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Consecutive monitoring of lifelong production of conidia by individual conidiophores of *Blumeria graminis* f. sp. *hordei* on barley leaves by digital microscopic techniques with electrostatic micromanipulation

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ABSTRACT

Conidial formation and secession by living conidiophores of *Blumeria graminis* f. sp. *hordei* on barley leaves were consecutively monitored using a high-fidelity digital microscopic technique combined with electrostatic micromanipulation to trap the released conidia. Conidial chains formed on conidiophores through a series of septum-mediated division and growth of generative cells. Apical conidial cells on the conidiophores were abstricted after the conidial chains developed ten conidial cells. The conidia were electrically conductive, and a positive charge was induced in the cells by a negatively polarized insulator probe (ebonite). The electrostatic force between the conidia and the insulator was used to attract the abstricted conidia from the conidiophores on leaves. This conidium movement from the targeted conidiophore to the rod was directly viewed under the digital microscope, and the length of the interval between conidial septation and secession, the total number of the conidia produced by a single conidiophore, and the modes of conidiogenesis were clarified. During the stage of conidial secession, the generative cells pushed new conidial cells upwards by repeated division and growth. The successive release of two apical conidia was synchronized with the successive septation and growth of a generative cell. The release ceased after 4-5 conidia were released without division and growth of the generative cell. Thus, the life of an individual conidiophore (from the erection of the conidiophore to the release of the final conidium) was shown to be 107 h and to produce an average of 33 conidia. To our knowledge, this is the first report on the direct estimation of life-long conidial production by a powdery mildew on host leaves.

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Introduction

The powdery mildew fungi are among the most ubiquitous plant pathogens. Their parasitism is characterized in part by the formation of superficial hyphae carrying dense layers of conidiophores in which generative cells successively produce abundant conidia (Jarvis *et al.* 2002). Mature conidia are abstricted from the apex of conidiophores and easily dispersed by wind to infect neighbouring host plants. Although the fungi reproduce both asexually and sexually, asexual reproduction is more important for the propagation of the species because of the high number of propagules produced. Furthermore, the asexual cycle is usually repeated several times during the season, whereas the sexual stage of many fungi is produced once a year or irregularly. In addition, spore production is important to plant pathologists because spores are the major vehicle for fungal infection of plants (Aylor 1990; Brown & Hovmøller 2002; Jarvis *et al.* 2002).

Considering the high fertility of the powdery mildew fungi, our interest has been directed toward analyzing the factors affecting the conidial production and release by the conidiophores, for the effective control of the pathogen. For this purpose, however, it was of basic importance to develop a method for consecutively monitoring and quantifying the production of progeny conidia by individual conidiophores throughout their life. Conidiophores on the leaf surface are the most suitable targets for investigating conidium production, but microscopic monitoring of individuals is often difficult because of the numerous conidiophores in the colonies. This problem is more serious in the powdery mildew fungi which form conidia in chains. The colonies of these powdery mildews develop large numbers of conidiophores at different ages and with a different number of conidial cells, especially at the middle or last stage of conidiogenesis (Agrios 1988). This makes it more difficult to elucidate developmental aspects in living conidiophores of the colonies. In the present study, we use powdery mildew of barley, *Blumeria graminis* f. sp. *hordei*, as a model for conidial chain-forming fungi and clarify the developmental process of conidiogenesis by monitoring living conidiophores during their lifetime.

One of the most basic and effective analyses of conidiogenesis is the consecutive observation of living conidiophores on host leaves over time. For this purpose, we have used a high-fidelity digital microscope (Matsuda *et al.* 2005; Oichi *et al.* 2004) to obtain high magnification with high resolution and to dispense with both bleaching the leaves and staining the fungus. With this type of microscopy, it was possible to observe septation in conidiophores and the successive formation of individual conidia of *Oidium neolycopersici* on living tomato leaves (Oichi *et al.* 2004), as well as to distinguish the appressorial germ tubes produced by living conidia of different powdery mildews on the same leaves (Matsuda *et al.* 2005). Moreover, because the leaf is not covered with a glass coverslip, the living fungus on the leaf surface can be manipulated directly under the digital microscope (Matsuda *et al.* 2005). These advantageous techniques can also be successfully combined with an electrostatic micromanipulation technique to collect the mature conidia abstricted from target conidiophores. The technique is fundamentally different from conventional electrostatic precipitation methods for collecting airborne microorganisms aerosolized by spraying ionizing agents (Lee *et al.* 2004; Mitchell *et al.* 2002).

Cell walls of bacteria, yeast and plant cells act as an electrical conductor under an electrostatic field (Mizuno & Washizu 1995). A negatively polarized insulator causes free electrons to move within cell walls to produce a dipolar charge (electrostatic induction): a positive charge on the side of the insulator and a negative charge on the opposite side (Griffith 2004; Halliday *et al.* 2002). Fungal spores can act as electrical conductors and can be statically charged using negatively polarized insulators. The electrostatic force between the charged spore and a polarized insulator can be harnessed to collect airborne microorganisms. In this study, a dielectrically polarized insulator (ebonite) probe is used as an electrified spore-collector to trap the conidia released from the conidiophores as they are viewed with the digital microscope. We could therefore collect individual conidia one at a time without altering their infectivity. The method reported here is a powerful tool to trace the lifelong secession of the conidia from selected conidiophores, to determine the total production of conidia on leaves and to evaluate the fertility of barley powdery mildew fungi during their lifetime.

Materials and methods

Plant, pathogen and inoculation

Seeds of barley (*Hordeum vulgare* cv. 'Gose-shikoku') were germinated on a water-soaked filter paper and placed into a sponge cube (1 cm^3). The sponge cube and seed were then inserted into the top portion of a 10 ml test tube containing autoclaved fertilizer-soaked vermiculite and incubated for 12 d in a growth chamber that was controlled at 20 ± 0.5 °C under continuous illumination at 4000 lux with fluorescent lights. Fully developed primary leaves of 10 d-old seedlings were inoculated with newly produced conidia of barley powdery mildew (*Blumeria graminis* f. sp. *hordei*, race I) according to the method described by Toyoda *et al.* (1987). Voucher material of the fungus used is preserved in Herbarium Preservation Section of Kinki University.

