Effect of Temperature and Relative Humidity on Germination and Germ Tube Development of *Mycosphaerella fijiensis* var. *difformis*

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ABSTRACT

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Conidia (three isolates) and ascospores (one isolate) of *Mycosphaerella fijiensis* var. *difformis* germinated at 20–35 C. Germination followed a quadratic response function on temperature ($R^2 = 0.90$–0.98), with an estimated optimum at 26.5 C. Maximum germination was observed in free-water and decreased at lower relative humidities (RH). Conidia germinated over a wider range of RH (92–100%) than ascospores (98–100%). Ascospores germinated earlier than conidia. The highest number of germ tubes per conidium was at 25 C for two isolates and at 30 C for the third isolate in free-water after 24 h. The number decreased at lower RH. Maximum length of germ tubes from ascospores occurred at 30 C (91–95 μm) followed by 25 C (68–75 μm), with an estimated optimum at 27.7 C. Germ tube length was similar at 100% RH and in free-water, but decreased at lower RH. Differences among the isolates and the possibility of some degree of natural variability in the response of the fungus to environmental factors are discussed.

Additional keywords: adaption, Black Sigatoka, disease management.

Banana leaf spot or Black Sigatoka, caused by *Mycosphaerella fijiensis* Morelet var. *difformis* Mulder and Stover (13,21) is a very destructive disease of banana (*Musa acuminata*) in the tropics (6,9,21). In Central America, Black Sigatoka is by far the most damaging and costly disease of bananas, accounting for 27% of production costs (20). The cost of chemical control of the disease in Central and South America is approximately $10 million annually (23).

At present, there is little information available on the physiology of *M. f. difformis*, although several factors have been shown to influence infection and disease development under field conditions. Ascospores have been considered the main propagules of *M. f. difformis* in banana plantations (18), however, conidia can also be involved in infection (L. H. Jacome and W. Schuh, unpublished). Stover (19), studied the effect of temperature on ascospore germ tube growth of *M. f. difformis* and observed maximum growth at 26–28 C after 24 h of incubation. However, the study did not examine the effect of relative humidity (RH) on ascospore germination, and conidia were not considered. In the West Indies, temperature and daily evaporation were used by Ganry and Laville (7) to establish a set of favorable and unfavorable conditions for disease development due to *Mycosphaerella musicola*. The precise influence of temperature and relative humidity on the developmental stages of *M. f. difformis* has not been determined. Better understanding of the factors favoring infection process may lead to the development of reliable disease warning systems, or to the refinement of existing ones. This study was undertaken to evaluate the influence of relative humidity and temperature on germination of conidia and ascospores, and germ tube development of *M. f. difformis* using isolates from Honduras.

MATERIALS AND METHODS

Conidial production. Three isolates of *M. f. difformis* obtained from naturally infected banana leaves collected in Honduras, Central America, were used throughout this study. One of the isolates was from Santa Barbara (SB), a northwestern region with no history of chemical control of banana diseases (a wild subpopulation); and the other two isolates were from commercial banana plantations, Cobb (CO) and Omonita (OM) farms, both under known chemical disease control programs.

Fungal cultures were obtained from ascospores discharged from infected banana leaf tissue using the technique described by Stover (17). Single germinating ascospores from each location were transferred from 2.0% water agar to 3.6% Mycophil agar (11445, Baltimore Biological Laboratory, Baltimore, MD) plates. All cultures were incubated at 25 C under 2.5 W m^{-2} of continuous, cool-white, fluorescent light. The resulting anamorph conidial stage of the fungus, known as a *Cercospora* species (13) and later described as *Paracercospora fijiensis* (4,14), was used as the inoculum source.

Mycophil agar plates for conidial production were inoculated with a conidia suspension from each isolate (SB, CO, or OM) obtained by placing one 14- to 18-day-old fungal colony into a test tube containing 0.05% Tween 80 in distilled water, then vortexing for 1 min. Each plate was inoculated by uniformly spreading 0.25 ml of a conidia suspension (adjusted to 2×10^3 conidia ml^{-1}) with a sterile glass rod. Cultures were incubated for 14–18 days at 20 C under 2.5 W m^{-2} of continuous, cool-white, fluorescent light. After this period, 5 ml of distilled water (0.05% Tween 80) was poured into each plate, and conidia were dislodged by gentle agitation with a camel's hair brush. Spore concentration was adjusted to 2×10^4 conidia ml^{-1} using a hemacytometer.

