



# Innovative assessment of the fungicide sensitivity of the melon powdery mildew fungus, *Podosphaera xanthii*, on host leaves using electrostatic and digital microscopy techniques

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## Abstract

Powdery mildew (PM) fungi expand their infection of host plants by releasing progeny conidia, which form colonies that become subsequent sources of infection. In this study, 36 commercial fungicides and one biofungicide were sprayed on single 7-day-old colonies of melon PM fungi (*Podosphaera xanthii*) infecting the leaves of host seedlings (*Cucumis melo* L.). To quantitatively estimate the effectiveness of fungicides on Japanese melon PM isolates (*P. xanthii* KMP-6N) and to establish a fungicide sensitivity evaluation method, asexual conidia produced from single KMP-6N colonies spray-treated with different types of fungicides in a natural environment were collected using a dielectrically polarized insulator plate. Most of the tested fungicides strongly inhibited the production and/or germination of asexual conidia, determined as the amount of attraction to the insulator plate. However, the attracted conidia exhibited high germination rates in the presence of kresoxim-methyl, a quinone outside inhibitor fungicide; fenarimol and triforine, which are demethylation inhibitor fungicides; and thiophanate-methyl, a methyl benzimidazole carbamate fungicide. Moreover, the electrostatically collected conidia of fungicide-resistant KMP-6N colonies developed normally on host leaves previously spray-treated with the same fungicide. The morphologic and cytological characteristics of the conidiophores of single KMP-6N colonies spray-treated with the different fungicides were observed. Abnormal conidiophores were classified into five types based on their characteristics. These results demonstrate that methodologies assessing the number of conidia released from single colonies, conidial germination rates, and colony development in the early fungal growth stages can facilitate the screening of effective fungicides against cucurbit PMs, as well as the estimation of PM fungicide sensitivity.

**Keywords** Conidiophores · DMI-fungicides · Electrostatic spore collectors · MBC-fungicides · QoI-fungicides

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## Introduction

Powdery mildew (PM) fungi are obligate biotrophic fungal pathogens belonging to order Erysiphales that infect approximately 10,000 plant species (Braun and Cook 2012). PM fungi include *Podosphaera xanthii* (syn. *Sphaerotheca fuliginea* and *Sphaerotheca fusca*), which poses a serious threat to Cucurbitaceae plants worldwide (Reifschneider et al. 1985; Mohamed et al. 1995; Del Pino et al. 2002; Křístková et al. 2004; Tomason and Gibson 2006; Pérez-García et al. 2009; Hong et al. 2018). Cucurbit PMs mainly occur on the surfaces of epigeal organs, including the leaves and stems (Cohen et al. 2004). A severe outbreak of PM on host plants can significantly reduce yields (McGrath and Thomas 1996; Pérez-García et al. 2009). In Japan, PM frequently occurs on the leaves of melon plants (*Cucumis melo* L.) cultivated hydroponically in greenhouses. In a previous study, we examined the pathogenicity of a Japanese melon PM isolate for other plant species (Takikawa et al. 2015). The results showed that Cucurbitaceae plants were susceptible to the isolate, whereas plants of other genera were resistant, suggesting a narrow host range of melon PM fungi.

Chemical methods are commonly used in Japan and elsewhere to control cucurbit PM diseases (Ishii et al. 2001; Matheron and Porchas 2007). Typically, farmers immediately spray-treat plant leaves with commercial fungicides to control PM following its detection. However, if fungicide treatment is insufficient, host plants will be incompletely protected. Eventually, the fungi produce progeny conidia from normal conidiophores. The wind-mediated dispersal of mature conidia to other host plants leads to the further dissemination of PM over large areas (Aylor 1990; Brown and Hovmöller 2002). On the other hand, frequent fungicide application can promote the appearance of resistant PM strains. Therefore, the use of fungicides as the principal management practice in the control of PMs must comply with the registered spray dose and recommended number of applications. Rotation of the fungicide with other fungicides with different modes of action, such as fungal respiration targeting, nucleic acid synthesis, sterol biosynthesis, and signal transduction (FRAC Code List 2024), is essential to avoid the development of resistance. However, many PM fungal strains have become resistant, or insensitive, to the active ingredients of the major commercial fungicides used on cucurbit plants both within and outside Japan (Huggenberger et al. 1984; Ishii et al. 2001; McGrath and Shishkoff 2003; Lebeda and Sedláková 2008). For example, isolates or samples of *P. xanthii* have been detected to be resistant to methyl benzimidazole carbamate (MBC) fungicides (benomyl and thiophanate-methyl) (Schroeder and Provvidenti 1969; Peterson 1973; Petsikos-Panayotarou 1977; McGrath et al. 1996; Bellón-Gómez et al. 2014), demethylation

inhibitor (DMI) fungicides (bitertanol, fenarimol, imazalil, triadimefon, triforine, myclobutanil, propiconazole, and triadimenol) (Schepers 1983; Huggenberger et al. 1984; McGrath et al. 1996; López-Ruiz et al. 2010), hydroxypyrimidines (dimethirimol) (Bent et al. 1971), phosphorothiolates (pyrazophos) (Dekker and Gielink 1979), quinone outside inhibitor (QoI) fungicides (azoxystrobin, kresoxim-methyl, and trifloxystrobin) (McGrath and Shishkoff 2003; Fernández-Ortuño et al. 2006), succinate-dehydrogenase inhibitors (SDHIs) (boscalid) (McGrath 2008), aza-naphthalenes (quinoxifen) (McGrath 2017), phenyl-acetamide (cyflufenamid) (Pirondi et al. 2014), and ditalimfos (Malathrakakis 1985). In Japan, *P. xanthii* has developed low sensitivity, i.e., resistance, to DMI fungicides (bitertanol, fenarimol, triadimefon, and triflumizole) (Ohtsuka et al. 1988), QoI fungicides (Ishii et al. 2001), and SDHIs (Miyamoto et al. 2010, 2020b; Ishii et al. 2011), as well as fungicides in the phenyl-acetamide group (cyflufenamid) (Hosokawa et al. 2006), cyano-methylene-thiazolidine group (flutianil), and aryl-phenyl-ketone group (pyriofenone) (Miyamoto et al. 2020a). DMI fungicides inhibit the C14 demethylation of lanosterol or eburicol, an important step in fungal sterol biosynthesis (ergosterol or methylene-24-cholesterol), by inhibiting cytochrome P450 14 $\alpha$ -demethylase, leading to the disruption of membrane function and dysregulation of the cell cycle (Lepesheva and Waterman 2007). QoI fungicides inhibit mitochondrial respiration by binding to the external quinone of the cytochrome *bc1* enzyme complex (complex III), which blocks electron transfer in the respiration pathway and thereby causes an energy deficiency due to a lack of ATP (Becker et al. 1981). SDHIs inhibit fungal respiration by suppressing a succinate dehydrogenase (complex II) in the mitochondrial electron transport chain (Matsson and Hederstedt 2001). Another concern is that plant leaves retain fungicides not completely decomposed by microorganisms. To avoid both resistance and retention requires the development of novel, eco-friendly control methods beyond the chemical methods currently used to control PMs. We developed a simple method to quantitatively estimate the sensitivity of melon PM isolate to commercial fungicides. Specifically, the number of asexual conidia released from single PM colonies on melon plants (*Cucumis melo* cv. ‘Earl’s Favourite’) spray-treated with individual fungicides was determined using a combination of digital microscopy and electrostatic techniques.

Takikawa et al. (2015) demonstrated the use of digital microscopy to observe the hyphal morphology and development of living PM fungi on host leaves without the need for fixation or histochemical staining. Matsuda et al. (2006) showed that asexual conidia in an electric field are attracted to and subsequently trapped by polarized dipole insulators. This strategy has been successfully applied to collect

asexual conidia from living single PM colonies, such as barley, melon, strawberry, and Japanese tomato PM isolates, without harming or damaging the conidia (Moriura et al. 2006; Nonomura et al. 2009; Suzuki et al. 2019; Ayabe et al. 2022). Recently Takahara et al. (2024) confirmed the fungicide-sensitivity of a Japanese strawberry PM isolate (*P. aphanis* KSP-7N) by collecting asexual conidia with the electrostatic technique (i.e., a dielectric insulator plate). Moreover, using microscopes, Takahara et al. (2024) confirmed colony development and conidiophore morphology of the strawberry PM isolate varied depending on the fungicides tested.

