Natural Woody Plant, *Mallotus japonicus*, as an Ecological Partner to Transfer Different Pathotypic Conidia of *Oidium neolycopersici* to Greenhouse Tomatoes

Teruo NONOMURA[1], Yoshinori MATSUDA[1], Shun YAMASHITA[1], Haruhiko AKAHOSHI[1], Yoshihiro TAKIKAWA[2], Koji KAKUTANI[3] and Hideyoshi TOYODA[1]

[1]Laboratory of Phytoprotection, Science and Technology, Faculty of Agriculture, Kinki University, Nara, Japan; [2]Plant Center, Institute of Advanced Technology, Kinki University, Wakayama, Japan; [3]Pharmaceutical Research and Technology Institute, Kinki University, Osaka, Japan

Abstract

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In our routine surveys for the powdery mildew disease in greenhouse tomatoes, we detected a new pathogen that forms pseudochains consisting of 12 conidia. To identify the original plant that dispersed this pathogen, wild plants infected with powdery mildew were monitored. The pathogen on Japanese mallotus, *Mallotus japonicus*, produced a similar type of pseudochain, and conidia were infectious to tomatoes. Inversely, the conidia on the tomato leaves infected *M. japonicus*. Infectivity assays and internal transcribed spacer (ITS)-based phylogenetic analyses indicated that the two pathogens on the tomato and *M. japonicus* were identical. These results suggest that the conidia on *M. japonicus* can be transmitted to greenhouse tomatoes. This work documents the ecological transmission of conidia between wild plants and greenhouse tomatoes.

Keywords: tomato powdery mildew; Japanese mallotus; conidial pseudochain; wild tomato species; *Oidium neolycopersici* teleomorph

Our greenhouse tomatoes have suffered from infection with tomato powdery mildews for 15 years. During this period, we have attempted to isolate the pathogens infecting various tomato cultivars at different periods of cultivation, to characterise physiological and morphological differences among the isolates, and to identify variation in their virulence to commercial tomato cultivars or wild tomato species and in their host ranges. We obtained some isolates of *Oidium neolycopersici* from our greenhouses: KTP-01 in 1998 (Kashimoto *et al*. 2003), KTP-02 in 2009 (Seifi *et al*. 2012), and KTP-03 and KTP-04 in 2012 (Nonomura *et al*. 2013). In the present paper, we describe the additional isolation of the pathogen KTP-05.

Our routine method for morphologically specifying powdery mildew pathogens uses digital microscopy to observe pathogens colonising tomato leaves. This approach is highly effective for collecting living conidia after observation, as no use of chemical treatments for fixation, decolorisation or staining of samples is required (Matsuda *et al*. 2005). We targeted conidiophores for microscopic observations, because of the easy and rapid detection of conidial pseudochains on them (Oichi *et al*. 2004, 2006). Pseudochain formation is common in the tomato powdery mildew pathogens (Jones *et al*. 2001; Kiss *et al*. 2001), and the number of conidia in the pseudochain is inherited (Oichi *et al*. 2006; Nonomura *et al*. 2009).

Examining isolated single conidia for their infectivity was the second step to specify the pathogens. Some authors (Lindhout *et al*. 1994; Ciccarese *et al*. 1998; Huang *et al*. 1998; Mieslerová *et*

al. 2000; Lebeda & Mieslerová 2002; Lebeda *et al*. 2002) have examined the infectivity of the *O. neolycopersici* isolates to wild tomato species. Particular lines of wild tomatoes were available as detector plants to select new isolates. We have also used *Solanum habrochaites* G1.1560 and *S. peruvianum* LA2172 to distinguish between KTP-01 and KTP-02 (Seifi *et al*. 2012) and *S. arcanum* LA1351 and *S. hirsutum* LA1777 to distinguish between KTP-03 and KTP-04 and among previous isolates (Nonomura *et al*. 2013).

In the present study, we investigated the mechanism of the repeated natural infection of our greenhouse tomatoes with different pathogenic isolates of tomato powdery mildew. Our hypothesis was that new pathogens may originate from wild plant colonies surrounding the greenhouse, i.e. there is an ecological relationship between pathogens in greenhouse tomatoes and those in the surrounding wild plants. Our essential approach was to identify wild plants infected with powdery mildew (partner plants) whose conidia can infect tomato plants. To this end, we intensively surveyed wild plants on our campus. Our main goal was to compare morphological and pathogenic characteristics between isolates obtained from wild plants and tomato plants. We focused on conidial pseudochains of the powdery mildews on both plants and their infectivity to wild tomato species.

