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The effect of environmental factors on infection of blueberry fruit by *Colletotrichum acutatum*

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Anthracnose fruit rot of blueberries caused by *Colletotrichum acutatum* is a serious problem in humid blueberry-growing regions of North America. In order to develop a disease prediction model, environmental factors that affect mycelial growth, conidial germination, appressorium formation and fruit infection by *C. acutatum* were investigated. Variables included temperature, wetness duration, wetness interruption and relative humidity. The optimal temperature for mycelial growth was 26°C, and little or no growth was observed at 5 and 35°C. The development of melanized appressoria was studied on Parafilm-covered glass slides and infection was evaluated in immature and mature blueberry fruits. In all three assays, the optimal temperature for infection was identified as 25°C, and infections increased up to a wetness duration of 48 h. Three-dimensional Gaussian equations were used to assess the effect of temperature and wetness duration on the development of melanized appressoria (R^2 = 0·89) on Parafilm-covered glass slides and on infection incidence in immature (R^2 = 0·86) and mature (R^2 = 0·90) blueberry fruits. Interrupted wetness periods of different durations were investigated and models were fitted to the response of melanized appressoria (R^2 = 0·95) and infection incidence in immature (R^2 = 0·90) and mature (R^2 = 0·78) blueberry fruits. Additionally, the development of melanized appressoria and fruit infection incidence were modelled in relation to relative humidity (R^2 = 0·99 and 0·97, respectively). Three comprehensive equations were then developed that incorporate the aforementioned variables. The results lay the groundwork for a disease prediction model for anthracnose fruit rot in blueberries.

Keywords: anthracnose fruit rot, relative humidity, ripe rot, temperature, *Vaccinium corymbosum*, wetting period

Introduction

Blueberries are a popular fruit with proven human health benefits (de Pascual-Teresa *et al.*, 2010). The United States is the world's leading producer of blueberries and Michigan is the number one blueberry-producing state (Anonymous, 2012). Anthracnose fruit rot, caused by *Colletotrichum acutatum*, is a serious problem in most blueberry-growing regions, with reported annual yield losses as high as 10–20% (Milholland, 1995). Most highbush blueberry cultivars are susceptible to this disease (Polashock *et al.*, 2005), which is typically managed with fungicides (Schilder *et al.*, 2002; Wise *et al.*, 2012). Sprays are usually initiated at pink bud or bloom and continued through fruit development and ripening (Miles & Schilder, 2008).

Colletotrichum acutatum overwinters in infected twigs, fruit trusses and bud scales from which conidia are rain-splash dispersed during the growing season (DeMarsay & Oudemans, 2003). Monitoring of *C. acutatum* conidia in Michigan over several years has shown one or two peaks in conidium dispersal, one around bloom and the other around fruit ripening (Wharton *et al.*, 2002). Other research in Michigan has documented up to three peaks of *C. gloeosporioides* spore dispersal: at bloom, green fruit, and ripe fruit, with spores present throughout the season (Hartung *et al.*, 1981). In that study, *C. acutatum* may have been misidentified as *C. gloeosporioides* because subsequent sampling in Michigan blueberry fields has confirmed *C. acutatum* to be the predominant pathogen (Schilder *et al.*, 2002).

In addition to spore dispersal by rain, environmental variables such as temperature and wetness duration are thought to play a role in the infection process. *Colletotrichum coccodes* formed melanized appressoria on tomato fruit and foliage in as few as 6 h at 16–28°C (Byrne *et al.*, 1997; Sanogo *et al.*, 2003). In strawberries, the incidence of fruit rot caused by *C. acutatum* was generally higher in mature fruit than in immature fruit and increased with longer wetness durations (up to 48 h) at temperatures between 10 and 30°C (Wilson *et al.*, 1990). The optimum temperature for infection was between 25 and 30°C (King *et al.*, 1997).

Researchers have also looked at interrupted wetting periods and the role of relative humidity on the development of melanized appressoria and infection in *Colletotrichum* pathosystems. In *Stylosanthes scabra*, foliar

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infection by *C. gloeosporioides* was relatively unaffected by brief interruptions (2–4 h) in wetness when they occurred after the initial 12 h and were followed by another 24 h of continuous wetness (Chakraborty *et al.*, 1990). However, they did not investigate longer periods of dryness or interruptions at different stages of the infection process. In a study on the effect of relative humidity (RH), *C. gloeosporioides* conidia were able to germinate and form appressoria on 1-month-old mango leaves at RH values between 95 and 100% even though free water was only visible at 100% (Dodd *et al.*, 1991).

In blueberries, a previous study indicated that temperatures below 5°C and above 30°C inhibited mycelial growth of *C. gloeosporioides* on potato dextrose agar (PDA) (Hartung *et al.*, 1981). Based on *in vitro* conidium germination studies, the authors suggested that 12 h of continuous wetness at 15–27°C would be required for fruit infection (Hartung *et al.*, 1981). A field study in British Columbia indicated that the optimum temperature for anthracnose fruit rot infection of blueberries was 20°C but infection was possible at temperatures between 7 and 30°C (Verma *et al.*, 2006). In studies with potted plants placed in commercial blueberry fields at weekly intervals with weather monitoring, fruit infection occurred after a minimum of 10 h of wetness at 11, 15 and 14°C in 2001, 2002 and 2003, respectively (Verma *et al.*, 2007). However, in these studies background infection by *C. acutatum* was consistently found to be a complicating factor.

A successful blueberry disease management programme depends on the grower's ability to recognize and anticipate problems. Knowing the critical periods when infection risk is highest can help in optimizing fungicide timing and effectiveness. The purpose of this study was to determine the effect of temperature, wetness duration, interrupted wetness and relative humidity on the infection of blueberries by *C. acutatum* under controlled conditions and to use the results to develop a disease prediction model.

Materials and methods

Fungal and plant material

A single-conidium isolate of *C. acutatum* from blueberry fruit collected in Grand Junction, MI, USA in August 2006 (isolate #0001) was used for all experiments except the initial temperature and wetness duration experiment on immature fruit. For this initial experiment, a single-conidium isolate from a blueberry bud collected from a commercial farm in Grand Junction was used. Both isolates were stored as previously described (Miles *et al.*, 2011) and cultures were grown on PDA for 14 days, after which conidia were harvested, recultured on one-quarter-strength PDA and allowed to microconidiate (produce conidia directly on conidia) for 3–4 days. For inoculum production, sporulating cultures were flooded with 3 mL of sterile deionized water (SDW) and microconidia were dislodged using a sterilized L-shaped glass rod. The conidium concentration was determined using a haemocytometer, and the appropriate concentration was achieved via dilution with SDW. To study the development of melanized appressoria on Parafilm-covered glass slides, a suspension of 1×10^5 conidia mL^{-1} was used. For all fruit infection experiments, a suspension of 1×10^6 conidia was applied directly to the fruit surface.