Temperature and humidity in relation to conidial germination and germ tube development. The level of relative humidity was controlled by using the agar dish isopiestic equilibration technique developed by Harris et al (8) and modified by Arauz and Sutton (2). About 44 ml of 2% water agar amended with sodium chloride was poured into the bottom of a 9-cm petri dish. The relative humidity in this chamber is related to the NaCl molality according to the values given by Lang (11). The relative humidities tested were 100, 99, 98, 95, 92, and 88.5%, which were obtained by

amending the agar with 0, 0.3, 0.6, 1.5, 2.2, and 3.1 M NaCl, respectively (2).

One drop of the conidia suspension of each isolate (SB, CO, or OM) was placed on each of three microscope cover glasses per isolate, and air-dried. One free-water treatment was also used, in which the drop of conidia suspension was not air-dried. The three cover glasses, corresponding to three assessment times, were placed on a clean microscope slide inside each relative humidity chamber. Each dish was sealed with Parafilm. These sealed relative humidity chambers were placed in controlled temperature incubators at 20, 25, 30, or 35 ± 1 C under continuous light. The sealed chambers were preconditioned at the desired temperature for at least 15 h before the spores were placed in them (2).

Conidial germination was evaluated at 8, 16, and 24 h after the spores were placed in the relative humidity chamber. After each prescribed incubation period, one cover glass from each relative humidity chamber was removed. The chamber then was resealed. The removed cover glass was inverted on a drop of cotton blue in lactophenol on a microscope glass slide to stop germination and preserve the spores and germ tubes for later observation. Percentage of germination was determined by observing at least 100 conidia, in a two-transect pattern, on each cover glass per treatment/replication. A conidium was considered to have germinated if the germ tube was at least one-fourth the length of the conidium (80–100 μm). Due to irregular growth habits, the germ tube length was not measured. However, the number of germ tubes per conidium and the percentage of them with a relative size one-half the length of the conidium were determined from 10 germinated conidia selected randomly from each cover glass.

Each set of treatments was replicated four times on different days. The experiment was conducted in a split-split-plot design with temperature as the whole plot, isolates as the subplot, and relative humidity as sub-subplot. A separate analysis was done for each assessment time.

Temperature and humidity in relation to ascospore germination and germ tube development. Naturally infected banana leaf tissue, bearing perithecia of the teleomorph stage of *M. f. difformis*, was used as the source of ascospores. Only leaf tissue collected from the Santa Barbara (SB) region was used. Pieces of infected leaf were incubated for 24 h in a humid chamber (in a sealed plastic bag containing damp paper towelling). Ascospores were discharged using a technique similar to the one described by Stover (17). Pieces of infected tissue were stapled to a disk of filter paper with the lower surface of the leaf outwards, then soaked in distilled water for 3–5 min. The wet, infected tissue was attached to the inside of a petri dish lid and allowed to discharge ascospores for 20–30 min. Ascospores were collected on cover glasses, and placed immediately after collection in the controlled relative humidity chambers described previously. Treatments for temperature and relative humidity were the same as those described for conidia. A free-water treatment was created by placing a drop of sterile, distilled water (0.05% Tween 80) on the cover glass, over which the ascospores were discharged.

Ascospore germination was evaluated as described for conidia. An ascospore was considered to have germinated if the germ tube length was ≥the diameter of the ascospore (4–6 μm). The germ tube length was determined from 10 germinated ascospores selected randomly from each cover glass, per treatment/replication.

Each set of treatments was replicated three times on different days. The experiment was conducted in a split-plot design with temperature as the whole plot and relative humidity as the subplots (10). A separate analysis was done for each assessment time.

Statistical analysis. Statistics were computed for the percentage of germination, number of germ tubes per spore, and length of the germ tubes. The proportion of germinated spores was transformed using the arcsine $\sqrt{x}$ transformation (16). The germination response was evaluated separately for each isolate. Analyses of variance were conducted using the SAS PROC GLM (15). Significance of the temperature effect was determined using the replication × temperature interaction as the error term. Regression analysis was conducted using SAS PROC GLM (15). Models were chosen based on the following criteria: randomness and normality of residuals; significance of estimated parameters; and R^2 values (15). Linear, quadratic, and cubic effects of temperature and relative humidity, and their interaction on spore germination and germ tube development were tested. Models were based on the results obtained after 24 h.

