Differences in early hyphal development of *Podosphaera xanthii* on *Cucumis sativus* leaves acclimatized to high or low relative humidity

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Abstract: This research investigated early hyphal development of cucurbit powdery mildew (*Podosphaera xanthii*) on cucumber (*Cucumis sativus* L.) leaves acclimatized to different relative humidities to clarify the factors that limit it. Cucumber seedlings were acclimatized to a low (10%) or high (90%) relative humidity (RH) at 30°C from germination. When the cotyledons had expanded and the first true leaf had begun to emerge, plants were inoculated, and initial conidial invasion, hyphal development, and secondary haustorial formation were evaluated. The initial invasion of *P. xanthii* into the epidermis was slower on the low-RH leaves than on the high-RH leaves, suggesting that structural properties, such as greater leaf mass per area, physically limited the development of *P. xanthii*. The hyphal development and secondary haustoria of conidia that had successfully infected the epidermis were also slower on the low-RH leaves. These post-invasion behaviors indicate that conidial development was also suppressed by non-structural properties of low-RH leaves such as nutrients which correlate with non-structural defense. Reduced leaf N content of the low-RH leaves may in part have inhibited the post-invasion behaviors of *P. xanthii*.

Key words. cucurbit powdery mildew, haustorium formation, initial invasion, leaf structure, leaf nitrogen content, plant–pathogen interactions

33 **Introduction**

34 Development of powdery mildews is influenced by atmospheric humidity, but response to
 35 humidity differs between different parts of the life cycle. The colonization, sporulation, and
 36 dispersal of powdery mildews are favored by low humidity (Yarwood 1957; Aust and v.
 37 Hoyningen-Huene 1986), whereas infection and survival are favored by high humidity
 38 (Reuveni and Rotem 1974). In addition to its direct effects on the pathogen, atmospheric
 39 humidity influences the host plants, which in turn influence the development of powdery
 40 mildew indirectly.

41 The authors previously reported that conidial development of cucurbit powdery mildew
 42 (*Podosphaera xanthii*) was suppressed on cucumber (*Cucumis sativus* L.) leaves acclimatized
 43 to low humidity (Itagaki et al. 2014). This previous research demonstrated that spore
 44 germination was not affected, but colony density and haustoria formation 7 days after
 45 inoculation were less on leaves whose epidermis had become thicker under lower humidity.
 46 Thus, the inhibition of *P. xanthii* development may have been due to changes in leaf structure,
 47 such as the thickness of epidermal cell wall. However, our knowledge of the early
 48 development of *P. xanthii* on leaves, which is important for understanding limitations on the
 49 infection process, is still incomplete.

50 The early development of *P. xanthii* after germination can be divided into initial invasion
 51 and subsequent development. The former can be limited by the structural properties of the
 52 host leaf, which physically resist penetration by the pathogen (Aust and Hoyningen-Huene
 53 1986). The latter can be limited by non-structural properties, which resist subsequent
 54 penetration and nutrient absorption by the pathogen (Aust and Hoyningen-Huene 1986;
 55 Bélanger et al. 2002; Pérez-García et al. 2009). Thus, the distinction of these processes by
 56 evaluating early hyphal development can reveal the factors that limit pathogen development
 57 (Itagaki et al. 2016, 2017).

The first objective of this study was to evaluate the early development of hyphae on leaves acclimatized to different relative humidities (RHs) so as to investigate the relationships between host-plant susceptibility and leaf properties. The authors focused on leaf mass per area (LMA) and nitrogen (N) content as the leaf properties, because these values correlate respectively with structural and non-structural defense (Walters and Bingham 2007; Toome et al. 2010) and can be altered under water stress conditions (Ben-Zioni et al. 1967; Salleo and Gullo 1990; Yin et al. 2009). The previous study tested seedlings only at the cotyledon stage (Itagaki et al. 2014), whereas the growth stage at which the effects of environmental factors on plant–pathogen interactions are described must be specified (Itagaki et al. 2015). So the second objective was to investigate the effect of acclimatization humidity on conidial development at the true leaf stage.

Materials and Methods

Expt. 1: Evaluation of early hyphal development of *P. xanthii* on cucumber seedlings acclimatized to different relative humidities

Cucumber cv. Hokushin (nonresistant cultivar; Takii & Co. Ltd., Kyoto, Japan) seeds were sown in plastic pots containing vermiculite, which were then placed in growth chambers maintained at a low (10%) or high RH (90%) at 30°C. Illumination was supplied at a photosynthetic photon flux density (PPFD) of 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ by fluorescent lamps with a 12-h photoperiod. The pots were placed in nutrient solution (the A-type recipe of OAT Solution; OAT Agrio Co. Ltd., Tokyo, Japan) 10–20 mm deep.