Multiple experiments were done using detached and attached immature and mature fruit of susceptible highbush blueberry cultivars but, because of background infections or suspected fungicide residues on the fruit, results from only a subset were considered acceptable for further analysis. Also, to avoid background infection as much as possible, fruit was collected from multiple sources, including non-irrigated fields and fields in drier areas of the state with low disease pressure. All experiments presented here had little or no evidence of background infection as indicated by the water controls.

For the temperature and wetness duration assays, 20 cm-long detached cv. Jersey shoots bearing immature fruit were collected in June 2006 and 2007 from a mature planting at the Michigan State University (MSU) Horticulture Teaching and Research Center in East Lansing, MI, USA. Detached mature cv. Jersey fruit were obtained from a commercial farm in Harrietta, MI, USA in September 2006 and cv. Bluecrop fruit were obtained from a commercial farm in Traverse City, MI, USA in September 2007.

For interrupted wetness period experiments, immature cv. Jersey fruits were obtained from MSU's Southwest Michigan Research and Extension Center in Benton Harbor, MI, USA in July 2011, and detached mature cv. Bluecrop fruit were obtained from a commercial farm in Traverse City, MI, USA in August 2011. For relative humidity assays, detached mature fruit were obtained from Sun Belle Berries (Miami, FL, USA) of a southern highbush blueberry cultivar (southern-adapted *Vaccinium corymbosum*, usually with some introgressed *Vaccinium darrowi* and/or *Vaccinium ashei*) in Florida. In a preliminary experiment, the cultivar was susceptible to *C. acutatum* with symptoms similar to those of cvs Bluecrop or Jersey.

Effect of temperature and wetness duration

The optimal temperature for the growth of isolate #0001 was investigated by transferring 5·5 mm mycelial plugs to one-quarter-strength PDA in 9 cm Petri dishes, and incubating the cultures in the dark at various temperatures for 10 days. Culture diameter (in mm) was then measured in two perpendicular directions. The results reported are the combined results of six experiments. Initially, experiments were done at 5°C intervals (5, 10, 15, 20, 25 and 30°C), whereas subsequent experiments targeted the 20 to 35°C range at 1 or 2°C intervals. The following temperatures were represented in these experiments: 5, 10, 15, 20, 22, 24, 25, 26, 28, 30, 31, 33 and 35°C. For each temperature, four replicate cultures per experiment were used to calculate the mean and each temperature was evaluated at least twice.

To determine the effects of temperature and wetness duration on the formation of melanized appressoria, 20 μL droplets of a conidial suspension (10^6 conidia mL^{-1}) were placed on Parafilm-covered glass slides and placed in Petri dishes (90 mm diameter, 15 mm deep) with wet Whatman filter paper to create a humidity chamber. Plates were incubated at 10, 15, 20, 25, or 30°C in the dark with 4, 6, 8, 12, 16, 20, 24, 36 or 48 h of wetness. For each temperature, five replicate droplets were used and 50 random conidia were counted per droplet (250 conidia in total). This experiment was conducted twice and data were normalized as a percentage of the highest number of melanized appressoria.

For experiments with immature cv. Jersey fruit, detached fruit clusters were collected from field-grown plants in East

Lansing, MI, USA, and were placed with the cut end in wet Florafoam® in 1 L plastic containers (one cluster per container). The fruit was spray-inoculated with *C. acutatum* until run-off (10^6 conidia mL^{-1}) and enclosed in a plastic bag. Fruit clusters wetted with SDW served as a control. Inoculated fruit clusters were incubated at 10, 15, 20, 25, or 30°C in the dark with 6, 12, 18, 24, 36 or 48 h of wetness. Four clusters were used per treatment combination. At the end of the wetness period, 10 green berries were removed from each cluster, surface sterilized in 10% bleach for 2 min, rinsed three times in SDW, cut in half and placed on PDA in Petri dishes. Berries were observed for characteristic hyphal growth and sporulation of *C. acutatum* over a 2 week period, at the end of which infection incidence was visually quantified. This experiment was conducted twice. Data were normalized as a percentage of the highest mean infection rate (%).

For experiments with mature fruit, 10 detached fruit per replicate were inoculated by placing a 30 μL drop of *C. acutatum* (10^6 conidia mL^{-1}) in the calyx cup of each fruit and were incubated on a metal mesh screen within a covered Petri dish. There were four replicates for each temperature–wetness duration combination (the same as described above for immature fruit). At the end of the wetness period, the fruit were surface sterilized as described above to inactivate any remaining inoculum, incubated at 100% RH, and observed for signs of *C. acutatum* infection over a 2 week period. Data were normalized as a percentage of the highest mean infection rate. This experiment was conducted in 2006 with cv. Jersey fruit and in 2007 with cv. Bluecrop fruit.

Effect of interrupted wetness periods

Initial experiments showed that there was little difference in melanized appressorium formation when dry periods were applied at different time intervals throughout a 12-h wetness period (data not shown). Therefore, to simplify the experiments, a dry period was applied in all experiments in the middle of the wetness period. To determine the effect of interrupted wetness on formation of melanized appressoria, 10 μL droplets of a conidial suspension (10^6 conidia mL^{-1}) were placed on Parafilm-covered glass slides as described above. Slides were then placed in humidity chambers and incubated at room temperature (22–24°C) with wetness durations of 12, 24 and 48 h. Interruptions of 1, 4 and 16 h were then applied in the middle of each wetting period (after 6, 12, and 24 h of wetness, respectively) by removing Parafilm-covered glass slides from humidity chambers and placing them in a laminar flow hood at room temperature (22–24°C and approximately 40% RH). Following the interruption, 10 μL droplets of SDW were applied to the dried droplet location and the slides were returned to the humidity chambers. For each treatment, four replicate droplets were used and 50 conidia were counted per droplet (200 conidia in total). The mean was calculated for each treatment, average values were standardized against the uninterrupted control and data were plotted in SigmaPlot for curve fitting.

For experiments with immature and mature fruit, a 10 μL droplet of a conidial suspension (10^6 conidia mL^{-1}) was placed on the side of detached cv. Jersey fruit placed equidistantly on wire-mesh screens. Fruit were incubated in humidity chambers at room temperature (22–24°C) with wetness durations of 12, 24 and 48 h. Interruptions of 1, 4 and 16 h were applied in the middle of each wetting period by removing fruit from the humidity chambers and placing them in a laminar flow hood at room temperature (22–24°C and approximately 40% RH). Four replicates of 10 fruit each were used per treatment for both immature and mature fruit maturity levels. Following the interruption, 10 μL droplets of SDW were applied to the sites of the dried droplets and the fruit were returned to the humidity chamber. At the end of the wetness period, immature and mature fruit were removed, surface sterilized as described above, cut in half and placed on PDA in Petri dishes. Infection incidence was visually rated after 10 days. The mean was calculated for each treatment, and data were expressed as a percentage of the uninterrupted control and plotted in SigmaPlot for curve fitting. The experiment was conducted twice.