The aims of the present study were (i) to determine the number of asexual conidia electrostatically collected from single melon PM colonies spray-treated with different commercial fungicides, (ii) predict the colony development of fungicide-resistant melon PM fungi on host leaves previously treated with the fungicides, and (iii) evaluate the effects of commercial fungicides on PM colony development and the morphology and cytology of conidiophores in melon PM colonies. Our novel method to estimate the fungicide sensitivity of PM fungi and examine whether PM isolates acquire resistance to fungicides was based on quantitative assessment of asexual conidia collected electrostatically from single melon PM colonies on the leaves of living spray-treated or control host seedlings.

## Materials and methods

### Cultivation of melon seedlings

Melon seeds (*Cucumis melo* L. cv. ‘Earl’s Favourite’) supplied by Yuasa Experimental Farm, Kindai University (Wakayama, Japan) were placed on wet filter paper in Petri dishes, and left to germinate for 3–4 days in a growth chamber (LH-240 N; Nippon Medical & Chemical Instruments, Osaka, Japan) under controlled conditions of  $25 \pm 2$  °C, 40–50% relative humidity (RH), and continuous illumination at  $22.2 \mu\text{mol m}^{-2} \text{s}^{-1}$ , provided by white (full-spectrum) fluorescent lamps (380–750 nm; FL40SS W/37; Mitsubishi, Tokyo, Japan). Cotyledonal seedlings were transferred to polyurethane cubic sponge supports (3 cm × 3 cm × 3 cm), which were then inserted into 30-mL cylindrical plastic containers (diameter: 3 cm; length: 5 cm) containing 20 mL of liquid fertilizer (Kyowa, Osaka, Japan) with a controlled pH of 6.0–6.5 and an electrical conductivity of  $1.2 \text{ mS cm}^{-1}$ . The seedlings were incubated for 14 days at  $25 \pm 1$  °C, 40–50% RH, and continuous illumination of  $59.5 \mu\text{mol m}^{-2} \text{s}^{-1}$ , as described by Takikawa et al. (2015). Light intensity was measured using a light meter (LI-250A; Li-COR,

Lincoln, NE, USA) fitted with a quantum sensor that measures photosynthetically active radiation (400–700 nm).

The 14-day-old healthy seedlings were transferred to a polystyrene plate (61.5 cm × 60.5 cm × 3.0 cm) floating in hydroponic nutrient solution contained in the hydroponic culture trough (67.0 cm × 65.5 cm × 21.0 cm) (Home Hyponica 303; Kyowa) of a cultivating table (height: 100 cm) installed in a pathogen-free nursery greenhouse (10.0 m × 6.0 m;  $26 \pm 3$  °C) (Matsuda et al. 2006). The seedlings were used to maintain the Japanese melon PM isolate and to treat single melon PM colonies by spraying the leaves with commercial fungicides.

### Inoculation and incubation of melon PM isolate

The Japanese melon PM isolate used in this study (*Podosphaera xanthii* KMP-6N) was described in Takikawa et al. (2015). The fungus was isolated from PM-infected leaves of melon plants (cv. ‘Earl’s Favourite’) cultivated hydroponically in our greenhouses in 2014. In the present study, mature conidia were collected from conidiophores on KMP-6N-infected melon leaves using an electrostatic spore collector. The collector consisted of an ebonite rod with a pencil-type electrostatic insulator probe (length: 7 cm; diameter: 4 mm; pointed tip diameter: 5  $\mu\text{m}$ ) and was mounted on a micromanipulator placed next to a high-fidelity digital microscope (DM) (KH-2700; Hirox, Tokyo, Japan). Well-developed young true leaves of 14-day-old healthy melon seedlings were inoculated with strain KMP-6N and observed under the DM (Takikawa et al. 2015). The KMP-6N isolate was incubated for 14 days in an electrostatic screen chamber that excluded the entry of airborne pathogens, which was installed in a greenhouse (10.0 m × 6.0 m) at  $26 \pm 2$  °C and 30–55% RH under an illumination of  $190.6\text{--}400.4 \mu\text{mol m}^{-2} \text{s}^{-1}$  (Matsuda et al. 2006), and in an LH-240 N growth chamber at  $25 \pm 2$  °C and 40–50% RH under continuous illumination of  $22.2 \mu\text{mol m}^{-2} \text{s}^{-1}$  (Takikawa et al. 2015), respectively.

### Fungicide testing

We examined the effects of 36 commercial fungicides and one biofungicide on the development of *P. xanthii* and on conidial release (Table 1). All tested fungicides (products) were purchased from agrochemical manufacturers in Japan and diluted based on their instructions. The leaves of 14-day-old melon seedlings were inoculated with single *P. xanthii* conidia using the insulator probe described above. Single 7-day-old *P. xanthii* colonies (fungal fleck) on leaves (Fig. 1A) were sprayed with solutions of the prepared fungicides and then incubated for 3 days in a greenhouse under the environmental conditions described above (Matsuda et

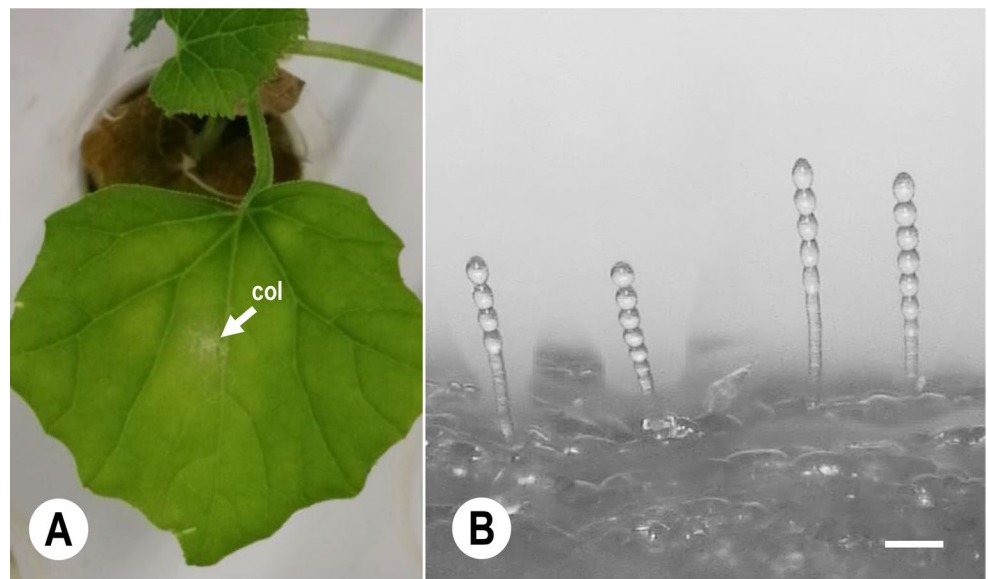
**Table 1** Mode of action of commercial fungicides used in this study to assess the fungicide-sensitivity of *Podosphaera xanthii* KMP-6N

