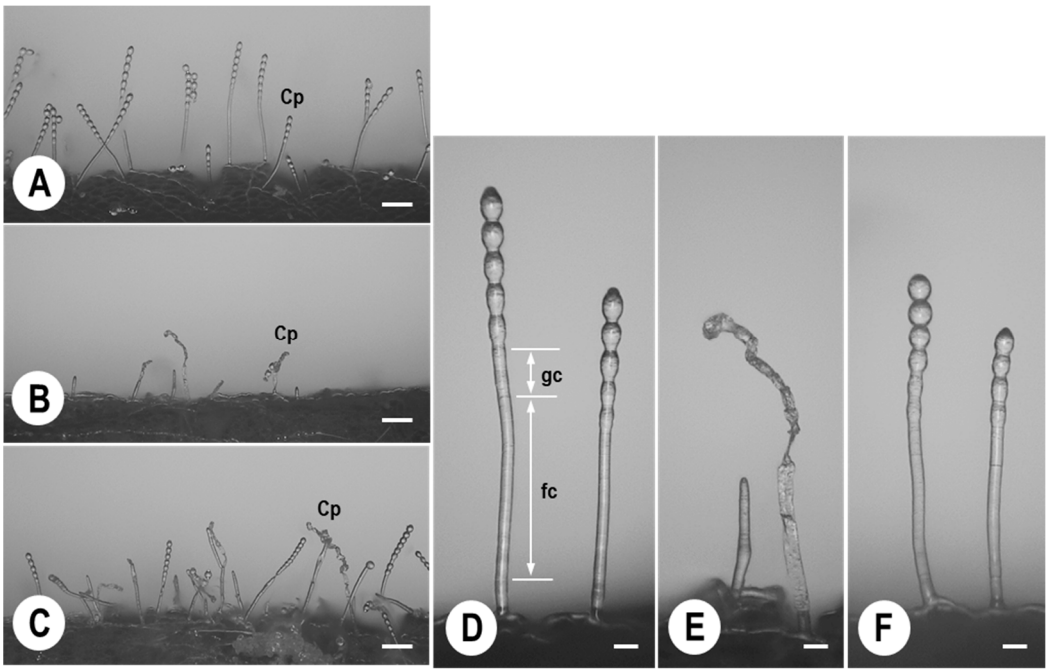

Figure 3. Digital micrographs of *Podospharea aphanis* colonies and conidiophores in the colonies spray-treated with tap water (**A**,**D**), pyraziflumid (**B**,**E**), and thiophanate-methyl (**C**,**F**). Note that normal *P. aphanis* conidiophores possess a maximum number of six conidial cells in full-length chains, a generative cell (gc), and a foot cell (fc) on hyphae under greenhouse conditions. The scale bars are 100 μm (**A**–**C**) and 20 μm (**D**–**F**). Cp: conidiophore.

Table 3. Conidiophore shape of *Podosphaera aphanis* after spray treatment with individual fungicides.

Active Ingredients	The Number of Normal or Abnormal Conidiophores (%)							
	A *	**B**	**C**	**D**	**E**	**F**	**G**	**H**
Thiophanate-methyl	70.0 ± 5.5 b	0.6 ± 0.5	3.8 ± 2.8	7.0 ± 6.0	1.6 ± 1.2	3.8 ± 2.6	18.8 ± 11.7	0
Pyriofenone	0.6 ± 0.4 i	3.0 ± 1.4	32.6 ± 11.6	7.0 ± 3.7	8.8 ± 3.1	41.8 ± 8.2	13.6 ± 8.8	0.6 ± 0.3
Fluopyram	1.8 ± 1.6 ghi	4.6 ± 3.5	24.0 ± 11.5	15.2 ± 8.3	28.6 ± 9.8	2.0 ± 0.8	20.2 ± 3.7	3.6 ± 1.9
Isopyrazam	0.8 ± 0.2 i	1.8 ± 0.7	2.6 ± 1.7	1.0 ± 0.2	11.4 ± 1.0	8.8 ± 4.8	67.0 ± 17.8	4.2 ± 1.8
Penthiopyrad	16.0 ± 3.8 d	5.2 ± 2.9	42.6 ± 17.5	5.6 ± 1.4	5.6 ± 1.4	1.4 ± 0.5	22.2 ± 9.8	1.0 ± 0.2
Pyraziflumid	0 j	18.2 ± 5.6	12.4 ± 7.8	2.0 ± 0.1	11.6 ± 5.5	3.4 ± 1.6	50.2 ± 32.1	1.6 ± 0.6

