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FULL PAPER

Conidia of *Erysiphe trifoliorum* attempt penetration twice during a two-step germination process on non-host barley leaves and an artificial hydrophobic surface

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Abstract In the present study, using a high-fidelity digital microscope, we observed the sequence of appressorial development on the germ tubes of a powdery mildew fungus isolated from red clover leaves. Based on its morphological characteristics and rDNA internal transcribed spacer (ITS) sequences, the fungus was identified as *Erysiphe trifoliorum*, and one of its isolates, designated as KRCP-4N, was used in this work. The conidial germination of isolate KRCP-4N was studied on host (red clover) and non-host (barley) leaves, as well as on an artificial hydrophobic membrane (Parafilm). More than 90% of conidia germinated synchronously and developed dichotomous appressoria (symmetrical double-headed appressoria) on all substrata used. On host leaves, all appressorium-forming conidia developed hyphae (colony-forming hyphae) from conidial bodies without extending germ tubes from the tips of the appressoria. On non-host leaves and on Parafilm-covered glass slides, however, all conidia extended germ tubes from one side of dichotomous appressoria (two-step germination). In addition to the dichotomous appressoria, we detected a few conidia that produced hooked appressoria and extended germ tubes from the tip of the appressorium. Penetration attempts by KRCP-4N conidia on barley leaves were impeded by papillae formed at penetration sites beneath these two types of appressorium. From these results, we conclude that the "two-step germination" of *E. trifoliorum* KRCP-4N conidia is the result of an unsuccessful penetration attempt, causing diversity in appressorial shape.

Keywords Appressorial formation · Barley · Papilla formation · Parafilm · Red clover

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Introduction

In the taxonomy of powdery mildew fungi, Braun et al. (2002) have proposed that anamorphic characteristics and DNA phylogeny are the basis for generic taxonomy and that teleomorphs are less important in this respect but provide useful features at the species level. Important anamorphic characteristics are the size and shape of conidia, position and type of germ tube, shape of appressoria, location of mycelia, production of conidia singly or in chains, and the presence or absence of fibrosin bodies (Boesewinkel 1980). Based on germination patterns (e.g., number, length, and width of germ tubes; shapes of appressoria), Cook and Braun (2009) classified the anamorphs belonging to the genus *Oidium* into nine groups, including the *longitubus* pattern within the *pseudoidium* type that is represented by *Erysiphe trifoliorum* (syn. *E. trifolii*) (Braun et al. 2010). In our previous paper (Matsuda et al. 2005), single appressorium-forming conidia on greenhouse tomato leaves were directly examined for their rDNA internal transcribed spacer (ITS) sequences, indicating that the tomato plants were infected with conidia of two different powdery mildew species with ITS sequences identical to those of *Oidium*

neolycopersici and *E. trifoliorum*, respectively. Moreover, one conidium (with *E. trifoliorum* ITS sequences) developed appressoria from which 'secondary' germ tubes were generated (two-step germination). However, this specific germination pattern was not involved in the groups exemplified by Cook and Braun (2009). So, we attempted to clarify the mode of appressorial germ tube formation in the *trifoliorum*-type powdery mildew fungus.

Isolation of powdery mildew showing the morphological and DNA sequence characteristics mentioned above was a prerequisite in the present study. *E. trifoliorum* was reported to be infectious to lentil and wild soybean (Attanayake et al. 2009), and red clover plants (Braun 1987, 1995). A preliminary inoculation of conidia obtained from infected red clover plants showed the two-step appressorial germination on tomato leaves. In the present study, we therefore attempted to obtain conidia of this powdery mildew as a purified isolate through repeated single conidium isolation. We then examined the isolate for its morphological and molecular characteristics and studied conidial germination following inoculation onto host (red clover) and non-host (barley) plants and an artificial hydrophobic membrane (Parafilm, American National Can, Menasha, WI, USA).

Materials and methods

Plants

Seeds of red clover (*Trifolium pratense* L. cv. Megium) were purchased from Takii Seed Company (Kyoto, Japan), and barley seeds (*Hordeum vulgare* L. cv. Gose-shikoku) were multiplied through self-pollination in our laboratory. Germinated seeds of both plants were planted in vermiculite in 15 cm pots and grown in a growth chamber (for non-inoculated plants) (continuous illumination of 4,000 lux at 20 ± 2°C) for 14 days (for red clover) and 12 days (for barley). The resulting seedlings were used for the following experiments.