Effect of relative humidity

To determine the effect of RH on formation of melanized appressoria, 10 μL droplets of a conidial suspension (10^6 conidia mL^{-1}) were placed on Parafilm-covered glass slides and allowed to dry in a laminar flow hood as described above. Slides were then placed in chambers with varying RH levels using different concentrations of glycerol (0, 10, 33 and 66%) in water. For each treatment, three replicates were used and 50 conidia were counted per replicate (150 conidia in total). Model 450 Watchdogs (Spectrum Technologies Inc.) were used to monitor relative humidity in the chambers. Relative humidity varied with temperature. The following RH values were achieved at 20°C: 100, 96, 86 and 56%; at 25°C: 100, 95, 84 and 54%; and at 30°C: 100, 94, 80 and 50%. Slides were incubated for 3 days and monitored daily for the production of melanized appressoria. The percentage of appressorium formation out of the total number of conidia was calculated for each treatment, and average values for the daily counts were plotted in SigmaPlot for curve fitting.

In the mature fruit experiment, a conidial suspension (10^6 conidia mL^{-1}) was sprayed until run-off on the surface of detached mature fruit and allowed to dry as described above. Fruits were then placed in humidity chambers and incubated at different temperatures (20, 25 and 30°C) with varying levels of relative humidity (same as described above) for 3 days. For each temperature–RH combination, 10 fruits were used, replicated four times (40 fruits total per RH level). At the end of the incubation period, fruits were removed, surface sterilized as described above, placed equidistantly on wire mesh grates and incubated at 100% RH. Fruits were observed for the presence of *C. acutatum* infection at 10 days post-inoculation. Mean values were then calculated and plotted in SigmaPlot for curve fitting.

Statistical analysis

All statistical analyses were performed with SigmaPlot v. 11 (SYSTAT Software). Regression analyses were used to develop quantitative models of the infection process in relation to temperature, wetness duration, interrupted wetness periods and relative humidity. Three-dimensional non-linear regressions, including planer, parabolic Gaussian and Lorentzian, were fitted to the data from two experiments in each case. Criteria for selecting the best statistical models included an examination of normality, equality of variance, R^2 values and residual plots (Table S1). Equations were chosen and three models were proposed predicting the development of melanized appressoria and infection levels of immature and mature blueberry fruits (Table 1). A summarized equation for each model was developed that best fit the data.

Results

Temperature and wetness duration

Mycelial growth of *C. acutatum* from blueberry increased with increasing temperature from 10 to 26°C and then declined sharply between 27 and 30°C (Fig. 1). Little to no growth was observed at 5 and 35°C. The optimal temperature for melanized appressorium formation was 25°C and development was significantly reduced at 10 and 30°C. At 25°C, a minimum of 8 h of continuous wetness was required for the development of melanized appressoria. However, a minimum of 12 h was required at 30°C and 24 h at 10°C (Fig. S1). No significant effect of experiment was observed in an ANOVA ($P = 0{\cdot}302$), therefore the data of the two experiments were pooled together for further analysis. A Gaussian model was found to be the best fit for the raw data for the combined experiments ($R^2 = 0{\cdot}89$) (Table 1; Fig. 2; Eqn 1).

In the infection assays of immature and mature fruit, low levels of infection were seen after wetness durations as short as 6 h (Fig. S2). However, the infection percentage increased considerably after 12–18 h of wetness at 20–25°C, 18–24 h at 30°C, 24 h at 15°C, and 36–48 h at 10°C (Fig. S2). The optimal temperature for fruit infection by *C. acutatum* was determined to be 25°C based on infection incidence. No significant effect of experiment was observed during an ANOVA for immature ($P = 0{\cdot}287$) or mature ($P = 0{\cdot}416$) fruit, therefore the data were pooled together for further analysis. For both immature and mature fruit experiments, a Gaussian model was found to be the best fit for the raw data ($R^2 = 0{\cdot}86$ and 0·90, respectively; Table 1; Fig. 3; Eqns 4 & 7). No background infections were observed in non-inoculated control fruit.

Interrupted wetness periods

In general, the longer the wetness period, the higher the percentage of melanized appressoria formed and the percentage of infected fruit. However, interruption of the wetness period significantly reduced successful appressorium formation and infection. In the experiments, dry periods of different durations were applied in the middle of each wetness periods (e.g. for a 12 h total wetness duration, the dry period was applied at 6 h). The average percentage of melanized appressoria produced on Parafilm-covered slides with no interruption in the wetness duration was 6, 30 and 63% after 12, 24 and 48 h of wetness, respectively. For all three incubation times, the longer the wetness interruption, the greater the reduction in the number of melanized appressoria produced (Figs S3a–c & S4). In order to compare the interrupted wetness

Table 1 Best-fit models for the development of melanized appressoria and the infection level of immature and mature blueberry fruits by *Colletotrichum acutatum* in response to different environmental variables in replicated growth chamber experiments. At least two experiments were conducted for each response

					Coefficients				
Equation	Dependent variable	Independent variables		Best-fit model	x_0	y_0	a	b	c
1	Melanized appressoria (%)	Wetness duration (h)	Temperature (°C)	Gaussian	48·668	21·702	68·513	16·682	6·870
2	''	Interruption (h)	Wetness duration (h)	Planer	N/A	8·715	−1·699	1·320	N/A
3	''	Relative humidity (%)	Temperature (°C)	Gaussian	99·709	23·872	50·022	2·294	2·299
4	Infection incidence in immature fruit (%)	Wetness duration (h)	Temperature (°C)	Gaussian	46·730	24·647	94·407	24·734	10·580
5	''	Interruption (h)	Wetness duration (h)	Planer	N/A	14·947	−0·095	1·814	N/A
6[a]	''	Relative humidity (%)	Temperature (°C)	Lorentzian	104·102	25·814	80·408	10·775	6·126
7	Infection incidence in mature fruit (%)	Wetness duration (h)	Temperature (°C)	Gaussian	42·527	23·549	101·605	17·447	6·645
8	''	Interruption (h)	Wetness duration (h)	Planer	N/A	54·276	−1·304	1·764	N/A
9	''	Relative humidity (%)	Temperature (°C)	Lorentzian	104·102	25·814	80·408	10·775	6·126

The following equations were used to fit the data: Planer: $f(Z) = y_0 + a*x+b*y$; Gaussian: $f(Z) = a*\exp(-0{\cdot}5*(((x-x_0)/b)^2 + ((y-y_0)/c)^2))$; and Lorentzian: $f(Z) = a/((1 + ((x-x_0)/b)^2)*(1 + ((y-y_0)/c)^2$. [a]Because the effect of relative humidity was not studied on immature fruit, Eqn 9, developed for mature fruit, is used here.