RESULTS

Temperature and humidity in relation to conidia germination and germ tube development. Conidia of *M. f. difformis* germinated at temperatures from 20 to 35 C. Maximum germination averaged over isolates occurred at 25 C (96.6%) followed by 30 C (95.9%) in free-water after 24 h. Germination of conidia at 20 and 35 C after 24 h was 81.1 and 67.0%, respectively (Fig. 1). Germination of conidia followed a quadratic response function for temperature ($R^2 = 0.90$–0.98), with an estimated optimum at 26.5 C. A similar pattern was observed at the three assessment times.

In general, conidial germination decreased drastically at lower relative humidities (Fig. 2) at all temperatures tested and the three assessment times. However, no conidial germination was observed for the isolate SB at 35 C after 8 h at 99, or 24 h at 95% RH. Less than 10% of germination occurred at 95% RH after 24 h, with the exception of isolate OM at 25 C (25.9%). After 24 h, only isolates OM (3.6%) and CO (1.2%) germinated at 25 C and 92% RH. No conidial germination was observed at 88.5% RH.

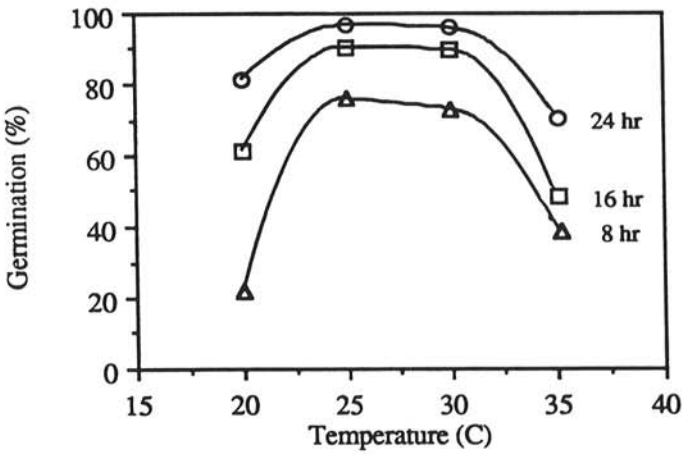

Fig. 1. Effect of temperature on germination of conidia of *Mycosphaerella fijiensis* var. *difformis* in free-water for three assessment times. Percentage of germination averaged over isolates.

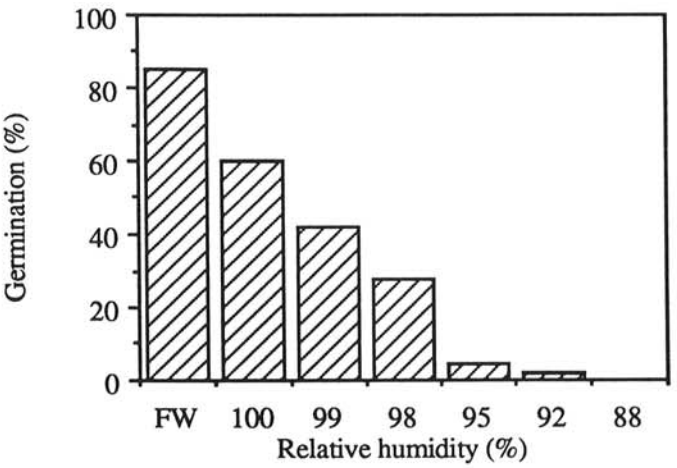

Fig. 2. Effect of relative humidity on germination of conidia of *Mycosphaerella fijiensis* var. *difformis*. FW = free-water. Percentage of germination averaged over isolates.