We identified some plants infected with powdery mildew pathogens. Among them, a euphorbiaceous plant, Japanese mallotus, *Mallotus japonicus* (JM) was highly susceptible to powdery mildew and formed conidial pseudochains on its leaves. The present paper compares the characteristics of isolates from tomato (KTP-05) and JM (KMP-01) obtained in this study to clarify their relationship.

MATERIAL AND METHODS

Hydroponic culture of tomato. One-month-old seedlings of the common tomato plant (*Solanum lycopersicum* L. var. *lycopersicum*, cv. Moneymaker) were hydroponically cultured in a window-open greenhouse according to previous methods (Nonomura *et al*. 2001). A total of 200 Moneymaker (MM) seedlings (50 seedlings per hydroponic trough) were grown in 4 troughs for 2 months to allow natural infection to occur and replaced with non-infected, young seedlings at 2-month intervals.

Microscopic specification of pathogens collected from greenhouse tomatoes. In the survey of greenhouse tomatoes, all colony-forming leaves were collected to assay powdery mildew pathogens. Leaves were excised at the petiole, and the edge of the petiole was covered with moistened cotton wool. Excised leaves were placed in a Petri dish and incubated in a growth chamber for 7 days at 25 ± 1°C and 50–70% relative humidity under continuous illumination of 4000 lux. Conidiophores on the leaves were observed using a KH-2700 high-fidelity digital microscope (Hirox, Tokyo, Japan) according to methods described previously (Matsuda *et al*. 2005).

Mature conidia were collected from pseudochains of conidiophores under digital microscopy using an electrostatic spore collection probe (Nonomura *et al*. 2009) and transferred to healthy leaves of 14-day-old tomato seedlings that had been placed in an electrostatic screen chamber (ES-chamber) (an apparatus for preventing airborne pathogens from entering the chamber) (Matsuda *et al*. 2006) to prepare pathogen-free seedlings. After repeating this inoculation procedure three times, the obtained cultures were maintained as a new isolate (KTP-05).

Collection of powdery mildews colonising wild plants. A total of 852 wild and cultivated woody and herbaceous plants on our campus were surveyed, and the leaves on which fungal colonies were formed were collected. To maintain the pathogens, conidia were collected from pseudochains as above, and inoculated onto leaves of ES-chamber-guarded, healthy seedlings of the plants from which infection was originally detected. After three rounds of single-colony isolation, purified conidia were used for the following experiments.

Inoculation with isolated conidia. The wild tomato species *S. chilense* LA0468, *S. hirsutum* LA0386 and LA1738, *S. peruvianum* LA2172, and *S. parviflorum* G1.1601 and MM, as well as JM were used for inoculation assays. Seeds of G1.1601 were obtained from the Centre for Genetic Resources (CGN, Wageningen University, the Netherlands), and the others were obtained from the Tomato Genetic Resource Center (TGRC, University of California, Davis, USA). Germinated seeds of wild tomato and MM (cultivated tomato) were incubated in a growth chamber for 2 weeks, and cotyledons were used for inoculation. Young JM seedlings growing in a field were separated by cutting their subterranean stems, and cuttings with two leaves

were potted for multiplication. The leaf surface was surveyed with a high-fidelity digital microscope to confirm no conidia that had naturally infected the plants. Potted healthy cuttings were grown in an ES-chamber for one month to prepare pathogen-free seedlings. Successfully grown pathogen-free seedlings were used for inoculation.

Conidia of the recent isolate ex tomato (KTP-05) and the recent isolate ex JM (KMP-01) were used for inoculation. In addition, four isolates ex tomato (KTP-01 to KTP-04) maintained in our laboratory were used for comparison. Single conidia were collected and transferred to cotyledons of wild tomato plants and MM or upper young leaves of JM. Inoculated plants were placed in a growth chamber under the same conditions for 2 weeks. Infectivity of the pathogens was evaluated according to the criteria shown in the footnote of Table 1.