Staining of conidiophores with fluorescent brightener and fluorescent microscopy

A fluorescent brightener, Calcofluor white (CFW; Sigma, MO), was used to stain conidiophores on barley leaves according to a previous method (Mcintyre *et al.* 2001). A few drops of distilled water containing CFW at 300 $\mu g\,ml^{-1}$ were placed onto inoculated barley leaves and incubated at room temperature for 5 min. Fluorescence-stained conidiophores were observed with an Olympus fluorescence microscope BX-60 (U excitation with BP330-385 excitation filter and BA420 absorption filter).

Digital microscopic observation

The test tube with an inoculated seedling was placed on the stage of a high-fidelity digital microscope KH-2700 (Hirox, Tokyo) that was set in a growth cabinet (20 ± 1 °C; relative humidity, 45-55 %; continuous illumination of 4000 lux with

fluorescent lights), and conidiophores on the inoculated leaf were observed directly using an objective zoom lens, either MX-2525CS (1000×) or MX-5030RZII (50×) (Fig 1A). Digitized images of the conidiophores were automatically obtained at 5-min intervals at the same focal point with a 1/2″ Interline Transfer CCD camera and produced on a computer with Adobe Photoshop software (version 5.0) (Adobe, San Jose, CA). For consecutive tracing of the formation of a chain of conidial cells, new conidiophores at the outer edge of the colonies (boxed area S1, S2 and S3 in Fig 3) were selected as the targets for observation.

Dielectric polarization of insulator probe

The insulator probe used for the spore collector was a pencil-type, ebonite rod with a pointed tip (length, 5.5 cm; 2 mm diam; tip 30 μm diam). The insulator probe was held onto the micromanipulator on the digital microscope (Fig 1A) with its flat base in contact with an aluminum film (electrical conductor) (Fig 1B), which was negatively charged by a van de Graaff electrostatic generator VG (Kenis, Osaka). The contact with the negatively charged aluminum film created a positive polarization on the film side of the probe and a negative polarization on the opposite, pointed end (dielectric polarization of the insulator probe). The static electricity at the pointed end of the probe was measured by touching the point to the probe (tip 50 μm diam) of a coulometer NK-1001 (Kasuga Denki, Tokyo). The electricity level was controlled by changing the voltage loaded on the aluminum film. The voltage was measured with an electrostatic field meter FMX-002 (Simco, Kobe).

Relationship between static electricity and attractive force of the insulator probe

Different quantities of static electricity were supplied to the tip surface of the insulator probe, and the relation between

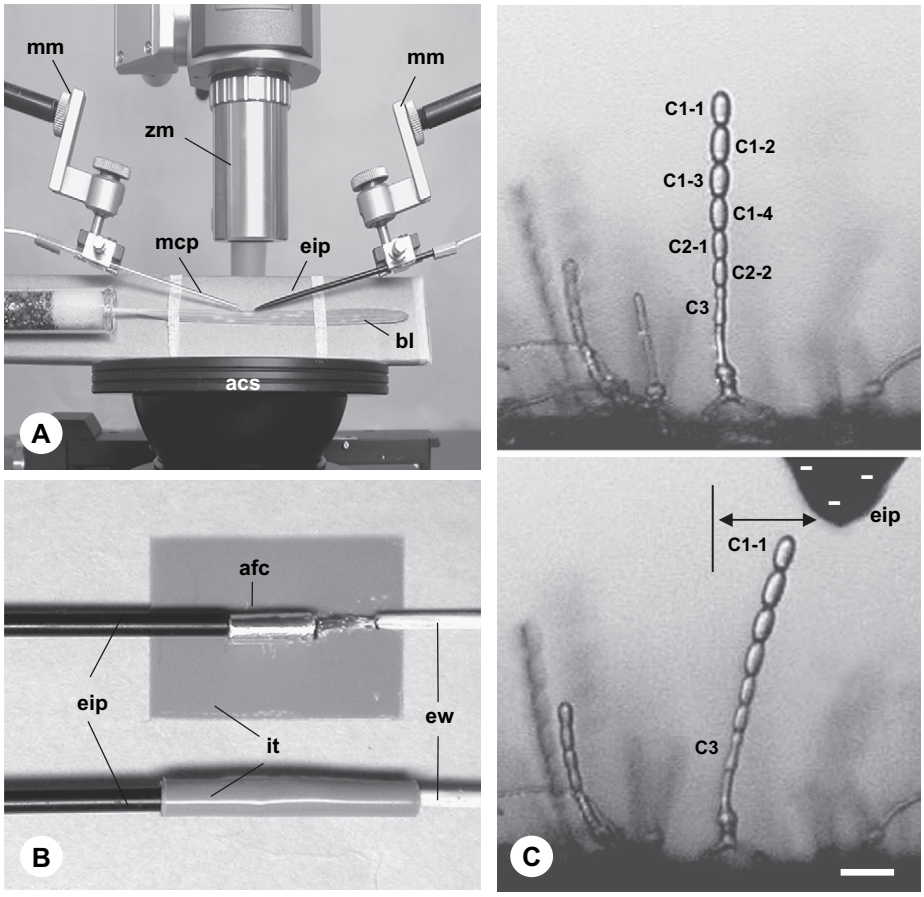

Fig 1 – Electrostatic manipulation system used to collect conidia released from conidiophores of *Blumeria graminis* f. sp. *hordei* on barley leaves (A-B) and a conidiophore attracted toward an electrostatically activated insulator (ebonite) probe (C). (A) Barley seedling growing in a test tube containing fertilized vermiculite that was placed horizontally on a stage of the digital microscope with a zoom lens (zm) for directly observing conidiophores on the inoculated leaf (bl) and a pointed insulator probe (eip) held by a manipulator (mm) on the microscope. The insulator probe was negatively polarized at the tip to deliver an electrostatic force toward the targeted conidiophore. The static electricity of the probe was measured by touching the tip of the probe to the probe (mcp) of a microcoulometer. (B) Interface between the opposite side of the pointed insulator probe and an aluminum film conductor (afc) linked to an electrostatic generator by a switch-containing electric wire (ew) (upper). The joint was covered with insulation tape (it) (lower). (C) Conidiophore before (upper) and after the approach of the polarized insulator probe (lower). Note successful attraction of the erect conidiophore to the probe placed at 40 μm from the apex of the conidiophore (arrow). See Fig 4 for conidial cells C1-1 to C3 of the conidiophore. Bar = 20 μm.