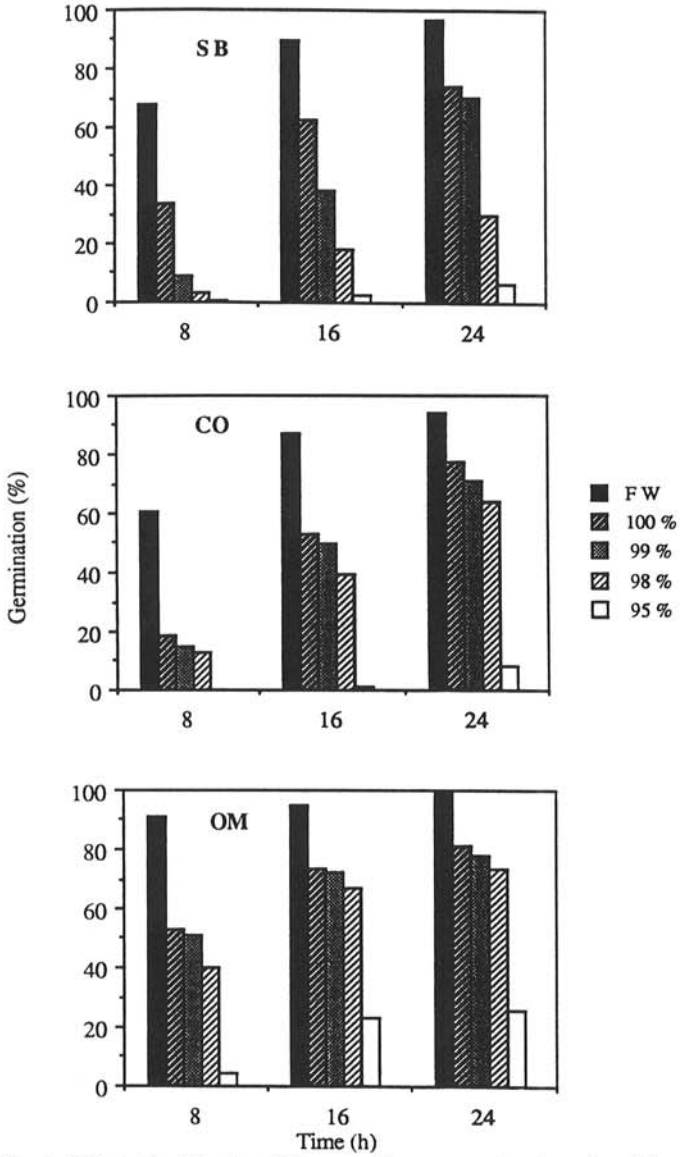

Fig. 3. Effect of relative humidity over time on germination of conidia of three isolates of *Mycosphaerella fijiensis* var. *difformis*. SB = Santa Barbara (top), CO = Cobb (middle), and OM = Omonita (bottom) at 25 C. FW = free-water.

However, evidence of germ tube development (about 10% of conidium length) was observed in conidia from isolates CO (0.5%) and OM (0.3%) at 92% RH and 30 C; and for OM (2.2%) and CO (0.4%) at 88.5% RH and 25 C. Because this length was below the established criteria for germination, they were not considered in further analysis.

Highly significant ($P < 0.001$) effects of temperature, relative humidity, isolates, and their interactions on germination were observed. Interactions involving replication effects were not significant. Therefore, data presented are combined means. Germination was higher for isolate OM at all relative humidities, followed by isolate CO at ≤100% RH, and by isolate SB in free-water (Fig. 3). A quadratic response on temperature ($R^2 = 0.78$–0.99) with maximum germination at 25 C was observed on the 15 combinations (three isolates × five humidity levels) tested. Lower and higher coefficients of variation were observed in free-water and at 95% RH, respectively. Differences in germination were observed among temperatures at each humidity level. Germination was similar at 25 C and 30 C for isolates SB and CO in free-water and for isolates CO and OM at 100% RH. The effects of temperature, relative humidity, and their interaction on germination of conidia are shown for each of the isolates in Table 1. Germination in free-water was determined by a quadratic response on temperature.

Maximum number of germ tubes per conidium occurred at 25 C for the isolates SB (2–6) and CO (2–4), and at 30 C for the isolate OM (2–8) in free-water after 24 h. The average number of germ tubes per conidium followed a quadratic response function on temperature. The average number of germ tubes per conidium decreased drastically at lower relative humidities over all temperatures and assessment times. The average number of germ tubes per conidium was higher for isolates OM at all relative humidities, followed by isolate CO at ≤100% RH, and by isolate SB in free-water (Fig. 4).

Maximum germ tube development was observed in free-water for isolate OM, followed by isolate SB and CO. The proportion of long germ tubes decreased at lower relative humidities (Fig. 4). Germ tubes of the isolates SB and CO were more sensitive to relative humidity than those of the isolate OM. About 48% of the germ tubes of the isolate OM were ≥one-half the length of the conidium at 98% RH, while similar values for the isolates SB and CO were only observed at 100% RH. At 25 C, 56–74% of the germ tubes were ≥one-half the length of the conidium; isolates SB and OM showed the lowest and the highest proportion, respectively.