Phylogenetic analysis of the pathogen. DNAs of KTP-05 and KMP-01 were extracted from conidia using a Qiagen DNeasy plant mini kit (Qiagen, Hilden, Germany), and ribosomal DNA internal transcribed spacer (ITS) sequences were amplified by polymerase chain reaction (PCR), using the ITS1F (Gardes & Bruns 1993) and ITS4 fungal-specific primers (White *et al*. 1990). PCR was carried out with an iCycler (Bio-Rad, Hercules, USA) according to the protocols of Szentiványi *et al*. (2005). The nucleotide sequences of both isolates were deposited at the DNA Data Bank of Japan (DDBJ) under the accession numbers AB808656 for KTP-05 and AB808657 for KMP-01.

For phylogenetic analysis, the ITS sequences of different powdery mildews including the present isolates were obtained from the DDBJ database and aligned using the CLUSTALW software. The Njplot software was used to create distance matrices and to infer dendrograms using neighbour-joining analysis. The strength of groupings was estimated using bootstrap analysis with 1000 replicates (Felsenstein 1985).

Observation of chasmothecia. Leaves of potted JM plants were inoculated with KTP-05 conidia in September, 2012. Two months later, the leaves forming chasmothecia were collected for a microscopic assay. Chasmothecia were gently scraped from the leaves with a glass needle, transferred to a glass slide and pressed over a cover glass to push asci and ascospores out of the chasmothecia.

RESULTS AND DISCUSSION

To detect newly emerging tomato powdery mildews, we routinely surveyed the occurrence of powdery mildew colonies on greenhouse tomatoes. Once symptoms occurred on tomato leaves, infected leaves were collected to analyse the mycological and phytopathological characteristics of the pathogens. Excised leaves were kept in a Petri dish to ensure windless conditions. These conditions were essential for the pathogens to produce conidial pseudochains of a maximum length (Oichi *et al*. 2006; Nonomura *et al*. 2009). In fact, pseudochain conidia of the present isolates fell down from the conidiophores when they were exposed to a wind stronger than 0.4 m/s.

In a previous study (Nonomura *et al*. 2008), we monitored the occurrence of wind-borne conidia of tomato powdery mildews in a window-open greenhouse and reported limited numbers of conidia (5–10 conidia per day). In the present

Table 1. Infection indices for isolates of tomato powdery mildew *O. neolycopersici* on wild and common tomato plants

Isolates	Date of inoculation	Number of conidia forming pseudochain	Plants of inoculation[a]						
			LA0468	LA0386	LA1738	LA2172	G1.1601	MM	JM
KTP-05	2012	12	0	0	1	0	0	3	3
KMP-01	2012	12	0	0	1	0	0	3	3
KTP-01	1998	4	2	0	1	0	3	3	0
KTP-02	2009	4	2	2	0	2	2	3	0
KTP-03	2012	8	3	1	2	0	2	3	0
KTP-04	2012	8	1	2	0	3	1	3	0

0 – no growth with no visible host response; 1– limited colony growth with slight host necrosis; 2 – moderate colony growth with host necrosis surrounding fungal colonies; 3 – vigorous colony growth with no host necrosis; [a]see the text in Material and Methods for scientific names of inoculated plants

study, we obtained similar results: about 8.2 (± 0.5) colonies per day were found on separate leaves of different tomato plants. Directly observing colonies formed by these conidia was a rapid way to obtain isolates producing pseudochains with different numbers of conidia.

We tested a total of 12 021 colonies during the experimental period and detected a new type of pathogen forming different pseudochains in 185 colonies, which were distinguishable from the prevalent powdery mildews in our greenhouses. Figure 1A shows colony forming pseudochains with 1–9 conidia in the same colony on a tomato leaf in a greenhouse. Actually, these pseudochains were different from those of pathogens that produce pseudochains of 1–6 conidia in greenhouse conditions (Figure 1B). These results indicated that colonies forming pseudochains of 1–6 conidia are dominant in our greenhouse tomatoes and that pathogens forming pseudochains of 1–9 conidia newly appeared in this period. Our recent work (Oichi *et al*. 2006; Seifi *et al*. 2012; Nonomura *et al*. 2013) has revealed that the population of powdery mildew pathogens in our greenhouses has changed over the last 5 years. Here, we report the occurrence of the new powdery mildew pathogen (KTP-05).