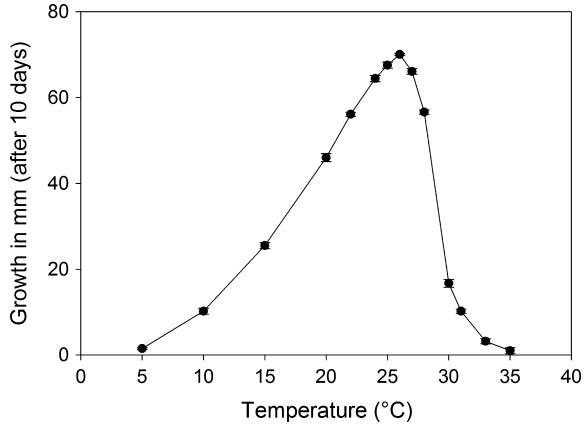

Figure 1 The effect of temperature on mycelial growth of *Colletotrichum acutatum* (Michigan blueberry isolate #0001) on quarter-strength potato dextrose agar. Colony diameter was measured in two perpendicular directions and averaged after a 10-day incubation at each temperature. Values are the average of three experiments and error bars denote the standard error of the mean.

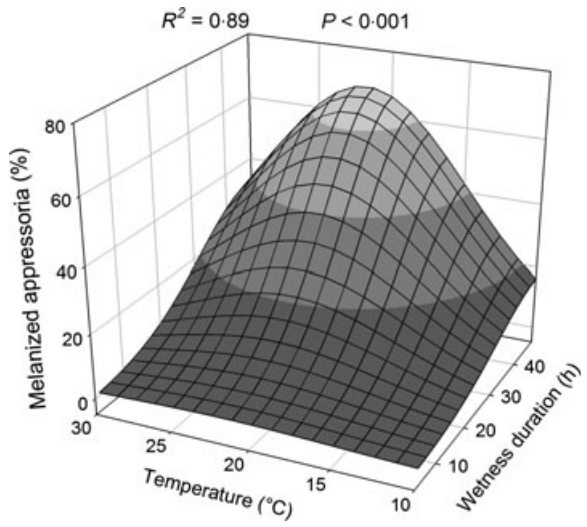

Figure 2 The effect of temperature and wetness duration on the predicted development of melanized appressoria of *Colletotrichum acutatum* on Parafilm-covered glass slides. A Gaussian equation (Table 1; Eqn 1) was used to fit the data. Shading intensity of the surface indicates changes in the percentage of melanized appressoria at 20% intervals starting at zero.

treatments with the continuous wetness duration treatments, the continuous wetness control from each incubation time was used to standardize the number of melanized appressoria relative to no interruption. No significant effect of experiment was observed in an ANOVA (P = 0·091), therefore the data of the two experiments were pooled together for further analysis. A planer model was found to be the best fit for the raw data (R^2 = 0·95; Table 1; Fig. 4; Eqn 2).

The percentage of infected immature fruit was 15, 17 and 30%, respectively, after 12, 24 and 48 h of continuous wetness. In the 12 and 24 h incubation treatments, interruptions of any duration, after 6 and 12 h incubation, respectively, dramatically reduced the percentage of infected fruit (Figs S3d,e & S5a). No reduction in the percentage of infected immature fruit was observed when the 48 h incubation time was interrupted for any length of time after 24 h (Figs S3f & S5b). In order to compare the interruption treatments with the continuous wetness duration treatments, the continuous wetness control from each incubation time was used to standardize the amount of infected fruit relative to no interruption. No significant effect of experiment was observed in an ANOVA (P = 0·126), therefore the data of the two experiments were pooled together for further analysis. A planer model was found to be the best fit for the raw data (R^2 = 0·90; Table 1; Fig. 5a; Eqn 5).

The percentage of infected mature fruit was 25, 28 and 38%, respectively, after 12, 24 and 48 h of continuous wetness. At the 12 h incubation time, there was a reduction in fruit infection but the duration of the interruption did not seem to matter (Fig. S3g). No reduction in the percentage of mature fruit infection due to interrupted wetness was observed at the 24 and 48 h incubation times (Figs S3h,i & S5b). In order to compare the interruption treatments with the continuous wetness duration treatments, the continuous wetness control from each incubation time was used to standardize the amount of infected fruit relative to no interruption. No significant effect of experiment was observed in an ANOVA (P = 0·136), therefore the data of the two experiments were pooled together for further analysis. Planer, parabolic and Gaussian models were used to fit the data and a planer model was found to be the best fit for the raw data (R^2 = 0·78; Table 1; Fig. 5b; Eqn 8).

Relative humidity

Conidia had germinated and formed melanized appressoria on Parafilm-covered glass slides at 100% RH after 48 h at all temperatures tested, with a further increase in germination evident after 72 h of incubation. At 25°C, the percentage of melanized appressoria was 44, 5, 1 and 0% after 3 days at 100, 95, 84 and 54% RH, respectively (Fig. S6a). No significant effect of experiment was observed in the ANOVA (P = 0·069), therefore the data were pooled together for further analysis. A Gaussian model was found to be the best fit for the raw data (R^2 = 0·99; Table 1; Fig. 6a; Eqn 3).

Mature fruit was infected at all temperatures tested (20, 25 and 30°C), and increasing RH led to higher infection incidence. At 20°C, the percentage of infected fruit was 43, 20, 7 and 3% after 3 days at 100, 96, 86 and 56% RH, respectively. At 25°C, the percentage of infected fruit was 67, 50, 17 and 3% after 3 days at 100, 95, 84 and 54% RH, respectively. At 30°C, the percentage of infected fruit was 47, 30, 13 and 0% after 3 days at 100, 94, 80 and 50% RH, respectively (Fig. S6B). No significant effect of experiment was observed in the ANOVA (P = 0·109), therefore the data were pooled