Highly significant ($P < 0.01$) effects of temperature, humidity, isolates, and their interactions were identified in the analysis of

TABLE 1. Estimated parameters ($P = 0.05$) for the effect of temperatures (T) and relative humidity (RH) on germination (conidia and ascospores), number of germ tubes (conidia), and germ tube length (ascospore) of *Mycosphaerella fijieusis* var. *difformis*

Isolate	Response variable	I[a]	T	RH	T^2	T^3	RH^2	RH^3	T*RH	T^3*RH	RH^2*T	R^2
SB	Germination (%)	−26.746 (2.663)[b]	6.624 (0.559)	0.106 (0.015)	−0.068 (0.009)	8.078×10^{-4} (1.110×10^{-4})	···	···	−0.098 (0.010)	···	5.092×10^{-4} (5.429×10^{-5})	93.0
CO	Germination (%)	−25.418 (3.336)	0.736 (0.124)	0.234 (0.033)	−0.005 (7.912×10^{-4})	···	···	···	−0.005 (0.001)	···	···	81.9
OM	Germination (%)	−23.974 (2.255)	0.667 (0.084)	0.212 (0.022)	−0.006 (5.349×10^{-4})	···	···	···	−0.003 (8.176×19^{-4})	···	···	91.7
SB	Average number of germ tubes per conidia	−13.467 (2.050)	··· ···	0.148 (0.021)	0.029 (0.009)	···	···	···	−0.009 (0.003)	3.227×10^{-4} (3.656×10^{-5})	1.014×10^{-4} (3.656×10^{-5})	73.4
CO	Average number of germ tubes per conidia	−5.841 (0.632)	0.540 (0.703)	···	···	-2.814×10^{-5} (6.520×10^{-6})	···	7.939×10^{-6} (7.200×10^{-7})	(7.328×10^{-4})	···	···	64.3
OM	Average number of germ tubes per conidia	−5.025 (0.731)	··· ···	··· ···	0.014 (0.002)	1.426×10 (1.913×10^{-5})	···	6.860×10^{-6} (8.700×10^{-7})	···	-9.301×10^{-5} (2.841×10^{-5})	···	64.2
SB	Ascospore germination (%)	−358.097 (70.796)	0.374 (0.177)	7.016 (1.452)	··· ···	··· ···	−0.034 (0.007)	··· ···	−0.003 (0.001)	··· ···	··· ···	93.7
SB	Average germ tube length per ascospore	28,471.462 (7,237.168)	−2,291.866 (540.144)	−289.528 (73.047)	41.770 (9.750)	··· ···	··· ···	··· ···	23.320 (5.452)	−0.424 (0.098)	···	76.2

[a] Intercept.
[b] Standard deviation in parentheses.

variance of the average number of germ tubes per conidium. The mean of the average number of germ tubes per conidium (over all temperature and humidity combinations) of isolate OM was the highest (3.5), followed by isolates SB (2.9) and CO (2.3). Differences among humidity levels were found at each temperature. However, the number of germ tubes was similar in free-water and 99 and 100% RH for the isolates SB and CO at 20 C, and for the isolates CO and OM at 30 C. At the other eight combinations (isolate × temperature), similar values were observed at 99 and 100% RH. The average number of germ tubes per conidium at 95 and 98% RH was similar and consistently the lowest. The effect of temperature became less pronounced as relative humidity increased from 95 to 100%. Higher values of the average number of germ tubes per conidium were observed at 20/25 C compared to 30/35 C for isolates SB and CO, and at 25/30 C compared to 20/35 C for isolate OM. In free-water, the average number of germ tubes per conidium was similar at 25 and 30 C for the three isolates, but different from 20 and 35 C. The effects of temperature, relative humidity, and their interaction on the average number of germ tubes per conidium are shown in Table 1.