When the cotyledons had expanded and the first true leaf had begun to emerge, seedlings were inoculated. Inoculum was prepared by gently transferring *P. xanthii* conidia on cucumber leaves into distilled water with a brush. The suspension (2.1×10^6 spores mL^{-1}) was then sprayed onto the adaxial surface of the cotyledons with a hand sprayer. The

83 inoculated seedlings were placed in another growth chamber at 28°C, 50% RH, and a PPFD
84 of 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

85 Immediately after acclimatization, 10 uninoculated seedlings were sampled from each
86 RH treatment for evaluation of leaf properties. Dry mass and leaf area were measured, and
87 then LMA was calculated by dividing the dry mass by the leaf area. In addition, the N
88 contents of the dry matter in each treatment were determined with a CN Elemental Analyzer
89 (2400 II; PerkinElmer Inc., Waltham, MA, USA).

90 Five inoculated seedlings were sampled at 24, 48 and 72 h after inoculation, and then
91 conidial invasion, hyphal development, and haustorial formation of *P. xanthii* on their leaves
92 were evaluated according to the methods of Itagaki et al. (2016, 2017). The formation of
93 conidial germ tubes and hyphal growth were observed 24 h after inoculation in each of 50
94 randomly selected conidia per seedling under an optical microscope (BX-50; Olympus Corp.,
95 Tokyo, Japan). The percentage of conidial invasion was calculated as Σ (conidia with germ
96 tube and primary hyphae) $\times$ 100 / { Σ (conidia with germ tube only) + Σ (conidia with germ
97 tube and primary hyphae)}. Hyphal growth in each of 30 randomly selected conidia per
98 seedling that had successfully infected leaves were then evaluated as the total number of
99 primary, secondary, and tertiary hyphae (which were formed respectively from conidia,
100 primary hyphae and secondary hyphae) per conidium 24, 48 and 72 h after inoculation. The
101 total number of secondary haustoria and hyphal cells per conidium were also evaluated 24 and
102 48 h after inoculation. The number of colonies on the adaxial surface of 10 seedlings in each
103 treatment was counted at 5 d after inoculation, and then colony density was determined by
104 dividing the number of colonies by the leaf area to evaluate conidial development.

105

106 **Expt. 2: Inoculation test at the first-true-leaf stage**

107 Twenty-five seedlings in each RH treatment (10% and 90%) were grown as in Expt. 1. When

the first true leaf had expanded and the second true leaf had begun to emerge, the adaxial surface of the first true leaf and cotyledons of 15 seedlings were spray-inoculated in each treatment with a spore suspension (8.4×10^5 spores mL⁻¹). Colony densities on the adaxial surface were determined at 7 d after inoculation. Immediately after acclimatization, 10 uninoculated seedlings were sampled from each RH treatment for evaluation of LMA.

Statistical analysis

In Expt. 1, the differences in leaf properties and hyphal development between treatments were analyzed by Student's *t*-test. In Expt. 2, the effects of RH and leaf type (i.e. cotyledons or true leaves) and their interaction were determined by two-way analysis of variance (ANOVA). Each seedling was considered as a biological replicate. All analyses were performed in Statcel 2 software (OMS Publishing Inc., Tokorozawa, Japan).

Results

Expt. 1: Evaluation of early hyphal development

The percentage of conidial invasion on the low-RH leaves 24 h after inoculation was $\times 0.74$ that of the high-RH leaves (Fig. 1A). The leaves acclimatized to low RH had a higher LMA ($\times 1.24$) than high-RH leaves (Table 1). The colony density on the low-RH leaves at 5 d after inoculation was $\times 0.68$ that on the high-RH leaves (Fig. 1B).

The low-RH leaves had fewer primary (Fig. 2A), secondary (Fig. 2B), and tertiary (Fig. 2C) hyphae than the high-RH leaves. The numbers of hyphal cells (Fig. 2D) and secondary haustoria (Fig. 2E) per conidium on the low-RH leaves were $\times 0.81$ and $\times 0.72$, respectively, compared to those on the high-RH leaves at 48 h. The leaf N content of the low-RH leaves was $\times 0.91$ that of the high-RH leaves (Table 1).

Expt. 2: Inoculation test at the first-true-leaf stage.

Conidial development was inhibited on the low-RH leaves at the first-true-leaf stage also (Fig. 3A). The colony densities on the cotyledons and first true leaves acclimatized to low RH were $\times 0.39$ and $\times 0.58$ the respective densities on high RH-leaves. There was a significant interaction between acclimatization RH and leaf type, with a stronger RH effect on cotyledons than on first true leaves as described above. In addition, the effect of RH appeared stronger in the cotyledons at the first-true-leaf stage (Fig. 3A) than in those at the cotyledon stage in Expt. 1 (Fig. 1B). LMA increased at low RH in both leaf types: LMA on cotyledons and first true leaves at low RH were $\times 1.16$ and $\times 1.28$ the respective LMA at high RH (Fig. 3B). LMA was greater in cotyledons than in first true leaves, and there was no significant interaction between leaf type and acclimatization RH.