We consecutively observed colony development of KTP-05 on a tomato leaf *in vitro*. Colonies first appeared 3–4 days after inoculation, and initial conidiophores formed on the leaf-surface hyphae 5 days after inoculation. Conidiophores increased as the colony developed; eventually 600–700 conidiophores formed in the same colony within 2 weeks of inoculation. These fungal developments were similar between KTP-05 and KTP-03 or -04. Conidiophores developed mature conidia on their apical end and continued to generate more conidia without releasing the initial ones. This process was repeated until 12 mature conidia had accumulated on the conidiophore (Figure 1C). At this point, under windless conditions, the pseudochains tumbled down the leaf surface. From these results we conclude that this is the maximum number of pseudochain conidia produced by KTP-05. However, these conidia were very sensitive to wind (0.2–0.4 m/s). In fact, longer pseudochains were rarely present on plants at the windy site of the ventilated greenhouse. Conversely, other pathogens formed pseudochains with a maximum of only eight conidia under windless conditions *in vitro* (Figure 1D). In these samples, the pseudochains also tumbled once eight conidia had formed. These

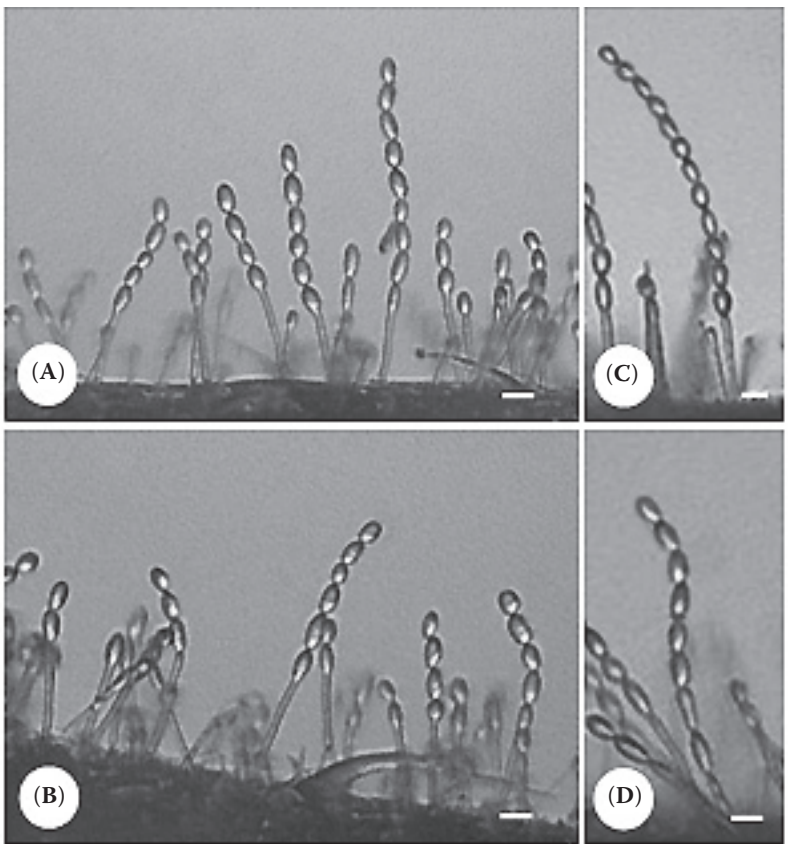

Figure 1. Digital micrographs of powdery mildew colonies on tomato leaves in a greenhouse **(A, B)** and pseudochains possessing 12 **(C)** and 8 **(D)** mature conidia on conidiophores under windless conditions in a growth chamber; **A** and **C** – KTP-05, **B** and **D** – KTP-03 (bars represent 20 μm)