| Mode of action                                                        | Code  | Target site                                                         | Group name                                       | Active Ingredient                                                                                                                           | Products                                                               | FRAC Code (FRAC 2024)          |
|-----------------------------------------------------------------------|-------|---------------------------------------------------------------------|--------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------|--------------------------------|
| Cytoskeleton and motor protein                                        | B1    | $\beta$ -tubulin assembly in mitosis                                | Methyl-Benzimidazole Carbamates (MBC fungicides) | Thiophanate-methyl                                                                                                                          | Topsin-M                                                               | 1                              |
|                                                                       | B6    | Actin/myosin/fimbrin function                                       | Aryl-phenyl-ketones                              | Pyriofenone                                                                                                                                 | Property                                                               | 50                             |
| Respiration                                                           | C2    | Complex II: succinate-dehydrogenase (SdhB, SdhC and SdhD)           | Succinate dehydrogenase inhibitors (SDHIs)       | Isofetamid<br>Isopyrazam<br>Penthiopyrad<br>Pyraziflumid                                                                                    | Kenja<br>Nexter<br>Afet<br>Parade 20                                   | 7<br>7<br>7<br>7               |
|                                                                       | C3    | Complex III: cytochrome bcl at Qo site (Cytb)                       | Quinone outside inhibitors (QoI-fungicides)      | Azoxystrobin<br>Kresoxim-methyl                                                                                                             | Amistar 20<br>Storoby                                                  | 11<br>11                       |
| Amino acids and protein synthesis                                     | D1    | Methionine biosynthesis (proposed <i>cgs</i> gene)                  | Anilinopyrimidines (AP-fungicides)               | Mepanipyrim                                                                                                                                 | Frupica                                                                | 9                              |
| Sterol biosynthesis in membranes                                      | G1    | C14-demethylase in sterol biosynthesis ( <i>erg11/cyp51</i> )       | Demethylation inhibitors (DMI-fungicides)        | Difenoconazole<br>Fenarimol<br><br>Imibenconazole<br>Myclobutanil<br>Triflumizole<br>Triforine                                              | Score<br>Nissan<br>Rubigen<br>Manage DF<br>Rally<br>Trifmine<br>Saprol | 3<br>3<br><br>3<br>3<br>3<br>3 |
| Cell wall biosynthesis                                                | H4    | Chitin synthase                                                     | Polyoxins                                        | Polyoxin                                                                                                                                    | Polyoxin                                                               | 19                             |
| Not specified                                                         | –     | Unknown                                                             | Diverse                                          | Pottasium bicarbonate<br>Sodium bicarbonate                                                                                                 | Kaligreen<br>Harmomate                                                 | NC<br>NC                       |
| Chemicals with multi-site activity                                    | M     | Multi-site contact activity                                         | Inorganic                                        | Dodecylbenzenesulfonic acid bisethylenediamine copper complex salt (II) (DBEDC)<br><br>Nonyl phenol sulfonic acid copper (II)-salt (NPS-Cu) | Sanyol<br><br>Yonepon                                                  | M01<br><br>M01                 |
|                                                                       |       |                                                                     | Chloronitriles                                   | Chlorothalonil                                                                                                                              | Dakonil 1000                                                           | M05                            |
|                                                                       |       |                                                                     | Bis-guanidines                                   | Iminoctadine albesilate                                                                                                                     | Bellkute                                                               | M07                            |
|                                                                       |       |                                                                     | Quinoxalines                                     | Chinomethionate                                                                                                                             | Kanesho morestan                                                       | M10                            |
| Biologicals with multiple modes of action: microbial                  | BM    | Multiple effects described                                          | Microbial                                        | <i>Bacillus subtilis</i>                                                                                                                    | Botokiller                                                             | BM02                           |
| Amino acids and protein synthesis, chemicals with multi-site activity | D3, M | Protein synthesis, multi-site contact activity                      | Hexopyranosyl antibiotic, Inorganic              | Kasugamycin, copper                                                                                                                         | Kappasin                                                               | 24, M01                        |
| Chemicals with multi-site activity                                    | M     | Multi-site contact activity                                         | Inorganic                                        | Sulphur, Copper                                                                                                                             | Engei bordeaux                                                         | M01, M02                       |
| Chemicals with multi-site activity, sterol biosynthesis in membranes  | G1, M | Multi-site contact activity, C14-demethylase in sterol biosynthesis | Dithiocarbamates and relatives, DMI-fungicides   | Mancozeb, Simeconazole                                                                                                                      | Theek                                                                  | M03, 3                         |
| Chemicals with multi-site activity, nucleic acids metabolism          | M, A1 | Multi-site contact activity, RNA polymerase I                       | Chloronitriles, Phenylamides (PA-fungicides)     | Chlorothalonil, Metalaxyl-M                                                                                                                 | Foriogold                                                              | M05, 4                         |
| Chemicals with multi-site activity, respiration                       | M, C2 | Multi-site contact activity, Complex II: succinate-dehydrogenase    | Chloronitriles, SDHIs                            | Chlorothalonil, Penthiopyrad                                                                                                                | Vegesaver                                                              | M05, 7                         |
| Chemicals with multi-site activity, respiration                       | M, C3 | Multi-site contact activity, Complex III: cytochrome bcl at Qo site | Chloronitriles, QoI-fungicides                   | Chlorothalonil, Azoxystrobin                                                                                                                | Amistar opti                                                           | M05, 11                        |

**Table 1** (continued)

| Mode of action                                                     | Code  | Target site                                                         | Group name                                                 | Active Ingredient                         | Products     | FRAC Code (FRAC 2024) |
|--------------------------------------------------------------------|-------|---------------------------------------------------------------------|------------------------------------------------------------|-------------------------------------------|--------------|-----------------------|
| Chemicals with multi-site activity, respiration                    | M, C4 | Multi-site contact activity, Complex III: cytochrome bc1 at Qi site | Chloronitriles, Quinone inside inhibitors (QiI-fungicides) | Chlorothalonil, Cyazofamid                | Docious      | M05, 21               |
| 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      | Funbell      | 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 | Diamerit     | M07, 19               |
| Chemicals with multi-site activity, cell wall biosynthesis         | M, H4 | Multi-site contact activity, chitin synthase                        | Bis-guanidines, Polyoxins                                  | Iminoctadine triacetate, Polyoxin complex | Polybelin    | 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      | Lamic        | M07, 50               |
| Unknown, sterol biosynthesis in membranes                          | U, G1 | Unknown, C14-demethylase in sterol biosynthesis                     | Phenyl-acetamide, DMI-fungicides                           | Cyflufenamid, Triflumizole                | Pancho TF    | U06, 3                |
| Unknown, amino acids and protein synthesis                         | U, D1 | Unknown, methionine biosynthesis                                    | Thiazolidine, AP-fungicides                                | Flutianil, Mepanipirim                    | Shochinosuke | U13, 9                |

**Fig. 1** Single powdery mildew (PM) colonies that developed on true leaves of 14-day-old melon seedlings. The colonies were used for fungicide testing and electrostatic collection of asexual conidia 3 days after spray treatment of the colonies with commercial fungicides. **(A)** Melon seedling grown hydroponically in a greenhouse, with a single 7-day-old *Podosphaera xanthii* colony (col) on the leaf. **(B)** Digital micrograph of conidiophores formed in 7-day-old *P. xanthii* colonies. Scale bar: 60  $\mu$ m



al. 2006). Each fungicide spray treatment involved 10 test plants. Fungal development on melon leaves and conidiophores (Fig. 1B) was monitored using the DM. As controls, 10 fungal colonies on melon seedlings were sprayed with tap water.

### Electrostatic conidial collector

Asexual conidia from single KMP-6N colonies sprayed with the fungicides were collected using an electrostatic conidial collector consisting of a conductor plate (aluminum, 60 mm  $\times$  30 mm) between two insulator plates (polyethylene

terephthalate, 80 mm  $\times$  50 mm) (Fig. 2). The conductor plate was connected to a direct current electrostatic voltage generator (HVA 10K202NA; Logy Electric, Tokyo, Japan). The outer surface of the insulator plate (conidial collection side) was negatively polarized by supplying an electric current from the voltage generator to the conductor plate. The negative charge on the surface of the electrified insulator plate generated an electrostatic field and thus an attractive force that trapped conidia released from the conidiophores (Takahara et al. 2024). To collect the released asexual conidia, the insulator plate was charged with static electricity ( $5.2 \times 10^{-1}$  nC) and placed at a distance of approximately 2 cm from

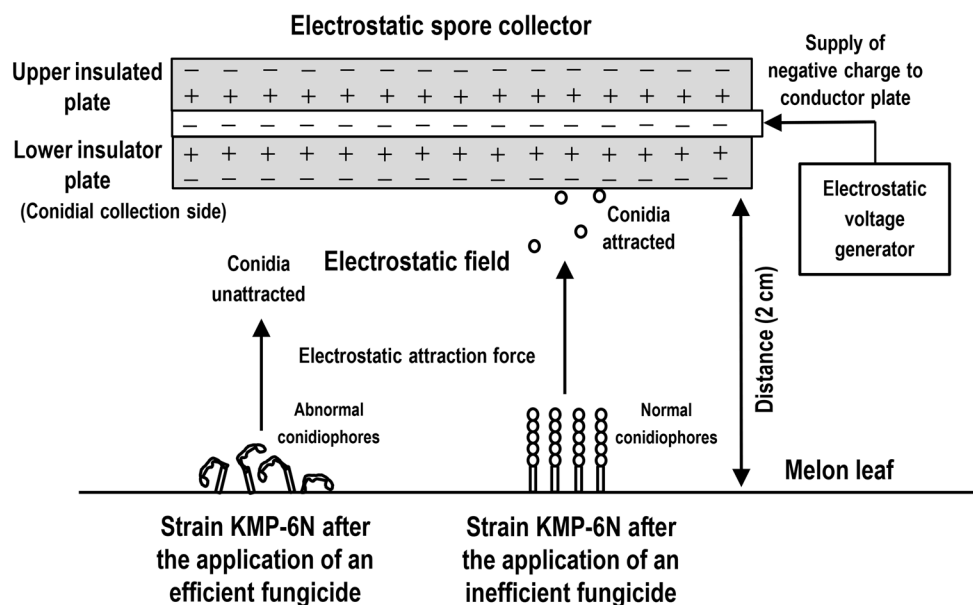


**Fig. 2** Schematic diagram of the electrostatic spore collector system (cross-sectional view) used to collect asexual conidia released from single *P. xanthii* colonies sprayed with commercial fungicides. When a negative charge is supplied from the electrostatic voltage generator to the conductor plate, an attractive force is created on the conidial collection side of the transparent lower insulator plate that traps the conidia. Dielectric polarization produces a negative surface charge on