Azoxystrobin	38.8 ± 4.3 c	2.6 ± 0.9	29.2 ± 11.0	5.2 ± 1.2	5.4 ± 1.6	1.6 ± 0.8	28.0 ± 10.4	1.2 ± 0.3
Kresoxim-methyl	0.8 ± 0.2 i	0.6 ± 0.2	23.0 ± 4.3	2.4 ± 0.9	0.4 ± 0.3	11.6 ± 7.4	29.2 ± 11.9	30.0 ± 12.9
Mepanipyrim	4.2 ± 1.7 fg	3.8 ± 1.8	14.6 ± 6.0	5.4 ± 2.8	23.2 ± 10.5	3.6 ± 1.3	40.4 ± 21.8	0.8 ± 0.2
Difenoconazole	2.0 ± 0.5 h	17.0 ± 7.4	24.2 ± 10.8	20.4 ± 7.5	12.8 ± 9.5	2.6 ± 0.6	19.4 ± 8.0	0.6 ± 0.3
Fenarimol	0 j	17.6 ± 8.1	35.6 ± 11.8	30.4 ± 8.0	2.0 ± 0.1	0	14.4 ± 8.8	0
Myclobutanil	0.6 ± 0.3 i	17.8 ± 11.1	8.8 ± 4.9	14.8 ± 8.7	3.6 ± 1.2	16.8 ± 4.6	31.6 ± 10.5	0
Simeconazole	1.8 ± 0.4 h	0	17.0 ± 1.1	0	30.8 ± 4.8	1.0 ± 0.4	45.4 ± 11.4	0
Triflumizole	3.4 ± 1.3 gh	0	5.2 ± 1.6	9.8 ± 3.2	26.2 ± 12.0	11.4 ± 1.8	39.2 ± 11.3	0.8 ± 0.2
Triforine	1.6 ± 0.2 h	7.2 ± 1.9	5.2 ± 2.2	3.6 ± 2.1	2.0 ± 0.7	2.0 ± 0.5	76.8 ± 12.1	0
Polyoxin	1.8 ± 0.8 h	5.6 ± 2.5	14.4 ± 4.9	5.2 ± 1.1	2.2 ± 0.9	5.0 ± 1.8	54.6 ± 17.8	1.2 ± 0.8
Pottasium bicarbonate	0.9 ± 0.1 i	16.4 ± 8.6	23.0 ± 10.7	4.0 ± 1.0	2.2 ± 1.9	17.2 ± 6.4	28.2 ± 7.4	2.6 ± 0.8
Sodium bicarbonate	31.4 ± 5.7 c	4.0 ± 0.2	11.4 ± 5.7	5.2 ± 1.7	7.6 ± 2.0	4.0 ± 1.2	30.4 ± 15.4	0.2 ± 0.2
DBEDC	15.0 ± 2.5 d	9.8 ± 6.9	18.8 ± 5.9	5.2 ± 2.4	8.0 ± 5.1	2.6 ± 1.5	41.6 ± 9.0	1.0 ± 1.0
Sulfur	4.6 ± 1.6 fg	0.4 ± 0.2	21.4 ± 7.5	6.6 ± 3.5	28.2 ± 13.6	0.4 ± 0.3	31.6 ± 14.5	0
Iminoctadine albesilate	0 j	2.8 ± 1.0	11.6 ± 1.3	14.0 ± 7.3	6.2 ± 1.3	0	61.6 ± 15.8	3.8 ± 1.4
Chinomethionate	4.2 ± 1.0 g	10.6 ± 4.8	18.4 ± 6.1	6.6 ± 1.3	10.8 ± 1.6	14.8 ± 6.6	33.2 ± 9.8	1.4 ± 0.9
Bacillus subtilis	3.4 ± 1.0 gh	2.4 ± 0.4	12.4 ± 1.8	14.2 ± 9.8	5.6 ± 2.1	15.6 ± 8.4	39.4 ± 14.1	2.4 ± 0.6