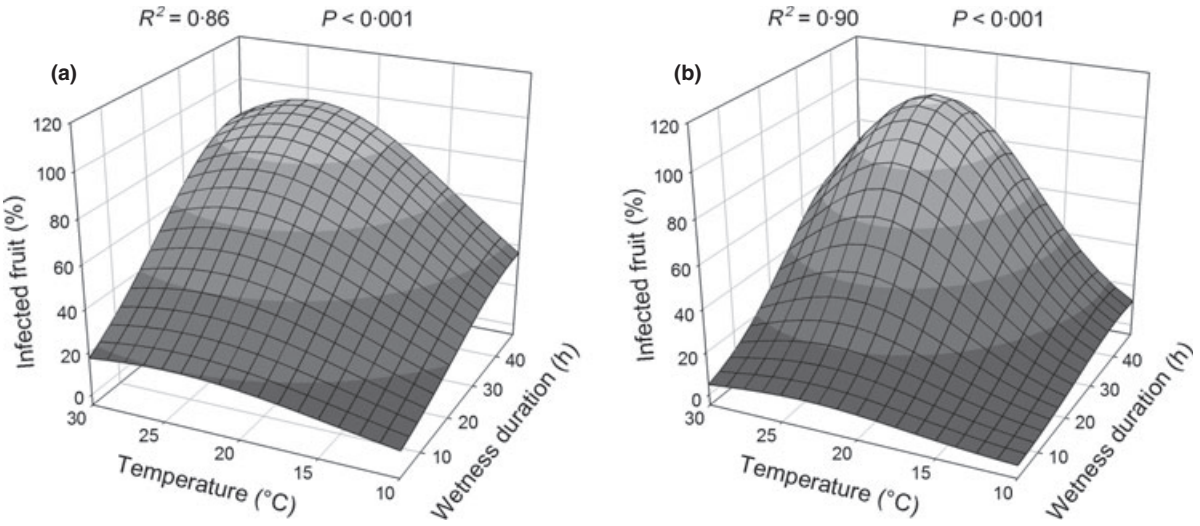

Figure 3 The effect of temperature and wetness duration on the predicted infection level of immature (a) and mature (b) blueberry fruits by *Colletotrichum acutatum*. Separate Gaussian equations were fitted to the data for immature fruit (Table 1; Eqn 4) and mature fruit (Table 1; Eqn 7). Shading intensity of the surface indicates changes in the percentage of infected fruit at 20% intervals starting at zero.

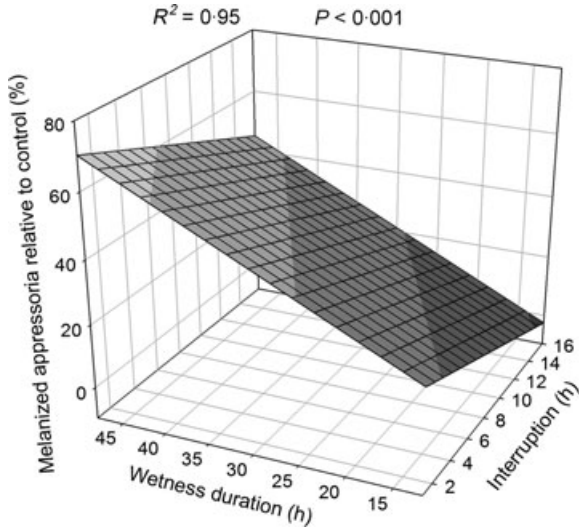

Figure 4 The effect of wetness duration and interrupted wetness periods on the predicted development of melanized appressoria of *Colletotrichum acutatum* on Parafilm-coated glass slides. Dry interruptions occurred in the middle of the wetness duration in all experiments. Values are calculated as the percentage melanized appressoria relative to the continuous wetness treatment. A planer equation (Table 1; Eqn 2) was fitted to the data. Shading intensity of the surface indicates changes in the percentage of melanized appressoria at 20% intervals starting at zero.

together for further analysis. A Lorentzian model was found to be the best fit for the raw data ($R^2 = 0{\cdot}97$; Table 1; Fig. 6b; Eqns 6 & 9). Because immature fruit were not tested, this model was included in the proposed comprehensive model for immature fruit infection (Table 1; Eqn 6).

Comprehensive models for appressorium development and infection of immature and mature fruit

Three models were constructed based on three non-linear regressions described above for temperature in relation to wetness duration and relative humidity, and wetness duration in relation to wetness interruption (Table 1). For the formation of melanized appressoria, each component of the proposed model had R^2 values greater than 0·89 (Table S1). For the percentage of infected immature fruit, each component of the proposed model had R^2 values >0·86. For the percentage of infected mature fruit, each component of the proposed model had R^2 values >0·78. For all models, an analysis of residuals did not reveal outliers or particular patterns. Therefore three general equations were developed for the development of melanized appressoria and infection in immature and mature fruit using the equations in Table 1. These equations are proposed because the combined effect of wetness duration and temperature will be multiplied by the inhibitory effect of wetness interruptions. Also, the effect of relative humidity was added to show the small added contribution of humidity to the overall infection process.

For the development of melanized appressoria:

$$\text{Melanized appressoria}\,(\%) = [(\text{Eqn}\,1/100 \times \text{Eqn}\,2/100) + (\text{Eqn}\,3/100)] \times 100.$$

For infection level of immature fruit:

$$\text{Immature infected fruit}\,(\%) = [(\text{Eqn}\,4/100 \times \text{Eqn}\,5/100) + (\text{Eqn}\,6/100)] \times 100.$$

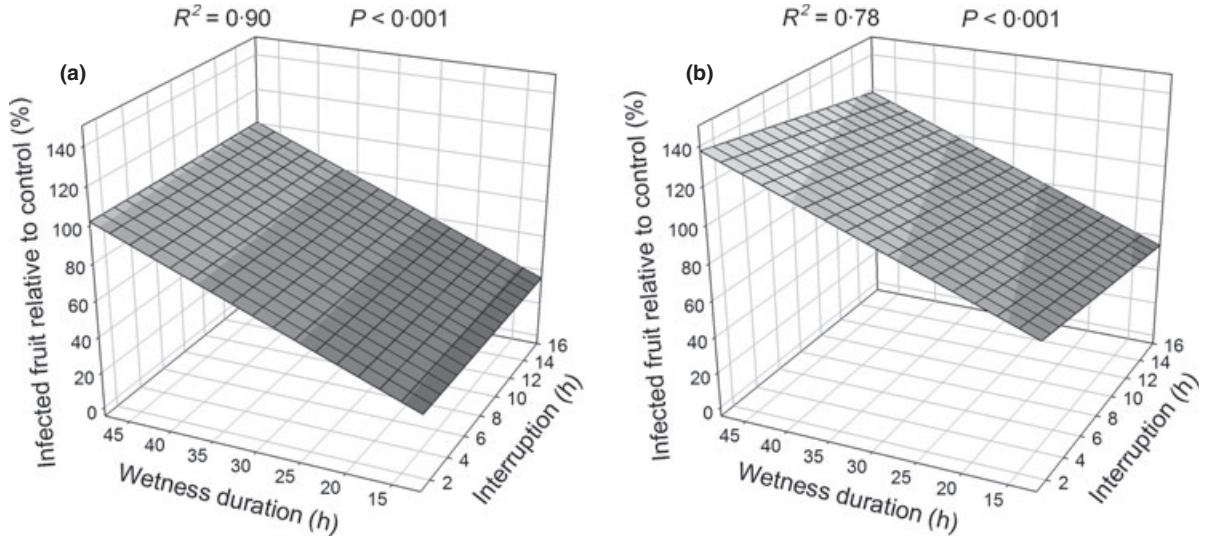

Figure 5 The effect of wetness duration and interrupted wetness periods on the predicted infection level of immature (a) and mature (b) blueberry fruit by *Colletotrichum acutatum*. Dry interruptions occurred in the middle of the wetness periods in all experiments. Values are calculated as the percentage infected fruit relative to the continuous wetness control. Planer equations (Table 1; Eqn 5) were fitted to the data for immature fruit and mature fruit (Table 1; Eqn 8). Shading intensity of the surface indicates changes in the percentage of infected fruit at 20% intervals starting at zero.