the opposite side of the insulator plate, and an electrostatic field forms around the dielectrically polarized insulator plate. Asexual conidia from normal *P. xanthii* conidiophores are directed to and collected on the conidial collection side of the lower insulator plate by electrostatic attraction. The lower insulator plate ( $5.2 \times 10^{-1}$  nC) is placed <2 cm from a *P. xanthii* colony on a melon leaf

a single *P. xanthii* colony on a melon leaf, as described previously (Suzuki et al. 2018). Asexual conidia released from 10 individual KMP-6N colonies on each leaf in each experiment were collected electrostatically 3 days after fungicide treatment of single 7-day-old KMP-6N colonies. The conidia were counted under the DM. As a control, asexual conidia were electrostatically collected from single 10-day-old KMP-6N colonies incubated for 3 days after spraying with tap water. The data are presented as means and standard deviation (SD) of 10 measurements.

### Numbers and germination rates of asexual conidia collected electrostatically from single melon PM colonies sprayed with fungicides

Asexual conidia on each melon leaf were electrostatically collected from individual single colonies 3 days after fungicide application, as described above. The conidia were counted under the DM; the results are expressed based on one replicate per colony. The data are presented as means and SD of 10 replicates per fungicide. The insulator plates and conidia were placed in a humid box (RH, 95–99%) and incubated for 24 h at  $25 \pm 1$  °C. Germinated conidia were counted under the DM, and germination rates were determined. The data are presented as means and SD of 10 replicates per fungicide. To test the infectivity of conidia on host leaves, asexual conidia electrostatically collected from

single fungicide-treated colonies were inoculated onto the leaves of healthy 14-day-old melon seedlings previously sprayed with the same fungicides. The inoculated leaves were then incubated in the growth chamber. Colony development was observed using the DM, as well as a light microscope (LM) (BX-60; Olympus, Tokyo, Japan).

### Microscopic observation of conidiophores in single colonies sprayed with fungicides

A melon seedling with a single 7-day-old KMP-6N colony on a leaf was spray-treated with each fungicide at the concentrations shown in Table 2 and grown for 3 days under the greenhouse conditions described above. Three days later, conidiophores of the KMP-6N colonies were observed using the DM and LM. The conidiophores were photographed using the half-inch interline-transfer charge-coupled device camera of the DM. The photographs were analyzed using image-processing software (Adobe Photoshop v5; Adobe Systems, Mountain View, CA, USA). Asexual conidia from single 10-day-old KMP-6N colonies sprayed with each fungicide were collected electrostatically to count the number of all conidia released from the single colonies and measure the conidial germination rates, and then ten individual leaf segments (*ca.* 3 cm × 3 cm) cut from the infected seedlings were prepared for histochemical colony staining as follows. Leaf segments were fixed and their chlorophyll was

**Table 2** Colony development of *P. xanthii* after treatment with fungicides containing different active ingredients, assessed by counting asexual conidia trapped on electrostatically activated insulator plates, and rates of conidial germination

| Active ingredients                        | Concentrations ( $\mu\text{g mL}^{-1}$ ) | Numbers of conidia collected from single colonies | Germination rates (%) | Colony areas ( $\text{mm}^2$ ) |
|-------------------------------------------|------------------------------------------|---------------------------------------------------|-----------------------|--------------------------------|
| Thiophanate-methyl                        | 466.6                                    | 710.4 $\pm$ 45.5                                  | b                     | 36.3 $\pm$ 9.2                 |
| Pyriofenone                               | 89.3                                     | 16.0 $\pm$ 2.3                                    | h                     | 5.4 $\pm$ 0.9                  |
| Isofetamid                                | 239.9                                    | 57.4 $\pm$ 8.5                                    | g                     | 16.0 $\pm$ 2.7                 |
| Isopyrazam                                | 187                                      | 16.0 $\pm$ 6.3                                    | h                     | 0                              |
| Penthiopyrad                              | 100                                      | 0                                                 | i                     | N/A                            |
| Pyraziflumid                              | 100                                      | 0                                                 | i                     | N/A                            |
| Azoxystrobin                              | 100                                      | 0                                                 | i                     | N/A                            |
| Kresoxim-methyl                           | 221                                      | 648.2 $\pm$ 37.5                                  | b                     | 55.4 $\pm$ 6.9                 |
| Mepanipyrim                               | 200                                      | 712.0 $\pm$ 38.0                                  | b                     | 15.5 $\pm$ 3.7                 |
| Difenoconazole                            | 50                                       | 97.2 $\pm$ 5.5                                    | f                     | 0.6 $\pm$ 0.2                  |
| Fenarimol                                 | 12                                       | 684.0 $\pm$ 47.6                                  | b                     | 40.5 $\pm$ 5.3                 |
| Imibenconazole                            | 150                                      | 0                                                 | i                     | N/A                            |
| Myclobutanil                              | 16.6                                     | 0                                                 | i                     | N/A                            |
| Triflumizole                              | 99.9                                     | 0                                                 | i                     | N/A                            |
| Triforine                                 | 90                                       | 428.0 $\pm$ 29.1                                  | c                     | 22.3 $\pm$ 2.4                 |
| Polyoxin                                  | 100                                      | 0                                                 | i                     | N/A                            |
| Pottasium bicarbonate                     | 1000                                     | 97.0 $\pm$ 1.8                                    | f                     | 2.9 $\pm$ 2.3                  |
| Sodium bicarbonate                        | 1000                                     | 202.8 $\pm$ 4.5                                   | d                     | 5.8 $\pm$ 3.1                  |
| DBEDC                                     | 400                                      | 58.6 $\pm$ 9.4                                    | g                     | 10.9 $\pm$ 5.8                 |
| NPS-Cu                                    | 800                                      | 0                                                 | i                     | N/A                            |
| Chlorothalonil                            | 571.4                                    | 0                                                 | i                     | N/A                            |
| Iminoctadine albesilate                   | 400                                      | 209.5 $\pm$ 4.5                                   | d                     | 1.5 $\pm$ 0.4                  |
| Chinomethionate                           | 125                                      | 0                                                 | i                     | N/A                            |
| <i>Bacillus subtilis</i>                  | 1 $\times$ 10 <sup>8</sup> CFU/g         | 153.0 $\pm$ 7.5                                   | e                     | 26.3 $\pm$ 4.7                 |
| Kasugamycin, Copper                       | 50, 450                                  | 0                                                 | i                     | N/A                            |
| Sulphur, Copper                           | 625, 875                                 | 0                                                 | i                     | N/A                            |
| Mancozeb, Simeconazole                    | 1083.3, 39.9                             | 0                                                 | i                     | N/A                            |
| Chlorothalonil, Metalaxyl-M               | 400, 41.3                                | 0                                                 | i                     | N/A                            |
| Chlorothalonil, Penthiopyrad              | 400, 64                                  | 0                                                 | i                     | N/A                            |
| Chlorothalonil, Azoxystrobin              | 400, 51                                  | 0                                                 | i                     | N/A                            |
| Chlorothalonil, Cyazofamid                | 400, 32                                  | 0                                                 | i                     | N/A                            |
| Iminoctadine albesilate, Pyribencarb      | 150, 100                                 | 7.8 $\pm$ 3.7                                     | h                     | 13.2 $\pm$ 9.6                 |
| Iminoctadine albesilate, Polyoxin complex | 83.3, 99.9                               | 0                                                 | i                     | N/A                            |
| Iminoctadine triacetate, Polyoxin complex | 33.3, 99.9                               | 0                                                 | i                     | N/A                            |
| Iminoctadine albesilate, Pyriofenone      | 150, 40                                  | 3.6 $\pm$ 1.2                                     | h                     | 0                              |
| Cyflufenamid, Triflumizole                | 17, 75                                   | 0                                                 | i                     | N/A                            |
| Flutianil, Mepanipyrim                    | 9, 100                                   | 0                                                 | i                     | N/A                            |
| Control (water)                           | —                                        | 2010.2 $\pm$ 69.2                                 | a                     | 80.6 $\pm$ 3.3                 |

Data represent numbers of electrostatically collected asexual conidia and colony areas measured in 10-day-old colonies 3 days after fungicide treatment. Single colonies were sprayed with tap water as a control. All data are means and standard deviation of 10 replicates. Different letters in each column indicate significant differences ( $P < 0.05$ , Tukey's test).

removed by boiling for 1–2 min in an alcoholic lactophenol solution (10 mL glycerol, 10 mL phenol, 10 mL lactic acid, 10 mL distilled water, and 40 mL 99.8% ethanol) followed by staining with 0.1% aniline blue (Nacalai Tesque, Tokyo, Japan) dissolved in distilled water, as described previously (Takahara et al. 2024). The stained KMP-6N colonies were observed under the LM and photographed using a digital camera (EOS KISSX6i; Canon, Tokyo, Japan) mounted on the microscope. Mycelial areas of the colonies were

measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The data are presented as means and SD of 10 single colonies. The total numbers of normal and abnormal conidiophores in 10 colonies from each leaf were also determined; the data are expressed as mean percentages and SD for 10 single colonies.