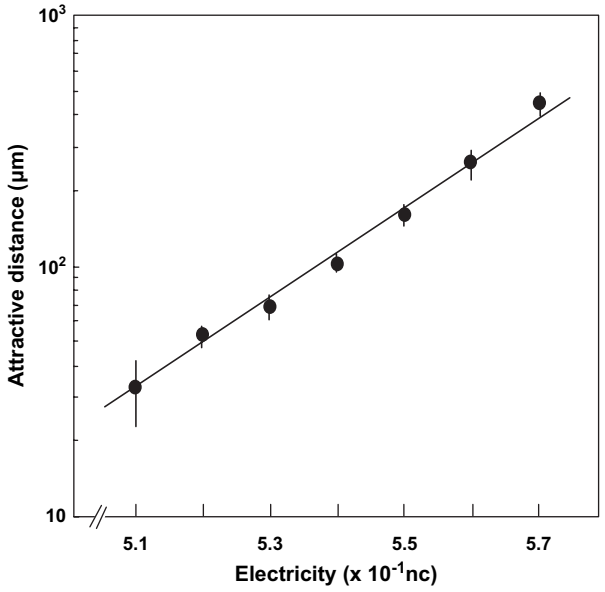

Fig 2 – Relationship between static electricity and attractability of the tip surface of an insulator (ebonite) probe. The tip of the probe was negatively polarized and was brought close to the apex of conidiophores that were eight conidial cells while being observed with a high-fidelity digital microscope. The distance between the probe and conidiophore when the conidiophore was first attracted was recorded and plotted (means and standard deviation for 20 conidiophores are given for each point).

the electricity level and conidiophore-attractive force was examined under the digital microscope. In the present experiment, the electrostatic level of the pointed tip was kept between 5-6 $\times 10^{-1}$ nanocoulomb (nC) under an electrostatic voltage of 0.8 to 6.8 kV. The probe was gradually brought to the apex of a conidiophore while viewing it with the digital microscope, and the distance at which the conidiophore was initially attracted toward the probe was recorded as a possible limit to the attractive force. Twenty conidiophores were used to test each electricity level.

Electrostatic collection of mature conidia

The conidiophores located at the edge of the colony (S3; Fig 3C) were selected as a target for the consecutive collection of the conidia. The tip of the insulator probe was positioned 40-60 μm distant from the targeted conidiophore. When the septum of the apical conidial cell in a full-length conidial chain was fully constricted, the probe was polarized by switching on the current between the aluminum film and the generator. After an apical conidium on the conidiophore was trapped with the probe, the polarity was earthed. The conidium trapped on the probe was gently transferred onto a fresh non-inoculated barley leaf to test its infectivity according to the method described by Matsuda *et al.* (2005). The collection procedure was repeated, and the interval between

