Effects of Temperature, Wetness Duration, and Moisture on the Conidial Germination, Infection, and Disease Incubation Period of *Glomerella cingulata*

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Abstract

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Glomerella leaf spot (GLS) caused by *Glomerella cingulata* is a newly emergent disease that results in severe defoliation and fruit spots. Currently, GLS is not effectively controlled in China due to a lack of understanding of its epidemiology. Therefore, the effects of temperature, wetness duration, and moisture on conidial germination, infection, and the disease incubation period of GLS were examined by inoculating cv. Gala apple leaves with a conidial suspension and performing in vitro germination assays. Conidia could germinate and form appressoria at temperatures ranging from 5 to 35°C, with an optimum temperature of 27.6°C. The germination of conidia required free water or a nearly saturated relative humidity, with only a few conidia germinating and forming appressoria when the RH was less than 99%. The conidial germination dynamics at 10, 25, and 30°C were well represented by three logistic models. The infection of cv. Gala apple leaves by conidia occurred at temperatures ranging from 15 to 35°C. The minimum wet-

ness duration required for infection by conidia at different temperatures was described using a polynomial equation, and the lowest minimum wetness duration was 2.76 h, which occurred at 27.6°C according to the polynomial. Successful infection by conidia was represented by the number of lesions per leaf, which increased with extended wetness durations at the conidial infection stage for six tested temperatures, with the exception of 10°C, when the minimum wetness durations were satisfied. The associations of successfully infected conidia with wetness duration at temperatures of 15, 20, 25, and 30°C were described by four logistic models. Conidia infections developed into visible lesions at temperatures ranging from 15 to 30°C, and the shortest incubation period of 2 days was observed at 25°C. These data and models can be used to construct forecasting models and develop effective control systems for *Glomerella* leaf spot.

Glomerella leaf spot (GLS) of apple (*Malus × domestica* Borkh.) is caused by *Glomerella cingulata* (Stoneman) Spauld. & H. Schrenk and has become a widespread and major public concern in China (29). GLS was first reported in 1988 in Brazil (17) and is now widespread in countries with apple production areas (28). The disease was noticed in August 1998 in two orchards in eastern Tennessee in the United States (7). In China, GLS was first detected in August 2011 in Fei-xian, Jiangsu Province (29), and currently, the disease has spread to seven provinces, which include almost all of the apple production areas.

The GLS pathogen infects apple leaves and causes many small black lesions with indistinct edges at the start of infection. Under warm and humid conditions, the lesions can expand rapidly and result in defoliation after 5 to 7 days of development (17,27–29). Infections on the fruit result in a large number of small brown to black colored lesions with diameters between 1 and 5 mm, and these lesions stop growing 5 to 7 days after infection. Gala, Golden Delicious, Qinguan (a cultivar bred in China), and several other apple cultivars are highly susceptible, and GLS has resulted in greater than 90% defoliation and diseased fruits in susceptible cultivars prior to harvest in the disease-stricken regions of China. However, the Fuji and Red Star cultivars are highly resistant to the disease.

Epidemics of GLS are mainly influenced by temperature and humidity and cause heavy losses in regions with humid subtropical climates, such as southern Brazil, southeastern United States, and eastern China (28). GLS often causes extensive defoliation in July and August in China, when the average monthly temperature

ranges from 25 to 30°C, and defoliation is especially common after a long period of rain. The spread of GLS is rapid and can lead to more than 90% defoliation within 1 to 2 months after primary infection. The diseased leaves from primary infections have been observed in June, whereas most of the leaves drop in mid-July to early August due to the rapid spread of the disease. Moreover, in August and September, *G. cingulata* can produce perithecia and ascospores in the leaf lesions, which can be dispersed over long distances and infect apple trees in orchards far from the source of infection.

To date, no approach has been able to effectively control GLS outbreaks. Traditional spraying systems do not prevent the defoliation of susceptible cultivars in China. Because the epidemiology of GLS is poorly understood and forecasting methods have not been produced, the extensive application of several fungicides, such as prochloraz, thiophanate-methyl, tebuconazole, and pyraclostrobin in numerous orchards has not effectively controlled the disease. The development of effective management systems to control the GLS epidemic is urgently required. An understanding of the epidemiology of the disease is an essential step for developing effective management systems and disease prediction models.