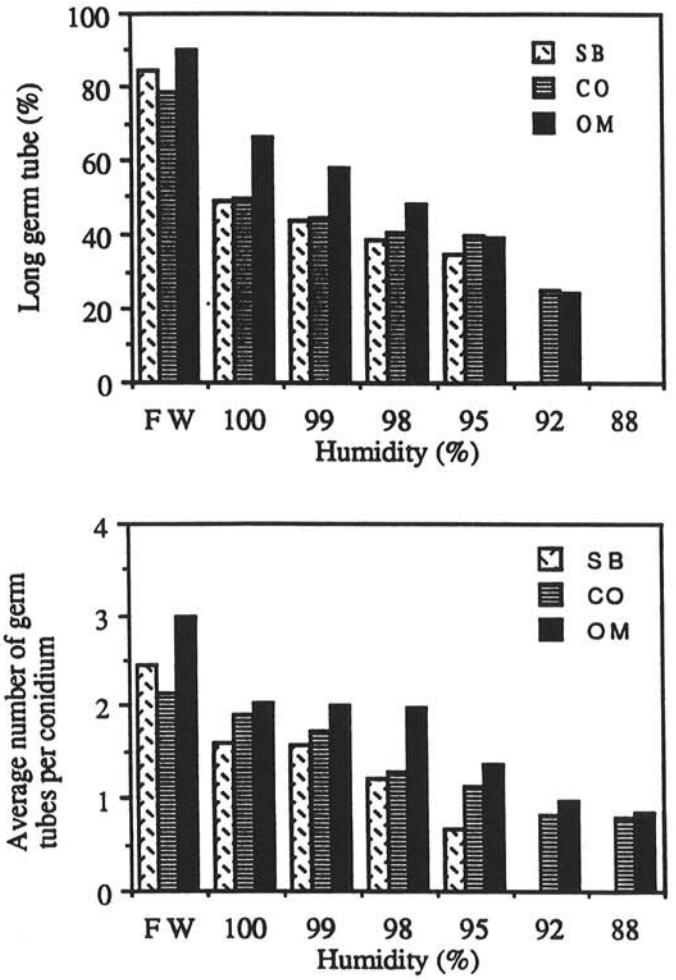

Fig. 4. Effect of relative humidity on the proportion of long germ tubes (≥one-half the length of the conidium) (top), and the average number of germ tubes per conidium (bottom) for three isolates of *Mycosphaerella fijiensis* var. *difformis*. FW = free-water.

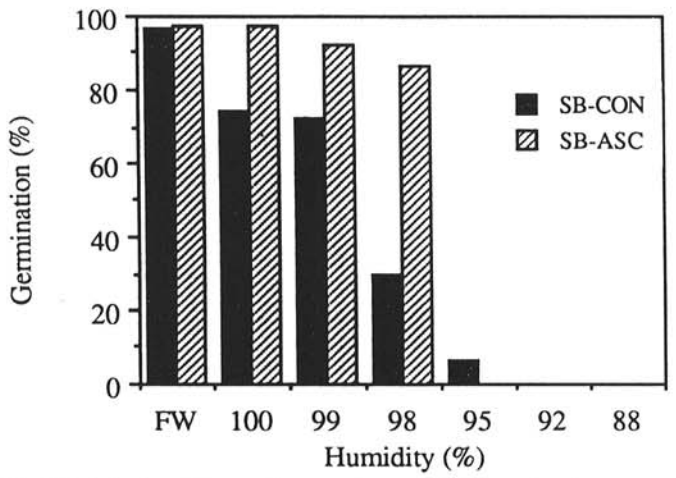

Fig. 5. Relative humidity requirements for conidia (SB-con) and ascospore (SB-asc) germination for isolate SB (from Santa Barbara, Honduras) of *Mycosphaerella fijiensis* var. *difformis*. FW = free-water.

Temperature and humidity in relation to ascospore germination and germ tube development. Ascospores of *M. f. difformis* germinated at temperatures from 20 to 35 C. Average maximum germination of ascospores occurred at 25 C (97.5%) followed by 30 C (95.8%) in free-water after 24 h. Germination of ascospores at 20 and 35 C was 92.5 and 94.7%, respectively. Ascospore germination was similar in free-water and at 100% RH. It slightly decreased at 99 and 98% RH. No ascospore germination was observed at ≤95% RH after 24 h (Fig. 5). Similar patterns were observed after 8 and 16 h.

