Temperature, Leaf Wetness, and Isolate Effects on Infection of Minneola Tangelo Leaves by *Alternaria* sp.

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ABSTRACT

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Alternaria brown spot causes necrotic lesions on immature leaves, twigs, and fruit of tangerines and their hybrids, reducing yield and fruit quality. The effect of temperature, leaf wetness, and isolate was evaluated in an in vitro system using immature detached leaves of Minneola tangelo. Infection was greatest at 27°C, decreased gradually as the temperature declined to 24, 20, and 17°C, and dropped sharply at 32°C. Levels of infection were low at 4 and 8 h of leaf wetness and continued to increase with longer wetting periods up to 36 h. A polynomial equation was developed that provided a good fit for the data (adjusted $R^2 = 0.93$). Isolates differed in aggressiveness, but there was no significant difference among isolates in their response to temperature and leaf wetness duration.

Alternaria brown spot is an important disease of tangerines (*Citrus reticulata*) and their hybrids. The causal agent was originally designated as *Alternaria citri* Ellis & N. Pierce (9) and later renamed *A. alternata* (Fr.:Fr.) Keissl. pv. *citri* (12), but the proper classification of this pathogen remains in doubt (19). In addition to the tangerine pathogen, a genetically distinct strain or species attacks rough lemon and Rangpur lime. Both strains produce well-characterized host-specific toxins, each with a different mode of action (6). The tangerine cultivars affected in Florida include Murcotts, Dancy tangerines, and Minneola tangelos, as well as other hybrids of Dancy tangerines. The pathogen attacks immature leaves, twigs, and fruit, causing small necrotic lesions that may appear as early as 24 h after infection. Once the fungus is established and produces toxin, many leaves and fruit abscise, and twigs die back. The fungus also produces lesions on more mature fruit, ranging from small, necrotic spots to large, sunken pockmarks. Leaves become resistant when they mature, and fruit become resistant when they reach a diameter of about 3 cm, usually

late July in Florida. The disease was first described in Australia (9), first reported in Florida in 1976 (17), and also occurs in Colombia (2,3), Israel (13-15), South Africa (11), and Turkey (1).

Little is known about the epidemiology of *Alternaria* brown spot (17,18). Sporulation occurs primarily on lesions on mature leaves (15), and free moisture or even high relative humidity (RH) brings about sporulation on such lesions (16). Spore release is triggered by abrupt changes in RH or by rainfall, but not by vibration or red/infrared irradiation. Infection of susceptible trap plants placed in the field weekly failed to reveal which environmental factors were essential for infection of leaves (16). In recent studies in Israel, the optimum temperature for lesion expansion was determined to be 20 to 25°C (14).

Alternaria brown spot is difficult to control, and frequent applications of iprodione or copper fungicides are used to prevent fruit drop and to produce blemish-free fruit for the fresh market. In Florida, applications are made on a 10- to 14-day schedule from late February to mid-July to achieve acceptable control in heavily infested orchards. In semiarid areas, such as Israel and South Africa, six to eight sprays are required (11,13). In Colombia, where rainfall and temperatures are high, weekly sprays year-round were required to achieve control (3). In certain areas, production of Minneola tangelo and other susceptible cultivars has been largely abandoned due to this disease.

Currently, fungicide applications are based on a calendar schedule or on tree phenology. Predictive models that use duration of leaf wetness and temperatures required for infection have been useful in

predicting disease and timing fungicide applications on similar diseases of tree crops (4,7,10). Predictive models should be helpful for reducing the number of fungicide applications and for improving control of brown spot. In this study, we investigated the effect of temperature, leaf wetness duration, and isolates on the infection of detached leaves of Minneola tangelo with the goal of developing models useful in the field.