The germination and infection of spores are key steps in the epidemiology of this disease. Spores of pathogenic fungi are sensitive to temperature and moisture, and determining the effects of temperature, wetness duration, and moisture on the germination and infection of spores is required to understand the epidemiology of GLS and develop forecasting models based on weather conditions. In the mid-1920s, Keitt and Jones published studies focused on the temperature and minimum hours of wetness required for infection by ascospores of *Venturia inaequalis* (13,15). Mills constructed a table based on these studies to describe the duration of leaf wetness required for light, moderate, and severe infection by ascospores of *V. inaequalis* and used this table to guide fungicide applications in scab management (24). The use of Mills' table led to great success in the management of apple scab, and this method has been used throughout the world (5,6,11,12,26,30). Microcomputer-based

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instruments and computer programs were developed based on Mills' table to predict the infection periods and quantity of apple scab (13,30). The concept of predicting infection periods and quantities of infected individuals based on the temperature and minimum duration of wetness required for infection by pathogens has been widely practiced in epidemiological studies and the management of plant diseases, such as pear scab, pear rust, and cucurbit downy mildew (1,3,4,18–20,22,23,25).

GLS is an emerging disease of apple trees, and only a few studies have focused on the epidemiology of this disease. *G. cingulata* produces cylindrical conidia with rounded ends and morphological characteristics the same as those of *Colletotrichum gloeosporioides* (8,9). Conidia are the primary infection source in the early stages of disease epidemics. Velho et al. (28) determined that GLS was favored by conditions of high humidity and temperatures between 23 and 28°C. Luciano et al. (21) investigated the effect of wetness duration on the infection of apple leaves by the conidia of *G. cingulata* in a growth chamber and found that disease severity increased proportionally to increases in temperature from 14 to 28°C. However, infection was completely inhibited at 34°C. The duration of leaf wetness required for infection ranged from 2 h at 30°C to 32 h at 16°C (21).

In the present study, we conducted several experiments with *G. cingulata* conidia and detached cv. Gala shoots under controlled environments to determine the epidemiology of GLS, with an emphasis on the following: 1) the effects of temperature on conidia undergoing in vitro germination, conidial infection on leaves, and disease incubation period on leaves; 2) the moisture required for conidial germination and the effects of wetness duration on conidial infection; and 3) the dynamics of germination and infection by conidia. These data can be used to develop models to predict the infection periods and quantity of individuals infected by *G. cingulata* based on weather data.

Materials and Methods

Inoculum. Apple leaves of cv. Gala with fresh GLS lesions were sampled in September 2011 from an orchard located in Laixi, Shandong Province, China. The leaves were sanitized in 75% ethyl alcohol for 10 s, washed with sterile distilled water, transferred to petri dishes, and then incubated at 25°C for 3 to 5 days to promote sporulation of the pathogen. Single conidial isolates were obtained by collecting and transferring single conidia to potato dextrose agar medium (PDA) according to the methods described by Dong (2). The pathogenicity of the isolates was tested by inoculating cv. Gala leaves with the conidial suspension and confirming that the infection was consistent with the pathogen on the sampled leaves. The isolates were stored in a refrigerator at 5°C. One isolate was coded GLS010101 and grown in PDA medium before each inoculation.

To reproduce the conidia, mycelia were transferred to several petri dishes containing PDA medium and cultured for 5 days at 25°C; the aerial mycelia were then scraped off and cultivated for another 5 days under UV light (365 nm). Newly formed conidia were collected using sterilized bamboo toothpicks and mixed with distilled water to prepare the conidial suspensions. The conidial suspensions used for the in vitro germination tests were adjusted to approximately 10^6 conidia ml^{-1} using a hemocytometer, and the suspensions used for inoculation of the apple leaves were adjusted to approximately 10^4 conidia ml^{-1} .