Boscalid, kresoxim-methyl	0.4 ± 0.2 i	6.6 ± 2.1	7.6 ± 3.2	19.8 ± 5.3	16.2 ± 9.3	24.2 ± 11.3	25.4 ± 13.3	0.2 ± 0.1
Copper, sulfur	1.0 ± 1.0 hi	8.4 ± 5.8	11.4 ± 3.8	3.0 ± 2.1	11.0 ± 5.2	20.8 ± 4.4	44.4 ± 12.9	0
Iminoctadine albesilate, pyribencarb	2.0 ± 1.5 ghi	19.6 ± 8.1	18.6 ± 10.8	10.0 ± 8.8	3.2 ± 2.6	6.2 ± 3.5	38.2 ± 10.8	1.2 ± 0.8
Iminoctadine albesilate, polyoxin complex	0.6 ± 0.2 i	1.0 ± 1.0	6.2 ± 2.6	0.2 ± 0.2	1.0 ± 1.0	0.8 ± 0.6	84.4 ± 13.5	1.8 ± 1.6
Iminoctadine albesilate, pyriofenone	2.8 ± 1.4 gh	9.4 ± 7.9	11.8 ± 9.5	3.6 ± 3.0	4.0 ± 1.5	10.2 ± 4.1	57.0 ± 13.6	1.2 ± 1.2
Sodium hydrogen carbonate and copper wettable powder	9.4 ± 2.2 e	8.4 ± 3.2	16.6 ± 8.8	0	4.0 ± 3.9	0	59.2 ± 16.6	0
Cyflufenamid, triflumizole	7.2 ± 1.6 ef	0	14.6 ± 9.3	0	1.4 ± 1.1	0	76.8 ± 31.8	0
Flutianil, mepanipyrim	0.4 ± 0.4 i	4.2 ± 2.4	18.2 ± 6.3	36.0 ± 14.1	0.6 ± 0.5	4.0 ± 4.0	33.8 ± 8.9	2.8 ± 1.9
Sorbitan fatty acid ester emulsion	2.8 ± 1.3 gh	5.8 ± 2.3	17.0 ± 3.7	9.6 ± 2.8	2.0 ± 1.9	31.0 ± 14.7	26.8 ± 11.3	3.0 ± 3.0
Polyglycerol fatty acid ester	0.6 ± 0.5 hi	0.4 ± 0.4	20.4 ± 11.7	9.0 ± 6.0	10.4 ± 2.1	14.0 ± 7.9	44.6 ± 12.2	0.6 ± 0.5
Propylene glycol fatty acid monoester	2.4 ± 1.7 ghi	4.2 ± 1.3	33.4 ± 9.1	4.6 ± 1.1	27.8 ± 19.8	3.0 ± 1.7	17.8 ± 8.8	3.6 ± 1.1
Sodium oleate	12.0 ± 2.0 de	10.0 ± 8.0	10.8 ± 8.0	5.4 ± 1.4	3.2 ± 1.6	1.4 ± 0.5	49.2 ± 18.1	0
Decanoyl octanoyl glycerol	13.8 ± 3.1 de	18.2 ± 6.8	8.2 ± 4.5	9.0 ± 2.7	5.2 ± 3.9	12.2 ± 5.9	35.6 ± 19.1	1.8 ± 1.8
Control (water)	98.8 ± 0.4 a	–	–	–	–	–	–	–