Pathogen and inoculation

Powdery mildew-infected leaves of red clover plants grown in our campus field were detached and gently tapped to drop conidia onto the leaves of non-inoculated healthy red clover seedlings. The inoculated seedlings were placed in a growth chamber (for inoculated plants) under the conditions mentioned above. Pseudochain conidia were collected from individual conidiophores of colonies formed on red clover leaves at 10 days after inoculation, using an electrostatic spore collector (Nonomura et al. 2009) and transferred to fresh leaves of uninfected red clover plants by gently moving conidia on the probe tip to the leaf. Newly produced single mature conidia on conidiophores were similarly obtained 1 week after inoculation. After repeating this single conidium isolation procedure three times, the powdery mildew colonies finally obtained were designated as isolate KRCP-4N of *E. trifoliorum* and used for the present study. The isolate was maintained on 14-day-old seedlings in the growth chamber described above.

Sequencing of the rDNA ITS region

To determine the rDNA ITS sequence in isolate KRCP-4N, conidia were collected from inoculated red clover leaves and DNA was extracted using a Qiagen DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was carried out in an iCycler (Bio-Rad, Hercules, CA, USA) using the ITS1F and ITS4 fungal-specific primers designed by Gardes and Bruns (1993) and White et al. (1990), respectively. PCR was performed according to the protocols described by Szentiványi et al. (2005): 5 min denaturation at 95°C and 35 cycles, consisting of a 30 s denaturation at 95°C, annealing at 52°C, and extension at 72°C, were followed by a final extension cycle of 5 min at 71°C. The nucleotide sequence of the amplified region was determined using the BigDye Terminator Cycle Sequencing Ready Reaction Kit from Applied Biosystems (Tokyo, Japan) on an ABI Prism 3130 Genetic Analyzer (Applied Biosystems) in the Pharmaceutical Research and Technology Institute, Kinki University, Japan. Genetyx-Mac software (Version 11.2; a sequence analysis software package; Genetyx, Tokyo, Japan) was used for sequence analysis, and BLAST software was accessed through the DNA Data Bank of Japan (DDBJ). The nucleotide sequence of KRCP-4N was deposited at DDBJ under the accession number AB591735.

Microscopy

Inoculated leaves were observed under a light microscope (BX-60; Olympus, Tokyo, Japan) to examine the shape and size of mature conidia, shapes of appressoria, and lengths of the foot cells and generative cells of conidiophores. Experiments were conducted using 50 non-germinated conidia for conidial shape and size, 50 appressorium-forming conidia for appressorial shapes, and 50 conidiophores (selected at random from 10 colonies on red clover leaves) for the lengths of the foot cells and germinative cells. The data obtained were compared with those reported for *E. trifoliorum* previously (Braun 1995; Attanayake et al. 2009).

For an assay of the germination pattern of KRCP-4N, mature conidia on conidiophores were collected with a

Table 1 Development of infection structure of KRCP-4N conidia following inoculation on different substrata

Substrata inoculated	Percentage of conidia forming							
	Germ tubes		Appressoria		Colony-forming hyphae from conidial bodies		Extension germ tubes from appressoria	
	3	4	5	6	12	24	6	9[a]
Red clover (host)	78.0 ± 5.7 z	90.0 ± 5.7 y	86.8 ± 5.3 x	97.8 ± 1.7 w	69.4 ± 1.8	93.3 ± 2.5	ND	ND
Barley (non-host)	84.0 ± 8.5 z	92.0 ± 5.7 y	94.2 ± 5.2 x	96.6 ± 1.9 w	ND	ND	55.8 ± 1.1 v	90.7 ± 0.9 u
Parafilm	73.0 ± 4.2 z	88.0 ± 2.8 y	81.7 ± 1.1 x	90.7 ± 5.2 w	ND	ND	46.9 ± 9.8 v	84.2 ± 4.0 u

Pseudochain conidia from conidiophores on red clover leaves were used for inoculation. Data are means and standard deviations of three replications. The same letters on mean values in each column indicate no significant difference ($p < 0.05$, Tukey's method)

ND not detected

[a] Hours after inoculation

spore collector and transferred to primary leaves of potted red clover and barley plants and also to Parafilm-covered glass slides. Inoculated leaves and membranes were incubated for 3 h at 95–100% relative humidity (RH) and continuous illumination of 4,000 lux (Kashimoto et al. 2003), and then transferred to a temperature-controlled room (20 ± 1°C, 45–50% RH, 3,500 lux) for consecutive microscopic observations. The observations were carried out using a high-fidelity digital microscope (KH-2700; Hirox, Tokyo, Japan) according to a previously described method (Matsuda et al. 2005).