Effects of temperature and RH on in vitro conidial germination. The effect of temperature on the conidial germination of *G. cingulata* was tested at eight different temperature levels (5, 10, 15, 20, 25, 30, 35, and 40°C). For each level, four treatments at different relative humidities (RH = 100, 99, 97, and 95%) and one treatment using free water were prepared. Thus, there were a total of 40 treatments of various temperature and moisture combinations. The temperature was controlled using eight incubators (BluePard MGC-250BP-2, Shanghai Bluepard Instruments Co. Ltd.), and the RH was achieved by amending the aqueous agar (Qing-dao Agar Powder Factory, China) with 0, 0.3, 0.9, and 1.5 mol NaCl in sealed dishes (10,16).

Two petri dishes containing approximately 30 ml of aqueous agar amended with the appropriate amount of NaCl (as described above) were prepared for each temperature $\times$ moisture combination. One slide was placed in the lid of each of the 80 petri dishes. Two 10- μl drops of conidial suspension were separately placed on each of the two ends of the glass slide using a micropipette. After the two droplets were air dried at room temperature (ranging from 22 to 27°C), the petri dish was immediately sealed with Parafilm (National Can, Chicago, USA). On average, it took approximately 10 to 20 min for the conidia suspension to dry. Glass slides for the free water treatments were placed in petri dishes containing 30 ml of aqueous agar without NaCl, and the droplets of conidial suspension were not allowed to air dry before the petri dishes were sealed with Parafilm. All the petri dishes were immediately placed into an incubator set to the appropriate temperature and allowed to incubate for 12 h prior to treatment.

After 12 h of incubation, all of the petri dishes were removed from the incubators, and a drop of cotton blue in lactophenol (lactic acid/phenol/glycerin/distilled water = 1:1:2:1 [v/v/v/v]) was added to each spore suspension droplet to stop germination and preserve the conidia. The percentages of germinated conidia and conidia forming appressoria were estimated by randomly examining 50 conidia in the center of each inoculum point by microscopy (100 $\times$ magnification; Leica DM500, Leica Microsystems, Wetzlar, Germany). The conidia were considered germinated when the germ tube was longer than half of the total length of the conidia and were considered to have formed appressoria when a distinct appressorium was formed on the tip of any of the germinated tubes. The lengths of the germinated conidia tubes in the free water treatments were measured by selecting the longest germination tube in each of five randomly selected visual fields at 100 $\times$ magnification at each inoculum point. The experiment was repeated four times on different dates, and each incubator was randomly assigned to one of the tested temperatures in each replication.

Effect of temperature on the temporal dynamics of in vitro conidial germination. The effects of temperature on the temporal dynamics of the conidial germination of *G. cingulata* were examined at 5, 10, 15, 20, 25, 30, and 35°C by estimating the germination rate at different assessment times. For each temperature, 3 to 10 incubation treatments, ranging from 3 to 48 h with intervals of 1, 2, 3, 4, 10, 12, or 24 h, were used (Fig. 1), for a total of 51 temperature $\times$ incubation period combinations.

One glass slide was placed in each lid of two petri dishes. Each dish contained approximately 30 ml of aqueous agar to maintain 100% RH inside the dish, and two dishes were used for each of the 51 combinations. Two 10- μl drops of conidial suspension were separately placed on each of the two ends of the glass slide using micropipettes. The petri dishes were then sealed with Parafilm without air drying the droplets, and the dishes were immediately placed in the appropriate incubator.

At the end of each incubation period, a drop of cotton blue in lactophenol was added to each inoculum droplet to stop any further germination. The percentage of germinated conidia was estimated by randomly examining 50 conidia in each inoculum droplet under 100 $\times$ magnification. The experiment was repeated four times on different dates.

Effects of temperature and wetness duration on infection. The effects of temperature on infection by *G. cingulata* conidia were examined at five temperature levels: 10, 15, 20, 25, 30, and 35°C. Seven to 12 wetness duration treatments were arranged for each temperature, and there were 60 temperature $\times$ wetness duration combinations (Fig. 2). Two detached shoots of cv. Gala, each with four leaves, were inoculated for each combination.