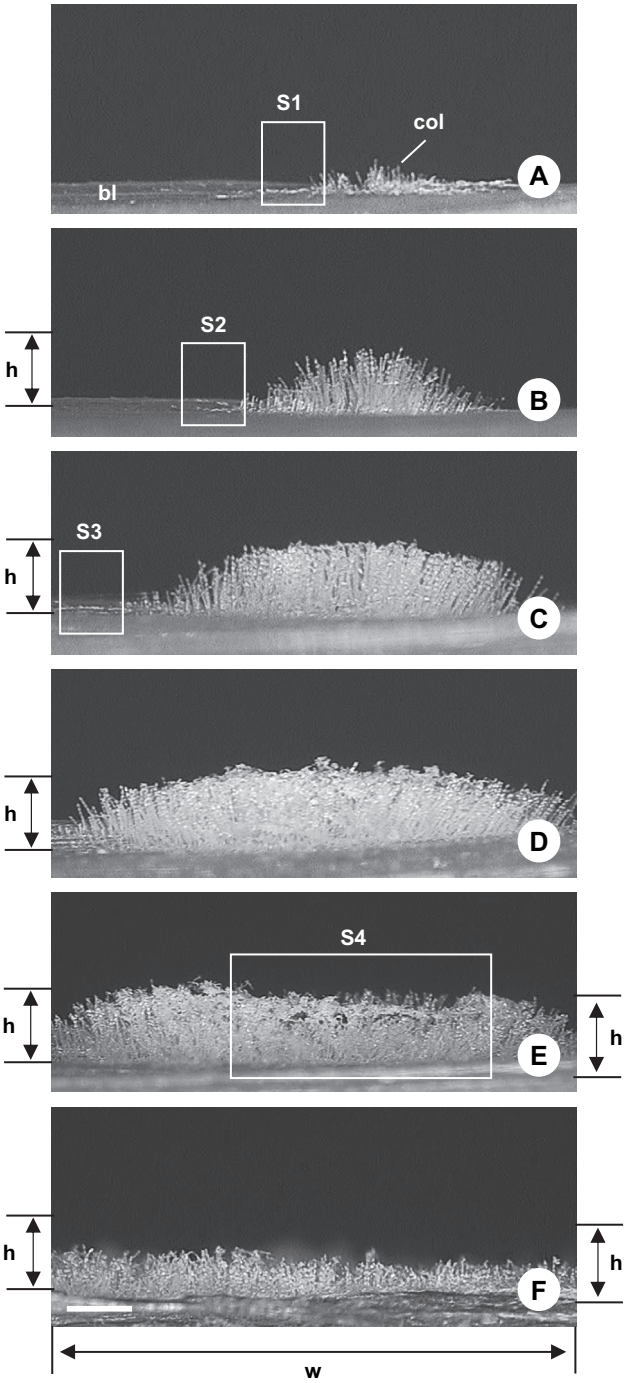

Fig 3 – Development of powdery mildew colony on barley leaf. Digital micrographs (A to F) of the same colony (col) on the leaf (bl) were taken 3, 6, 7, 8, 9, and 12 d after inoculation, respectively. Arrows h and w represent maximum height of the conidiophores and greatest width of the colony, respectively. Boxed areas S1 and S2 are the sites of the consecutively observed conidiophores to trace conidial chain formation, S3 is the site for electrostatic trapping of conidia released from full-length conidial chains, and S4 is where colony height first decreased. Bar = 1000 μm.

each release of a conidium was timed, until no more conidia were released from the conidiophore.

Results

Relationship between electricity and attractive force of the insulator probe

The idea of the present work began with the discovery of a potential attraction of the conidiophores and abstricted conidia to an electrically activated insulator probe. In Fig 1C, a conidiophore was attracted towards a probe that had a negatively polarized tip. Using our microscopic method with the electrostatic micromanipulation technique, the relationship between the static electricity at the tip of the insulator probe and its attractive capability was examined first (Fig 2). In this study, the probe was successfully polarized when the voltage level on the aluminum film was kept at 1.1-6 kV. Within this range, the distance that a conidiophore was attracted to the probe increased in proportion to an increase in the static electricity in the probe. On the other hand, voltages higher than 6.0 kV caused a discharge of electricity from the probe that repulsed the conidiophore, and lower voltages did not generate an attractive force. In the following experiment, therefore, the insulator probe with static electricity of 5.2×10^{-1} nC was placed 40-60 μm from the conidiophore apex to collect the released conidia.

Digital microscopy for colony development and conidiophore maturation

Prior to the release of conidia, the fungal growth on barley leaves was monitored with the high fidelity digital microscope (Fig 3). Colonies were first detected 3 d after inoculation. The area occupied by the colonies increased as the hyphae grew and produced conidiophores (Fig 3A-C) and reached a maximum 8 d after inoculation (Fig 3D-F). The maximum height of the chain-like conidiophores was reached by 6 d after inoculation (Fig 3B), when release of the conidia from the conidiophores began. Conidiophores at the centre of the colony were the first to reach the maximum height (Fig 3B), followed by the surrounding conidiophores 9-10 d after inoculation (Fig 3C-E). At the same time, i.e. 9 d after inoculation, the height of the central conidiophores (box S4; Fig 3E) decreased. By 12 d after inoculation, the height of all conidiophores had decreased to less than half of the maximum height (Fig 3F).

To clarify the process of conidiophore maturation, we selected new immature conidiophores (Fig 4A) that had not developed slender cells from the upper portion and which were located at the margin of the colonies (boxed area S1 to S3; Fig 3). These conidiophores were suitable for observing the complete process of maturation, because they were unobstructed by recently formed conidiophores. The conidiophores first formed on somatic hyphae 3 d after inoculation. With our microscope, we did not need any stains to observe septation of

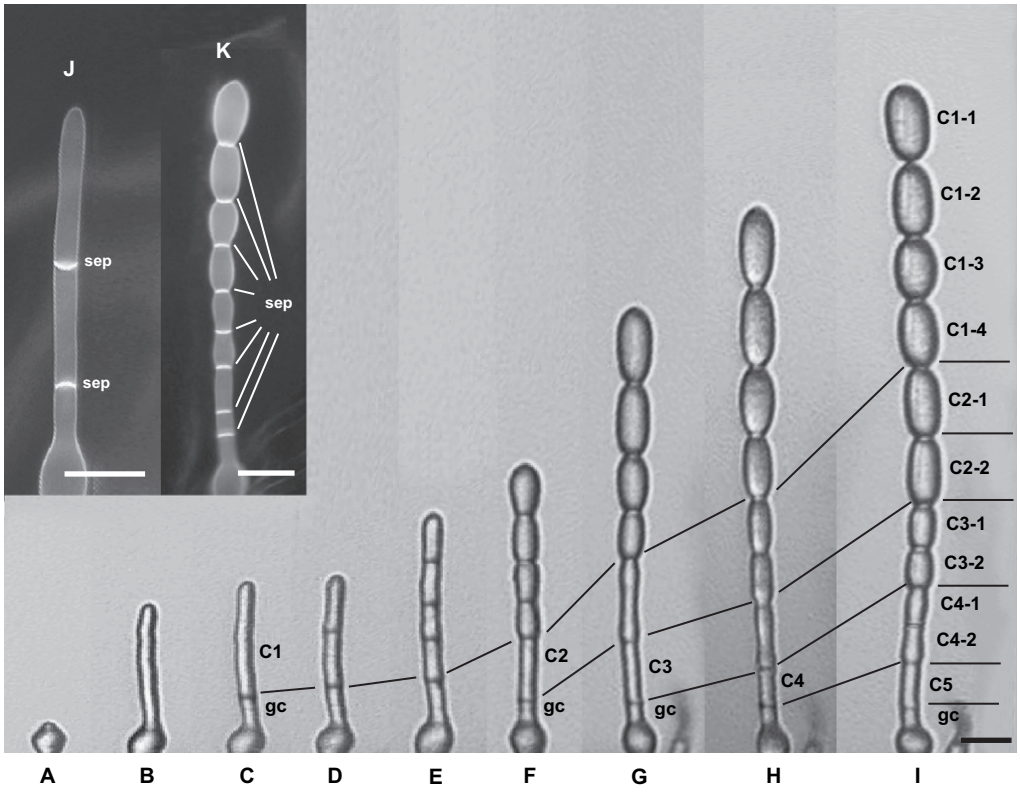

Fig 4 – Digital microscopic monitoring of conidiogenesis by *Blumeria graminis* f. sp. *hordei* on barley leaf and electrostatic trapping of released conidia. The same conidiophore (A) was consecutively observed until it formed full-length chains of 10 conidial cells (C1-1 to C4-2), undivided conidial cell (C5) and generative cell (gc) (A-I). Fluorescent micrographs of CFW-stained conidiophores (J and K, inserts) confirm the position of septa (sep) in the conidiophores. The conidiophores in J and K correspond to those at stages D and H, respectively. Bar = 10 μm.