Fluorescence microscopic observations were conducted to determine whether barley leaves formed papillae against penetration attempts by KRCP-4N conidia. Leaves were detached from the plants at various stages after inoculation, decolored in a boiling alcoholic lactophenol solution for 1–2 min, and stained with aniline blue. The specimen was mounted with 50% (v/v) glycerin and observed with an Olympus light and fluorescence microscope (BX-60; B excitation, B absorption filter, and O-515 barrier filter) according to a previously described method (Sameshima et al. 2004).

Results

Visible colonies of isolate KRCP-4N appeared on red clover leaves 4–5 days after inoculation, and during this period, conidiophores were initiated. The first mature conidia were formed on conidiophores 7 days after inoculation and then removed under windy conditions (wind of more than 0.5 m/s provided by an electric fan placed 1 m from the plant in a temperature-controlled room). Under this condition, no conidial pseudochains were formed. Alternatively, mature conidia piled up (three mature conidia maximum) on conidiophores for the following 3–4 days under conditions of still air or low-strength winds.

For the experiments, we collected conidial pseudochains 10 days after inoculation and examined their germination rates on red clover and barley leaves (Table 1). Conidia showed synchronous germination with very high germination rates and subsequent appressorial formation on red clover (Fig. 1a, b) and barley plants (Fig. 1e, f). On both plants, more than 90% of the observed conidia produced dichotomous appressoria (symmetrical double-headed appressoria), as shown in Fig. 1b, d. On host leaves (red clover), conidia initiated the development of hyphae from conidial bodies 12 h post-inoculation (Fig. 1c), and these then elongated further (Fig. 1d) and finally formed hyphal colonies. In contrast, conidia on non-host leaves (barley) extended a germ tube from one side of dichotomous appressorial heads 6–7 h post-inoculation (Fig. 1g). Elongation of these germ tubes ceased within 1 h. Occasionally, a few conidia produced very short hyphal outgrowths from the conidial bodies (Fig. 1h). These outgrowths did not elongate, even when the incubation period (additional 48 h) was further prolonged. Similar developmental changes were detected following conidial germination on Parafilm (Fig. 1i, j).

In addition to the dichotomous appressorial shape shown in Fig. 1, a minority (9.8 ± 0.2%) of conidia showed a hooked appressorial shape that was similar on red clover (Fig. 2a), barley leaves (Fig. 2b), and Parafilm (Fig. 2c). Also, in this type of appressorium, we observed the extension of germ tubes from the tips (Fig. 2d).

Barley leaves produced papillae at the first penetration site of the typical dichotomous appressorium (Fig. 3a) and at the second penetration site at the apex of the newly developed germ tube (Fig. 3b). Papilla formation was also detected at the penetration sites of hooked appressoria of this pathogen (Fig. 3c, d). These papillae were effective in suppressing penetration by the conidia of isolate KRCP-4N.

Some morphological characteristics (conidial shape and size, appressorial shapes, and the lengths of foot cells and