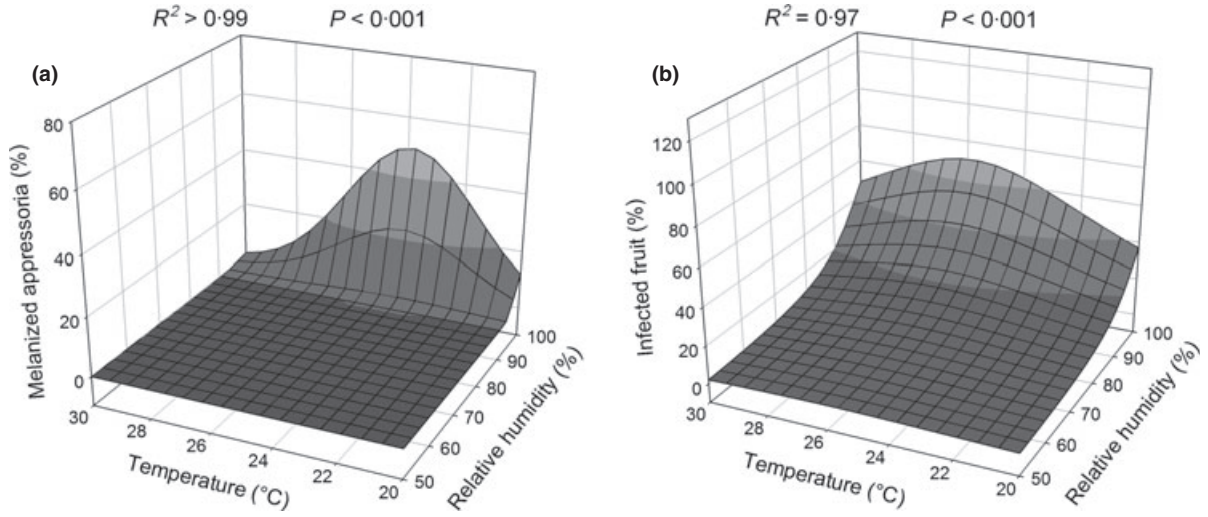

Figure 6 The effect of temperature and relative humidity on the predicted development of melanized appressoria of *Colletotrichum acutatum* (a), and the predicted infection level of mature blueberry fruits (b) by *C. acutatum* after 3 days. A Gaussian equation (Table 1; Eqn 3) was fitted to the data for melanized appressoria and a Lorentzian equation (Table 1; Eqns 6 & 9) was fitted to the data for mature fruit. Shading intensity of the surface indicates changes in the percentage of melanized appressoria and percentage infected fruit at 20% intervals, respectively, starting at zero.

For infection level of mature fruit:

$$\text{Mature infected fruit}\,(\%) = [(\text{Eqn}\,7/100 \times \text{Eqn}\,8/100) + (\text{Eqn}\,9/100)] \times 100.$$

Discussion

This study represents the first comprehensive analysis of the interaction of environmental variables on appressorium development and infection of both immature and mature blueberry fruits by *C. acutatum* under controlled conditions. Specific variables included in the study were temperature, wetness duration, interrupted wetting periods and relative humidity. The relationship between temperature and wetness conforms to previous studies of *Colletotrichum* spp. on other fruit crops (Wilson *et al.*, 1990; Monroe *et al.*, 1997; Sanogo *et al.*, 1997). Temperature effects were also consistent with the mycelial growth assay. While the optimum temperature was found to be 26°C for mycelial growth, the effect of

temperature was not investigated in such small increments for appressorium formation and infection, for which 25°C was found to be optimum when using 5°C increments.

The development of melanized appressoria on Parafilm-covered microscope slides was observed as early as 8 h after the start of incubation, and infection of immature and mature fruit as early as 6 h after inoculation, at the optimum temperature of 25°C. Previous microscopy studies by Wharton & Schilder (2008) conducted on detached, ripe Jersey fruit incubated at room temperature (22–24°C) showed that 4 h after inoculation, 10·6% of conidia had germinated. At 8 h post-inoculation, 41·5% of conidia had formed unmelanized appressoria and 11·4% had formed melanized appressoria with an internal light spot, which is thought to indicate formation of a penetration peg (Wharton & Schilder, 2008). It is therefore possible that a small percentage of conidia could have successfully infected the fruit as early as 6 h after inoculation under optimal conditions. Differences in the minimum environmental requirements for infection and for the development of melanized appressoria on Parafilm-covered glass slides may be due to specific features of plant-derived waxes on the surface of blueberry fruits, which may stimulate infection more so than Parafilm. The surface wax of avocado fruit has been shown to induce germination and appressorium formation in conidia of *C. gloeosporioides* (Podila *et al.*, 1993). Additionally, disease incidence was generally higher in mature fruit than in immature fruit, indicating greater tissue susceptibility, which is similar to other work in strawberries (Wilson *et al.*, 1990).

This study also demonstrates that wetness interruptions play an inhibitory role in the infection process under conditions where leaf wetness is limiting. Previous research on *Cercospora kikuchii* on soyabean leaves has shown that interruptions of different durations within 24 h wetness periods have a significant effect on overall disease severity and the number of infections. In addition, interruptions during which the RH was high had less of an effect than those during which the RH was low (Schuh, 1993). In the present study, infection was inhibited more at lower initial wetness durations and after longer interruptions. This is probably because the fungus had not yet developed protective structures or penetrated the host tissue and was therefore more vulnerable to desiccation. The study illustrates that when interruptions occur after 24 h of continuous wetness, the interruption has a negligible effect on infection, regardless of the length of the interruption.

This study also identifies and quantifies RH as an important environmental factor in the infection process of *C. acutatum* on blueberry. The results agree with previous work on *C. gloeosporioides* on mango which showed that conidia are capable of germinating and developing appressoria, albeit very slowly, between 95 and 100% RH (Dodd *et al.*, 1991). Furthermore, research on *C. gloeosporioides* on citrus fruits has shown that sustained high RH for 3 days dramatically increases disease incidence in the absence of wetness (Brown, 1975). This component of the model is relevant in humid growing regions such as Michigan where average daily RH can remain above 90% for several days during the growing season. High RH may also reduce the impact of wetness interruptions on appressorium formation and infection.