the conidiophores. With fluorescent microscopy, the sites of septation coincided with those in the CFW-stained specimen (see the fluorescent micrographs in Fig 4). Fig 4 shows the sequential changes in a conidiophore that was consecutively monitored with the digital microscope. The first septum formed in the upper portion of an immature conidiophore within 5 h after a slender, erect cell had elongated from the tip of the conidiophore (A-C). This septation separated the first conidial cell (C1) from the generative cell (swollen portion of the conidiophore). Within 3 h of the formation, of the first septum, the upper C1 cell divided by a second septation into two cells, then each of these two cells was divided into two by a subsequent septation (D and E). These new cells then enlarged during maturation (F-I) to produce the first four conidial cells (C1-1 to C1-4). The generative cell then produced additional conidial cells successively. First, the slender apical portion of the generative cell elongated, and then a septum formed that divided the cell into two cells. Again, the apical cell was divided by septation to produce two conidial cells. By repeating this process four times, the conidiophores produced full-length chains of ten conidial cells (C1-1 to C4-2), one undivided conidial cell (C5), and one generative cell (gc) (I). At this stage, the first mature conidia were released from the conidiophores, an event that could be predicted by the fully constricted septum between the apical and second apical cells.

Application of the electrostatic micromanipulation method to the consecutive collection of conidia released from target conidiophores

Successful collection of the conidia released from targeted conidiophores is seen in Fig 5. First, we collected C1-2 and C1-3 cells after the release of the C1-1 cell from the mature conidiophore (A-F), using the electrostatic micromanipulation technique. The non-polarized insulator probe did not attract conidia (A). The probe was negatively polarized when the septum between C1-2 and C1-3 cells constricted fully (approx. 2.5 h after the release of C1-1) (B-C). The conidiophore was attracted towards the probe (B), and then the apical conidial cell (C1-2) was collected with the probe (C). The probe was then depolarized, and the conidiophore returned to its original position (D). The next apical cell (C1-3) was similarly attracted to the probe 2 h later by a repolarization at the time of complete constriction of C1-3 cell septum (E-F). During this stage of conidial secession, the generative cells pushed new conidial cells upwards by repeated growth and division (A-F). At the final stage of conidial secession, however, the generative cells ceased division and growth, although some conidia were still being released (G-I). The secession of the conidial cells remaining on the conidiophores was not detected even when the observation was prolonged for another 12 h. By collecting all the released conidia, we could determine the lifelong production of conidia by individual conidiophores and the timing of conidial secession (Table 1). Individual conidiophores released an average of 33 conidia at an interval of approximately 2.7 h during their life-time.

After their release from the conidiophore, the conidia trapped on the ebonite probe were transferred onto fresh barley leaves to test their infectivity. All conidia (261 conidia; Table 1) germinated within 3-4 h, produced appressoria within 5-6 h, and formed primary haustoria within 16-18 h after inoculation. This infection behaviour was comparable to those of conidia dusted from fresh colonies on barley leaves.

The entire process of conidiogenesis

The conidiogenesis in *Blumeria graminis* f. sp. *hordei* on barley leaves consists of the maturation of the conidiophores (A), successive release of conidia driven by repeated division and growth of generative cells (B), and conidial secession after the cease of division and growth of the generative cells (C) (Fig 6). During conidiophore maturation, repeated elongation and septation by the generative cell was essential to successively produce progeny conidia (A). The stage after maturation was characterized by the successive release of two apical conidia (B). This stage was synchronized with the septation and growth of the generative cell, and the original height of the conidiophore was regained after the release of the apical conidium. The final stage was characterized by conidial release without division and growth of the generative cells (C). This type of conidial secession reduced the length of the conidial chains, and consequently the conidiophores became shorter. Thus, the life of an individual conidiophore (from the erection of the conidiophore to the release of the final conidium) was shown to be 107 h.

Discussion

The use of light and fluorescent microscopy with decolourization and aniline blue staining of leaf specimens enabled the clear observation of epiphytic powdery mildew fungi (Kita *et al*. 1981), but not the consecutive observation of living conidiophores on intact host leaves. Using digital microscopy methods allowed us to eliminate the use of chemicals to decolourize leaves and to stain the fungus. More importantly, we achieved the high magnifications and resolution essential to observe septum formation in conidiophores (Oichi *et al*. 2004) which are not attainable with conventional stereomicroscopes with lower limits of resolution. And, without the need for a glass coverslip, we manipulated conidia released from living conidiophores on leaves with the electrostatically activated insulator probe. Thus, the high-fidelity digital microscopy combined with the electrostatic micromanipulation technique enabled us to count the total number of conidia produced by individual conidiophores throughout their life-time.