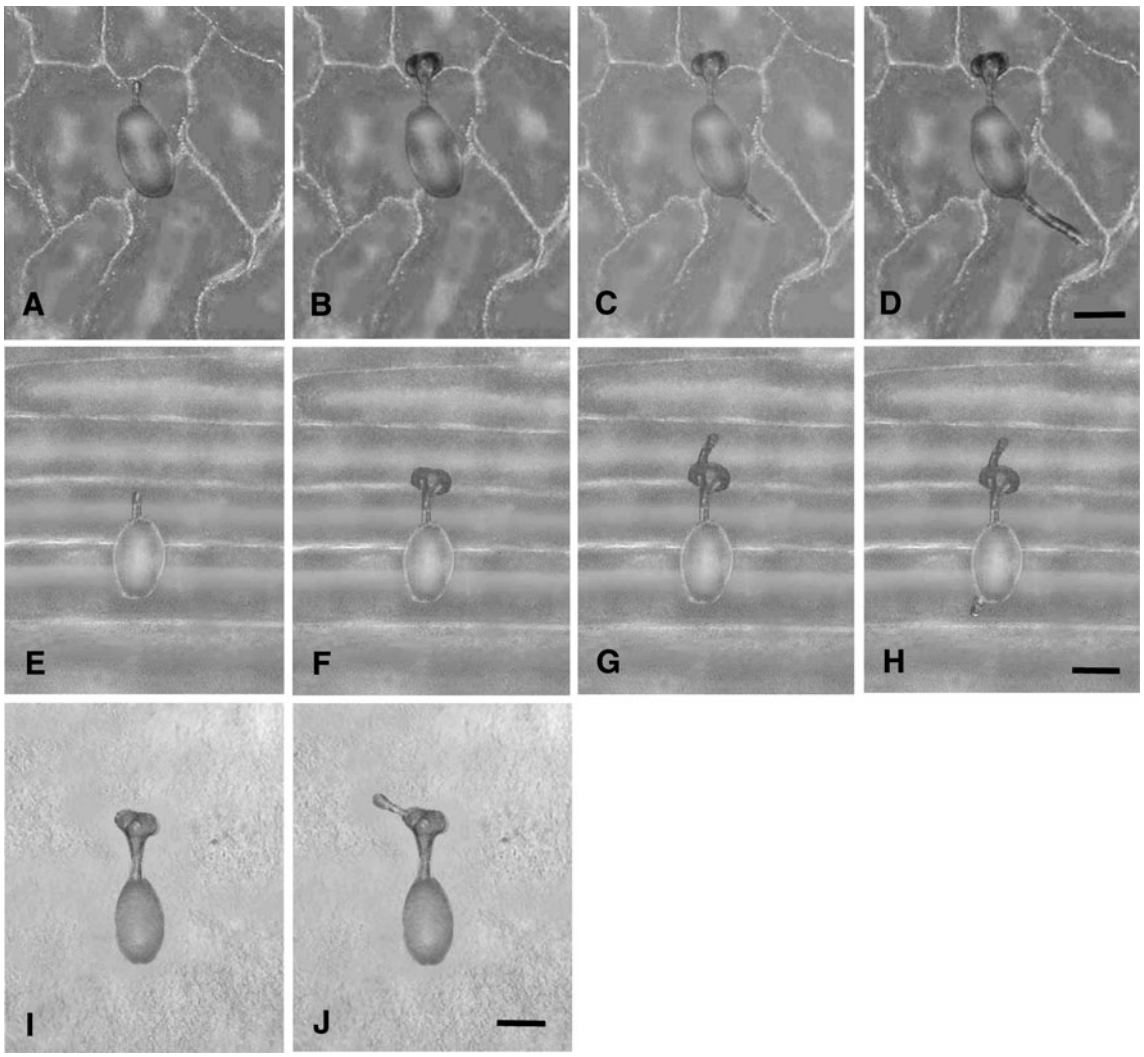

Fig. 1 Conidia of isolate KRCP-4N of *Erysiphe trifoliorum* observed with a high-fidelity digital microscope after germination on different substrata. Conidia on a red clover leaf after 3 h (for germination) (**a**), 5 h (for appressorial formation) (**b**), and after 12 h (**c**) and 14 h (**d**) (for elongation of colony-forming hyphae). Conidia on a barley leaf after 3 h (**e**), 5 h (for appressorial formation) (**f**), and after 6 h (**g**) and 8 h (**h**) (for extension of germ tube). Conidia on a Parafilm-covered glass slide after 6 h (for appressorial formation) (**i**) and after 8 h (for extension of germ tube) (**j**). *Bars* represent 10 μm

germinative cells in conidiophores) of the powdery mildew isolate KRCP-4N were examined to compare them with characteristics of *E. trifoliorum* previously reported by Attanayake et al. (2009) and Braun (1995). Eventually, the tested KRCP-4N characters were found to be close to the data for *E. trifoliorum*, although the conidia of KRCP-4N were slightly smaller than those of *E. trifoliorum* (data not shown). BLAST analysis indicated that the nucleotide sequence of the rDNA ITS region (645 bp) of isolate KRCP-4N was identical to the ITS sequences determined earlier for *E. trifoliorum* (DDBJ accession numbers, AB015913, AB163926, AB167523, AB167524, and AF298542). This supported the identification of isolate KRCP-4N as *E. trifoliorum.*