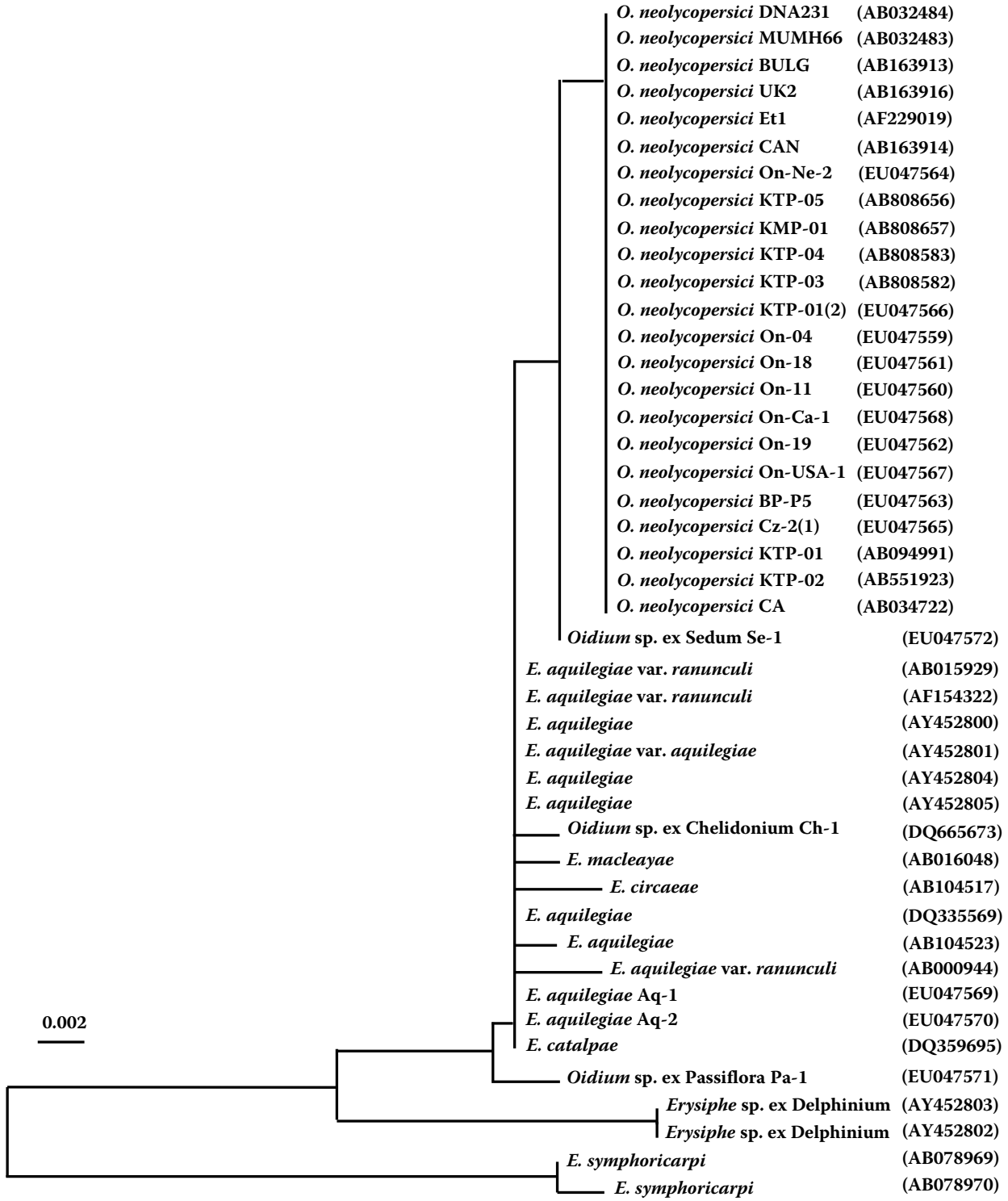

Figure 2. Phylogenetic tree of different powdery mildew species based on the internal transcribed spacer sequence. The newly discovered powdery mildew isolates KTP-05 and KMP-01 belong to the *Oidium neolycopersici* clade

characteristics are similar to those of KTP-03 and KTP-04 (Nonomura *et al*. 2013).

The major focus of the present work was to clarify partner plants that could multiply new tomato powdery mildews and transfer them to greenhouse tomatoes. In the present survey, we detected 14 wild plants found on the campus that were infected with powdery mildews: Japanese mallotus (*M. japonicus*), red clover (*Trifolium pratense*), Japanese mugwort (*Artemisia princeps*),