Highly significant ($P = 0.05$) effects of temperature, humidity, and their interaction on germination were observed. Ascospore germination was similar among temperatures in free-water after 24 h. However, ascospore germination followed a quadratic

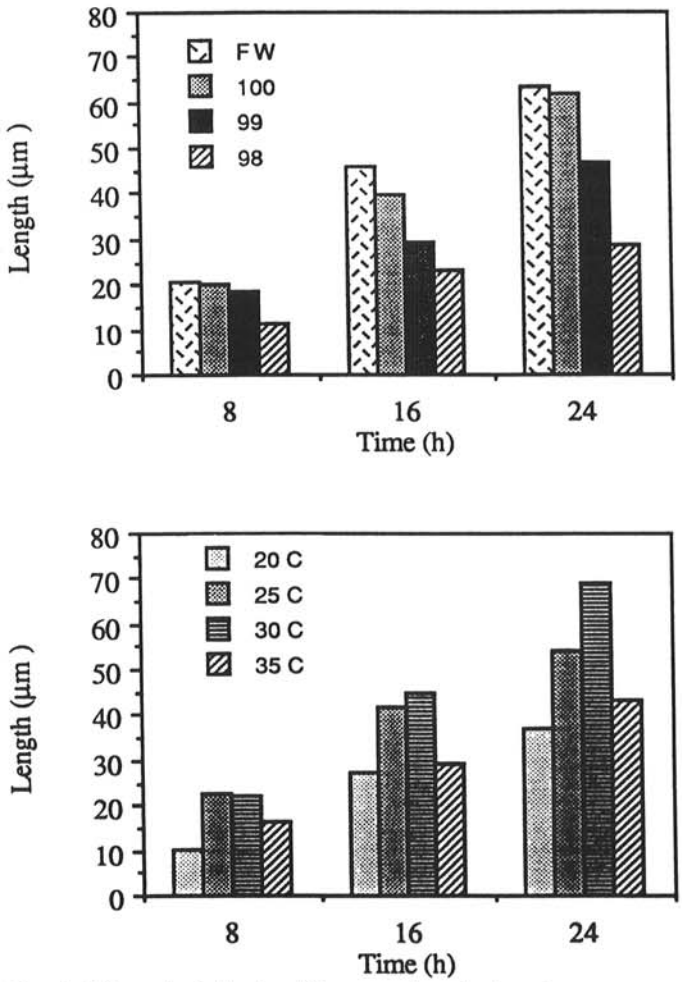

Fig. 6. Effect of relative humidity over time (top), and temperature (bottom) on germ tube length of ascospores of *Mycosphaerella* var. *difformis*. FW = free-water.

response function on temperature ($R^2 = 0.927$) after 16 h in free-water, with a maximum observed germination at 25 C (95.7%). At 98–100% RH, the percentage of ascospore germination was slightly lower at 20 C. The percentage of germination was similar at 25, 30, and 35 C after 24 h. Different germination levels among humidity levels were found at each temperature. Ascospore germination was the lowest at 98% RH for the four temperatures. Percentage of germination at 99% RH was lower than that at 100% RH and in free-water at 25 and 30 C. Germination was similar in free-water and at 100% RH at the four temperatures. The effects of temperature, relative humidity, and their interaction on germination of ascospores are shown in Table 1.

Germinated ascospores developed one or two germ tubes. No effect of relative humidity was found, but most ascospores developed two germ tubes at 25 and 30 C. Maximum length of germ tubes from ascospores was observed at 30 C (91–95 μm) followed by 25 C (68–75 μm). Germ tube elongation at 20 and 35 C was slower. Germ tube growth in free-water and at 99 and 100% RH followed a quadratic response function on temperature ($R^2 = 0.81$–0.87), with an estimated optimum at 27.7 C. Germ tube elongation was similar at 100% RH and in free-water, and drastically decreased at 99 and 98% RH (Fig. 6). It was also similar among temperatures at 98% RH, and between 25 and 30 C in free-water. Germ tube growth at 98% RH was the lowest at the four temperatures. Germ tube elongation was comparable between 100% RH and in free-water among the four temperatures, and between 98 and 99% RH at 20 and 25 C. The combined effect of temperature and relative humidity on the average length of the germ tube of ascospores is shown in Table 1.

DISCUSSION

In this study, spore germination and germ tube development of *M. f. difformis* responded strongly to temperature, humidity, and isolate. Both conidia and ascospores of *M. f. difformis* germinated at 20–35 C, with an observed maximum at 25 C. Maximum spore germination occurred in free-water. Lower relative humidities reduced germination. Ascospores germinated at 98% RH or higher. Conidia, in contrast, showed a wider range of relative humidity required for germination. However, the proportion of ascospores that germinated at 98–100% RH was higher than that of conidia (Fig. 5). Most ascospores (67–92%) had germinated after 8 h in free-water. Conidia germinated slower than ascospores during the first 16 h, but reached similar levels after 24 h. In the related pathosystem *M. musicola*, conidia have been identified as the spore form causing disease during the dry season (February–May) (12,18). We suggest a similar situation for *M. f. difformis*. The weather conditions in Honduras during this period are not conducive for ascospore infection with their requirement for free-water or nearly saturated environment. In addition, ascospores are not generally trapped with Kramer-Collins spore traps during the dry period, but disease pressure continues. Infection by conidia with their ability to germinate over a wider range of relative humidities and in the absence of free water would explain these observations. Conidia are able to cause significant amounts of disease, and the disease symptom is identical to the one caused by ascospore infection (L. H. Jacome and W. Schuh, *unpublished*).