In June and July, growing shoots with 6 to 10 leaves were cut from cv. Gala trees in a 4-year-old orchard to which fungicides had never been applied. Four of the youngest fully expanded, healthy leaves that were similar in size on each shoot were selected and surface-disinfected by soaking in a 0.08% NaOCl solution for 2 to 3 min before rinsing with distilled water. Leaves that were not selected were cut off. After the leaves were air dried, two shoots

for each combination were cultivated with their base inside a flask full of distilled water. All of the shoots were placed in the assigned incubators with 12 h of light (12,000 lx) at the proper temperatures for 12 h before inoculation. The shoots were then inoculated by spraying the conidial suspension on the leaves using a hand sprayer until the suspension flowed down from the leaves; the leaves were then separately covered with polythene bags to maintain the humidity and subsequently placed back into the incubators. All of the shoots were inoculated within 20 min of the conidia being placed into the water, which was also used as the start time of the wetness duration.

At the end of each wetness duration, the covered bags of two shoots were removed, and the shoots remained in the incubator to air dry for approximately 10 to 20 min. All of the inoculated shoots

were transferred to large chambers (MGC-800B, Shanghai Bluepard Instruments Co. Ltd.) maintained at 25°C, with 75% RH and 12 h of light (20,000 lx), for 48 h after inoculation. The shoots were kept in these chambers for an additional 7 days.

The lesions on each leaf were visually examined, and the number was recorded on the third, fifth, and eighth days after inoculation. Because the lesions could coalesce during growth, the highest number of lesions on each leaf among the three assessments was used as the final lesion number, and this number represented the proportion of successfully infected conidia. The experiments were repeated three times at 10°C and six times at the other temperatures.

The shortest wetness duration that induced disease in 5% of leaves (each diseased leaf had at least three lesions) was defined as

Fig. 1. Percentage of *Glomerella cingulata* conidia germinated in vitro after incubation in free water at seven tested temperatures for different periods of time. The symbols represent the observed values in four replications, and the solid lines are the curves of the fitted logistic models; their parameter values are listed in Table 3.

the minimum wetness duration required for successful infection by conidia of *G. cingulata* (IMWD) in this study. At each temperature, the shortest wetness duration that induced more than three lesions (the maximum number was approximately 300 lesions per leaf) on at least two leaves (24 leaves in total) among the six repetitions was considered as the IMWD at that temperature.

Effect of temperature on incubation period. The effects of temperature on the GLS incubation period were tested at six temperatures: 10, 15, 20, 25, 30, and 35°C. The experiment was conducted in July, and two growing cv. Gala apple shoots, each with three young, fully expanded, healthy leaves, were detached and inoculated for each temperature treatment. All of the shoots were moved into large chambers kept at 25°C, and high humidity was maintained by covering the shoots with polythene bags after inoculation. After 24 h, the shoots were moved into six incubators that had been set to the appropriate temperatures 12 hours prior; the plants were kept under a 12-h light cycle (12,000 lx). The number of lesions on each leaf was assessed visually every day beginning on the third day after inoculation until the tenth day. The experiment was repeated three times on different dates.

Data analysis. ANOVA analysis was conducted using generalized linear models (GLM) to assess the effects of temperature and RH on conidial germination, appressorium formation, and germ tube growth. Replications were treated as block factors, temperature was treated as a whole plot factor, and RH was treated as a sub-plot factor in ANOVA. The percentage data at each of the

observed points, which were assumed to follow a binomial distribution in the GLM, and the germ tube lengths of each conidium, which were assumed to follow a Gaussian distribution in the GLM, were applied in the data analysis and used to calculate means and standard deviations. Regression analysis was used to fit the means of conidial germination percentages obtained in four replications in the free water treatment and the IMWD to temperature. In the regression analysis, temperature (T in °C) was considered the independent variable and was divided by 10 to avoid small values of estimated parameters. A logistic model was used to describe the conidial germination dynamics and infection dynamics:

$$p = K / \{1 + \exp[-\beta(t - M)]\} \quad (1)$$

where p is the proportion of germinated conidia or the lesion number per leaf and t is time in hours. The observed data were used to estimate the parameters K , M , and β : K is the maximum of p ; M is the length of elapsed time until $p = K / 2$, and β is the rate of germination or infection.

