Environmental and inoculum effects on epidemiology of bacterial spot disease of stone fruits and development of a disease forecasting system

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Abstract

Bacterial spot disease of stone fruits, caused by *Xanthomonas arboricola* pv. *pruni*, is of high economic importance in the major stone-fruit-producing areas worldwide. A better understanding of disease epidemiology can be valuable in developing disease management strategies. The effects of weather variables (temperature and wet/dry period) on epiphytic growth of *X. arboricola* pv. *pruni* on *Prunus* leaves were analyzed, and the relationship between inoculum density and temperature on disease development was determined and modeled. The information generated in this study, performed under controlled environmental conditions, will be useful to develop a forecasting system for *X. arboricola* pv. *pruni*.

Optimal temperature for growth of epiphytic populations ranged from 20 to 30ºC under leaf wetness. In contrast, multiplication of epiphytic populations was not only interrupted under low relative humidity (RH) (< 40%) at 25ºC, but also resulted in cell inactivation, with only 0.001% initial cells recovered after 72 h incubation. A significant effect of inoculum density on disease severity was observed and 10^6 CFU/ml was determined as the minimal infective dose for *X. arboricola* pv. *pruni* on *Prunus*. Infections occurred at temperatures from 15 to 35ºC, but incubation at 25 and 30ºC gave the shortest incubation periods (7.7 and 5.9 days respectively). A model for predicting disease symptom development was generated and successfully evaluated, based on the relationship between disease severity and the accumulated heat expressed in cumulative degree day (CDD). Incubation periods of 150, 175 and 280 CDD were required for 5, 10 and 50% of disease severity, respectively.

Introduction

Xanthomonas arboricola pv. *pruni* (Vauterin et al. 1995) (synonym, *Xanthomonas campestris* pv. *pruni* [Smith] Dye) is a Gram-negative plant-pathogenic bacterium that causes bacterial spot disease in stone fruits (EPPO/CABI 1997; Palacio-Bielsa et al. 2010). The pathogen can affect all cultivated *Prunus* species and their hybrids, but the most severe epidemics have been reported in Japanese plum (*Prunus salicina*), *P. japonica* and hybrids, and in peach and nectarines, *P. persica* and hybrids (Ritchie 1995). The disease, first described for Japanese plum in the USA in 1903 (Smith 1903), is today distributed throughout the major stone-fruit-producing areas of the world (EPPO 2017).

X. arboricola pv. *pruni* is a harmful organism relevant to the European Union (EU), according to the Council Directive 2000/29/EC, and a quarantine pathogen, in the A2 list, for the European Plant Protection Organisation (EPPO) (EPPO/CABI 1997). The pathogen is spreading in many European countries, which have reported local outbreaks (EPPO 2017; Scortichini 2010).

Disease symptoms include necrotic angular spots on leaves, spots or sunken lesions on fruit and stem canker. Heavy infections lead to severe defoliation, mainly on peach and nectarine (Stefani 2010), resulting in weakened trees. The disease produces a negative economic impact because of the decrease in quality and marketability of affected fruits, a reduction of tree productivity and an increase in production costs (Janse 2012).

As no effective chemical control is available for this disease, quarantine measures are needed to avoid the introduction and dissemination of *X. arboricola* pv. *pruni*. These include phytosanitary certification of plant propagation material, nursery and orchard inspections and elimination of contaminated plant material. In affected areas, control of this pathogen is currently limited to preventative copper spray applications at late dormant stage and early in the growing season (Ritchie 2004; Stefani 2010; Wert et al. 2006). The use of antibiotics, mainly oxytetracycline and streptomycin, is restricted to countries where they are registered for agronomic uses (Stockwell and Duffy 2012; Vanneste et al. 2005). Appropriate and rational timing of copper sprays is critical to increase the efficacy of disease control and to overcome the limitations that have been related to the use of this compound: moderate efficacy, plant phytotoxicity (Lalancette and McFarland 2007) and risk of development of copper resistance in the bacterium (Giovanardi et al. 2016; Vanneste et al. 2005). Therefore, an effective control strategy for this disease should take into account the use of a reliable disease forecasting model. The forecaster would be used as a decision support system to guide disease surveillance tasks for early detection of outbreaks or spread of the disease, and copper applications for disease management.

Some forecasting systems for plant diseases caused by bacteria, such as Maryblyt (Lightner and Steiner 1992), CougarBlight (Smith 1993), and Billing's integrated system (BIS95) (Billing 1999) for fire blight in apple and pear, caused by *Erwinia amylovora*, and the risk model for bacterial canker of kiwifruit, caused by *Pseudomonas syringae* pv. *actinidiae* (Beresford et al. 2017) are based in two separate processes. The first involves bacterial multiplication to provide inoculum, which is temperature-dependent and requires the presence of free water (surface wetness or high RH). The second process is related to infection occurrences and disease development, and depends on weather parameters, mainly temperature, leaf wetness, and RH. A similar approach is proposed to develop a forecasting system for bacterial spot disease of stone fruits.

Previously, we determined and modeled the effect of temperature on growth of *X. arboricola* pv. *pruni in vitro* (Morales et al. 2017). The model can be used to predict the inoculum potential of this pathogen. However, *in vitro* assays do not reflect the natural conditions for epiphytic bacterial growth on plant organs, and the model should be validated or refined on host plants. Some bacterial growth models included in disease forecasting systems consider bacterial multiplication only when moisture is present (Kim et al. 2014; Beresford et al. 2017). Although temperature is the most important weather parameter for bacterial growth, knowledge of the effect of dry periods (with low RH) on the dynamics of epiphytic populations could provide additional information for modelling the epiphytic growth of *X. arboricola* pv. *pruni*.

X. arboricola pv. *pruni* may survive as an epiphyte on *Prunus* hosts in orchards or nurseries, associated with buds and leaf scars, which act as overwintering sites and sources of primary inoculum for spring infections (Anonymous, 2006). The population density is critical to forecast disease development, and the minimum pathogen population size to cause infections, also known as the infection threshold, is a key parameter for estimating the inoculum potential. The infection threshold has been determined for bacterial plant pathogens: at least 10^5-10^6 bacterial cells/flower are needed for *Erwinia amylovora* infection on apple (Billing 1984), *Pseudomonas syringae* pv. *syringae* needed more than 10^4 bacterial cells/g leaf fresh weight for bean infection (Lindemann 1984), and the threshold for *X. campestris* pv. *vesicatoria* infection on hot pepper has been determined as 5×10^5 bacterial cells/leaf (Kim et al. 2014). Regarding *X. arboricola* pv. *pruni*, 16 - 18 bacterial cells were apparently required to induce a single lesion on peach leaves, and inoculum densities from 10^6 to 10^8 CFU/ml were necessary to cause generalized chlorosis and necrosis on peach leaves under controlled conditions (Civerolo 1975). However, the infective dose for this pathogen needs to be confirmed, as the efficiency of different inoculation techniques is variable (Socquet-Juglard et al. 2012). The infection threshold for *X.*

arboricola pv. *pruni* could be integrated into the bacterial growth model to predict when the inoculum potential is sufficient to initiate infections.

Weather parameters play an important role in the infection of *Prunus* by *X. arboricola* pv. *pruni* and in the disease development. Moderate temperatures and leaf wetness are required for this bacterium to penetrate the host cells through natural openings or wounds (EPPO/CABI 1997; Garcin et al. 2011a; Goodman 1976; Morales et al. 2016; Zehr et al. 1996). Thus, the combined effects of wetness period duration and temperature on infection of *Prunus* by *X. arboricola* pv. *pruni* were analyzed under controlled environment conditions and an infection risk model was developed (Morales et al. 2018). The model was successfully validated under greenhouse conditions and, after field evaluation, it could be used to forecast the *X. arboricola* pv. *pruni* infection events on *Prunus*. Plant disease forecasting models can not only predict the onset of a disease, but also predict disease symptom development. Since temperature affects the development rate of many organisms, the measure of accumulated heat (physiological time) has been used to predict the incubation period for disease symptom expression in forecasting systems such as Maryblyt (Lightner and Steiner 1992) and BIS95 (Billing 1999). As the incubation period for bacterial spot disease of stone fruits is mainly affected by temperature and inoculum dose (Battilani et al. 1999; Randhawa and Civerolo 1985; Zehr et al. 1996), it is important to know the relationship between these factors and disease progress and symptom development prior to inclusion into the forecasting system of this disease.