MATERIALS AND METHODS

Inoculum production, plant material, and inoculations. The SH-1 isolate of *Alternaria* sp. was obtained from a Minneola tangelo grove near Polk City, Florida. Conidial inoculum was produced by growing the fungus on potato dextrose agar (Difco, Detroit, MI) for 3 to 5 days at 27°C. The surface of the plates was scraped with a scalpel to remove aerial mycelium, and the colony was cut into approximately 4 mm² pieces. About 50 to 100 pieces were then transferred to each plate of a sporulation medium consisting of 30 g of CaCO₃, 20 g of sucrose and 20 g of agar per liter of distilled water. Two milliliters of sterile distilled water was added to each plate, and the plates were sealed with Parafilm and incubated with 12 h per day of fluorescent light for 3 to 4 days at 27°C. Conidia were collected by adding 10 ml of sterile distilled water to each plate and gently brushing the colonies and then filtering the suspension through two layers of cheesecloth to remove mycelial fragments. Conidia were washed twice in sterile distilled water by centrifugation to remove additional mycelium and any toxin carried over from the culture plates. Conidial suspensions were adjusted to 10⁴ conidia per ml using a hemacytometer. Preliminary experiments using conidial concentrations of 10³, 10⁴, 10⁵, and 10⁶ conidia per ml revealed that 10⁴ conidia per ml gave a scorable number of lesions on each leaf.

Leaves for inoculation were produced from 1- to 2-year-old Minneola tangelo (*Citrus reticulata* Blanco × *C. paradisi* Macf.) trees on rough lemon (*C. jambhiri* Lush) rootstock grown in the greenhouse. Succulent young leaves about 3 cm long were collected for inoculation. In the past, inoculations have generally been conducted by placing droplets or filter paper disks with conidia on detached leaves to evaluate cultivar susceptibility (6,9,14). In

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Table 1. Analysis of variance of the effect of temperature and duration of leaf wetness on the number of lesions per leaf of *Minneola tangelo* inoculated with *Alternaria* sp. isolate SH-1

Source	df	SS	MS	EMS	F test	F	P
Experiment	3	9.72	1.59	MSA	MSA/MSE	8.8	<0.0001
Temp	4	42.11	11.36	MSB	MSB/MSE	62.9	<0.0001
Time	4	86.97	21.00	MSC	MSC/MSE	116.2	<0.0001
Temp*time	16	14.45	0.90	MSBC	MSBC/MSE	5.0	<0.0001
Error	54	9.75	0.18	MSE			
Total	81	163.01

Fig. 1. Effect of temperature and time of leaf wetness on the number of lesions per leaf of *Minneola tangelo* inoculated with *Alternaria* sp. (A) Observed data, average of four experiments, (B) three-dimensional representation providing the best fit for the data, $z = 287.4 - 37.1x - 5.9y + 1.55x^2 + 0.05y^2 + 0.49xy - 0.02x^3 - 0.0003y^3 - 0.0016xy^2 - 0.009x^2y$, where x = temperature, y = duration of leaf wetness, and z = number of lesions per leaf.

our preliminary studies, droplets of conidial suspensions were placed on the abaxial surfaces of young leaves, and the moisture was removed with filter paper at different times after inoculation. This technique proved excessively severe, and lesions developed even after brief wetting periods (T. L. Peever, *unpublished*). Subsequently, an in vitro spray inoculation technique was developed. Petioles of immature leaves were immersed into microcentrifuge tubes filled with water, and the tops of the tubes were wrapped with Parafilm to hold the leaves in place and to prevent evaporation of water. Tubes with leaves were placed in racks inside plastic chambers about 30 × 23 × 10 cm with water in the bottom to maintain RH near 100%. Leaves were misted with the conidial suspension, and the chambers were covered and placed in incubators at various temperatures. After different periods of leaf wetness, six leaves were removed, dried with a fan, and incubated at room temperature (21 to 24°C) and RH (40 to 70%). Thirty-six hours after completion of the experiment, the number of lesions on each leaf was counted.

Experiments. Four experiments were conducted with the SH-1 isolate to evaluate the effect of temperature and duration of leaf wetness on infection. In two experiments, the temperatures tested were 17, 20, 23, 27, and 32°C, with leaf wetness durations of 4, 8, 12, 24, and 36 h. In a third experiment, the same temperatures and leaf wetness durations were evaluated, except that the 36-h treatment was omitted. In the fourth experiment, temperatures tested were 23, 27, and 32°C, with wetness durations of 4, 8, 12, and 24 h. A 35°C temperature was included in two experiments, but no results are presented since heat damage to leaves precluded evaluation of pathogen effects. Noninoculated control leaves were included for each temperature and exposed to the longest duration of wetness used in each experiment.