Results

Effects of temperature and RH on in vitro conidial germination. The ANOVA results showed that temperature, humidity, and the interaction of temperature with humidity had significant effects on in vitro germination and appressorium formation of *G. cingulata* conidia ($P < 0.01$). The variations in conidial germination and appressorium formation caused by RH accounted for 43.3 and

Fig. 2. Number of *Glomerella* leaf spots developed on each cv. Gala (*Malus × domestica*) apple leaf. The leaves were grown on detached shoots, which were water cultured and inoculated with a conidial suspension containing 10^4 *Glomerella cingulata* conidia per ml; the wetness was maintained for different durations after inoculation at six temperatures. The symbols represent the observed values of the six replications, and the solid lines represent the curves of the fitted logistic models; their parameter values are listed in Table 4.

49.8% of the total variation according to the GLM model, whereas the variations caused by temperature accounted for 23.7 and 29.1% of the total variation, and the interaction of temperature with RH accounted for 4.6 and 5.13% of the total variation.

Conidial germination and appressorium formation of *G. cingulata* required free water or a high RH. In the free water treatment, 53.5% of the conidia at eight tested temperatures germinated (Table 1) and 25.1% of the conidia formed appressoria (Table 2); this number was significantly higher than that in the 100% RH treatment ($P < 0.01$). The percentages of conidial germination and appressorium formation at 95% RH were 3.6 and 0.0%, respectively, which were significantly lower than the other moisture treatments ($P < 0.01$).

Temperature had a significant impact on conidial germination, appressoria formation, and germ tube growth (Tables 1 and 2, Fig. 3). In the free water treatment, the percentages of germinated conidia (CGP) at the examined temperatures (T) were well-fitted ($P < 0.001$) by a polynomial equation:

$$CGP = -(6.694 \pm 7.690) + (40.511 \pm 4.144)T^2 - (9.781 \pm 1.017)T^3 \quad R\text{-square} = 0.950 \quad (2)$$

The optimum temperature for conidia germination was 27.6°C according to the equation (Fig. 4).

The effect of temperature on the temporal dynamics of conidial germination. Germinated conidia were not detected at the 5°C treatment. The earliest times at which germinated conidia were observed were 8, 6, 6, 3, 4, and 5 h for the samples incubated at 10, 15, 20, 25, 30, and 35°C, respectively. The percentages of germinated conidia increased as the wetness periods increased (Fig. 1). The conidial germination process at temperatures of 20, 25, and 30°C was well-fitted ($P < 0.05$) by three logistic models (Eq. 1). The three models accounted for 56.4, 78.8, and 75.3% of the total

variance (Table 3). According to the models, the maximum germination percentages (K) at 20, 25, and 30°C were 92.30, 91.70, and 87.81%, respectively, and the incubation times required for $K/2$ conidia germination were 11.77, 7.56, and 7.52 h (Table 3).

Effect of temperature on temporal dynamics of conidial infection. Lesions were not observed on the leaves inoculated at 10°C, and the maximum number of lesions was observed on the

Fig. 3. Effects of temperature on the growth of *Glomerella cingulata* germination tubes. The average lengths of the germination tubes and the corresponding standard error (calculated from the observed data) at each temperature in the free water treatment (based on 40 conidia) is shown as a bar with an error bar. Different letters indicate that the germination tube lengths were significantly different ($P < 0.01$) according to multiple comparisons of predicted values from the generalized linear model.