beggar ticks (*Bidens frondosa*), harlequin glory bower (*Clerodendrun trichotomum*), snailseed (*Cocculus orbiculatus*), Japanese ash (*Fraxinus lanuginosa*), Indian lettuce (*Lactuca indica*), yellow sweet clover (*Melilotus officinalis*), Chinese plantain (*Plantago asiatica*), bamboo-leafed oak (*Quercus myrsinaefolia*), Canada goldenrod (*Solidago canadensis* var. *scabra*), common sowthistle (*Sonchus oleraceus*), and suckling clover (*Trifolium dubium*). The pathogens on two plants (JM and red clover) developed non-catenate conidiophores, i.e. conidia mature singly and then adhere together to form pseudochains on conidiophores, while all colonies on other plants formed catenate conidiophores, i.e. conidia mature gradually whilst still in a chain. We preliminarily tested the infectivity of the conidia from JM and red clover colonies to tomato. The JM powdery mildew was infectious to tomato plants, while the red clover powdery mildew was suppressed by papilla formation at penetration sites (data not shown). Based on these results, we considered JM a candidate partner plant for tomato. The pathogen of JM was purified and designated KMP-01. We confirmed by digital microscopic observation of KMP-01 that this powdery mildew also produced pseudochains of 12 conidia on both JM and MM.

We also tested the infectivity of KTP-05 and KMP-01. Table 1 summarises the results of the pathogenicity assay used to distinguish between powdery mildew isolates. All tomato isolates (KTP-01 to KMP-05) were distinguishable from one another, because they showed distinct levels of infection on the wild tomato plants used for inoculation. In contrast, KTP-05 and KMP-01 were weakly infectious to LA1738 only, and neither isolate was distinguishable from the present assay. Most importantly, reciprocal inoculation, i.e. inoculations of KTP-05 onto JM and KMP-01 onto MM, showed that MM and JM were highly susceptible to both pathogens. Figure 2 shows a ITS sequence-mediated phylogenetic tree for KTP-05, KMP-01 and previous tomato powdery mildew pathogens. The ITS sequence of KTP-05 was identical to KMP-01 and other *O. neolycopersici* isolates, indicating that KTP-05 and KMP-01 are new isolates of *O. neolycopersici*. Thus, our data strongly suggest that this type of powdery mildew is transmitted between JM and MM.

Consequently, it seems plausible that conidia that form on JM leaves could be carried by the wind to greenhouse tomatoes. To test this possibility, we traced the spread of JM powdery mildew among host plant clusters at various distances from each other. Figure 3 shows the locations of these clusters on the campus. At the initial survey (June, 2012), the disease was found only in one plant of a JM cluster (Figure 3A); however, this spread throughout the experimental period (Figures 3B and C). During the experimental period (from June to October), a mountain wind frequently blew from northwest to southeast (from top left to bottom right in the figure). These results support our presupposition that JM powdery mildew

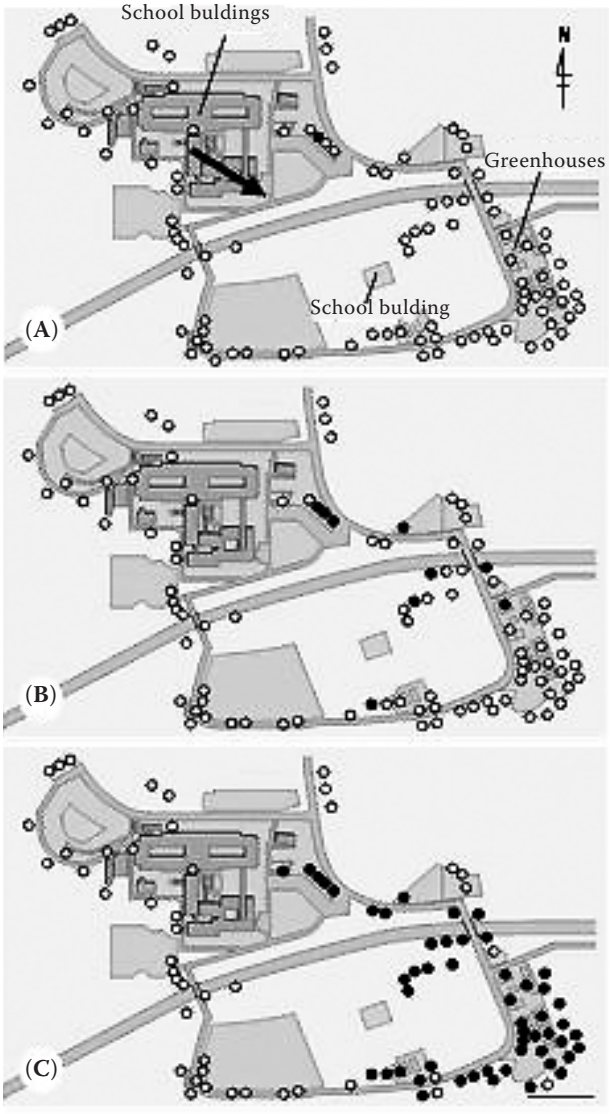

Figure 3. Occurrence of powdery mildew infection on *Mallotus japonicus* plants on our campus during surveys in June **(A)**, August **(B)** and October **(C)**, 2012. Open and closed circles represent non-infected and infected clusters, respectively. The arrow indicates the major direction of mountain wind blowing during the experimental period. Bar represents 100 m

conidia spread downwind to clustered JM plants near the tomato greenhouse.