Leaf lesion counts were square root transformed prior to analysis. Data were subjected to analysis of variance using an incomplete block design considering experiments as blocks using Minitab, Inc., programs (State College, PA). The ANOVA model was mixed, with time and temperature considered to be fixed factors and experiment a random factor. Data from the four experiments were pooled, and an equation and graph that fit the data were generated using the program Table Curve 3D (SPSS, Inc., Chicago, IL).

Two experiments were conducted to determine how different isolates responded to temperature and leaf wetness duration. Inoculum and leaves were produced, and inoculations were conducted as described above. Temperatures evaluated were 17, 20, 23, and 27°C, and durations of leaf wetness were 6, 10, and 24 h. Forty-two leaves were inoculated with each isolate

and maintained in separate humid chambers (one chamber for each temperature). Six leaves were removed and dried after each wetness period, and lesion counts were made about 36 h after completion of the experiment.

All isolates tested were recovered from *Alternaria* brown spot-affected *Minneola* tangelos in different citrus-producing areas of Florida. Two isolates, CPI (CPI-MIL-25) and IMK (IMK-MIL-IS), were from groves near Immokalee in southwestern Florida. Isolate SH (SH-MIL-30S) was from a grove near Polk City, and isolate EV (EV-MIL-17S) was from a grove near Lake Alfred, both in central Florida. Isolates chosen represented the range of population diversity of Florida isolates based on analysis of randomly amplified polymorphic DNA (T. L. Peever, *unpublished*). Lesion counts were square root transformed prior to analysis. Data were subjected to analysis of variance to test the effects of isolate, temperature, and leaf wetness using experiments as blocks (Minitab). A mixed model was employed, with temperature and time as fixed factors and isolate and experiment as random factors.

RESULTS

Temperature and leaf wetness effects.

The temperature and the time of leaf wetness significantly affected the amount of infection observed following inoculation (Table 1). Only low levels of infection were observed at 4 h of leaf wetness (Fig. 1A). With additional hours of leaf wetness, the amount of infection continued to increase up to 36 h, the maximum period evaluated. The greatest amount of infection was observed at 27°C with all periods of leaf wetness. The number of lesions was progressively less at 23, 20, and 17°C. Extended wetness periods were needed to achieve a high level of infection at 17°C. At higher than optimum temperatures, infection decreased rapidly, with little infection at 32°C even with extended wetness periods. Experiment was also a significant factor in the analysis (Table 1). Experiments differed in the average number of lesions per leaf, but the trends were the same in all four experiments. No symptoms developed on controls.

The polynomial equation $z = 287.4 - 37.1x - 5.9y + 1.55x^2 + 0.05y^2 + 0.49xy - 0.02x^3 - 0.0003y^3 - 0.0016xy^2 - 0.009x^2y$ provided a good fit for the observed data (adjusted $R^2 = 0.93$, Fig. 1B). Other more complex logistic and Gaussian equations provided equally good fits for the data, but calculations for predictions would be more difficult. The equation selected provided a good fit for the data over the range studied but would not apply for very low or high temperatures. Below 15°C, values predicted increase again, and at temperatures much above 32°C, values are zero or negative (Fig. 1B).

Isolate effects. In the two experiments where four isolates of *A. alternata* were evaluated (Figs. 2 and 3), temperature and leaf wetness duration were significant factors in the analysis (Table 2). The highest levels of infection occurred at 23 to 27°C with 24 h of leaf wetness. Isolate $\times$ temperature interaction was not significant; however, there was a significant isolate $\times$ time of wetness interaction, indicating possible differences in aggressiveness of isolates with longer wetting periods.

There were no significant differences observed among isolates (Table 2), and the rankings of isolates were not consistent between experiments (Figs. 2 and 3). The IMK isolate produced high numbers of lesions under most conditions in experiment 1, whereas the SH-1 isolate produced the highest number in experiment 2. The CPI isolate produced low numbers in both experiments, and the EV isolate produced

moderate number of lesions in both experiments.