This study was aimed at increasing knowledge of the effects of weather parameters and inoculum density on the dynamics of epiphytic populations of *X. arboricola* pv. *pruni* and on infection and disease symptom development on *Prunus* leaves, under controlled environmental conditions. The objectives were to: (i) determine the effect of temperature and relative humidity on *X. arboricola* pv. *pruni* growth on *Prunus* leaf surface; (ii) evaluate the effect of inoculum density of *X. arboricola* pv. *pruni* on infection on *Prunus* leaves; and (iii) analyze the effect of temperature and inoculum density on disease progress and symptom development.

Materials and methods

Plant material

Potted plants of the peach-almond hybrid (*Prunus persica* x *P. amygdalus*) GF-677, commonly used as rootstock, obtained by micropropagation (Agromillora Catalana, Subirats, Spain) and nectarine plants cv. Big Top (Certiplant, S.L., Lleida, Spain), both susceptible to bacterial spot disease of stone fruits, were used. Micropropagated GF-677 plants were used in whole plant assays, and in some detached leaf assays, whereas

nectarine plants were only used in detached leaf assays, depending on the supplier availability. Plants were grown in 0.5-l pots filled with a commercial peatmoss/vermiculite/perlite potting mix (type BVU, Prodeasa, Girona, Spain) in the greenhouse and fertilized once a week with a solution of 200 ppm N-P-K (20-10-20).

Pathogen

X. arboricola pv. *pruni* strain CFBP 5563 isolated from peach in France (CIRM-CFBP: International Center for Microbial Resources - French Collection for Plant-associated Bacteria, Beaucouzé, France) was used in this study. Bacteria were stored in stock tubes containing yeast-peptone-glucose broth (YPG) (Boudon et al. 2005) supplemented with glycerol (20% wt/vol) at -70ºC.

The strain *X. arboricola* pv. *pruni* CFBP 5563 was marked for rifampicin resistance (Rif^r) by growing on yeast-peptone-glucose agar (YPGA) (Boudon et al. 2005) supplemented with 50 µg/ml rifampicin (YPGA+R) for 48 h at 27ºC. Spontaneous mutant derivatives were selected and the stability of rifampicin resistance was confirmed by subculturing five times on YPGA in the absence of antibiotic selection and, finally, on YPGA+R. Mutants were routinely grown on YPGA+R (50 µg/ml rifampicin). The mutant strain CFBP 5563 Rif^r did not differ in growth from the wild-type strain, neither *in vitro* nor *ex vivo* (data not shown). The strain CFBP 5563 Rif^r was used in epiphytic growth assays.

Effect of temperature on epiphytic growth of *X. arboricola* pv. *pruni* on *Prunus* leaves under wetness

The epiphytic growth of *X. arboricola* pv. *pruni* at different temperatures under wetness was monitored on leaves of the peach-almond hybrid GF-677 in a detached leaf assay, under controlled environmental conditions.

Bacterial suspensions of *X. arboricola* pv. *pruni* CFBP 5563 Rif^r mutant were prepared from 24 h cultures grown at 27ºC on YPGA+R by scraping bacterial colonies from the culture and adjusting them with sterile distilled water to an optical density of 0.2 at λ = 600 nm (approximately 10^8 CFU/ml). The bacterial suspensions were serially diluted with sterile distilled water to obtain a final population density of 10^4 CFU/ml. Viable counts of the inoculum suspensions were determined by spreading 0.1 ml of appropriate 10-fold serial dilutions on YPGA+R plates and incubating for 72 h at 27ºC.

Leaves of the peach-almond hybrid GF-677 were collected from potted plants with actively growing shoots, surface disinfected by immersion in 5% sodium hypochlorite solution for 30 s and rinsed three times with sterile distilled water. The leaves were then inoculated by immersion in the bacterial suspension, placed on a grid in plastic boxes filled with wet filter paper and sealed in moistened transparent polyethylene bags to maintain leaf

wetness. Inoculated leaves were incubated for 14 days at constant temperatures of 5, 10, 15, 20, 25, 30 or 35ºC and a 12-h light photoperiod in controlled environment chambers (model MLR-350; Sanyo, Gunma, Japan), with a maximum variation of ±1°C for all temperatures. The temperature and RH inside the chamber was monitored and recorded using a HOBO® U23 Pro v2 temp/RH data logger (Onset Computer Corp, Pocasset, MA, USA). Leaves inoculated with sterile distilled water were used as negative control. Population densities of *X. arboricola* pv. *pruni* CFBP 5563 Rif^r on GF-677 leaf surface were assessed at 0, 12, 24 h and 2, 3, 4, 7, 9, 11 and 14 days after inoculation. Three replicates of five leaves were used for each sampling time and temperature. A completely randomized experimental design was used and the experiment was performed twice. For bacterial population density determination, each sample of five leaves was placed in a sterile plastic bag containing 50 ml of extraction buffer (7.10 g Na_2HPO_4, 2.72 g KH_2PO_4, and 1 g peptone per 1000 ml of distilled water) and ground in a lab blender (Stomacher, IUL Instruments, Germany) for 5 minutes. Aliquots and serial dilutions were subsequently plated in duplicate on YPGA+R supplemented with 10 µg/ml econazole nitrate (to prevent fungal growth) and incubated for 72 h at 27ºC. Results were expressed as CFU per gram of leaf fresh weight.

Viable count data were plotted versus time, and three growth curves were obtained at each temperature per experiment. The modified Gompertz model (Zwietering et al. 1990) described by equation 1 was used to estimate the maximum specific growth rate and lag time for *X. arboricola* pv. *pruni* at each temperature:

$$\log_{10} N_t = \log_{10} N_0 + A \cdot \exp\left\{-\exp\left[\frac{\mu_{max} \cdot e}{A} \cdot (lag - t) + 1\right]\right\} \quad \text{Equation 1}$$

where A is the logarithmic increase of bacterial population [$\log_{10}$ (CFU/g)], e is exp(1), lag is the lag time (h), N_0 is the initial population density (CFU/g), N_t is the population density (CFU/g) at time t (h), and μ_{max} is the maximum specific growth rate (h^{-1}). The modified Gompertz model was fitted to growth curves by nonlinear regression using R (R Development Core Team 2015) package nlstools (Baty et al. 2013). The goodness of fit of the model was assessed using the residual sum of squares (RSS). The effects of temperature on the growth rate was determined using the general linear model (GLM) procedure of SPSS v. 23.0 software (IBM Corp., Armonk, NY), after confirmation of the homogeneity of variance and normality.

Effect of low RH on epiphytic population dynamics of *X. arboricola* pv. *pruni* on *Prunus* leaves

The epiphytic population dynamics of *X. arboricola* pv. *pruni* on *Prunus* leaves under periods of low RH (< 40%) was analyzed under controlled environment conditions. As it was assumed that cell inactivation depends on the duration of the dry period, regardless of temperature (Kim et al. 2014), the experiment was performed at 25ºC, optimal temperature for *X. arboricola* pv. *pruni* growth (Morales et al. 2017).

Bacterial suspensions of strain CFBP 5563 Rif^r were obtained from 24 h cultures grown at 27ºC on YPGA+R as described above, and adjusted to an optical density of 0.5 at 600 nm (about 10^9 CFU/ml). Young leaves of peach-almond hybrid GF-677 potted plants were collected, disinfected in a 5% sodium hypochlorite solution, rinsed three times in sterile distilled water and inoculated by immersion in the bacterial suspension, as described previously. Inoculated leaves were left to surface dry inside a laminar flow cabinet and placed on sterile filter paper inside plastic boxes sealed in transparent polyethylene bags. Saturated calcium chloride ($CaCl_2$ 6 H_2O) was used as drying agent to maintain a low RH (< 40%) inside the plastic boxes (Dhingra and Sinclair 1985). Boxes with inoculated leaves were incubated for 72 h at 25ºC and 12-h light photoperiod in a controlled environment chamber (model MLR-350; Sanyo, Gunma, Japan), with a maximum variation of ±1°C. The temperature and RH inside the chamber were monitored and recorded as described above. Leaves inoculated with sterile distilled water were used as negative control. Three replicates of five leaves were sampled after 0, 6, 12, 24, 48 and 72 h of incubation and total viable counts were determined as described previously. The experiment was performed three times. A completely randomized experimental design was used. Differences between experiments on total viable counts were determined using the GLM procedure after confirmation of the homogeneity of variance and normality.