Many disease prediction models use regression equations, such as those based on polynomials (Schuh, 1991; Evans *et al.*, 1992), logistic equations (Bulger *et al.*, 1987; Schuh, 1991) and complex three-dimensional response surfaces (Broome *et al.*, 1995; Wu *et al.*, 1999; Carisse *et al.*, 2000). Wilson *et al.* (1990) used a regression model with the logit of disease incidence of *C. acutatum* on strawberries as the dependent variable that accurately described infection level as a function of wetness duration and temperature. In the *C. coccodes*–tomato pathosystem, fruit infection was correlated with the amount and duration of rain alone and in combination with other meteorological variables (i.e. temperature and RH), accounting for 72% of the variation in anthracnose incidence. Infection was also negatively correlated with the number of hours during which no rainfall occurred within 4-day intervals that tomato fruit were exposed to field conditions (Sanogo *et al.*, 1997). Another study used polynomial equations to describe the relationship between *C. orbiculare* infection on watermelon leaves in relationship to temperature and leaf wetness (Monroe *et al.*, 1997).

The models presented here focus on the environmental requirements for infection and point out that infection might be occurring as early as 6–8 h at the optimum temperature of 25°C. This is somewhat earlier than previously reported by Verma *et al.* (2007), who found that 10 h of wetness was the minimum needed for infection at 11–15°C. At those temperatures, the results here indicate that at least 18–24 h of fruit wetness are required for infection. This suggests that either there is significant pathogen variability between British Columbia and Michigan or that background infections or other factors, such as a continuation of the infection process during incubation of trap plants following retrieval from the field, may have influenced the infection outcome in the studies by Verma *et al.* (2007). It is also possible that weather station sensors do not accurately reflect the wetness duration of fruit clusters on the bush. Additionally, there are other epidemiological variables to consider such as regional climate differences, and pathogen dispersal and survival. It should also be noted that the experimental design is critical when conducting these types of experiments given that a large component of anthracnose-related yield loss occurs post-harvest due to latent infections. Future studies with field-inoculated plant that are monitored through the harvest may provide a better indicator of fruit infection under ambient weather conditions.

Colletotrichum spp. have been classically identified as rain-splash dispersed pathogens, and numerous studies link dispersal of conidia to rainfall (Yang *et al.*, 1990;

Bellaire *et al.*, 2000). Furthermore, survival of *Colletotrichum* spp. over the winter in soil, host tissue and plant debris has been described in numerous plant pathosystems (Eastburn & Gubler, 1992; Norman & Strandberg, 1997; Yoshida & Shirata, 1999; Freeman *et al.*, 2002; DeMarsay & Oudemans, 2003), while survival over the summer has been described on symptomless leaves of strawberry (Leandro *et al.*, 2001), which also may be an important factor when predicting disease risk. While the assumption for the models is that inoculum is uniformly distributed and unlimited, this may not be the case and inoculum availability needs to be taken into account when determining infection risk.

This study provides new and useful information on environmental requirements for infection of blueberry fruit by *C. acutatum*. The absence of or suppression of background infection, which is a confounding factor, is very important to obtain accurate results in fruit infection studies. The development of a disease prediction model which indicates infection risk is an important step towards integrated and sustainable management of anthracnose fruit rot in blueberries. Further research is needed to validate the model under field conditions using weather data provided by Michigan State University's Enviro-weather system (http://www.enviroweather.msu.edu/homeMap.php). This system includes automated weather stations situated approximately 10–20 km apart in the main blueberry-growing areas of Michigan and is an important information resource for growers, extension educators, crop consultants and researchers. Further studies are also needed to determine how fruit wetness relates to measurements by electronic wetness sensors, as fruit clusters may stay wet longer than an exposed metal grid. As one of the limiting factors in the use of disease prediction models based on real-time weather data is the availability of fungicides with good post-infection activity, future enhancements would include the use of forecasted weather data to increase flexibility in control decisions.

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References

Anonymous, 2012. U.S. blueberry production and utilization (cultivated and wild), selected states, 1980–2011. Noncitrus fruits and nuts summary. USDA National Agricultural Statistics Service. [http://usda.mannlib.cornell.edu/MannUsda/viewDocumentInfo.do?documentID=1765]. Accessed 10 April 2013.

Bellaire LD, Chillet M, Dubois C, Mourichon X, 2000. Importance of different sources of inoculum and dispersal methods of conidia of *Colletotrichum musae*, the causal agent of banana anthracnose, for fruit contamination. *Plant Pathology* **49**, 782–90.

Broome J, English J, Marois J, Latorre B, Aviles J, 1995. Development of an infection model for *Botrytis* bunch rot of grapes based on wetness duration and temperature. *Phytopathology* **85**, 97–102.

Brown GE, 1975. Factors affecting postharvest development of *Colletotrichum gloeosporioides* in citrus fruits. *Phytopathology* **65**, 404–9.

Bulger M, Ellis M, Madden L, 1987. Influence of temperature and wetness duration on infection of strawberry flowers by *Botrytis cinerea* and disease incidence of fruit originating from infected flowers. *Phytopathology* **77**, 1225–30.

Byrne JM, Hausbeck MK, Hammerschmidt R, 1997. Conidial germination and appressorium formation of *Colletotrichum coccodes* on tomato foliage. *Plant Disease* **81**, 715–8.

Carisse O, Bourgeois G, Duthie J, 2000. Influence of temperature and leaf wetness duration on infection of strawberry leaves by *Mycosphaerella fragariae*. *Phytopathology* **90**, 1120–5.

Chakraborty S, Ratcliff D, McKay F, 1990. Anthracnose of *Stylosanthes scabra*: effect of leaf surface wetness on disease severity. *Plant Disease* **74**, 379–84.

DeMarsay A, Oudemans PV, 2003. *Colletotrichum acutatum* infections in dormant highbush blueberry buds. *Phytopathology* **93**, S20.

Dodd JC, Estrada AB, Matcham J, Jeffries P, Jeger MJ, 1991. The effect of climatic factors on *Colletotrichum gloeosporioides*, causal agent of mango anthracnose, in the Philippines. *Plant Pathology* **40**, 568–75.

Eastburn D, Gubler W, 1992. Effects of soil moisture and temperature on the survival of *Colletotrichum acutatum*. *Plant Disease* **76**, 841–2.

Evans K, Nyquist W, Latin R, 1992. Model based on temperature and leaf wetness duration for establishment of *Alternaria* leaf blight of muskmelon. *Phytopathology* **82**, 890–5.

Freeman S, Shalev Z, Katan J, 2002. Survival in soil of *Colletotrichum acutatum* and *C. gloeosporioides* pathogenic on strawberry. *Plant Disease* **86**, 965–70.

Hartung J, Burton C, Ramsdell D, 1981. Epidemiological studies of blueberry anthracnose disease caused by *Colletotrichum gloeosporioides*. *Phytopathology* **71**, 449–53.

King WT, Madden LV, Ellis MA, Wilson LL, 1997. Effects of temperature on sporulation and latent period of *Colletotrichum* spp. infecting strawberry fruit. *Plant Disease* **81**, 77–84.

Leandro LFS, Gleason ML, Nutter FW Jr, Wegulo SN, Dixon PM, 2001. Germination and sporulation of *Colletotrichum acutatum* on symptomless strawberry leaves. *Phytopathology* **91**, 659–64.