DISCUSSION

The optimum temperature for infection of *Minneola* tangelo leaves was about 27°C. The optimum for most species of *Alternaria* is 25°C or above (10). Of course, optimum temperatures for pathogens of temperate crops, such as *A. brassicae* on oilseed rape (5), *A. mali* on apple (4), and *Stemphylium vesicarium* on pear (7) were closer to 20°C. In the present study, the optimum length of the wetting period for *A. alternata* on citrus was at least 36 h and may possibly be longer. In most previous studies on other crops, 24 h was the maximum period evaluated and gave the highest level of infection (5,7,10). However, Filajdic and Sutton (4) evaluated periods up to 48 h and found that maximum infection occurred at 36 h with *A.*

Fig. 2. Effect of isolate, temperature, and duration of leaf wetness on the number of lesions per leaf of *Minneola* tangelo inoculated with *Alternaria* sp. Experiment 1.

mali. The optimum temperature and leaf wetness periods correspond well with our field data (15), indicating that much of the infection occurs during the summer rainy

period in Florida when temperatures are near optimum for the fungus and extended periods of leaf wetness are common. The optimum temperature for lesion develop-

ment for *Alternaria* brown spot in Israel was 22 to 25°C, somewhat lower than observed with Florida isolates. These optima correspond well with the temperatures that occur during the major periods of reproduction of the pathogen in each location.

There were no significant differences observed among isolates used in this study, even though the isolates chosen were of maximum divergence genetically. This means that the model developed with these isolates should be generally applicable to the range of isolates found in Florida. Isolate response to temperature and wetness periods has not commonly been investigated. However, Filajdic and Sutton (4) attributed much of the variability in responses observed to differences in the aggressiveness of the isolates.

The descriptive model developed in this study has not yet been evaluated in the field. Fundamental models developed for similar diseases proved useful in predicting disease in field situations (4,5,7,10). The major component missing from laboratory-developed models for field use is the amount of inoculum available. In many Minneola tangelo groves in Florida, disease is severe and infection can take place almost any time of the year that susceptible tissue is available (15). Thus, predictive models based solely on temperature and leaf wetness may prove useful in these situations. However, in some groves and at certain times of the year, inoculum may be the limiting factor because of prevailing environmental conditions. The equation yields unrealistic predictions below 15°C and above 32°C. In field situations, this would not be a problem since temperatures are seldom below 15°C when susceptible tissue is available and would rarely exceed 32°C when moisture was present for infection.

Use of predictive models as a basis of fungicide applications for *Alternaria* brown spot control is complicated by the fact that disease develops so rapidly. Inoculated leaves that remained moist for 36 h often had already developed symptoms when they were removed from the chamber. Thus, there is no time to apply fungicides between an infection period and symptom development. Fungicide sprays, however, might be timed on the basis of cumulative daily severity values. Particularly in the spring in Florida, when extended dry periods may occur, risk advisories could allow growers to delay some fungicide sprays, resulting in fewer applications per year. Once the summer rainy season begins in June, regular sprays may be needed until the fruit become resistant in late July. In semiarid areas such as Israel and Turkey, where little or no rain occurs during the period of susceptibility (12,13), infection is the result of prolonged periods of dew. With more variable temperatures and no rain, outbreaks of *Alternaria* brown spot may be more easily forecast.

Fig. 3. Effect of isolate, temperature, and duration of leaf wetness on the number of lesions per leaf of Minneola tangelo inoculated with *Alternaria* sp. Experiment 2.

Table 2. Analysis of variance of the effect of isolate, temperature, and duration of leaf wetness on the number of lesions per leaf of Minneola tangelo inoculated with different isolates of *Alternaria* sp.

Source	df	SS	MS	EMS	F test	F	P
Experiment	1	0.22	0.22	MSX	MSX/MSE	0.5	0.470
Isolate	3	7.71	2.57	MSA	Approximate ^a	2.9	0.069
Temp	3	9.81	3.27	MSB	MSB/MSAB	23.6	<0.0001
Time	2	52.46	26.23	MSC	MSC/MSAC	29.8	0.001
Isolate*temp	9	1.25	0.14	MSAB	MSAB/MSABC	0.7	0.720
Isolate*time	6	5.28	0.88	MSAC	MSAC/MSABC	4.3	0.007
Temp*time	6	2.26	0.38	MSBC	MSBC/MSABC	1.8	0.147
Isolate*temp*time	18	3.68	0.20	MSABC	MSABC/MSE	0.5	0.950
Error	47	19.63	0.42	MSE			
Total	95	102.31					

^a An exact *F* test could not be obtained. The *F* and *P* values in the table were obtained from an approximate *F* test for isolate using MSA/MSAC. This test is appropriate since isolate × temp interaction is not significant. A Satterthwaite approximate *F* test (8) yielded a similar result (*F* = 2.10, *P* = 0.135).

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