Averaged viable count data of thee replicates in an experiment were plotted against time to give survival curves for *X. arboricola* pv. *pruni* CFBP 5563 Rif^r on *Prunus* leaves under dry periods. Different inactivation models have been successfully applied in predictive microbiology to fit different shapes of bacterial survival curves (Xiong et al. 1999), which can be used when pathogen populations decrease under dry conditions. Here, the Cerf's model (Cerf 1977) (equation 2), which is suitable for fitting lineal survival curves and curves with a tail (Xiong et al. 1999), was fitted to survival curves of *X. arboricola* pv. *pruni* CFBP 5563 Rif^r using the nonlinear regression procedure of SPSS v. 23.0 software (IBM Corp., Armonk, NY). The Cerf model is expressed as:

$$N_t = N_0 \times [\, f \times \exp(-k_1 t)] + (1 - f) \times \exp(-k_1 t) \qquad \text{Equation 2}$$

where f and (1 - f) are the initial proportion in the less resistant fraction and the more resistant fraction of the population, respectively; k_1 and k_2 ($k_1 > k_2 \geq 0$) are the death rate constants for the less and the more resistant fraction of the population, respectively; N_0 is the initial population density (CFU/ml); and N_t is the population density (CFU/ml) at time t (h). The model goodness of fit was assessed using the root mean square error (RMSE) and correlation coefficient (R^2) between experimental and predicted values.

Effect of inoculum density of *X. arboricola* pv. *pruni* on disease severity on *Prunus* leaves

A stock suspension on sterile distilled water of *X. arboricola* pv. *pruni* strain CFBP 5563 was obtained as described previously from LB agar cultures grown for 72 h at 27ºC and adjusted to an optical density of 0.5 at 600 nm (10^9 CFU/ml). The stock bacterial suspension was serially diluted 10-fold with sterile distilled water to obtain suspensions ranging from 1×10^1 to 1×10^9 CFU/ml. Viable counts of the stock bacterial suspension were determined by spreading 0.1 ml of appropriate dilutions on YPGA plates and incubating for 72 h at 27ºC.

Leaves of nectarine cv. Big Top were collected from actively growing shoots in potted plants and disinfected as described above. Three different inoculation methods were used: (i) leaf immersion in the bacterial suspension; (ii) local infiltration of 25-µl of bacterial suspension using a syringe without needle at four sites into the abaxial surface in a leaf, two at each side of the mid-vein; and (iii) deposition of four 25-µl drops of bacterial suspension onto the reverse of a leaf. The same methods were repeated with sterile distilled water on different leaves to serve as negative controls. Three replicates of three leaves were inoculated with each method per inoculum concentration. Inoculated leaves were placed inside plastic boxes on moistened filter paper, covered with a polyethylene bag to maintain high RH (> 98%) and incubated for 21 days at 25°C and 12-h light photoperiod in a controlled environment chamber (model MLR-350; Sanyo, Gunma, Japan) with a maximum variation of ±1°C. A completely randomized experimental design was used. The experiment was performed twice.

Disease severity was assessed 21 days after inoculation. Different disease severity indices (*I*) were established depending on the inoculation method. For immersion, *I* ranged from 0 to 3, corresponding to a leaf area affected by 0, 6, 12 or ≥ 24%, respectively (Battilani et al. 1999; Garcin et al. 2011b). For local infiltration and drop deposition *I* ranged from 0 to 3 with 0, no infection; 1, necrosis restricted to the inoculation point; 2, necrosis affecting the whole inoculated area; and 3, necrosis expanding through the leaf (Moragrega et al. 1998; Ruz et al. 2008). Finally, disease severity (*S*) was calculated according to the equation: $S = (\sum_{i=1}^{n} In \; / \; (N \; x \; 3)) \; x \; 100$, where I_n is the disease severity index in an inoculation site, *N* is the total number of inoculation sites in a leaf, and 3 is the maximum severity index. The effects of inoculum density and inoculation method were determined using the GLM procedure, after confirmation of the homogeneity of variance and normality, and Tukey's HSD test was used for mean comparison.

Effect of temperature and inoculum density on disease progress and symptom development

A total of six temperatures (10, 15, 20, 25, 30 or 35ºC) and three inoculum concentrations (1×10^4, 1×10^6 and 1×10^8 CFU/ml) were combined in a detached leaf assay performed under controlled environment conditions.

Bacterial suspensions in sterile distilled water of *X. arboricola* pv. *pruni* strain CFBP 5563 grown on LB plates for 72 h at 27ºC were obtained as described above and adjusted to an optical density of 0.2 at 600 nm (10^8 CFU/ml). Bacterial suspensions were serially 10-fold diluted in sterile distilled water to obtain inoculum concentrations of about 10^4, 10^6 CFU/ml, as well as the initial concentration of 10^8 CFU/ml. Cell density of inoculum suspensions was confirmed by dilution plating on YPGA and incubation for 72 h at 27ºC.

Young leaves of the peach-almond hybrid GF-677 were collected from potted plants with actively growing shoots, and disinfected with sodium hypochlorite (5%) for 10 min, followed by rinsing three times with sterile distilled water. Leaves were inoculated with bacterial suspensions of the corresponding inoculum concentration by local infiltration of 25-µl bacterial suspension, as described above. Four inoculations were performed in any single leaf. Leaves inoculated with sterile distilled water were used as negative controls. Inoculated leaves were placed on a grid in plastic boxes filled with wet filter paper, sealed in moistened transparent polyethylene bags to maintain a high RH (> 98%), and incubated for 21 days at the corresponding constant temperature and 12-h light photoperiod in a controlled environment chamber (model MLR-350; Sanyo, Gunma, Japan), with a maximum variation of ±1°C for all temperatures. The temperature inside the growth cabinet was monitored and recorded as described above. Disease severity was assessed daily on a 0 - 3 scale severity index (*I*) as previously described, and disease severity (S) was calculated per leaf according to the formula indicated above. A completely randomized experimental design was used. The treatment layout was a factorial arrangement with six temperatures (10, 15, 20, 25, 30 and 35ºC) and three inoculum densities (10^4, 10^6 and 10^8 CFU/ml). Five leaf replicates were used per temperature-inoculum density combination with four inoculation sites in a leaf. The entire experiment was conducted twice.

Disease progress curves were obtained per temperature, inoculum concentration and replicate by plotting the disease severity over time. The area under the disease progress curve (AUDPC) was calculated using the midpoint (trapezoidal) rule method and standardized (SAUDPC) by dividing its value by the total length of incubation (21 days) (Campbell and Madden 1990). The effects of experiment, temperature and inoculum density on the incubation period (the time between inoculation and symptom development), final disease severity (21 days after inoculation) and SAUDPC were determined using the GLM procedure and Tukey's HSD test was used for mean comparison. Previously, the homogeneity of variance and normality were tested.

Cumulative degree days (CDD) with a threshold temperature of 0ºC, were calculated daily as the sum of the daily mean temperature from day 1 of incubation to the current day. Disease progress curves against CDD were obtained for each temperature-inoculum density combination. The CDD-disease progress curves at optimal

temperatures (20, 25 and 30ºC) and highest inoculum density (10^8 CFU/ml) were used for modeling the symptom development of bacterial spot disease. Disease severity was standardized by dividing by 100 (y) and transformed according to the monomolecular, $z = \ln[1/(1-y)]$; exponential, $z = \ln(y)$; logistic, $z = \ln[y/(1-y)]$; and Gompertz, $z = -\ln[-\ln(y)]$ models (Table 3) (Campbell and Madden 1990) to obtain linear relationships between disease severity (z) and CDD (independent variable). Only data points with disease severity $0 < S < 1$ were included, since 0 and 1 are not defined in several of the model transformations. The four models (Table 3) were fitted to data by linear regression. The goodness of fit of models were assessed by R^2, mean square error (MSE), and R^2* obtained from the relationship between predicted back-transformed values and observed values (Campbell and Madden 1990). The upper and lower boundaries of the 95% confidence interval were also calculated. The best fit model was selected for predicting the disease severity as a function of CDD and proposed as a prediction model of symptom development.