Discussion

In Expt. 1, the lower percentage of conidial invasion on the low-RH leaves (Fig. 1A) means that initial invasion of *P. xanthii* was suppressed on the low-RH leaves. The initial invasion of *P. xanthii* is generally affected by the structural properties of host leaves (Aust and Hoyningen-Huene 1986). The higher LMA of low-RH leaves (Table 1), which functions to protect plants against water loss (Salleo and Gullo 1990), may correlate with an increase in structural defenses against pathogen invasion (Toome et al. 2010).

The difference in post-invasion behaviors of *P. xanthii* between treatments (Fig. 2) suggests that conidial development was also affected by non-structural properties of leaves. The reduced leaf N content of the low-RH leaves (Table 1) may in part have inhibited the post-invasion behaviors of *P. xanthii*, because reduced N generally correlated with plant defense through altered leaf nutritional quality (Walters and Bingham 2007). Several reports demonstrated that leaf N content is positively related to susceptibility to powdery mildews

(Hoffland et al. 2000; Olesen et al. 2003; Neumann et al. 2004). The reduction of leaf N content in the present study was in a range where this would have a significant effect on susceptibility (Hoffland et al. 2000). Leaf N content possibly decreases under water-stress conditions (Ben-Zioni et al. 1967; Yin et al. 2009). Therefore, the reduction of leaf N contents by low-RH acclimatization may be caused by moderate water stress due to high evaporative demand, although the true reasons are not elucidated in the present study.

In Expt. 2, it was confirmed that low-RH acclimatization inhibits development of *P. xanthii* also at the true leaf stage. The stronger RH effect on colony density in the cotyledons than in the first true leaf (Fig. 3A) indicates that effect of low RH on host-plant susceptibility was less on the first true leaf than on the cotyledon. This is probably due to developmental differences between the two types of leaves. In addition, the stronger RH effect in the cotyledons at the first-true-leaf stage (Fig. 3A) than in those at the cotyledon stage (Fig. 1B) may be caused by differences in acclimatization period or leaf maturity, because the cotyledons were already older than the first true leaves. The greater LMA of low-RH leaves (Table 1, Fig. 3B) may have partially explain these differences. However, relationships between LMA and host-plant susceptibility were not consistent between leaf types; the difference in LMA between low and high RH was larger for first true leaves than for the cotyledons, whereas the difference in colony density was greater in the cotyledons (Fig. 3). This implies that plant susceptibility to *P. xanthii* cannot be explained only by leaf structural properties, and that one or more other factors such as the nutrient components limit pathogen development.

In conclusion, both the initial and the post invasion behavior of *P. xanthii* were inhibited on the leaves acclimatized to low RH, suggesting that conidial development was affected by both structural and non-structural properties of host plant leaves. The main findings and experimental approach in the present study could be used effectively in future studies to

clarify the roles of atmospheric humidity in determining plant–pathogen interactions.

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236 Table 1 Leaf mass per area (LMA) and leaf nitrogen (N) contents of cucumber cotyledons
237 acclimatized to low (10%) or high (90%) relative humidity (RH) in Expt. 1.

Treatment	LMA (mg cm ⁻²)	Leaf N content (% DW)
Low RH	2.45 ± 0.07	7.12 ± 0.08
High RH	1.98 ± 0.05	7.79 ± 0.08
<i>P</i> value	< 0.001	< 0.001

238 Mean ± SE (*n* = 10).

239 *P* values were determined by Student's *t*-test.

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Figure captions

Fig. 1 (A) Initial conidial invasion of *P. xanthii* 24 h after inoculation ($n = 5$) and (B) colony density 5 d after inoculation ($n = 10$) on cucumber leaves acclimatized to either a low (10%) or high (90%) relative humidity (RH) in Expt. 1. Error bars represent SE. P values were determined by Student's t -test.

Fig. 2 Numbers of (A) primary, (B) secondary, and (C) tertiary hyphae, (D) total number of hyphal cells, and (E) number of secondary haustoria of *P. xanthii* on cucumber seedlings acclimatized to either low (10%) or high (90%) relative humidity (RH) in Expt. 1 ($n = 5$). Differences between treatments are significant at $*P = 0.05$, $**P = 0.01$, $***P = 0.001$ by Student's t -test; NS, not significant ($P > 0.05$).

Fig. 3 (A) Colony density of *P. xanthii* on cotyledons and first true leaves in cucumber seedlings acclimatized to either low (10%) or high (90%) relative humidity (RH) 7 days after inoculation ($n = 15$), and (B) leaf mass per area (LMA) of host plants before inoculation ($n = 10$) in Expt. 2. Error bars represent SE. Spores were inoculated on cotyledons and first true leaves when the first true leaves were fully expanded. The effects of RH and leaf type and their interaction were determined by two-way analysis of variance (ANOVA).

Fig. 1

Fig. 2

Fig. 3