Discussion

In the present study, we confirmed that the "two-step germination" pattern of *E. trifoliorum* conidia observed previously on tomato leaves (Matsuda et al. 2005) is a well-recognizable characteristic of this species on non-host surfaces. We detected no chasmothecia of this pathogen, so the identity of the present isolate was confirmed based on its ITS sequence and morphological characteristics. Cook and Braun (2009) presented a new method to classify powdery mildews on the basis of the germination patterns of conidia under defined conditions (on a hydrophobic surface, e.g., plastic at 97% RH) and illustrated possible appressorial shapes of the *pseudoidium* type (rod-shaped,

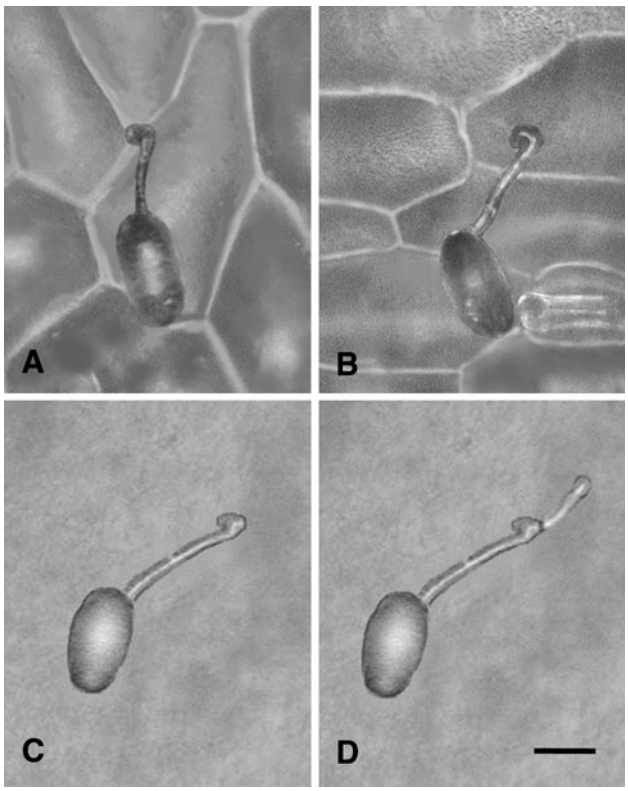

Fig. 2 Digital micrographs of hooked appressoria of germinated conidia of isolate KRCP-4N; after 5 h on leaves of red clover (**a**) and barley (**b**). On a Parafilm-covered glass slide, after 5 h (**c**) and 8 h (**d**). *Bar* represents 10 μm

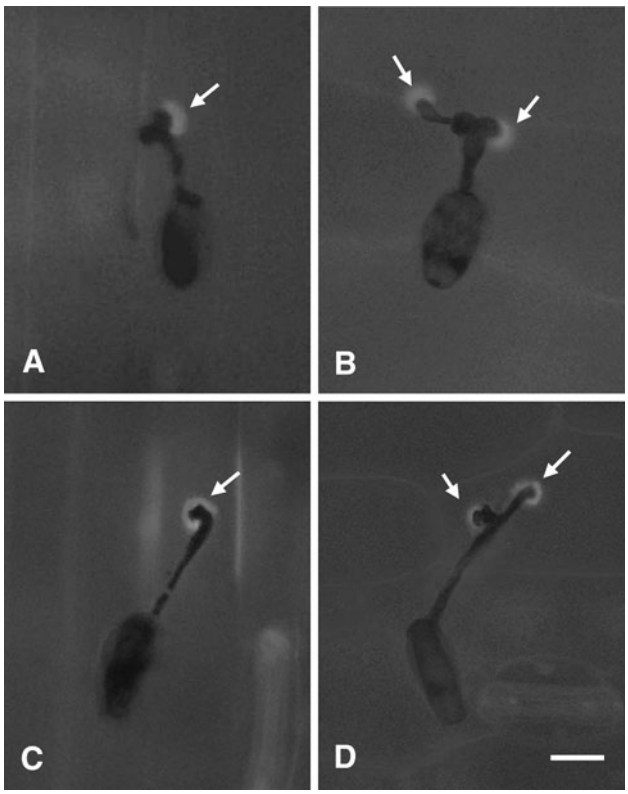

Fig. 3 Fluorescence micrographs of papillae (*arrows*) formed by barley leaves against penetration of KRCP-4N conidia. Papilla at the first penetration site beneath a dichotomous appressorium 6 h post-inoculation (**a**) and at the second penetration site at the apex of an extended germ tube after 8 h (**b**). Conidia forming hooked appressoria inciting a papilla at the first (**c**) and second (**d**) penetration sites, photographed after 6 and 8 h, respectively. *Bar* represents 10 μm