The capacity of the model for predicting symptom development was analyzed in two additional independent experiments on *Prunus* plants. Actively growing potted plants of the peach-almond hybrid GF-677 were inoculated by spraying 5×10^8 CFU/ml suspensions of *X. arboricola* pv. *pruni* strain CFBP 5563, obtained as described above, supplemented with 1 mg/ml diatomaceous earth (abrasive agent to favor bacterial infection). Plants inoculated with sterile distilled water with the addition of diatomaceous earth were used as negative controls. Inoculated plants were introduced into transparent plastic bags to maintain leaf wetness and incubated for 24 h at 25ºC under darkness in a controlled environment chamber (model MLR-350; Sanyo, Gunma, Japan). The plastic bags were then removed and plants were transferred for disease development to a biosafety greenhouse and incubated under a daily temperature range from 15 - 25ºC, 70 - 80% RH and natural photoperiod for 21 days. Weather parameters inside the biosafety greenhouse were monitored with a datalogger (CR10X, Campbell Scientific Ltd., UK) connected to combined temperature-relative humidity (model HMP35C) and leaf wetness (model 237) sensors. Three replicates of five plants were used in each of two independent experiments.

Disease severity was assessed 7, 14 and 21 days after inoculation in the five youngest completely developed leaves at the moment of inoculation in a plant. A 0-to-5 scale severity index (I) corresponding to a leaf area affected by 0, 1, 3, 6, 12 and $\geq$ 24%, respectively (Battilani et al. 1999; Garcin et al. 2011b) was used. Disease severity (S) was calculated for each plant according to the formula: $S = \lceil (\sum_{n=1}^{N} I_n)/N \times 5 \rceil \times 100$ where In is the severity index in a leaf, N is the number of leaves per plant, and 5 is the maximum severity index value on the scale. In order to compare the predicted and observed values, the disease severity (S) was standardized by

dividing by 100 (*y*). CDD was calculated as the sum of the daily mean temperature in the greenhouse for the 7, 14 and 21 days of plant incubation, corresponding to days when the disease was assessed. A linear regression analysis between predicted and observed disease severity values at each CDD was performed. The linear regression was analyzed using the coefficient of determination (R^2) and testing the significance of the difference in the intercept from 0 and the slope from 1.

Results

Effect of temperature on epiphytic growth of *X. arboricola* pv. *pruni* on *Prunus* leaves under high RH

The mean initial population density of *X. arboricola* pv. *pruni* strain CFBP 5563 Rif^r on inoculated leaves of peach-almond hybrid GF-677, recovered after inoculation (t = 0 h), was 1.33 x 10^4 CFU/g. The dynamics of bacterial population densities on leaf surface depended on the temperature and incubation period. The pathogen was able to grow epiphytically on GF-677 leaves at temperatures from 20 to 30ºC. At these temperatures no increase in the bacterial population was observed in the first 12 to 24 h of incubation, but after 24 h bacterial densities increased with time up to mean population densities ranging from 2.64 x 10^6 to 5.22 x 10^8 CFU/g 6-8 days after inoculation. In contrast, no increase in pathogen population was observed on leaves incubated at temperatures from 5 to 15ºC and 35ºC. The epiphytic pathogen population density on leaves incubated at 35ºC was reduced to < 10^2 CFU/g after 6 days incubation under wetness. Similarly, the initial population was reduced by 1-2 log units on leaves incubated at 10 and 15ºC. No viable bacterial cells were recovered from leaves corresponding to the negative control.

Population densities were used to generate the growth curves of *X. arboricola* pv. *pruni* strain CFBP 5563 Rif^r on *Prunus* at each temperature. Three growth curves were obtained per temperature and experiment. The modified Gompertz model (equation 1) was fitted to the growth curves obtained at temperatures at which bacterial growth was observed, corresponding to 20, 25 and 30ºC, and the maximum specific growth rate (μ_{max}) was estimated. The doubling time (DT= ln (2) / μ_{max}) was calculated. No significant effect of experiment replicate in growth rate was observed (P = 0.545). Mean values for the maximum specific growth rate and the doubling time at each temperature are given in Table 1. The maximum specific growth rate ranged from 0.073 to 0.141 h^{-1}, depending on temperature, and was higher (up to the double) at 25ºC than at 20 and 30ºC. However, no significant differences in growth rate (P = 0.419) were observed between these temperatures (Table 1). The mean doubling time was low (4.9 h) at 25ºC and higher at 30 and 20ºC (7.2 and 9.5 h, respectively).

Effect of low RH on epiphytic population dynamics of *X. arboricola* pv. *pruni* on *Prunus* leaves

The mean initial population density of *X. arboricola* pv. *pruni* strain CFBP 5563 Rif^r on nectarine cv. Big Top leaves was 3.9 x 10^8 CFU/g. Viable cell counts of pathogen recovered from the surface of 'Big Top' leaves at each sampling time (from 0 to 72 h incubation at 25ºC) were plotted against time to obtain the survival curves. Pathogen epiphytic populations decreased with time when incubated at 25ºC under dry conditions (RH < 40%) (Fig. 1). During the incubation period under dry conditions, 'Big Top' leaves became flaccid and finally desiccated. The survival curve for *X. arboricola* pv. *pruni* had a biphasic shape, with a rapid decline of pathogen population densities at the beginning of the dry period followed by a tail in which the population density maintained low. No significant differences were observed in bacterial population densities in experiment replicates according to GLM analysis (P = 0.846), and data of three independent experiments were pooled for modeling, to reduce data variability. The Cerf model (equation 2) fitted well to data (RMSE = 0.251 and R^2 = 0.931), with the following equation: $N_t = 8.59 \times [\, 0.173 \times \exp(-0.360t) \,] + (1 - 0.173) \times \exp(-0.003t)$ (Fig. 1). The first straight phase of the curve had a negative slope (k_1) corresponding to a death rate of -0.360 log CFU/g h. After 6 h incubation under dry conditions, a 2-log reduction was observed in the bacterial population density, which meant that less than 10% initial cells survived. The second phase of the model (tail), had a low slope (k_2 = -0.003 log CFU/g h), with a 1-log the reduction of bacterial epiphytic population density, slower than in the first phase, in the following 66 h exposure to dry conditions. Although the reduction in the pathogen population density on nectarine leaves was significant, with only 0.001% of cells surviving after 72 h incubation at 25ºC and low RH, not all cells were inactivated and approximately 10^6 CFU/g were recovered at the end of the incubation period.