* A to H represents the morphological and cytological characteristics of conidiophores mentioned in the legends of Figure 4. The numbers of normal and abnormal conidiophores in the five individual 10-day-old colonies from each leaf were counted and calculated on average. Single colonies were spray-treated with tap water, as a control. Data represent the mean and standard deviation of five replicates, calculated for each category (A–H). Different letters in each column indicate significant differences ($p < 0.05$, Tukey's test).

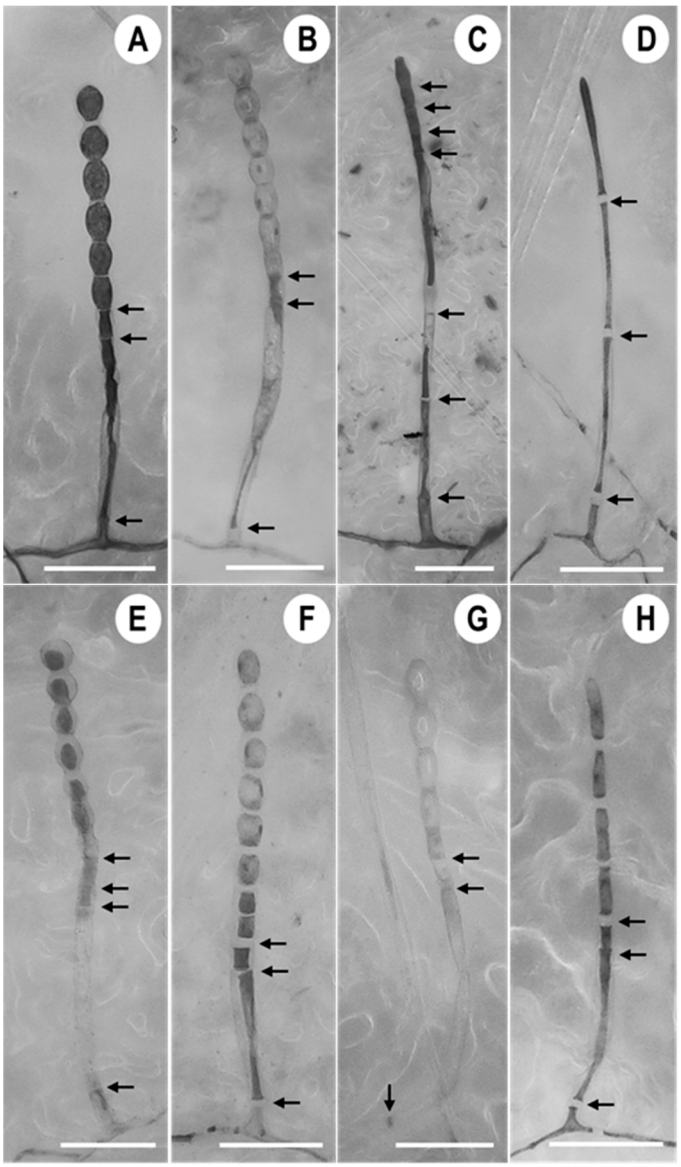

Figure 4. Light micrographs of *Podosphaera aphanis* conidiophores histochemically stained for observation of morphological and cytological characteristics of the fungal cells after spray treatment of tap water and fungicides. (**A**) Normal catenescent conidiophores (concatenated conidia in chains) successfully stained in *P. aphanis* colonies. (**B**) Collapse of cell membranes and granulated cells in conidiophores while maintaining the shape of ellipsoid-ovoid conidia. (**C**) Atrophy and collapse of conidiophores. (**D**) Linear conidiophores. (**E**) Separation of cell membranes while maintaining the shape of globular conidia. (**F**) Separation of cell membranes and granulated cells in conidiophores while maintaining the shape of ellipsoid-ovoid conidia. (**G**) Hyaline cells by collapse of cell membranes while maintaining the shape of ellipsoid-ovoid conidia. (**H**) Thin conidiophores without dividing and enlarging cells. Arrows show septa (septal wall). The micrographs of histochemically stained conidiophores were taken 3 days after spray treatment with tap water and fungicides. The scale bars are 60 μm.