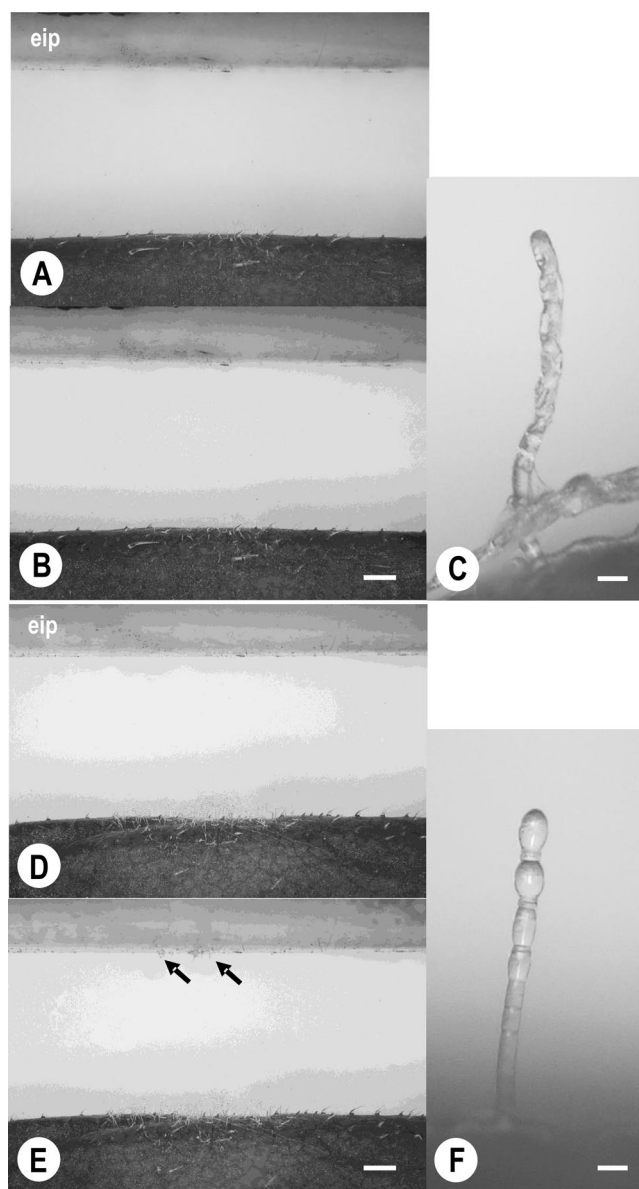


**Fig. 3** Collection of asexual conidia released from single 10-day-old *P. xanthii* colonies sprayed with fungicides on the true leaves of living melon seedlings using electrostatic spore collection (electrostatic insulator plate; eip). *Podosphaera xanthii* colonies sprayed with polyoxin and kresoxim-methyl as observed by digital microscopy before (A, D) and after (B, E) the supply of a negative charge ( $5.2 \times 10^{-1}$  nC) to the conductor plate. Asexual conidia (arrows at top of E) were successfully captured on the insulator plate from single kresoxim-methyl-treated colonies after negative electrification. The insulator plate was placed ~2,500 μm from the apex of the colonies. *Podosphaera xanthii* conidiophores (C, F) from polyoxin- and kresoxim-methyl-treated colonies (B, E) as observed by digital microscopy. Almost all *P. xanthii* polyoxin-treated conidiophores (C) were abnormal (disrupted), whereas most of those treated with kresoxim-methyl (F) were normal. Scale bars: 500 μm (B, E) and 20 μm (C, F)

## Statistical analysis

For single colonies treated with each fungicide, significant differences among the numbers of electrostatically collected asexual conidia, conidial germination rates, colony areas, and rates of normal conidiophore formation were determined using Tukey's test, conducted using the EZR v1.54 software (Jichi Medical University, Saitama, Japan).

## Results

### Electrostatic collection of asexual conidia from single KMP-6N colonies sprayed with commercial fungicides

The numbers of asexual conidia collected electrostatically from single colonies sprayed with each fungicide are reported in Table 2. For single colonies sprayed with each of the 21 fungicides, including polyoxin (polyoxins group), no conidia were attracted onto the electrostatic insulator plate, even when it was negatively polarized (Fig. 3A, B), because fungicide-treated KMP-6N conidiophores were completely destroyed, such that they were unable to scatter asexual conidia (Fig. 3C). By contrast, large numbers of asexual conidia ( $> 50$  conidia per single colony) were attracted onto the plate from single colonies sprayed with each of 12 fungicides, including thiophanate-methyl (an MBC), kresoxim-methyl (a QoI), mepanipyrim (an anilino-pyrimidines, AP), and fenarimol and triforine (DMIs), demonstrating the resistance of KMP-6N to these agents (Fig. 3D, E). The morphology of KMP-6N conidiophores sprayed with each of these 12 fungicides was the same as that of conidiophores sprayed with tap water, demonstrating the ability of KMP-6N to successfully scatter asexual conidia, fully confined to the top of the conidiophores (catenescence conidia), in single colonies (Fig. 3F). The numbers of asexual conidia released from melon PM conidiophores varied depending on the active ingredients of the fungicides (Table 2).

### Morphology and germination rates of asexual conidia electrostatically collected from KMP-6N colonies sprayed with commercial fungicides

Asexual conidia electrostatically collected from single colonies sprayed with kresoxim-methyl had a mean size of  $30.1 \pm 1.9 \mu\text{m} \times 18.6 \pm 1.8 \mu\text{m}$  and were ellipsoid-ovoid to doliiform–limoniform in shape (Fig. 4A), similar to their counterparts treated with tap water. Oil and fibrosin bodies were also observed in the collected conidia. Asexual conidia electrostatically collected from single colonies sprayed with kresoxim-methyl had the highest germination rate, followed

**Fig. 4** Characteristics of kresoxim-methyl-resistant *P. xanthii* conidia electrostatically attracted from a single *P. xanthii* colony to the insulator plate. (A) Light micrograph of *P. xanthii* conidia, showing oil bodies and fibrosin bodies (Fb) (arrow). (B) Digital micrograph of conidial appressoria (Ap) at the tip of germ tubes. Scale bars: 10  $\mu$ m (A, B)