The most important part of the present work was the application of an electrostatic force to collect the conidia released from the conidiophores. Leach (1976) described how many wind-dispersed fungal spores were violently discharged into the atmosphere and became electrically charged at the instant of release. McCartney *et al*. (1982) calculated the actual surface charge of conidia as they impacted charged cylinders, showing that spores carry charges of low magnitude, which are insufficient to influence their deposition on natural surfaces. In our study, however, the conidial cells were attracted to the negatively polarized insulator probe in both the pre- and post-abstriction stages. In addition, these cells were similarly attracted to the positively polarized insulator probe (data not shown). These results imply that conidial cells were

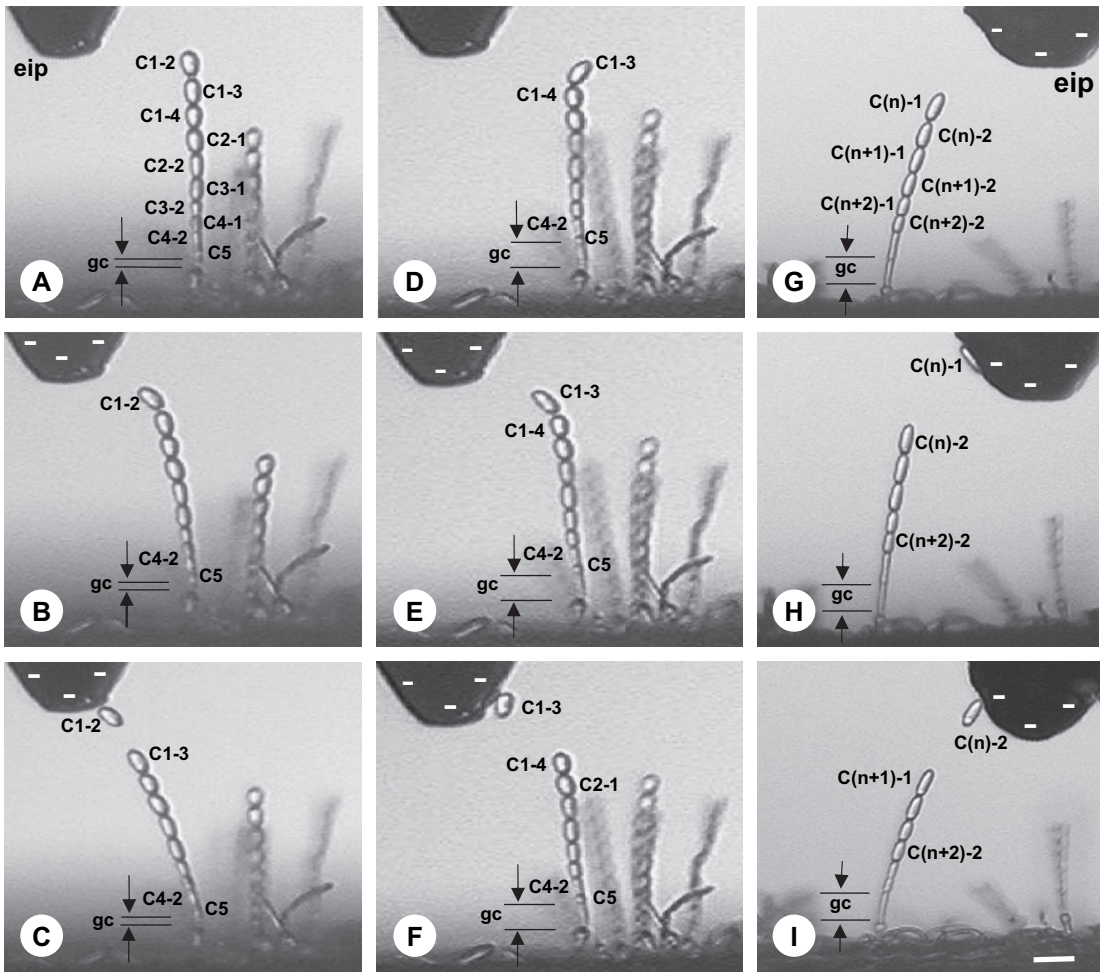

Fig 5 – Electrostatic trapping of conidia from targeted conidiophores on barley leaves during conidiogenesis (A-F) and at the final stage of conidiogenesis (G-I). (A-F) The ebonite probe (eip) was positioned 40 μm from a targeted conidiophore with a conidial cell (C1-2) at the apex (A), and the tip was negatively polarized when the septum constricted fully (B). The conidiophore was then attracted to the polarized probe, and the C1-2 conidium to the probe (B-C). After depolarization of the probe, the conidiophore returned to its original position (D). The next apical conidium (C1-3) was similarly trapped with the re-polarized probe (E-F). Note the expansion of a generative cell (gc) as a result of its growth. G to I: Nonpolarized ebonite probe was moved to an apical conidial cell ($C_{(n)}$-1) of a targeted conidiophore and negatively polarized to attract the apical conidium (G and H). The subsequent conidium ($C_{(n)}$-2) was trapped by the depolarization and re-polarization of the probe (I). Note the unchanged length of a generative cell during the subsequent abstriction of the conidia. Trapped conidia were transferred to fresh barley leaves to examine their infectivity. "-" represents negative polarity in the pointed tip of the probe. Bar = 20 μm.

uncharged, at least just before and after their release, and then became charged largely as a result of an electrostatic induction by the polarized insulators. In response to the presence of the electrically activated insulator, the conidia developed a charge opposite to that of the static electricity on the insulator plate on the plate-side of the cell from the intracellular movement of free electrons (Griffith 2004; Halliday *et al.* 2002; Mizuno & Washizu 1995). These opposite charges created an electrostatic force between the cell and the plate. Under our voltage conditions, the dielectrically charged insulator did not discharge to the conidia, and we expected to be able to collect the conidia without any detriment to their survival. In fact, these conidia had normal infectivity on host plants. Thus, electrostatic attraction did not detach immature conidia from the conidiophores.

Although abstricted conidia were easily separated from the probe soon after depolarization, they became sticky from their rapid secretion of a sticky substance(s) (data not shown). Some earlier investigators have reported the secretion of substances by non-germinated conidia of the powdery mildew immediately after their attachment to leaves or artificial surfaces (Carver *et al.* 1995, 1999; Nielsen *et al.* 2000; Wright *et al.* 2002). In our study, within 3 min on the polarized probe, the conidia became sticky. These results also suggest that our collection method did not affect the behaviour of the conidia when they first contact the host surface. From the results we obtained, the electrostatic attraction device can effectively collect viable conidia of the powdery mildew.