3.3. Collection of Asexual Conidia from Single P. aphanis Colonies Spray-Treated with Fungicides Using an Electrostatic Spore Collection Technique

Asexual conidia were collected using an electrostatic spore collection technique from individual single *P. aphanis* colonies, spray-treated with tap water or fungicides. In single colonies spray-treated with tap water, the asexual conidia, fully constricted at the top of conidiophores (catenescent conidia), were attracted to the plate when the insulator plate was negatively polarized (Figure 5). Figure 5A,B show digital micrographs of *P. aphanis*

colonies spray-treated with tap water, before and after negative electrification of the insulator plate, respectively. Asexual conidia (arrows at the top of picture in B) were successfully captured on the insulator plate from single tap water-spray-treated colonies after negative electrification. For example, in single *P. aphanis* colonies spray-treated with pyraziflumid, no conidia were attracted from the single colonies to the plate, even when the insulator plate was negatively polarized. In contrast, in single colonies spray-treated with thiophanate-methyl, conidia were attracted from the single colonies. Consequently, the number of asexual conidia released from PM conidiophores varied, depending on the active ingredients included in the fungicides.

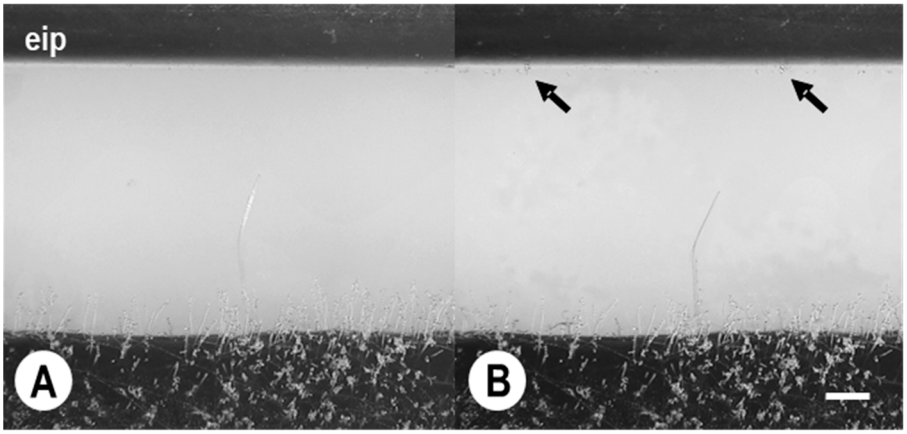

Figure 5. Collection of asexual conidia released from single 10-day-old *Podosphaera aphanis* colonies, spray-non-treated and spray-treated with fungicides, on true leaves of living strawberry seedlings using an electrostatic spore collection technique (an electrostatic insulator plate; eip). The insulator plate was placed approximately 2500 μm from the apex of colonies. (**A**,**B**) show collection of asexual conidia from *P. aphanis* colonies spray-treated with tap water, before and after negative electrification to eip, respectively. Bar represents 400 μm.

3.4. Germination Rates of Conidia Electrostatically Collected from P. aphanis Colonies Spray-Treated with Fungicides

The size and shape of asexual *P. aphanis* conidia electrostatically collected from single colonies spray-treated with thiophanate-methyl were 28.3 ± 2.3 μm × 19.8 ± 1.4 μm and ellipsoid–ovoid to doliiform–limoniform, respectively (Figure 6A). In addition, they contained oil and fibrosin bodies (Fb). After the conidia were electrostatically collected from individual single colonies spray-treated with fungicides, the germination rates were measured (Table 2). Particularly, asexual conidia collected from single colonies spray-treated with azoxystrobin next to thiophanate-methyl had high germinative capacity. Figure 6B shows germination of conidia electrostatically collected from single colonies spray-treated with thiophanate-methyl, and formation of appressoria (Ap) at the tip of germ tubes. On the other hand, conidia electrostatically collected from single colonies spray-treated with the other fungicides did not germinate at all.