Table 1. Average percentages of in vitro conidial germination of *Glomerella cingulata* (based on 800 conidia) and the corresponding standard deviations at various combinations of temperature and relative humidity assessed 12 h after inoculation

Temperature (°C)	Free water	Relative humidity (%) ^x				Mean
		100	99	97	95	
5	8.5 ± 15.5 ^y	6.3 ± 14.4	2.6 ± 4.4	3.8 ± 6.1	4.1 ± 9.1	5.1 ± 10.9 d
10	7.5 ± 12.5	1.4 ± 2.4	3.3 ± 7.0	1.1 ± 2.2	1.5 ± 2.8	3.0 ± 7.0 e
15	63.4 ± 24.4	21.8 ± 38.8	11.5 ± 20.2	4.9 ± 11.3	1.1 ± 2.6	20.5 ± 31.9 c
20	79.0 ± 13.5	14.5 ± 14.9	10.5 ± 13.9	10.0 ± 16.7	1.0 ± 1.8	23.0 ± 31.3 c
25	90.8 ± 10.5	33.3 ± 32.5	31.8 ± 23.9	10.4 ± 16.4	6.8 ± 17.3	34.6 ± 36.8 a
30	92.0 ± 9.6	42.8 ± 35.3	31.8 ± 31.9	13.9 ± 21.2	3.8 ± 6.2	36.8 ± 38.7 a
35	71.1 ± 24.2	30.1 ± 29.2	23.9 ± 22.1	17.6 ± 20.8	10.3 ± 19.4	30.6 ± 31.3 b
40	15.6 ± 24.4	0.13 ± 0.5	0.5 ± 1.2	0.1 ± 0.5	0.4 ± 1.1	3.4 ± 12.4 e
Mean	53.5 ± 38.7 A ^z	18.8 ± 28.7 B	14.5 ± 21.7 C	7.7 ± 15.0 D	3.6 ± 10.3 E	19.6 ± 30.6

^x Relative humidity was controlled by adding NaCl to the aqueous agar inside the sealed agar plates.

^y Means and standard deviations were calculated from the data obtained from four replications.

^z Different letters indicate that the percentages were significantly different ($P < 0.01$) according to multiple comparisons of values predicted by the generalized linear model. Upper case letters are for RH and lower case letters are for temperature.

Table 2. Average percentages of *Glomerella cingulata* conidia forming appressoria in vitro (based on 800 conidia) and the corresponding standard deviations at various combinations of temperature and relative humidity assessed 12 h after inoculation

Temperature (°C)	Free water	Relative humidity (%) ^x				Mean
		100	99	97	95	
5	0.5 ± 1.6 ^y	0.0 ± 0.0	0.1 ± 0.5	0.0 ± 0.0	0.1 ± 0.5	0.2 ± 0.8 d
10	0.4 ± 1.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.5 d
15	20.3 ± 24.5	11.9 ± 22.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	6.4 ± 16.7 c
20	53.0 ± 22.6	0.3 ± 1.0	0.1 ± 0.5	0.8 ± 2.5	0.0 ± 0.0	10.8 ± 23.4 b
25	72.0 ± 14.7	8.3 ± 12.0	1.3 ± 2.5	1.0 ± 3.1	0.0 ± 0.0	16.5 ± 29.3 a
30	53.4 ± 29.4	16.1 ± 23.2	5.6 ± 9.9	3.9 ± 9.1	0.0 ± 0.0	15.8 ± 26.2 a
35	0.9 ± 1.3	1.0 ± 2.2	0.1 ± 0.5	0.3 ± 1.0	0.0 ± 0.0	0.5 ± 1.3 d
40	0.3 ± 0.7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.3 d
Mean	25.1 ± 32.3 A ^z	4.7 ± 13.3 B	0.9 ± 4.0 C	0.7 ± 3.7 C	0.0 ± 0.2 DA	6.3 ± 18.4

^x Relative humidity was controlled by adding NaCl to the aqueous agar inside the sealed agar plates.

^y Means and standard deviations were calculated from the data obtained from four replications.