The major aim in the present work was to monitor cytological events during conidiogenesis of *B. graminis* f. sp. *hordei*: the

Table 1 – Time intervals of conidial secession by individual conidiophores of *Blumeria graminis* f. sp. *hordei* on barley leaves

Conidia released	Hours required for conidial secession[a]								Means ± S.D.
	Conidiophore-1	-2	-3	-4	-5	-6	-7	-8	
C1-2	1.7	2.2	3.3	2.0	2.3	2.8	2.6	1.7	2.3 ± 0.6
C1-3	1.7	2.9	2.5	1.7	2.0	1.8	0.8	2.0	1.9 ± 0.6
C1-4	3.3	2.3	3.3	3.3	1.8	2.8	2.0	2.6	2.7 ± 0.6
C2-1	3.7	3.8	2.3	2.9	2.6	1.6	1.6	2.6	2.6 ± 0.8
C2-2	1.6	1.9	3.3	2.5	1.4	3.0	3.0	1.8	2.2 ± 0.7
C3-1	3.1	2.7	2.7	3.5	1.6	1.8	2.8	3.1	2.7 ± 0.7
C3-2	2.7	2.8	2.8	2.6	2.5	2.8	2.6	2.7	3.0 ± 0.8
C4-1	2.5	2.7	2.8	2.4	2.8	2.3	2.6	2.3	2.5 ± 0.2
C4-2	1.3	1.3	1.3	2.3	1.7	2.0	2.5	2.7	2.1 ± 1.0
C5-1	3.8	3.8	3.8	4.1	3.1	2.3	2.8	3.1	3.1 ± 0.7
C5-2	3.7	2.5	4.0	1.8	2.5	2.2	2.7	2.7	2.8 ± 0.7
C6-1	1.9	1.9	1.5	2.3	2.3	2.6	1.8	2.5	2.1 ± 0.4
C6-2	2.3	2.5	3.2	1.8	2.3	1.3	2.9	3.2	2.5 ± 0.6
C7-1	2.3	2.3	1.5	2.0	3.1	2.2	2.2	2.9	2.5 ± 0.7
C7-2	2.4	2.4	2.8	3.8	1.8	3.1	1.9	2.0	2.4 ± 0.5
C8-1	2.0	2.1	2.5	2.5	1.3	1.0	2.3	3.4	2.1 ± 0.7
C8-2	2.1	2.0	2.6	2.0	2.2	2.4	2.9	2.1	2.4 ± 0.4
C9-1	2.9	2.9	1.4	3.0	1.1	4.1	2.9	2.2	2.4 ± 1.0
C9-2	1.8	1.9	2.0	1.8	1.9	4.1	2.8	2.2	2.4 ± 0.8
C10-1	2.6	1.7	2.7	2.1	2.5	1.5	3.9	2.7	2.5 ± 0.7
C10-2	3.0	2.7	2.7	2.7	2.7	3.1	3.8	2.6	2.9 ± 0.4
C11-1	2.0	2.3	3.3	2.4	2.7	3.1	1.8	1.5	2.5 ± 0.6
C11-2	2.8	2.8	2.8	2.3	3.3	2.8	2.4	3.5	2.8 ± 0.4
C12-1	2.5	2.4	1.9	3.4	2.1	2.6	2.7	2.0	2.5 ± 0.5
C12-2	2.3	2.3	1.2	2.3	2.0	1.0	3.1	2.3	2.3 ± 0.9
C13-1	3.7	2.3	2.3	3.2	2.0	1.3	2.5	3.3	2.4 ± 0.5
C13-2	1.8 [b]	2.5	2.9	2.5	2.6	3.0	2.2	2.9	2.5 ± 0.5
C14-1	1.6	2.7	1.9	1.8	2.6	2.6	3.0	3.2	2.8 ± 0.9
C14-2	4.6	3.5	1.9		3.8	2.2	2.7	2.2	3.0 ± 0.9
C15-1	4.1	4.2	3.7		1.0	3.5	2.1	4.0	3.4 ± 1.2
C15-2					3.5	3.2	4.0	2.1	3.1 ± 0.9
C16-1					2.3	3.7		3.1	2.5 ± 0.5
C16-2					3.5	2.2		3.5	3.1 ± 0.7
C17-1					2.4	2.3			2.4
C17-2					3.8				3.8
C18-1					4.5				4.5
C18-2					2.8				2.8
C19-1									
C19-2									
Total number of conidia released	31	31	31	29	38	35	32	34	32.6 ± 2.9
Finally developed conidial cells remained in the work-ended conidiophores	C17-1	C17-1	C17-1	C16-1	C20-1	C18-1	C17-1	C18-1	
Continuance (h) of conidial secession	77.8	76.3	76.9	71.0	90.4	84.3	79.9	86.7	80.4 ± 6.3

a The measurement of conidium release time was initiated immediately after C1-1 conidia were abstricted.
b Boundary between conidial secessions with and without division and growth of generative cells.

erection of the swollen generative cell of the conidiophore on somatic hyphae, the differentiation of generative cells and conidial cells, the maturation of conidiophore, the conidial secession driven by repeated division and growth of generative cells, and the cessation of generative cell growth. During these processes, the septum-mediated division of generative cells was a vital step to monitor, because it resulted in the concurrent production of new conidial cells at the basal portion of the conidiophore and the secession of mature conidia at the apex. The sites of conidiophore septation that we identified with the digital microscope were coincident with those we identified in CFW-stained conidiophores with fluorescent microscopy, indicating that the digital microscope is a useful tool to detect septa in living generative cells, even when the septum forms at a nonconstricted portion of the conidiophores. As a result of the consecutive observation of living conidiophores, we also determined the time interval between septations. Hirata (1967) observed chemically fixed samples of powdery-mildewed barley leaves collected at various stages after inoculation and reported developmental changes of conidiophores. Our results support his data on conidial differentiation, but we also clarified the precise cycle of growth and