Miles TD, Schilder A, 2008. Anthracnose fruit rot (ripe rot). Michigan State University Extension Bulletin E-3039.

Miles TD, Day B, Schilder AC, 2011. Identification of differentially expressed genes in a resistant versus a susceptible blueberry cultivar after infection by *Colletotrichum acutatum*. *Molecular Plant Pathology* **12**, 463–77.

Milholland RD, 1995. Anthracnose fruit rot (ripe rot). In: Caruso FL, Ramsdell DC, eds. *Compendium of Blueberry and Cranberry Diseases*. St Paul, MN, USA: APS Press, 17.

Monroe J, Santini J, Latin R, 1997. A model defining the relationship between temperature and leaf wetness duration, and infection of watermelon by *Colletotrichum orbiculare*. *Plant Disease* **81**, 739–42.

Norman D, Strandberg J, 1997. Survival of *Colletotrichum acutatum* in soil and plant debris of leatherleaf fern. *Plant Disease* **81**, 1177–80.

de Pascual-Teresa S, Moreno DA, García-Viguera C, 2010. Flavanols and anthocyanins in cardiovascular health: a review of current evidence. *International Journal of Molecular Sciences* **11**, 1679–703.

Podila GK, Rogers LM, Kolattukudy PE, 1993. Chemical signals from avocado surface wax trigger germination and appressorium formation in *Colletotrichum gloeosporioides*. *Plant Physiology* **103**, 267–72.

Polashock JJ, Ehlenfeldt MK, Stretch AW, Kramer M, 2005. Anthracnose fruit rot resistance in blueberry cultivars. *Plant Disease* **89**, 33–8.

Sanogo S, Pennypacker SP, Stevenson RE, MacNab AA, 1997. Weather variables associated with infection of tomato fruit by *Colletotrichum coccodes*. *Plant Disease* **81**, 753–6.

Sanogo S, Stevenson RE, Pennypacker SP, 2003. Appressorium formation and tomato fruit infection by *Colletotrichum coccodes*. *Plant Disease* **87**, 336–40.

Schilder A, Gillett J, Woodworth J, 2002. The kaleidoscopic nature of blueberry fruit rots. *Acta Horticulturae* **574**, 81–3.

Schuh W, 1991. Influence of temperature and leaf wetness period on conidial germination in vitro and infection of *Cercospora kikuchii* on soybean. *Phytopathology* **81**, 1315–8.

Schuh W, 1993. Influence of interrupted dew periods, relative humidity, and light on disease severity and latent infections caused by *Cercospora kikuchii* on soybean. *Phytopathology* **83**, 109–13.

Verma N, Macdonald L, Punja ZK, 2006. Inoculum prevalence, host infection and biological control of *Colletotrichum acutatum*: causal agent of blueberry anthracnose in British Columbia. *Plant Pathology* **55**, 442–50.

Verma N, Macdonald L, Punja ZK, 2007. Environmental and host requirements for field infection of blueberry fruits by *Colletotrichum acutatum* in British Columbia. *Plant Pathology* **56**, 107–13.

Wharton PS, Schilder AC, 2008. Novel infection strategies of *Colletotrichum acutatum* on ripe blueberry fruit. *Plant Pathology* **57**, 122–34.

Wharton PS, Dickman JS, Schilder AMC, 2002. Timing of spore release by *Colletotrichum acutatum* in Michigan blueberry fields. *Phytopathology* **92**, S86.

Wilson L, Madden L, Ellis M, 1990. Influence of temperature and wetness duration on infection of immature and mature strawberry fruit by *Colletotrichum acutatum*. *Phytopathology* **80**, 111–6.

Wise JC, Gut LJ, Isaacs R, *et al.*, 2012. *Michigan Fruit Management Guide 2013*. *Extension Bulletin E0154*. East Lansing, MI, USA: Michigan State University.

Wu L, Damicone J, Duthie J, Melouk H, 1999. Effects of temperature and wetness duration on infection of peanut cultivars by *Cercospora arachidicola*. *Phytopathology* **89**, 653–9.

Yang X, Wilson L, Madden L, Ellis M, 1990. Rain splash dispersal of *Colletotrichum acutatum* from infected strawberry fruit. *Phytopathology* **80**, 590–5.

Yoshida S, Shirata A, 1999. Survival of *Colletotrichum dematium* in soil and infected mulberry leaves. *Plant Disease* **83**, 465–8.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1. The effect of temperature and wetness duration on the development of melanized appressoria of *Colletotrichum acutatum* on Parafilm-covered microscope slides. Shading transitions indicate increasing levels of melanized appressorium formation at 20% intervals starting at zero.

Figure S2. The effect of temperature and wetness duration on the incidence of infection of immature (a) and mature (b) blueberry fruits by *Colletotrichum acutatum*. Shading transitions indicate increasing levels of infected fruit at 20% intervals starting at zero.

Figure S3. The effect of interrupted wetness periods (0, 1, 4, and 16 h of dry time in the middle of 12, 24, and 48 h wetness periods) on the development of melanized appressoria (%) of *Colletotrichum acutatum* (a–c) and the percentage immature (d–f) and mature (g–i) blueberry fruit infected by *C. acutatum*. Values are the average of four replications and error bars denote the standard error of the mean.

Figure S4. The effect of wetness duration and interrupted wetness periods on the development of melanized appressoria of *Colletotrichum acutatum* on Parafilm-covered microscope slides. Dry periods occurred in the middle of each wetness period in all experiments. Values are calculated as percentage of melanized appressoria relative to the continuous wetness control. Shading transitions indicate increasing levels of melanized appressoria at 20% intervals starting at zero.

Figure S5. The effect of wetness duration and interrupted wetness periods on the infection level of immature (a) and mature (b) blueberry fruits by *Colletotrichum acutatum*. Dry periods occurred in the middle of each wetness period in all experiments. Values are calculated as percentage of infected fruit relative to the continuous wetness control. Shading transitions indicate increasing levels of infected fruit at 20% intervals starting at zero.

Figure S6. The effect of temperature and relative humidity on the development of melanized appressoria (%) of *Colletotrichum acutatum* (a), and the infection level of mature blueberry fruits (b) by *C. acutatum*. Shading transitions indicate increasing levels of melanized appressorium formation and infection of mature fruit at 20% intervals starting at zero.

Table S1. Statistics for fitted three-dimensional models of the percentage of melanized appressoria and infection incidence of immature and mature blueberry fruits by *Colletotrichum acutatum* in response to temperature (°C), wetness duration (h), wetness interruption (h) and relative humidity (%). Nine equations were chosen based on the best fit to the raw data from a mixture of experiments, and models were examined for goodness of fit (R^2), standard error of the estimate (SEE), the P value of the regression and the mean squared error (MSE) of the residuals (* denotes a chosen equation).