swollen, hooked, symmetrical, and forked appressoria), while, on a hydrophilic surface, e.g., glass at 100% RH, the "*longitubus* pattern within the *pseudoidium* type" was commonly exhibited by *E. trifoliorum* under these conditions. The KRCP-4N conidia showed dichotomous appressoria (symmetrical double-headed appressoria) at the apex of short germ tubes on host red clover leaves (as well as on non-host barley and artificial hydrophobic surfaces). These results strongly support the identity of the present red clover powdery mildew KRCP-4N as *E. trifoliorum*.

In our previous paper (Nonomura et al. 2010), we inoculated conidia of the tomato powdery mildew (*O. neolycopersici*) onto barley leaves to detect their penetration attempts resulting in papilla formation at penetration sites. The examination under a fluorescence microscope was useful in determining the number and position of the clearly fluorescing papillae at penetration sites. One of the most important findings in the present work is the two-step germination of KRCP-4N conidia on the non-host barley leaves. Importantly, the pseudochain conidia collected from conidiophores were fully mature, independent of each other, and highly germinative. In fact, almost all conidia germinated synchronously and formed dichotomous appressoria within 5 h of inoculation. The duration of the process (from germination to appressorial development) and the appressorium shape (see Table 1; Fig. 1) were very similar in the conidia on both red clover and barley leaves. However, subsequent fungal development was remarkably different on the different substrates; conidia on host leaves developed and elongated superficial colony-forming hyphae from conidial bodies, while conidia on the non-host leaves extended germ tubes from one side of the forked appressoria. Obviously, the vigorous elongation of hyphae from the conidial body was due to the successful formation of functional primary haustoria in the invaded epidermal cells at the first penetration site. In contrast, the extension of a germ tube from the appressorium (two-step germination) was the result of failure of the conidia to invade at the first penetration site, leading to an additional penetration attempt beneath the tip of the extended germ tube. The present results reveal that both penetration attempts on the barley leaves were impeded by papilla formation (see Fig. 3).

In our previous work (Nonomura et al. 2010), we described the widely variable shape of appressoria

(non-lobed; nipple-shaped; moderately, multi-, and highly lobed) of the tomato powdery mildew conidia as a result of unsuccessful penetration attempts. Also in the present study, the KRCP-4N conidial appressoria changed shape on the non-host plant, but the change only involved the extension of a short germ tube from one side of the fork of a dichotomous appressorium. Powdery mildew conidia appear to repeat penetration attempts until they succeed in forming functional primary haustoria. The number of penetration attempts may be specific to the powdery mildew, e.g., twice in the present powdery mildew and up to seven times in *O. neolycopersici* (Nonomura et al. 2010).

An additional important finding of the present work is the two-step germination of KRCP-4N conidia on Parafilm (hydrophobic surface). No clear differences were observed between conidia on barley leaves and those on Parafilm surfaces in the germination rate, appressorial shape, or length of time to the end of the two-step germination process. These results strongly suggest that the conidia attempted to penetrate Parafilm on a glass slide twice due to failure of haustorial formation, although we still need to confirm, using scanning electron microscopy, that these conidia produced infection pegs on the membrane.

According to the above assumption, an assay to determine conidial germination patterns on a plastic or glass surface, as recommended by Cook and Braun (2009), indicates a condition in which they never form haustoria. The two-step germination pattern (dichotomous appressoria with an extension germ tube) of isolate KRCP-4N of *E. trifoliorum* is not included in patterns presented for this pathogen in Cook and Braun (2009). The reason why we missed the relevant groups for our isolate may be due to the experimental protocol used here. The most recent extensive study, by Cook et al. (2011), revealed that some species of *Erysiphe* powdery mildews showed the two-step germination pattern with wide diversities of appressorial shapes. We plan further studies of other powdery mildews to clarify the diversification in their germination patterns.

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