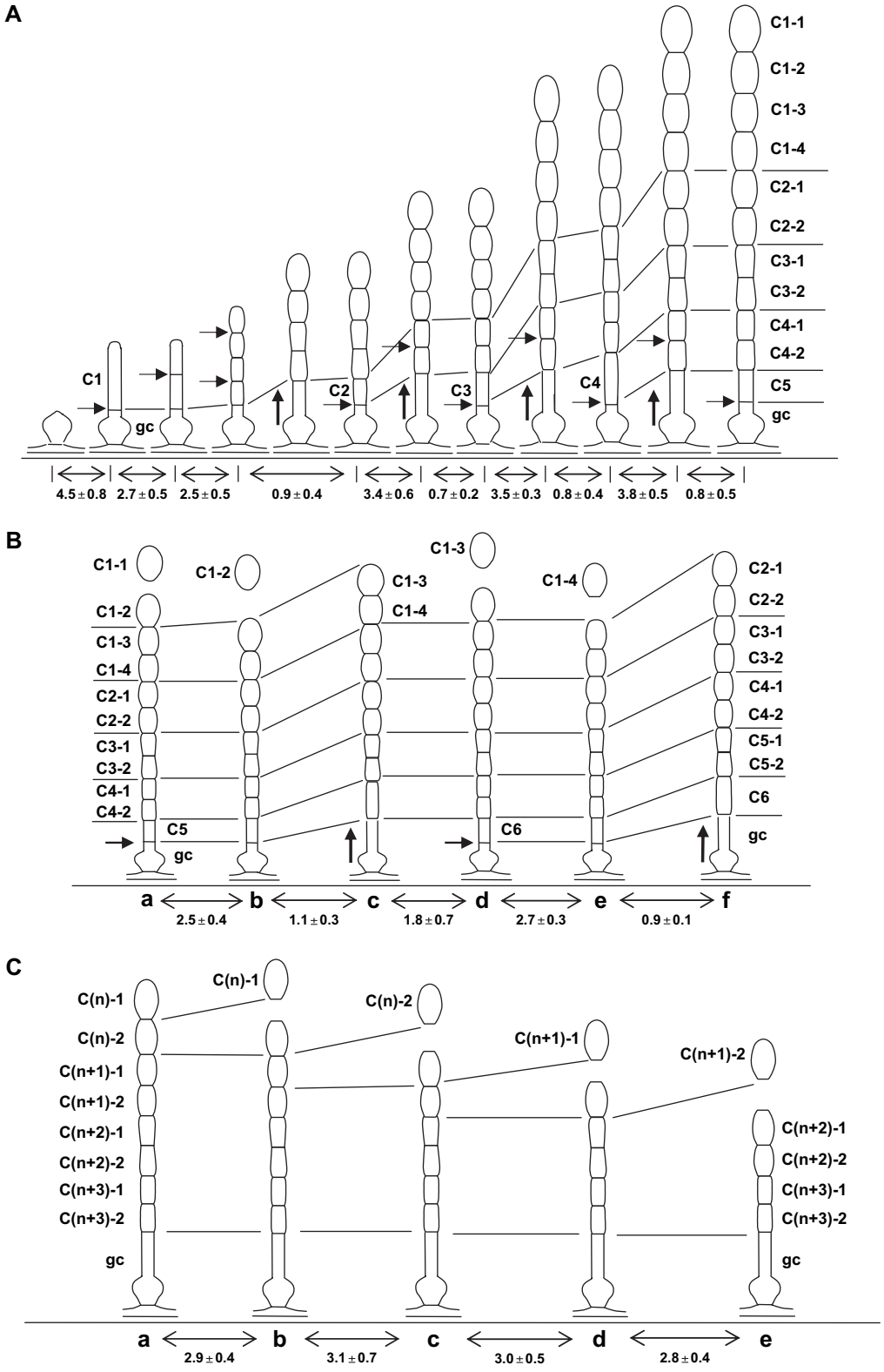

Fig 6 – Diagrams of the developmental process of full-length conidial chain formation and successive secession of mature conidia in conidiogenesis. (A) Conidial formation, with a focus on the growth and septation of the generative cell. Perpendicular and horizontal arrows represent the stage of growth and of septation of a generative cell, respectively. (B) Concurrent events during conidial secession, with an emphasis on elongation of the conidial chain and growth and septation of the generative cell. Perpendicular and horizontal arrows represent growth and septation of the generative cell, respectively. (C) Conidial secession at the final stage. Note the decrease in conidial cells in the chain after growth and division of the generative cell have stopped. C(n + 2) -1 to C(n + 3) -2 were conidial cells that remained in a conidiophore. Time intervals (h) for septation and conidial secession are given at the bottom of the figure. 30 conidiophores were observed, and data are given as means and standard deviation.

division by the generative cells and the subsequent septation of the conidial cells. Using digital microscopy to observe the division of the generative cell and the electrostatic micromanipulation to collect the released conidia, we counted precisely the conidia produced by the barley powdery mildew.

Aylor (1990) reviewed the factors that affect the conidiogenesis in the powdery mildew: wind, relative humidity, air temperature, short and long wavelength radiation, and wetting or dryness of fungus or host. The present system could be exploited to analyze the effect of such environmental factors on the consecutive formation and release of the conidia by living pathogens infecting host leaves. Future work should examine each environmental factor singly while other factors were fixed, using our system.

To our knowledge, we are the first to determine directly the time interval between conidial releases and to count the total conidia that seceded from individual living conidiophores during their life-time. The present method should prove useful for analyzing the details of conidiogenesis, life-long production of conidia, and longevity of the conidiophores of other powdery mildew pathogens on crop plants.

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