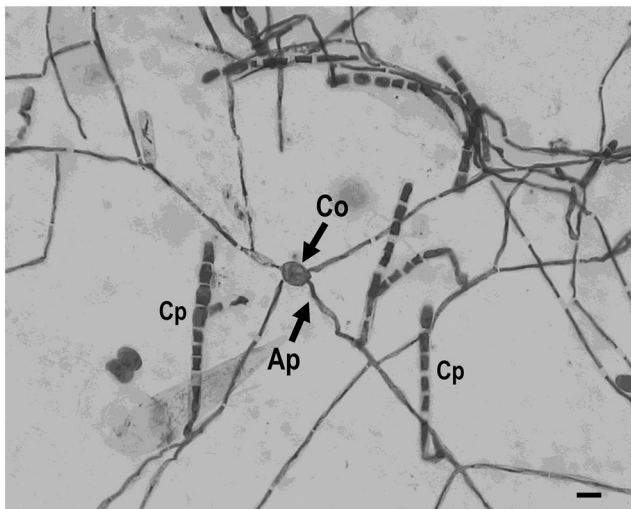
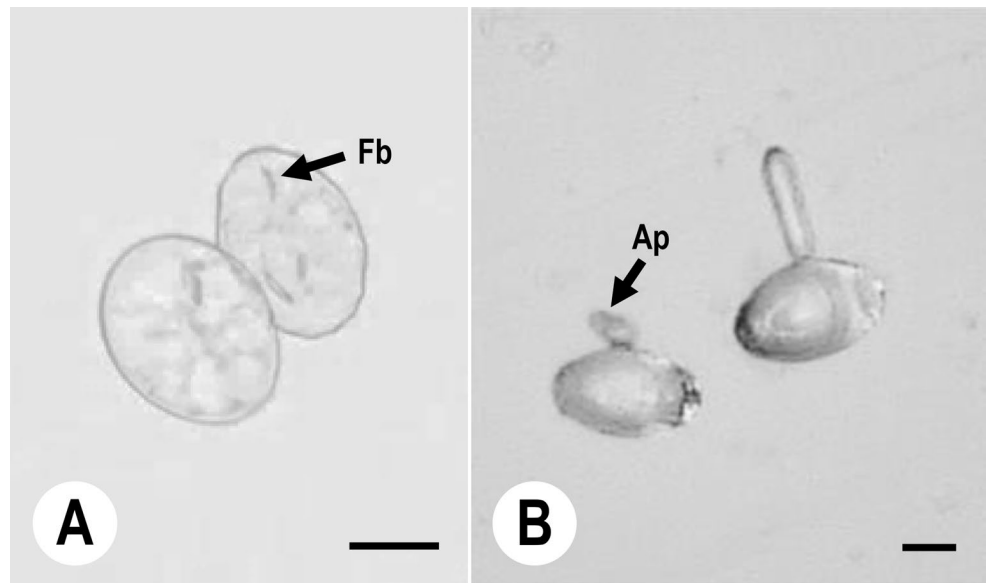


**Fig. 5** Light micrograph of hyphae from kresoxim-methyl-resistant *P. xanthii* conidia electrostatically collected and then inoculated onto healthy melon leaves previously sprayed with kresoxim-methyl (2,000-fold dilution; 221  $\mu$ g mL<sup>-1</sup>). Conidiophores formed from the highly elongated hyphae. Scale bar: 20  $\mu$ m. Co conidium, Ap appressorium, Cp conidiophore

by fenarimol (Fig. 4B and Table 2). Asexual conidia electrostatically collected from single colonies sprayed with isopyrazam (SDHIs), and iminoctadine albesilate + pyriofenone, did not germinate.

#### Development of single KMP-6N isolates on kresoxim-methyl-treated melon leaves

KMP-6N conidia germinated 3–5 h after inoculation on kresoxim-methyl-treated melon leaves and formed primary appressoria 6–8 h after inoculation. Colony-forming hyphae produced from the conidial bodies were first observed 24 h

after inoculation, and continued to elongate on the leaves. The isolate began to form conidiophores (catenated conidia) from the elongated hyphae 5–6 days after inoculation (Fig. 5).

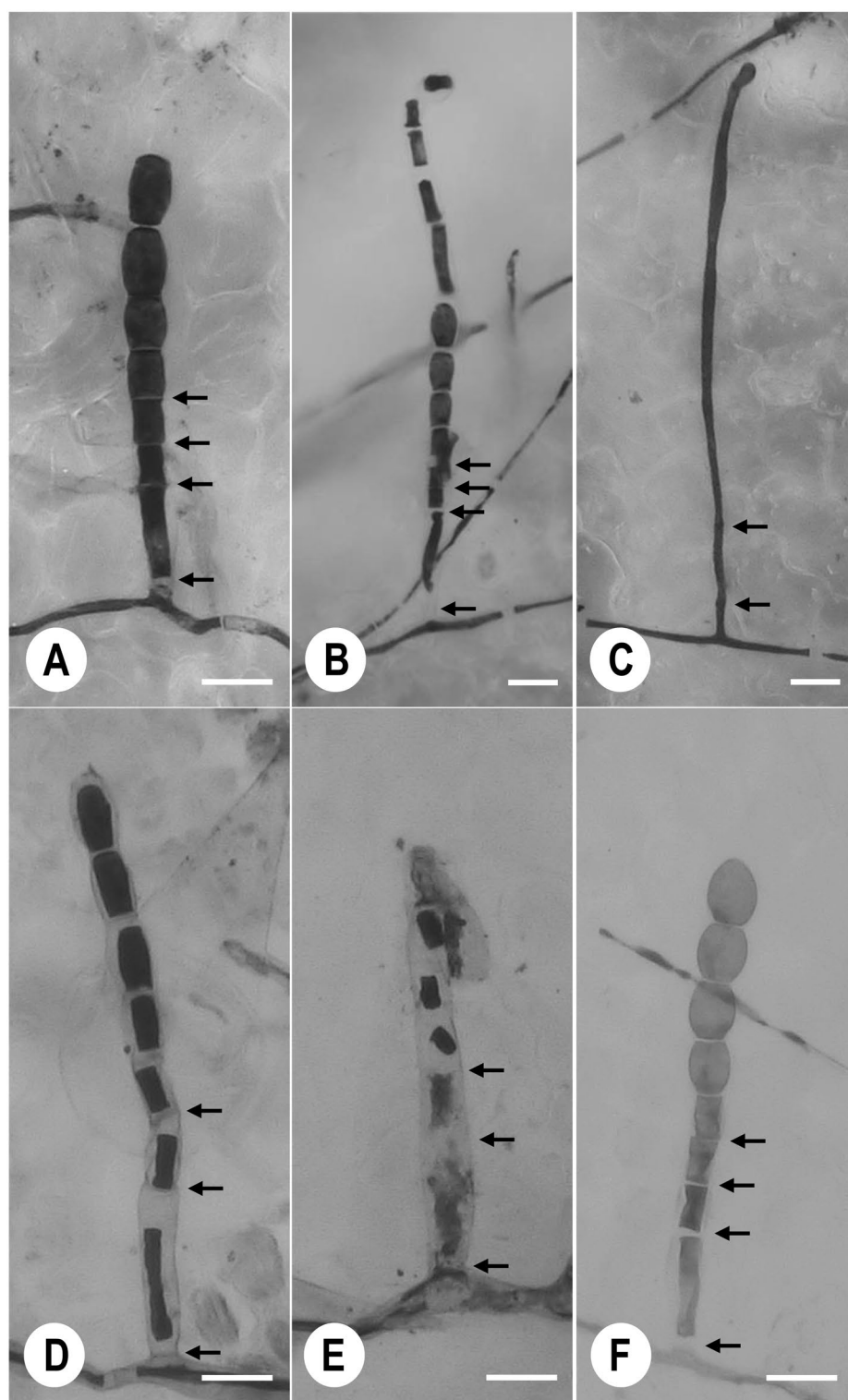
#### Effects of commercial fungicides and biofungicide on KMP-6N colony development

The four fungicides that included isofetamid (an SDHI), azoxystrobin (a QoI), mepanipyrin, and polyoxin as active ingredients significantly inhibited KMP-6N colony development. By contrast, colony development in the presence of the biofungicide that included *Bacillus subtilis*, and the two fungicides that included kresoxim-methyl and myclobutanil (a DMI) as active ingredients was similar to that of KMP-6N colonies sprayed with tap water (Table 2). Thiophanate-methyl, potassium bicarbonate, mancozeb + simeconazole, chlorothalonil + cyazofamid, and flutianil + mepanipyrin caused only moderate inhibition (Table 2). According to these results, the KMP-6N isolates were insensitive to fungicides in Fungicide Resistance Action Committee (FRAC) groups 3 (DMI fungicides), 11 (QoI fungicides), and BM02 (microbial fungicides).