^z Different letters indicate that the percentages were significantly different ($P < 0.01$) according to multiple comparisons of values predicted by a generalized linear model. Upper case letters are for RH and lower case letters are for temperature.

leaves inoculated at 25°C. The IMWDs were 6, 5, 3, 3, and 12 h for the samples incubated at 15, 20, 25, 30, and 35°C, respectively. The maximum number of lesions per inoculated leaf increased with increasing durations of wetness at the infection stage after the IMWDs were satisfied (Fig. 2). The lesion numbers per leaf inoculated at temperatures of 15, 20, 25, and 30°C were well-expressed ($P < 0.05$) by the four logistic models (Eq. 1). According to the models, the maximum number of lesions per leaf inoculated at 15, 20, 25, and 30°C were 110.12, 110.94, 191.74, and 123.89, respectively, and the wetness duration required for conidial infection resulting in $K/2$ lesions per leaf was 30.34, 23.42, 19.07, and 20.87 h (Table 4).

The IMWD (in hours) at each treated temperature (T) can be well described ($P < 0.01$) using a polynomial equation:

$$IMWD = (6.8940 \pm 0.2112) - (0.1666 \pm 0.0082)T^5 + (0.0504 \pm 0.0023)T^6 \quad R\text{-square} = 0.998 \quad (3)$$

This equation accounted for 99.80% of the total variance (Fig. 5). According to Equation 3, the optimum temperature for conidia infection was 27.6°C, which was equal to the optimum temperature of germination. The IMWD was 2.76 h at the optimum temperature.

Effect of temperature on incubation period. Lesions did not develop on the inoculated leaves that were incubated at 10 and 35°C. More lesions were observed in the leaves incubated at 25 and 20°C (Fig. 6) compared with those of the other temperature

Fig. 4. Effects of temperature on conidial germination. Conidial germination percentage means in the free water treatment at each temperature were obtained from four replications and are shown as circles, and the fitted model curves (Eq. 2) are shown as a solid line.

Table 3. Parameter estimates of the logistic models in Figure 1 describing the temporal dynamics of *Glomerella cingulata* conidia in vitro germination and the corresponding R-square values at three temperatures.

Temperature	K^2	M^2	β^2	R-square
20°C	92.30 ± 7.453	11.77 ± 0.9488	0.3206 ± 0.0798	0.564
25	91.70 ± 3.738	7.56 ± 0.2614	0.7448 ± 0.1012	0.788
30	87.81 ± 3.816	7.52 ± 0.2635	0.8646 ± 0.1415	0.753

² In the fitted logistic model $p = K / \{1 + \exp[-\beta(t - M)]\}$, P is the percentage of conidia germinated (%) at time t , K is the maximum germination percentage (%), M is the length of time (h) elapsed until $P = K/2$ and β is the germination rate (% h⁻¹).

Table 4. Parameter estimates of the logistic models in Figure 2 describing the temporal dynamics of *Glomerella cingulata* conidial infection of cv. Gala (*Malus × domestica*) apple leaves and the corresponding R-square values at four temperatures.

Temperature	K^2	M^2	β^2	R-square
15°C	110.12 ± 10.88	30.34 ± 2.75	0.1286 ± 0.02150	0.520
20	110.94 ± 5.70	23.42 ± 0.80	0.1857 ± 0.01808	0.689
25	191.74 ± 8.00	19.07 ± 0.59	0.2402 ± 0.01739	0.820
30	123.89 ± 5.98	20.87 ± 0.70	0.2059 ± 0.01655	0.747

² In the fitted logistic model $p = K / \{1 + \exp[-\beta(t - M)]\}$, P is the number of lesions on each leaf (lesions) inoculated with a conidial suspension containing 10⁴ *G. cingulata* conidia per ml and incubated under wet conditions for time t , K is the maximum number of lesions per leaf (lesions), M is the wetness duration (h) until $P = K/2$ and β is the infection rate (lesions h⁻¹).

treatments. The first lesions to appear were observed on the third day after inoculation on leaves incubated at 25°C. The minimum incubation periods, i.e., elapsed days from inoculation to the first lesions were observed, were 5, 4, 2, and 4 days at temperatures of 15, 20, 25, and 30°C, respectively. The lesion number increased within 5 days after inoculation. However, the lesion number was reduced with the expansion of lesions, and lesions coalesced after 5 days.