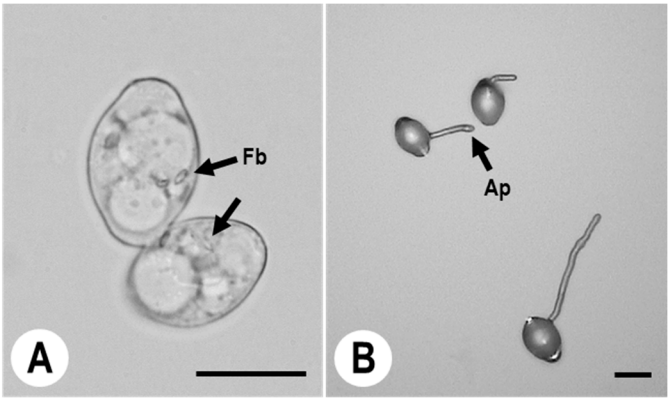

Figure 6. Characteristics of thiophanate-methyl-resistant *Podosphaera aphanis* conidia attracted from a single *P. aphanis* colony to the insulator plate with an electrostatic technique. The scale bars are 20 μm (**A**,**B**).

3.5. Development of Single P. aphanis Conidia on Thiophanate-Methyl-Treated Strawberry Leaves

Conidia germinated 3 to 5 h after inoculation on thiophanate-methyl-treated strawberry leaves and formed primary appressoria 6 to 8 h after inoculation. They began to produce colony-forming hyphae from the conidial bodies 24 h after inoculation, and hyphae continued to elongate on the leaves. The isolate began to form conidiophores from elongated hyphae 5 to 6 days after inoculation (Figure 7).

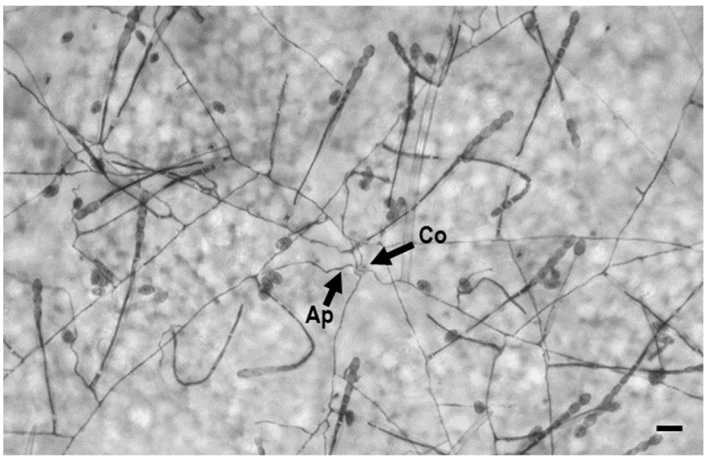

Figure 7. Light micrograph of hyphae developed from thiophanate-methyl-resistant *Podosphaera aphanis* conidia after asexual conidia electrostatically collected were inoculated onto healthy strawberry leaf spray-treated with thiophanate-methyl (1500 dilutions; 466.6 μg mL^{-1}) in advance. The scale bar is 40 μm. Co: conidium; Ap: appressorium.