#### Effects of commercial fungicides on KMP-6N conidiophore shape

Figure 6 shows the morphologies and cytological characteristics of fungal cells that formed normal (Fig. 6A) and abnormal (Fig. 6B–F) conidiophores. The abnormal conidiophores (catenated conidia) were classified into five types based on their morphological and cytological characteristics. Table 3 shows the distributions of normal and abnormal morphologies of the conidiophores in single colonies

**Fig. 6** Light micrographs of *P. xanthii* conidiophores histochemically stained to reveal the morphological changes and cytological characteristics of fungal cells after treatment with tap water or fungicides. **(A)** Normal catenescence conidiophores (concatenated conidia in chains) are apparent in *P. xanthii* colonies. **(B)** Thin cells that maintain the ellipsoid–ovoid shape of the conidia are evident in the lower half and divided cells in the upper half of the conidiophores. **(C)** Linear conidiophores with undivided cells. **(D)** Separation of cell membranes in conidia that have maintained their ellipsoid–ovoid shape. **(E)** Cell membrane atrophy and granulated cells in conidiophores. **(F)** Hyaline cells resulting from cell membrane collapse in conidia that have maintained their ellipsoid–ovoid shape. Arrows indicate septa (septal walls). Histochemically stained conidiophores were imaged 3 days after treatment with tap water or fungicides. Scale bars: 30  $\mu$ m



sprayed with each fungicide, as determined by histochemical staining. The numbers of normal conidiophores in a single colony were significantly lower following polyoxin treatment than following tap water treatment. The numbers of normal conidiophores were higher in treatments with fungicides containing thiophanate-methyl, kresoxim-methyl,

mepanipyrim or myclobutanil than in those containing other fungicides. For example, in KMP-6N colonies sprayed with kresoxim-methyl or myclobutanil, abnormal conidiophores accounted for *ca.* 36% and 17% of all conidiophores, respectively; the highest percentage (> 70%) was determined in the pyriofenone (an aryl-phenyl-ketone) treatment (C in

**Table 3** Formation rates of normal and abnormal conidiophores in single *P. xanthii* colonies after treatment with fungicides including different active ingredients

| Active ingredients                        | The number of normal or abnormal conidiophores (%) |    |         |           |           |           |           |
|-------------------------------------------|----------------------------------------------------|----|---------|-----------|-----------|-----------|-----------|
|                                           | A                                                  |    | B       | C         | D         | E         | F         |
| Thiophanate-methyl                        | 37.1±6.7                                           | b  | 0.5±0.3 | 11.4±7.0  | 3.9±1.7   | 44.1±10.5 | 5.2±3.6   |
| Pyriofenone                               | 14.7±8.8                                           | c  | 0       | 70.2±7.9  | 2.1±1.8   | 6.4±3.7   | 4.8±3.1   |
| Isofetamid                                | 2.7±1.6                                            | d  | 0       | 31.3±7.7  | 13.4±9.8  | 33.2±8.4  | 18.4±7.4  |
| Isopyrazam                                | 1.2±1.1                                            | d  | 3.0±1.7 | 20.6±9.7  | 5.2±2.8   | 10.9±9.7  | 51.5±9.4  |
| Penthiopyrad                              | 10.6±5.5                                           | cd | 0       | 38.1±8.4  | 28.4±9.4  | 11.3±4.1  | 48.9±9.8  |
| Pyraziflumid                              | 2.9±2.3                                            | d  | 0       | 34.5±8.4  | 3.6±3.5   | 45.8±9.0  | 13.1±9.9  |
| Azoxystrobin                              | 10.6±6.8                                           | cd | 0       | 25.3±4.1  | 3.3±1.8   | 28.8±9.5  | 31.4±9.9  |
| Kresoxim-methyl                           | 42.5±9.9                                           | b  | 0       | 36.2±8.1  | 0         | 16.4±6.0  | 5.1±2.1   |
| Mepanipyrin                               | 37.5±9.4                                           | b  | 0       | 12.5±3.1  | 0.4±0.4   | 45.3±8.7  | 4.3±3.7   |
| Difenoconazole                            | 2.6±2.1                                            | d  | 0.1±0.1 | 44.9±8.0  | 5.9±1.8   | 19.1±9.9  | 29.0±6.2  |
| Fenarimol                                 | 8.3±4.1                                            | cd | 0       | 32.7±7.8  | 0.7±0.6   | 17.4±9.8  | 41.0±7.1  |
| Imibenconazole                            | 2.3±1.6                                            | d  | 0.1±0.1 | 17.6±7.9  | 34.3±9.5  | 38.2±6.0  | 7.7±3.8   |
| Myclobutanil                              | 40.5±9.8                                           | b  | 0       | 17.1±8.6  | 12.4±6.5  | 20.9±7.6  | 3.8±2.0   |
| Triflumizole                              | 8.9±6.6                                            | cd | 0       | 43.8±7.6  | 1.8±1.2   | 19.3±9.4  | 26.0±8.5  |
| Triforine                                 | 12.3±6.8                                           | c  | 0       | 16.9±5.4  | 6.9±2.3   | 36.3±9.6  | 27.6±9.1  |
| Polyoxin                                  | 0                                                  | e  | 0       | 0         | 52.1±9.4  | 33.3±8.1  | 13.0±5.4  |
| Pottasium bicarbonate                     | 11.9±4.3                                           | c  | 0.4±0.3 | 6.3±2.5   | 5.4±2.3   | 71.6±9.3  | 8.5±4.0   |
| Sodium bicarbonate                        | 1.3±1.0                                            | d  | 0.3±0.3 | 8.5±3.7   | 78.5±8.6  | 4.7±4.4   | 5.5±2.5   |
| DBEDC                                     | 3.1±1.1                                            | d  | 0       | 4.0±2.5   | 1.7±0.7   | 86.8±6.8  | 5.7±3.6   |
| NPS-Cu                                    | 0                                                  | e  | 0       | 4.1±1.3   | 75.0±10.2 | 6.1±3.9   | 14.6±4.5  |
| Chlorothalonil                            | 1.3±1.0                                            | d  | 2.4±1.9 | 21.8±8.6  | 5.2±3.8   | 31.8±9.0  | 38.9±8.9  |
| Iminoctadine albesilate                   | 5.8±5.5                                            | cd | 0       | 31.9±9.0  | 18.6±8.5  | 19.8±6.7  | 25.8±9.2  |
| Chinomethionate                           | 0                                                  | e  | 0       | 3.7±1.1   | 56.3±3.0  | 36.8±9.6  | 2.9±1.2   |
| <i>Bacillus subtilis</i>                  | 10.0±4.9                                           | cd | 0.4±0.4 | 21.0±7.7  | 3.9±1.9   | 28.8±9.0  | 36.0±7.9  |
| Kasugamycin, Copper                       | 0                                                  | e  | 4.4±2.4 | 34.6±9.6  | 23.5±7.2  | 28.1±6.5  | 33.0±3.5  |
| Sulphur, Copper                           | 0                                                  | e  | 0       | 12.0±9.8  | 41.9±10.1 | 17.7±6.1  | 12.1±8.6  |
| Mancozeb, Simeconazole                    | 4.5±2.5                                            | cd | 1.9±1.9 | 17.7±8.2  | 4.9±2.2   | 29.8±10.9 | 47.4±4.4  |
| Chlorothalonil, Metalaxy1-M               | 0                                                  | e  | 0       | 19.6±5.9  | 46.1±9.6  | 31.5±9.1  | 2.8±2.8   |
| Chlorothalonil, Penthiopyrad              | 0                                                  | e  | 0       | 12.4±8.1  | 41.4±9.2  | 11.1±2.4  | 40.1±9.1  |
| Chlorothalonil, Azoxystrobin              | 1.0±0.8                                            | d  | 0.3±0.1 | 6.6±4.7   | 5.6±3.6   | 61.9±12.9 | 28.4±11.0 |
| Chlorothalonil, Cyazofamid                | 0                                                  | e  | 0       | 11.7±3.4  | 39.2±4.9  | 32.6±4.7  | 16.4±4.2  |
| Iminoctadine albesilate, Pyribencarb      | 1.6±0.8                                            | d  | 0       | 16.6±7.6  | 12.1±5.3  | 42.1±10.5 | 17.2±8.0  |
| Iminoctadine albesilate, Polyoxin complex | 0                                                  | e  | 0       | 9.1±8.3   | 41.2±7.8  | 33.2±6.0  | 16.5±7.9  |
| Iminoctadine triacetate, Polyoxin complex | 0                                                  | e  | 0       | 0         | 52.1±9.7  | 34.8±10.2 | 13.0±5.4  |
| Iminoctadine albesilate, Pyriofenone      | 0.4±0.2                                            | d  | 0.9±0.6 | 34.0±10.6 | 11.2±6.1  | 33.1±10.5 | 17.7±9.6  |
| Cyflufenamid, Triflumizole                | 4.4±3.3                                            | cd | 0.1±0.1 | 17.6±6.8  | 7.5±3.7   | 34.7±5.9  | 38.5±6.6  |
| Flutianil, Mepanipyrin                    | 0                                                  | e  | 0       | 22.6±9.7  | 14.4±4.1  | 34.8±10.5 | 31.0±7.0  |
| Control (water)                           | 97.5±2.4                                           | a  | 0       | 0         | 0         | 1.5±0.3   | 0         |

As a control, single colonies were sprayed with tap water. Columns A–F refer to the morphological and cytological conidiophore characteristics described for Fig. 6. Normal and abnormal conidiophores in 10 individual 10-day-old colonies from each leaf were counted and expressed as percentages. Data are means and standard deviation of 10 replicates. Different letters in each column indicate significant differences ( $P < 0.05$ , Tukey's test).