Discussion

The favorable temperature for *G. cingulata* conidial germination, conidial infection, and disease development was between 20 and 30°C, with an optimum temperature of approximately 27.6°C. This was consistent with experimental results from Brazil (21,28). When the temperature was lower than 15°C or higher than 30°C, only a few conidia were able to successfully infect the plant, and a small number of infections developed into visible lesions, although many conidia germinated and formed appressoria. Therefore, 15°C can be considered the lowest temperature that supports disease development, and 20°C is the baseline favorable temperature for disease epidemics. The overwintered pathogen would begin to grow and produce conidia when daily mean temperature was above 15°C. When the daily mean temperature was above 20°C for 1 month (approximately 4 to 6 generations), the disease would reach epidemic status and result in the defoliation of the apple. Obviously, rain and moisture are important factors in disease epidemiology.

The conidial germination of *G. cingulata* required free water or a high moisture level. When the RH was lower than 99%, only a few conidia germinated or formed appressoria, which is similar to what has been observed for many other fungal pathogens. Because the release and dispersion of *G. cingulata* conidia and ascospores require rain water, rain or irrigation by spraying may be the only trigger factor for pathogen infection in nature. The minimum wetness duration required for *G. cingulata* conidia infection was 2.76 h at an optimum temperature of 27.6°C. This minimum wetness duration was slightly longer than that reported by Luciano et al. (21), which was only 2 h at 30°C, and shorter than what is required for many other pathogens, such as *V. inaequalis*, *V. nashicola*, and *Gymnosporangium asiaticum*. However, the minimum wetness duration was similar to that required by *P. cubensis* (2 h). Thus, the infection rate was faster than the causal pathogens *V. inaequalis*, *V. nashicola*, and *G. asiaticum* and similar to *P. cubensis*, the causal agent of cucumber downy mildew. Rain that causes more than 3 h of leaf wetness could cause infection by *G. cingulata* conidia at the most favorable temperature, and this information can be used to estimate whether *G. cingulata* conidia might cause successful infections during rain.

Lesions on leaves of different ages did not show distinct differences in these experiments. In orchards that are seriously diseased, more than 95% of the leaves defoliate and the disease severity did not show an obvious difference on leaves of different ages. This result indicated that the susceptibility of all leaves of the susceptible to *G. cingulata* was not substantially different based on their age. The minimum wetness duration required for conidial infection was shorter than that required for conidial germination under the same temperature. This phenomenon indicates that there may be some instances that stimulate or promote germination and lead to faster conidia germination on apple leaves than in pure water.

The incubation periods of GLS were short, and the infection required 2 to 5 days to develop into visual lesions. This result sug-

Fig. 5. Minimum wetness duration required for successful infection by *Glomerella cingulata* conidia, resulting in lesions on the leaves of cv. Gala (*Malus domestica*), at different temperatures. The leaves were grown on detached shoots, which were water cultured and inoculated with a conidial suspension containing 10^4 *Glomerella cingulata* conidia per ml; the wetness was maintained for different durations. The symbols represent the mean of the six replications, and the solid line represents the curve of the fitted model (Eq. 3).

Fig. 6. Average number of *Glomerella* leaf spots developed on each leaf of cv. Gala (*Malus domestica*) and its corresponding standard error after incubation for different periods of time at six temperatures. The leaves were grown on detached shoots, which were water cultured and inoculated with a conidial suspension containing 10^4 *Glomerella cingulata* conidia per ml; the wetness was maintained for 24 h at 25°C, and the leaves were then incubated at different temperatures. The bars represent the average number of lesions per leaf from the three replications; six leaves were inoculated for each replication, and the error bars show the standard errors.

gests that GLS is very difficult to control after pathogen infection. Because the incubation period is very short, the grower would not have adequate time to apply the fungicide after a long period of rain, during which a large number of conidia can be activated. However, GLS could be primarily controlled by the application of fungicides or the eradication of the pathogens in the early epidemic stages, especially during the primary infection stages. A forecasting system to predict the infection periods and quantity in the early epidemic stages, especially the primary infection stages, would be helpful in controlling this disease. The present study provides the data and models needed to develop such a system.

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