Germination of spores at 92% relative humidity at 20 C or higher may be due to an increase in the atmospheric moisture content with increasing temperature (2). Germination of spores placed at lower relative humidity was delayed and may be associated with a required longer water absorption period. The ability of spores to germinate at lower relative humidity has been related to high osmotic pressure of the spore (22), which allows spores to absorb water from the air. Conidia showed more tolerance to moderate humidity conditions than ascospores. High temperature (35 C) or low relative humidity (40–70%) during daytime hours curtails germ tube elongation and infection by conidia (L. H. Jacome and W. Schuh, *unpublished*). Thus, moderate (20–25 C) night temperature could favor spore germination, whereas germ tube elongation could continue during warmer (25–30 C) morning or daytime conditions, provided the relative humidity is high. The observed effect of temperature on germ tube length of ascospores (Fig. 6) corresponds closely with previous work (19).

The number of germ tubes per conidium may be correlated with increase in stomatal penetration or multiple penetrations from a single conidium. Multiple germ tubes would offer an advantage to a particular isolate when relative humidity decreases. In vivo studies to investigate stomatal penetration and lesion induction under conditions favorable for germination and germ tube development are being conducted. Multiple penetrations from a single conidium have been reported for *Cercospora arachidicola* (1).

The mean of conidial germination of isolate OM was the highest at the three assessment times. The differences observed among isolates suggest some degree of natural variability in the response of *M. f. difformis* to environmental factors. This differential ability in germination and germ tube development could provide certain survival advantage in the field under tropical conditions. For example, isolate OM was from a warmer and drier region and had higher temperature requirements. It germinated faster and developed in a wider range of humidity as compared to the isolate SB from a colder and wetter region (Fig. 3). Variation in temperature requirements among isolates has also been found in other fungi (2). The validity of this hypothesis regarding the adaptability of the fungus to different environments should be investigated using isolates from different geographical areas in Central and South America.

The models developed for estimating the germination of conidia of the three isolates show some common characteristics (Table 1). A significant negative quadratic effect of temperature can be seen, resulting in minimum, optimum, and maximum temperatures, as can be expected from a biological system. A negative interaction parameter of temperature and relative humidity is significant for all three isolates. This parameter causes the increases in germination due to increases in relative humidities to become smaller. The effect of temperature in the interaction term is less due to lower values of this term. However, it indicates that higher temperatures cause smaller increases in germination at the same relative humidities. Additionally, it amplifies the effect of the negative quadratic effect of temperature. A similar situation is found for the equation describing the germination of ascospores. The effect of increasing relative humidities on the germination is even smaller with the inclusion of a negative squared term for relative humidity. This can be explained by the similar germination rate in 100% RH and free-water, and the only slight decrease when RH was 99 and 98%. The equations describing the average number of germ tubes (conidia) and germ tube length (ascospore) are harder to interpret. The lower R^2 value observed may be due to higher inherent variability of the estimated responses as compared to germination, which is basically a binary variable.

A model to predict the rate of disease development based on previous diseases present, accumulated precipitation, and days of RH at 90% was developed in Taiwan by Chuang and Jeger (3). The model did not consider the effect of temperature and relative humidity at each stage of the epidemic. Our results indicated that a narrower range of relative humidity is required for germination and germ tube development for isolates in Honduras. The effect of temperature on pathogen and disease development often becomes distinct only when optimum humidity conditions prevail at the same time (5).

Chemical control strategies rely on the use of protectants and systemic fungicides with limited curative action. The availability of fungal inoculum during the wet season is not considered to be a limiting factor. The relationships between temperature, relative humidity, and germination developed in this study should be useful in determining time periods when favorable combinations of these parameters result in infection, and therefore are a vital component in developing a disease forecasting system to optimize the timing of fungicide applications. Additionally, results from this study should point to the potential role of conidia in the disease epidemiology, especially during the dry season.

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