Table 3). Thus, the morphological and cytological characteristics of melon PM conidiophores varied even when the colonies were sprayed with the same active ingredients of commercial fungicides. The distributions clearly differed depending on the active ingredients in the fungicides.

## Discussion

The potential for effective control of cucurbit PM diseases using fungicides can be assessed by performing sensitivity tests. This study evaluated the efficacy of different commercial fungicides in controlling *P. xanthii*, by determining the fungicide sensitivity of the fungus. Previous studies of cucurbit PMs focused on the incidence or severity of disease on host leaves, as a measure of disease progression (McGrath

et al. 1996; Fernández-Ortuño et al. 2006; Miyamoto et al. 2020a). However, the criteria used to evaluate fungicide sensitivity (i.e., scale of values and days after fungicide treatment) differ among studies. For example, Fernández-Ortuño et al. (2006) conducted a leaf disc assay in which PM symptoms on each leaf disc were recorded according to a 4 level scale. Miyamoto et al. (2020a) also recorded PM development on each leaf disc but at 10–11 days after inoculation and using a 0–5 scale: 0 (no visible PM development), 1 (> 0–5%), 2 (> 5–25%), 3 (> 25–50%), 4 (> 50–75%), and 5 (> 75% of the disc surface covered with PM). In these studies, sensitivity assays were conducted after inoculating *P. xanthii* onto fungicide-treated leaf discs. In the present study, PM colonies sprayed with each fungicide were applied directly to the leaves of living melon seedlings. The results showed that the development of *P. xanthii* isolate KMP-6N varied depending on the applied fungicide. Thus, in addition to leaf disc assays, effective fungicides can be identified based on the evaluation of colony size on leaves. On the other hand, although fungicides containing pyriofenone, isofetamid, isopyrazam, mepanipyrim, difenoconazole, fenarimol, triforine, sodium bicarbonate, iminoctadine albesilate, or iminoctadine albesilate+pyribencarb had highly inhibitory effects on colony development, asexual conidia were collected from single KMP-6N colonies sprayed with their fungicides. This means that the asexual conidia collected can infect neighboring host plants if the conidia retain the germinative capability.

PM fungi produce numerous conidia, allowing disease spread over a large area by dispersal of the mature conidia from PM-infected plants (Aylor 1990; Brown and Hovmöller 2002). Suzuki et al. (2019) investigated the ecological mechanism underlying conidial dispersal from single PM colonies, and thus the expansion of infection, by counting all conidia released from single colonies over the lifetime of melon PM fungi, using an electrostatic technique based on an electrostatically activated insulator drum. The Japanese isolate KMP-6N of *P. xanthii* was found to release an average of  $12.6 \times 10^4$  conidia from each single colony during its lifespan (ca. 744 h) under greenhouse conditions; the authors also used electrostatic spore collection to quantitatively assess the production of progeny conidia dispersed day and night from living single colonies of *P. xanthii* ex *C. melo* L. cv. 'Earl's Favourite' throughout their lifetime. Matsuda et al. (2006) applied electrostatic forces to successfully attract PM conidia to both negatively and positively polarized insulator cylinders; the collection method had no detrimental effect on conidial survival, even in fungi exposed to the electrostatic force over their lifetime, as shown by the formation of elongated hyphae and conidiophores, and the subsequent production of living progeny conidia (Nonomura et al. 2009). Importantly, the dielectrically polarized

insulators did not forcibly detach immature conidia from conidiophores (Suzuki et al. 2019). These observations, together with those of the present study, recommend the use and underline the potential of electrostatic techniques in the quantitative early assessment of the fungicide sensitivity of melon PM fungi. Moreover, the same electrostatic technique enables us to use not only in the case of this melon PM fungus but also with other PM fungi.

Asexual conidia electrostatically collected from fungicide-resistant KMP-6N colonies germinated normally, developed typical elongated hyphae, and formed normal conidiophores on host leaves previously sprayed with the same fungicides. Thus, the collected asexual conidia maintained their germinative capacity and were not affected by electrostatic forces or the active ingredients of the fungicides. Asexual conidia collected from colonies treated with the other fungicides (e.g., isopyrazam) did not germinate. A comparison of the numbers and germination rates of asexual conidia electrostatically collected from fungicide-treated PM colonies showed that KMP-6N isolate was resistance to the QoI fungicide kresoxim-methyl (oximino-acetates group), the DMI fungicides fenarimol (pyrimidines group) and triforine (piperazines group), the MBC fungicide thiophanate-methyl (thiophanates group), and the microbial fungicide *Bacillus subtilis* (bacterial *Bacillus* spp. group), based on their germination rates (> 20%). On the other hand, KMP-6N isolate showed different germination rates among difenoconazole, fenarimol, and triforine treatments, depending on the number of asexual conidia collected electrostatically. In the current study, our electrostatic spore collection method allows efficient estimation of the spread of viable PM conidia resistant to fungicides.

Conidiophores are infectious structures that release asexual conidia, thereby facilitating the spread of PM diseases. In nature, *P. xanthii* KMP-6N forms a maximum of six conidia in a chain (catenated conidia) on conidiophores (Takikawa et al. 2015). However, to our knowledge, the morphological characteristics of conidiophores in cucurbit PM colonies sprayed with fungicides have not previously been observed in detail. Most of the fungicides led to morphological changes in conidiophores and conidial cells of *P. xanthii*. Based on these changes, the conidiophores were classified into five groups. Despite their different modes of action, most of these fungicides had a curative effect, although thiophanate-methyl, kresoxim-methyl, mepanipyrim, and myclobutanil caused fewer morphological changes in the fungal conidiophores, consistent with ca. 40% population survival rate within the population of normal conidiophores. Interestingly, no asexual conidia were collected electrostatically on insulator plate from KMP-6N colonies sprayed with myclobutanil (see Table 2). It seemed that conidial cells on the tops of conidiophores were not fully

constricted, and as a result, the conidia could not scatter from the conidiophores. In another study, the morphological characteristics of conidiophores in strawberry PM colonies (*P. aphanis* KSP-7N isolate) sprayed with commercial fungicides were observed in detail (Takahara et al. 2024). The treatment resulted in morphological changes similar to those of KMP-6N conidiophores but also included collapsed cell membranes and granulated cells in the conidiophores, albeit with a normal ellipsoid–ovoid conidial shape; separation of cell membranes and granulated cells in conidiophores, with no changes in conidial shape; atrophy and collapse of conidiophores; and thin conidiophores without dividing and enlarging cells.

To our knowledge, this is the first study describing methods to precisely evaluate the total number of asexual conidia released from single cucurbit PM colonies sprayed with commercial fungicides, and thus accurately estimate the sensitivity of *P. xanthii* to fungicides. In the present study, differences in the numbers of asexual conidia collected electrostatically from single colonies and treated with a wide range of different fungicide types were investigated. Our novel method for the early detection of fungicide-resistant PM fungi will be useful for confirming their appearance, as it allows quantitative assessment of conidial release from single PM colonies, and aid in the selection of highly effective fungicides for cucurbit PM control. It may also lead to a better understanding of the ecological distribution of fungicide-resistant cucurbit PM fungi and elucidation of the ecological mechanisms supporting their infection of healthy hosts plants.

## Conclusions

In this study, an insulator plate was used as an electrostatic spore collector to determine the numbers of asexual conidia released from single *P. xanthii* colonies (KMP-6N isolate) sprayed with fungicides with different modes and mechanisms of action. Asexual conidia collected from individual *P. xanthii* colonies sprayed with kresoxim-methyl (a QoI fungicide), fenarimol and triforine (DMI fungicides), and thiophanate-methyl (an MBC fungicide) germinated > 20% and subsequently infected host leaves previously sprayed with the same fungicides. Thus, the electrostatic spore collection method allowed the early detection of fungicide-resistant PM fungi. As next steps, analyses of the expression of mutated  $\beta$ -tubulin, cytochrome *b* (*cyt b*), and cytochrome P450 sterol 14 $\alpha$ -demethylase (*cyp51*) genes will provide insights into the site-specific fungicide-resistance of single conidia electrostatically collected from fungicide-resistant colonies of cucurbit PM fungi.

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