Effect of inoculum density of *X. arboricola* pv. *pruni* on disease severity on *Prunus* leaves

Two independent experiments were performed to evaluate the effects of bacterial concentration on infection and disease severity on detached nectarine leaves inoculated by three different methods. Analysis of variance indicated a significant effect of inoculum density and inoculation method on disease severity (P < 0.001), but no significant effect of experiment (P > 0.110), so data from the two independent experiments were pooled for further analysis. The effect of inoculum dose on disease severity was similar for the three inoculation methods (immersion, infiltration and drop deposition), although the disease severity differed (Fig. 2). Low disease severity (< 33%) was observed on leaves inoculated with densities ranging from 10^1 to 10^5 CFU/ml, whatever

359 the inoculation method. Higher inoculum concentrations, from 10^6 to 10^9 CFU/ml, resulted in significantly
360 higher disease severity (from 45 to 100% depending on the inoculation method). The local infiltration of
361 bacterial suspensions at concentrations from 10^6 to 10^9 CFU/ml produced the highest disease severity (80-
362 100%). For this method of inoculation, Tukey's HSD test separated inoculum concentrations into three groups
363 from 10^1 to 10^5, 10^6 and higher than 10^6 CFU/ml resulting in low (S < 45%), high (S = 80%) and very high (S =
364 100%) disease severity (Fig. 2). Drop deposition of bacterial suspensions on leaf surface gave the lowest
365 severity levels: 1-20% at inoculum densities from 10^1 to 10^5 and 50-70% at higher inoculum concentrations. A
366 similar dose-response effect was observed in leaves treated by immersion in bacterial suspensions, but the
367 disease severity was slightly higher, 10-30% for low and 60-80% for high inoculum densities. Tukey's HSD test
368 for immersion and drop inoculation methods grouped inoculum densities in two groups with significantly
369 different disease severity levels: one low (10^1-10^5 CFU/ml) and the other high (10^6-10^9 CFU/ml) (Fig. 2).
370
371 **Effect of temperature and inoculum density on disease progress and symptom development**
372 Disease progress curves of two independent experiments for each temperature-inoculum density combination
373 are shown in Fig. 3. Infections occurred at temperatures from 15 to 35ºC, whereas no disease symptoms were
374 observed at 10ºC for any inoculum density tested. For densities of 10^6 and 10^8 CFU/ml, when the temperature
375 was increased from 15 to 30ºC the disease severity also increased. A temperature of 30ºC was optimal for
376 disease development, since the symptoms appeared earlier than at other temperatures with inoculum densities of
377 10^6 and 10^8 CFU/ml, and higher disease severity was reached 21 days after inoculation (Table 2, Fig. 3). The
378 minimum temperature at which symptoms were observed was 15ºC, but low disease severity (12.5%) was
379 obtained with the highest inoculum concentration (10^8 CFU/ml), the final disease severity of 10^6 CFU/ml was
380 very low (0.8%), and no disease symptoms were observed at 10^4 CFU/ml. Leaves inoculated with bacterial
381 suspensions of 10^6 and 10^8 CFU/ml and incubated at 35ºC also developed low disease severity. In general,
382 disease incubation period decreased and final disease severity increased when increasing the inoculum density at
383 optimal temperatures (from 20 to 30ºC) (Fig. 3 and Table 2), whereas the lowest inoculum density (10^4 CFU/ml)
384 gave the lowest final disease severity and the longest incubation period (16-18 days).
385 The analysis of variance indicated no significant effect of experiment replicate (P = 0.072), but a significant
386 effect of temperature and inoculum density (P < 0.001) on disease-related parameters; incubation period, final
387 disease severity and SAUDPC. The Tukey HSD mean comparison was performed for each inoculum density
388 and disease-related parameter (Table 2). SAUDPC was significantly higher in nectarine leaves incubated at

30ºC than in those incubated at other temperatures, whatever the inoculum density. Similar SAUDPC values were observed in leaves incubated at 15, 20, 25 and 35ºC and inoculated with bacterial suspensions of 10^4 (from 0.2 to 1.6) and 10^6 CFU/ml (from 0.3 to 4.6). However, for inoculum densities of 10^8 CFU/ml differences in SAUDPC were observed with temperature, being low at 15ºC (2.48), medium at 20 and 35ºC (26.1 and 19.3, respectively) and high at 25 and 30ºC (41.5 and 60.9, respectively) (Table 2). No significant differences were observed among temperatures in the incubation period at inoculum concentrations of 10^4 and 10^6 CFU/ml; whereas for 10^8 CFU/ml, incubation at 25 and 30ºC gave the shortest incubation period (7.7 and 5.9 days respectively) and incubation at 15, 20 and 35ºC resulted in longer incubation periods, from 11- 14 days (Table 2).

To obtain the symptom development model, the measure of accumulated heat (physiological time) was used, expressed in CDD and calculated as the sum of daily mean temperature for a given time period with a threshold temperature of 0ºC. Temperatures from 20 to 30ºC and the inoculum density of 10^8 CFU/ml were the most favorable for symptom development according to the SAUPCD, and when disease severity was plotted against CDD disease progress curves overlapped (Fig. 4). Consequently, data from treatments with inoculum density of 10^8 CFU/ml and 20, 25 and 30ºC temperatures were used for symptom model development. The reason for discarding the curves obtained at 15ºC was that, after 21 days of incubation, only 315 CDD were accumulated at 15ºC in comparison to the 420, 525 and 630 CDD at 20, 25 and 30ºC, respectively (Fig. 4c and 4f); although the incubation period at 15 and 20ºC did not differ. For disease severity, no significant differences were observed between experiments (P = 0.184) and temperatures (P = 0.105) for inoculum density of 10^8 CFU/. So, data from the two experiments and the three temperatures were pooled to analyze the relationship between the disease progress and the CDD (Fig. 5). The linearized form of the monomolecular, exponential, logistic and Gompertz models (Table 3) were fitted to transformed severity data (z). The best model fit was obtained using the linearized form of the Gompertz model (R^2 = 0.715, MSE = 0.511 and R^{2*} = 0.778), with parameter estimates b_0 = -2.7869 and r_G = 0.0112. Parameter b_0 and values predicted by the linear model (z) were back-transformed to the original equation of Gompertz using: $B = \exp(-b_0)$ and $y = \exp[-\exp(-z)]$. The Gompertz equation describes the S-shaped curve of the dependent variable over CDD: $y = \exp[-B \cdot \exp(-r_G \cdot CDD)]$, where y is the disease severity (0-1); B is a constant of integration ($-\ln(y_0)$) and r_G is the slope. According to the obtained Gompertz model, 281.6 CDD were necessary for a disease severity of 50%, while 150 and 174 CDD were necessary to reach 5 and 10% disease severity, respectively.

418 The predictive capacity of the symptom development model was evaluated in two additional experiments, in
419 which *Prunus* plants were inoculated with bacterial suspensions containing 1×10^8 CFU/ml, exposed for 24 h at
420 25ºC under wetness to induce infection, and then, incubated in the greenhouse for symptom development at a
421 daily temperature range of 15-25ºC. Disease severity was assessed 7, 14 and 21 days after inoculation and the
422 corresponding CDD was calculated. Observed disease severity and disease severity predicted by the Gompertz
423 model at each CDD were compared. A significant correlation was obtained between observed and predicted
424 values of disease severity ($P < 0.01$), with a Pearson coefficient $R = 0.909$. The linear regression of the predicted
425 against the observed disease severity is shown in Fig. 6, with a coefficient of determination $R^2 = 0.83$ and the
426 intercept and the slope not significantly different from 0 ($P = 0.963$) and 1 ($P = 0.815$), respectively.
427

428 ## Discussion

429 A better understanding of bacterial spot disease epidemiology can be valuable in developing disease
430 management strategies based on the use of disease forecasters in decision support systems to guide copper
431 applications and disease surveillance tasks for early detection of outbreaks or spread of the disease. This study
432 contributes to increase the knowledge on some epidemiological aspects of the bacterial spot disease of stone
433 fruits and provides new information that will be the basis for the development of a forecasting system for this
434 disease.
435 Epiphytic growth of *X. arboricola* pv. *pruni* on *Prunus* leaves was only observed at temperatures of 20, 25 and
436 30ºC, being maximal at 25ºC. In previous *in vitro* studies, the bacterium was able to grow at temperatures from
437 5 to 35ºC, with a maximum at 30ºC (Morales et al. 2017). As expected, the maximum specific growth rate and
438 the doubling time for the epiphytic growth of *X. arboricola* pv. *pruni* on *Prunus* leaves differed from those
439 determined *in vitro.* For a given temperature, the maximum specific growth rate for strain CFBP 5563 on
440 *Prunus* leaves was lower than that obtained for the same strain when grown *in vitro*, in LB broth. These
441 differences can be attributed to the growth conditions, mainly nutrient and free water availability. The growth of
442 epiphytic bacteria on plant surface is limited by the availability of nutrients, and the lack of carbon sources on
443 the leaf surface has been reported (Mercier and Lindow 2000), Additionally, only a few sites on the leaf surface,
444 such as veins and trichomes, offer conditions that allow bacterial growth. These specific sites protect bacteria
445 from water stress since they retain water longer than other parts, which may increase the local availability of
446 nutrients such as sugars, which are used by bacteria on the leaf surface (van der Wal et al. 2013). Consequently,
447 although the pathogen was initially uniformly distributed onto the leaf surface by leaf immersion in the bacterial

suspension, only specific areas were colonized by the bacterium. In contrast, *X. arboricola* pv. *pruni* growth *in vitro* was not limited by nutrients or free water since LB broth is a nutritionally rich medium with high water activity (a_w = 0.975). Detached leaves appear to be a useful approach for analyzing the potential epiphytic bacterial growth since they more closely reflect natural conditions, with limited carbon sources, a reduction of water activity, and interaction with host factors (Lebeaux et al. 2013). However, it should be taken into account that,, under natural conditions, phyllosphere microbial communities are diverse and their diversity and population size are influenced by environmental conditions and host factors (plant species, plant cultivar, and stage of growth) (Gnanamanickam and Immanuel 2007). Consequently, diversity of microbial communities present in the phylloplane may affect host colonization by epiphytic populations of *X. arboricola* pv. *pruni*. Information obtained in this study could be combined with the model for predicting *X. arboricola* pv. *pruni* growth as a function of temperature developed under *in vitro* conditions and used to forecast the inoculum potential for this pathogen.