4. Discussion

In several previous studies, *P. aphanis* developed resistance to DMI fungicides (nuarimol, fenarimol, tetraconazole, triadimefon, and triadimenol), SDHI (penthiopyrad), and aza-naphthalenes (quinoxyfen) [21,22,24,34,35]. In Japan (Nara, Miyagi, and Shiga Prefecture), strawberry PM fungi show low sensitivity (i.e., resistance) to DMI fungicides (bitertanol, fenarimol, triforine, and triadimenol) [23,36]. Recently, Ishii [37] reported the development of resistance to quinone outside inhibitors (QoI fungicides) in strawberry PM fungi. In the present study, we clarified that *P. aphanis* has developed resistance to both thiophanate-methyl (MBC fungicides) and azoxystrobin (QoI fungicides) using the electrostatic technique. Therefore, it is important to clarify the effects of various fungicides against strawberry PM fungi via sensitivity tests. We evaluated the sensitivity of *P. aphanis* to different fungicides to determine the effectiveness of the treatments. After fungicide

application, colony areas of *P. aphanis* isolates varied depending on the fungicide treatment. Six fungicides, including pyraziflumid, triflumizole, triforine, polyoxin, sodium hydrogen carbonate + copper wettable powder, and flutianil + mepanipyrim, were highly effective at inhibiting colony development. Therefore, the evaluation of colony size may be useful for identifying effective fungicides.

The morphological and ecological characteristics of conidiophores in PM colonies sprayed with fungicides were observed in detail, as the conidiophores are infectious structures that release asexual conidia, thus facilitating the spread of PM diseases. In the present study, most of the tested fungicide applications resulted in abnormal morphological changes in conidiophores and conidial cells in the conidiophores of *P. aphanis* isolates. However, thiophanate-methyl, azoxystrobin, sodium bicarbonate, and DBEDC were less effective. Taken together, these results indicate that most of the fungicides, despite their different modes of action, have a temporary effect against *P. aphanis*. To the best of our knowledge, no study has detailed the morphological characteristics of conidiophores of strawberry PM fungi spray-treated with fungicides. In nature, *P. aphanis* (KSP-7N) forms sets of six linked conidia on conidiophores, with a straight and non-bulky generative cell [14].

The germination rates of asexual conidia collected electrostatically from single colonies spray-treated with thiophanate-methyl were the highest among the fungicides tested in this study. This means that the collected asexual conidia survived successfully without largely affecting the electrostatic forces or the effective components (active ingredients) of the fungicides. In addition, we clarified that the conidia collected on the insulators maintain their high germinative capacity [29]. Thus, this electrostatic spore collection method appears to be a superior method for estimating the spread of germinable PM conidia that do not respond to fungicides. In actual, a negatively charged dielectric body (insulator plate) is used to electrostatically induce the PM fungi, instead of charging the surface (surrounding) of the PM fungi (i.e., conidia) with static electricity, by spray-treating with the fungicides (some ingredients) having anti-static properties. Consequently, the PM fungi become net positive and, due to Coulomb force, are captured by a plate that is oppositely charged [38]. Eventually, using the electrostatic technique, the asexual conidia are successfully collected from fungicide-spray-treated PM colonies without being affected by some ingredients (i.e., ionic surfactant sodium oleate) of the fungicides having anti-static properties. Thus, we suggested that the collection of asexual conidia was inhibited by lack of conidial releases from unconstructed conidial cells at the upper parts of conidiophores or abnormal conidiophores (i.e., atrophied conidiophores).