The final aim of the present work was to examine whether the JM leaves were possible sites of chasmothecia formation (sexual stage) of the tomato powdery mildew pathogens. To examine this possibility, we inoculated KTP-05 conidia onto 200 leaves of 10 JM plants (5–10 conidia per leaf) and placed inoculated plants outside the greenhouse. We conducted the inoculation in September to expose the pathogens to lower temperatures in late October and November; average temperatures in our district were 22.9 ± 5.3, 16.6 ± 5.6, and 11.1 ± 5.4°C in September, October and November, respectively. Within 2 months of inoculation, numerous chasmothecia formed on inoculated leaves (Figure 4A): 50–60 per leaf on 98 of 200 inoculated leaves. Each chasmothecium tested possessed two asci (Figure 4B), each ascus had six ascospores (Figure 4C) and the appendages had a rod-like tip (Figure 4D). These morphological characteristics are similar to those of the *Erysiphe* genus (Braun 1987; Braun & Cook 2012). From these results we concluded that the wild JM plants surrounding our greenhouses are possible sites of mating for the tomato powdery mildews.

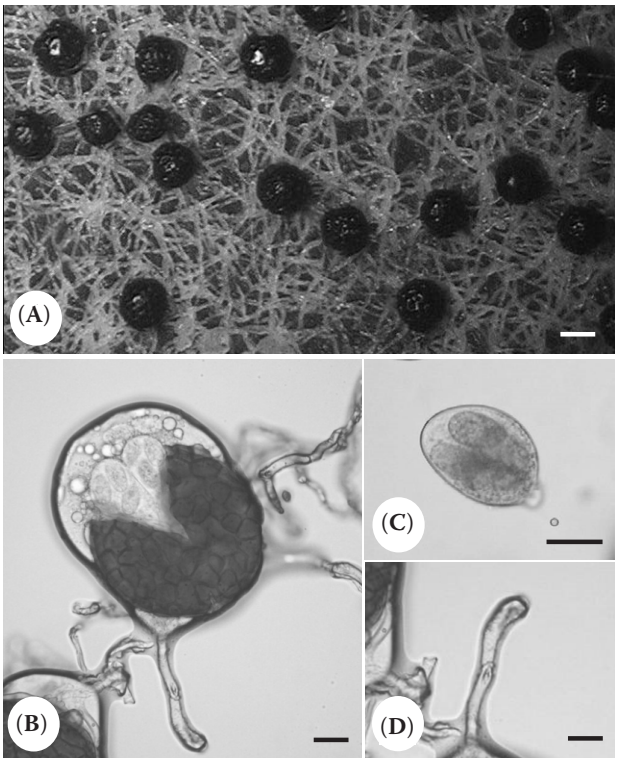

Figure 4. Light micrographs of KTP-05 chasmothecia on *Mallotus japonica* leaves **(A)**, asci in a broken chasmothecium **(B)**, ascospores in asci **(C)** and appendages **(D)**. Bars represent 100 μm **(A)** and 20 μm **(B–D)**

Despite successfully tracing the most recently appearing pathogen by equating KTP-05 ex tomato with KMP-01 ex JM, we have failed to identify the source of the powdery mildew pathogens (KTP-01 to KTP-04) that have prevailed in our greenhouse tomatoes. Apparently, JM was not the source of these pathogens due to their unsuccessful infection of JM (Table 1). Our next step is to search partner plants that could multiply and transfer powdery mildews of tomato. This approach is essential to clarify the mechanism of ecological transfer of tomato powdery mildews.

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Corresponding author:

Prof Hideyoshi Toyoda, Kinki University, Faculty of Agriculture, Laboratory of Phytoprotection Science and Technology, 3327-204 Nakamachi Nara 631-8505, Japan; E-mail: toyoda@nara.kindai.ac.jp