Although bacterial growth is a temperature-dependent process, it requires the presence of free water, provided by rain, dew or irrigation, a situation that does not always occur on host tissues under field conditions (Agrios 2005; Garcin et al. 2007; Moh et al. 2011). The multiplication of the pathogen, as well as the infection process, may be interrupted by a dry period (Magarey and Sutton, 2007). In this research, variations in the density of *X. arboricola* pv. *pruni* epiphytic populations under different wetness conditions were analyzed. The population density on *Prunus* leaves decreased when incubated under low RH (< 40%) at optimal temperature for growth (25ºC) in contrast to the population increase observed under wetness. The survival curve under dry conditions contained two separate phases, a rapid decline of population during the first 6 h of dryness followed by a slow and continuous inactivation of bacterial cells on increasing the dry period. Similar results were observed in experiments with lower initial bacterial concentrations (10^6 CFU/ml); a biphasic curve with the turning point after 6 h of dryness was also obtained (data not shown). Accordingly, 6 h may be the time required for *X. arboricola* pv. *pruni* to activate the mechanisms to respond and adapt to dry conditions. The presence of these two phases has been considered to represent a mix of two fractions or sub-populations of different head resistance, in which the first phase describes the inactivation of the less resistant cells and the second phase corresponds to the more resistant ones (Xiong et al. 1999). *Xanthomonads* are able to synthesize large amounts of lipopolysaccharides and the extracellular polysaccharide xanthan. The abundant xanthan slime layer aids in bacterial adhesion with biofilm formation, survival and infection (Crossman and Dow 2004; Ryan et al. 2011; Schubert et al. 2001), and probably protects against dehydration, which could explain the survival of 0.001%

478 bacterial population after 72 h incubation under low RH. A combined model for predicting the epiphytic
479 inoculum potential of *X. arboricola* pv. *pruni* based on temperature and wet/dry periods could be developed by
480 integrating results obtained here and in previous studies (Morales et al. 2017), in a similar way to *X. campestris*
481 pv *vesicatoria* (Kim et al. 2014) and *Pseudomonas syringae* pv *actinidiae* (Beresford et al. 2017) prediction
482 models.
483 The relationship between inoculum density and infection of *Prunus* by *X. arboricola* pv. *pruni* was determined
484 on detached leaf assays using three inoculation methods. All inoculation methods were effective for bacterial
485 infection and disease symptom development despite quantitative variance in disease expression. Leaves
486 inoculated by infiltration expressed the highest disease severity, explained by the fact that bacterial cells were
487 introduced directly into the leaf mesophyll. Although the severity was lower in leaves inoculated by immersion
488 or drop deposition, these methods may reflect natural infections more accurately because bacterial cells had to
489 enter leaves through natural openings or wounds (Battilani et al. 1999; Garcin et al. 2011a; Morales et al. 2016,
490 2017). Nevertheless, a similar pattern of the effect of inoculum dose on disease severity was observed for the
491 three inoculation methods. Low severity obtained at inoculum densities below 10^6 CFU/ml and high disease
492 severity recorded at inoculum densities from 10^6 to 10^9 CFU/ml agreed with previous reports (Civerolo 1975;
493 Socquet-Juglard et al. 2012) and could be related to quorum sensing. Several regulatory systems depend on
494 quorum sensing mechanisms, widely studied for *X. campestris* (Dow et al., 2003; He and Zhang, 2008),
495 whereby bacteria monitor their local population density before expressing a phenotype or to control
496 pathogenicity genes (von Bodman et al. 2003; Whitehead et al. 2001). Supporting this idea, *X. arboricola* pv.
497 *pruni* has been detected on symptomless peach twigs over a year with a maximum of 6.1 x 10^4 CFU/g of fresh
498 weight (Shepard and Zehr 1994), and from 10^2 to 10^5 CFU/ml bacterial densities have been detected in
499 asymptomatic leaves of *Prunus* field samples, whereas at least 10^6 CFU/ml were present in symptomatic leaves
500 (Palacio-Bielsa et al. 2011). Our results confirm that 10^6 CFU/ml can be considered the minimum concentration
501 for *X. arboricola* pv. *pruni* to cause infections, and seems to be the threshold for bacterial cells to activate
502 pathogenesis on host leaves and change from the epiphytic phase through an endophytic phase. Additionally, the
503 inoculum density was also related to the incubation time, since longer incubation periods and lower final disease
504 severity values were observed in leaves inoculated with doses below 10^6 CFU/ml.
505 Once infections occur, temperature is probably the major conditioning factor for disease progress and symptom
506 development, but it also depends on the specific host-pathogen combination (Agrios 2005; Dickson and Holbert
507 1928). In our study, disease progress and symptom development were observed at temperatures from 15 to

35ºC, with the optimum at 30ºC, which agree with the optimal temperatures for the pathogen growth (Morales et al. 2017; Young et al. 1977). The cumulative degree day, CDD, as predictor of the physiological time, has been used to forecast symptom development in plant disease forecasting models, such as Maryblyt (Lightner and Steiner 1992) and BIS95 (Billing 1999). The same concept was applied in our study to predict symptom development of bacterial spot disease of stone fruits. The relationship between the progress of disease severity and CDD was described by the Gompertz model, whereby 150, 175 and 280 CDD with a temperature base of 0ºC were required for disease severity of 5, 10 and 50%, respectively. The biofix to initiate the computation of CDD is the date when infections occur. An incubation period of 250 CDD (with a threshold temperature of 10.8ºC) had been determined for bacterial spot disease of stone fruits in a previous study performed under field conditions, in French peach orchards naturally affected by the disease (Garcin et al. 2011b). Symptom development under field conditions may be affected by temperature, but also by other weather variables, such as leaf wetness or RH (Zehr et al. 1996), as well as the inoculum density of natural pathogen populations (Randhawa and Civerolo 1985). The incubation period for bacterial spot disease reported on Italian peach orchards varied from 6 to 26 days in warm and cold weather, respectively (Battilani et al. 1999). Variation on the CDD found in our study and those performed under natural conditions may be partially attributed to pathogen inoculum density, which was probably lower in the orchards than in our experiments performed under controlled conditions (10^8 CFU/ml). The model for predicting the incubation period developed and validated in the work presented here needs to be evaluated under field conditions before its practical application.