Most studies have assessed the progress of strawberry PMs by recording the incidence or severity of disease on leaves [9,17,39–41]. For example, of the strawberry PM isolates that received fungicide application, Okayama et al. [17] inoculated the leaves and plants with strawberry PM fungi and recorded the number of leaflets that were infected by the disease and the severity of the disease in each leaflet; each leaflet was scored from one (no visible symptoms) to five (more than 50% of the surface of the leaf infected). Recently, Palmer and Holmes [35] defined the disease incidence as the presence of a sporulating colony on a leaflet and assigned one of five categories, depending on the disease incidence for each fungicide treatment: entirely sensitive (0%), sensitive (0.1–33.2%), somewhat sensitive (33.3–66.6%), resistant (66.7–99%), and entirely resistant (100%). Thus, most of the sensitivity tests in these earlier studies were conducted with the inoculation of *P. aphanis* after fungicide application to the leaflets, leaves, or leaf discs [17,24]. In contrast, with our method, inoculation was conducted before fungicide application. However, in general, there is little information on the best way to measure the progress of strawberry PM disease, as pointed out by Menzel [42], who emphasized the need to determine the variation in the severity of PM within and across experiments and the best way to record the development of the disease. In the present study, we included colony growth and the number of asexual conidia released from single PM colonies spray-treated with fungicides to assess fungicide sensitivity. In nature, PMs expand the infection source by releasing

progeny conidia from conidiophores [43,44]. Previously, using an electrostatic technique (electrostatically activated insulator drum), Ayabe et al. [29] conducted a quantitative analysis of the number of progeny conidia dispersed from living single colonies of *P. aphanis* ex *Fragaria* × *ananassa* Duchesne ex Rozier cv. Sagahonoka throughout their lifetime. In the present study, by collecting the asexual conidia released from single fungicide-treated *P. aphanis* colonies with an electrostatic technique (electrostatically activated insulator plate), we clarified the existence of normal *P. aphanis* conidiophores and the production of asexual conidia by individual single colonies, and then quantitatively evaluated the sensitivity of strawberry PM fungi to fungicides. We observed sharp differences among the number of asexual conidia released from fungicide-treated colonies, based on the electrostatically collected data. Notably, isolates of strawberry PM colonies that the fungicides did not control released asexual conidia. Moreover, we confirmed that thiophanate-methyl (MBC fungicides) and azoxystrobin (QoI fungicides) do not adequately control colony size of *P. aphanis* isolates, because the isolates produce germinable conidia on normal conidiophores, regardless of the fungicide treatment. Consequently, we clarified that *P. aphanis* has developed resistance to both thiophanate-methyl and azoxystrobin. In the *P. aphanis* Japanese isolates (KSP-7N), no active fungal growth to aryl-phenyl-ketones, SDHIs, anilino-pyrimidines (AP fungicides), DMIs, polyoxins, bis-guanidines, or quinoxalines was observed.

To the best of our knowledge, this study reports the first methodology to precisely count the total number of asexual conidia released from single *P. aphanis* colonies spray-treated with individual commercial fungicides for estimating the sensitivity of *P. aphanis* to fungicides.

5. Conclusions

This study clarified the number of asexual conidia released from single *P. aphanis* colonies, spray-treated with individual fungicides (fungicides with different modes and mechanisms of action), using an electrostatic spore collector (an insulator plate). Thus, the electrostatic technique used in this study is expected to be useful for quantitatively analyzing conidial releases from single PM colonies, spray-treated with equivalent fungicide concentrations, to identify the most effective fungicides against strawberry PM disease. Also, more importantly, this electrostatic spore collection technique enables the accurate assessment of the presence or absence of infection expansion from PM colonies spray-treated with individual fungicides. The asexual conidia collected from *P. aphanis* colonies, spray-treated with thiophanate-methyl, germinated, and then infected with host leaves spray-treated with the same fungicides in advance. Based on our results, we suggest that electrostatic and digital microscopic techniques are useful for the early detection of PM fungi. As next steps, we will analyze the gene expression of the mutated β-tubulin gene, to gain insight into site-specific fungicide resistance in single conidia electrostatically collected from PM colonies for which the fungicides did not work, to determine the fungicide-resistant mechanisms of PM fungi. From the perspective of estimating the fungicide sensitivity of PM fungi by collecting germinable conidia with our electrostatic technique, this study provides new information on the ecological characteristics of strawberry PM fungi (*P. aphanis* var. *aphanis*) in host plants and the acquired fungicide resistance.

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Conflicts of Interest: The authors declare no conflicts of interest.

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