The information generated in this study, performed under controlled environment and greenhouse conditions, will be useful in the development of a disease forecasting system for *X. arboricola* pv. *pruni*. Our approach for the development of a forecaster for bacterial spot disease of stone fruits was focused on three crucial stages of the disease cycle: host colonization by epiphytic populations of the pathogen, host infection and disease progress and symptom development. Consequently, the forecasting system will be composed of three components: i) the epiphytic inoculum potential, ii) the infection model and, iii) the disease symptom development model. The first component of the forecasting system, which refers to pathogen potential inoculum, will be based on the model for *X. arboricola* pv. *pruni* growth *in vitro* as a function of temperature (Morales et al. 2017) and the results of epiphytic growth under wet/dry periods from this study. The relationship between the inoculum dose of *X. arboricola* pv. *pruni* and the onset of the infection process is an essential parameter for operation of the forecasting system, since it links the epiphytic inoculum potential with the infection model. The infection threshold of 10^6 CFU/ml should be included in the forecasting system to link the

epiphytic inoculum potential and the infection model. Therefore, the infection model (second component) developed and validated previously (Morales et al. 2018) will start working when the inoculum potential predicted by the growth model is high enough to cause infections. Therefore, if the infection model predicts favorable weather conditions for initiating an infection process, the symptom model will run for prediction of disease symptoms appearance on the basis of daily mean temperature and CDD. The whole forecasting system proposed needs to be evaluated under field conditions in experimental or commercial orchards before being used in decision support systems (DSS) for management of the bacterial spot disease of stone fruits.

Compliance with Ethical Standards

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698

Tables

Table 1 Growth parameters estimated by the modified Gompertz model for epiphytic populations of *X. arboricola* pv. *pruni* on *Prunus* leaves at different temperatures under high RH

Temperature (ºC)	Maximum specific growth rate (h^{-1})[y]	Doubling time (h)[y]
5	-[z]	-
10	-	-
15	-	-
20	0.073 ± 0.027	9.54 ± 3.6
25	0.141 ± 0.034	4.92 ± 1.2
30	0.099 ± 0.033	7.02 ± 2.3
35	-	-

[y] Maximum specific growth rate (μ_{max}) was estimated from the modified Gomperzt model fitted to growth curves at different temperatures. Doubling time = ln(2) / μ_{max}. Values are the mean of two experiments.

[z] -: no growth was observed.

Table 2 Effect of temperature (T) and inoculum density (ID) on bacterial spot disease development on *Prunus* leaves inoculated with *X. arboricola* pv. *pruni*

ID (CFU/ml)	T (ºC)	Disease development parameters [w]		
		Disease severity (%) [x]	SAUDPC [y]	Incubation period (days)
10^4	10	- [z]	-	-
	15	-	-	-
	20	7.5 ± 5.0 b	1.01 ± 0.69 b	18.0 ± 1.0 a
	25	5.8 ± 2.8 b	1.57 ± 0.95 b	16.3 ± 1.7 a
	30	27.5 ± 9.0 a	6.29 ± 2.26 a	17.0 ± 1.0 a
	35	1.7 ± 1.7 b	0.20 ± 0.20 b	18.0 ± 7.5 a
10^6	10	-	-	-
	15	0.8 ± 0.8 C	0.30 ± 0.30 B	14.0 ± 0.0 A
	20	20.0 ± 5.2 BC	2.38 ± 1.07 B	15.8 ± 2.7 A
	25	23.3 ± 6.1 B	4.44 ± 1.61 B	12.4 ± 3.0 A
	30	81.7 ± 8.9 A	28.77 ± 4.16 A	10.8 ± 0.5 A
	35	15.0 ± 5.4 BC	4.56 ± 1.65 B	12.4 ± 0.2 A
10^8	10	-	-	-
	15	12.5 ± 4.5 C’	2.48 ± 1.27 D’	14.1 ± 1.9 A’
	20	98.3 ± 1.1 A’	26.11 ± 2.36 C’	12.2 ± 0.9 A’B’
	25	93.3 ± 3.7 A’	41.67 ± 4.65 B’	7.7 ± 0.7 C’
	30	100.0 ± 0.0 A’	60.95 ± 1.45 A’	5.9 ± 0.2 C’
	35	47.5 ± 13.2 B’	19.35 ± 5.91 C’	11.7 ± 1.1 B’

[w] Values are the mean of two experiments, with five leaf-replicates per temperature-inoculum density combination and experiment. For each ID means followed by the same letter did not differ significantly (P = 0.05) according to the Tukey’s HDS mean comparison test.

[x] Disease severity at the end of incubation period (21 days after inoculation).

[y] SAUDPC: Standardized area under the disease progress curve (AUDPC divided by the length of incubation; 21 days).

[z] -: disease symptoms were not observed.

718 Table 3 Description and parameters of models fitted to the bacterial spot disease progress curves

Model [y]	Linearized equation [z]	b_0	r	R^2	MSE	R^{2*}
Monomolecular	$\ln\left(\frac{1}{1-y}\right) = b_0 + r_M CDD$	-1.4633	0.0083	0.670	0.343	0.688
Exponential	$\ln(y) = b_0 + r_E CDD$	-3.2572	0.0074	0.545	0.462	0.538
Logistic	$\ln\left(\frac{y}{1-y}\right) = b_0 + r_L CDD$	-4.7206	0.0157	0.699	1.075	0.784
Gompertz	$-\ln[-\ln(y)] = b_0 + r_G CDD$	-2.7869	0.0112	0.715	0.511	0.778

719 [y] Models were fitted to pooled disease severity data from *Prunus* leaves inoculated with 10^8 CFU/ml
720 suspensions of *X. arboricola* pv. *pruni* CFBP 5563 and incubated for 21 days at 20, 25 and 30ºC for
721 disease development. Data from two independent experiments were used.
722 [z] Adapted from Campbell and Madden (1990). *y*: disease severity (0-1); b_0 and *r*: intercept and slope
723 parameters of the linearized forms of the models; *CDD*: cumulative degree day; R^2 and *MSE*:
724 coefficient of determination and mean square error of linear regression; R^{2*}: coefficient of
725 determination between predicted back-transformed values and observed values.
726
727

Figure captions

Fig. 1 Survival curve of epiphytic populations of *X. arboricola* pv. *pruni* strain CFBP 5563 Rif^r on nectarine cv. Big Top leaves incubated for 72 h at 25ºC under low HR (< 40%). Values are the mean of three five-leaf replicates per sampling time for three independent experiments. Cerf model is represented with continuous line. Error bar (upper right corner) corresponds to the mean standard error

Fig. 2 Effect of inoculum density on infection of nectarine cv. Big Top leaves by *X. arboricola* pv. *pruni* using three different inoculation methods: leaf immersion in the bacterial suspension (a); local infiltration of 25 µl of bacterial suspension in the leaf (b), and deposition of a 25-µl drop bacterial suspension onto the leaf surface (c). Bars are the mean disease severity after 21 days incubation at 25ºC of two independent experiments and three replicates per experiment. Error bars are the standard error. Different letters indicate significant differences according to Tukey's mean separation test ($P = 0.05$)

Fig. 3 Effect of temperature and inoculum density on bacterial spot disease development on peach-almond hybrid GF-677 leaves inoculated with *X. arboricola* pv. *pruni* strain CFBP 5563. Three inoculum densities were tested: 10^4 CFU/ml (a and d), 10^6 CFU/ml (b and e), and 10^8 CFU/ml (c and f). Values are the mean of five-leaf replicates. Global mean standard error was ±1.32. The experiment was performed twice (Exp. 1 and Exp. 2)

Fig. 4 Bacterial spot disease progress as a function of cumulative degree days (CDD) in leaves of the peach-almond hybrid GF-677 inoculated with *X. arboricola* pv. *pruni* strain CFBP 5563 at different inoculum densities 10^4 CFU/ml (a and d), 10^6 CFU/ml (b and e), and 10^8 CFU/ml (c and f) and incubated at different temperatures. The experiment was performed twice. Values are the mean disease severity of five-leaf replicates. Global mean standard error was ±1.32

Fig. 5 Relationship between bacterial spot disease severity and cumulative degree days (ºC) on peach-almond hybrid GF-677 plants inoculated with *X. arboricola* pv. *pruni* strain CFBP 5563 10^8 CFU/ml and incubated under controlled environment conditions at 20 (●), 25 (○) and 30ºC (▲). Regression lines were calculated by combining the results for the three temperatures in two independent experiments. Each data point is the mean of five-leaf replicates. a, Linearized form of the Gompertz model ($z = -2.7869 + 0.0112*CDD$, $R^2 = 0.71$). b, Gompertz model, back-transformed from the linearized form ($y = \exp [-16.2306 \cdot \exp(-0.0112 \cdot CDD)]$)

Fig. 6 Validation of the symptom development model for bacterial spot disease in potted plants of the peach-almond hybrid GF-677 inoculated with *X. arboricola* pv. *pruni* strain CFBP 5563 (10^8 CFU/ml) and incubated under greenhouse conditions for 21 days. Values are the mean disease severity of three replicates of five plants for each of two independent experiments (black and white circles). a, Gompertz model, back-transformed from the linearized form ($y = \exp [-16.2306 \cdot \exp (-0.0112 \cdot CDD)]$). b, observed disease severity versus predicted severity by the linearized form of the Gompertz model. The regression line was not different from a line with an intercept of 0 and a slope of 1

table 1

Table 1 Growth parameters estimated by the modified Gompertz model for epiphytic populations of *X. arboricola* pv. *pruni* on *Prunus* leaves at different temperatures under high RH

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35	-	-

[y] Maximum specific growth rate (μ_{max}) was estimated from the modified Gomperzt model fitted to growth curves at different temperatures. Doubling time = ln(2) / μ_{max}. Values are the mean of two experiments.

[z]-: no growth was observed.

table 2

Table 2 Effect of temperature (T) and inoculum density (ID) on bacterial spot disease development on *Prunus* leaves inoculated with *Xanthomonas arboricola* pv. *pruni*

ID (CFU/ml)	T (ºC)	Disease development parameters [w]		
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	25	5.8 ± 2.8 b	1.57 ± 0.95 b	16.3 ± 1.7 a
	30	27.5 ± 9.0 a	6.29 ± 2.26 a	17.0 ± 1.0 a
	35	1.7 ± 1.7 b	0.20 ± 0.20 b	18.0 ± 7.5 a
10^6	10	-	-	-
	15	0.8 ± 0.8 C	0.30 ± 0.30 B	14.0 ± 0.0 A
	20	20.0 ± 5.2 BC	2.38 ± 1.07 B	15.8 ± 2.7 A
	25	23.3 ± 6.1 B	4.44 ± 1.61 B	12.4 ± 3.0 A
	30	81.7 ± 8.9 A	28.77 ± 4.16 A	10.8 ± 0.5 A
	35	15.0 ± 5.4B C	4.56 ± 1.65 B	12.4 ± 0.2 A
10^8	10	-	-	-
	15	12.5 ± 4.5 C’	2.48 ± 1.27 D’	14.1 ± 1.9 A’
	20	98.3 ± 1.1 A’	26.11 ± 2.36 C’	12.2 ± 0.9 A’B’
	25	93.3 ± 3.7 A’	41.67 ± 4.65 B’	7.7 ± 0.7 C’
	30	100.0 ± 0.0 A’	60.95 ± 1.45 A’	5.9 ± 0.2 C’
	35	47.5 ± 13.2 B’	19.35 ± 5.91 C’	11.7 ± 1.1 B’

[w] Values are the mean of two experiments, with five leaf-replicates per temperature-inoculum density combination and experiment. For each ID means followed by the same letter did not differ significantly (P = 0.05) according to the Tukey’s HDS mean comparison test.

[x] Disease severity at the end of incubation period (21 days after inoculation).

[y] SAUDPC: Standardized area under the disease progress curve (AUDPC divided by the length of incubation; 21 days).

[z] -: disease symptoms were not observed.

Table 3

Table 3 Description and parameters of models fitted to the bacterial spot disease progress curves

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Gompertz	$-\ln[-\ln(y)] = b_0 + r_G CDD$	-2.7869	0.0112	0.715	0.511	0.778

[y] Models were fitted to pooled disease severity data from *Prunus* leaves inoculated with 10^8 CFU/ml suspensions of *X. arboricola* pv. *pruni* CFBP 5563 and incubated for 21 days at 20, 25 and 30ºC for disease development. Data from two independent experiments were used.

[z] Adapted from Campbell and Madden (1990). *y*: disease severity (0-1); b_0 and *r*: intercept and slope parameters of the linearized forms of the models; *CDD*: cumulative degree day; R^2 and *MSE*: coefficient of determination and mean square error of linear regression; R^{2*}: coefficient of determination between predicted back-transformed values and observed values.

Figure 1

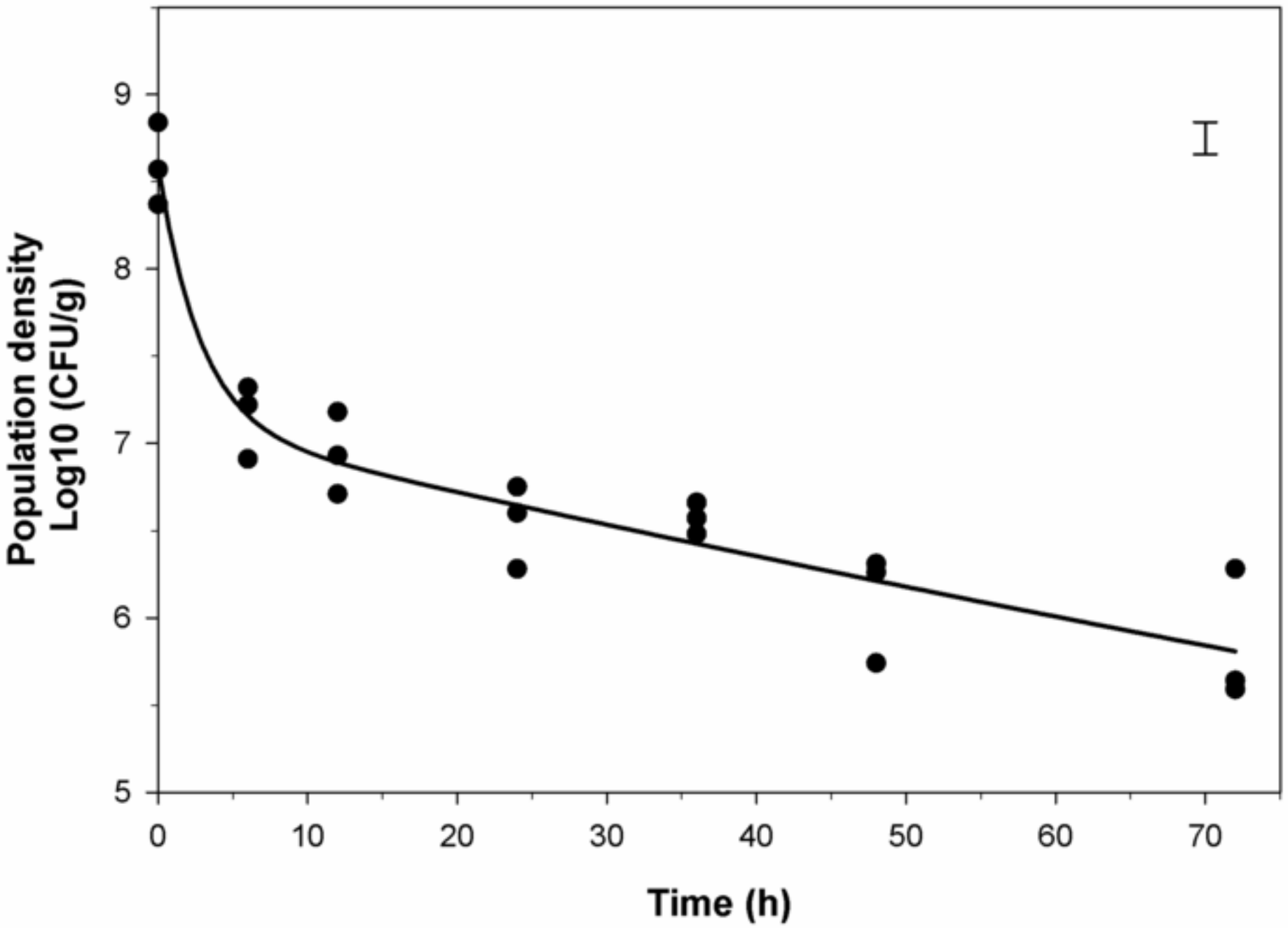

Population density
Log10 (CFU/g)
9
8
7
6
5
0
10
20
30
40
50
60
70
Time (h)

Figure 2

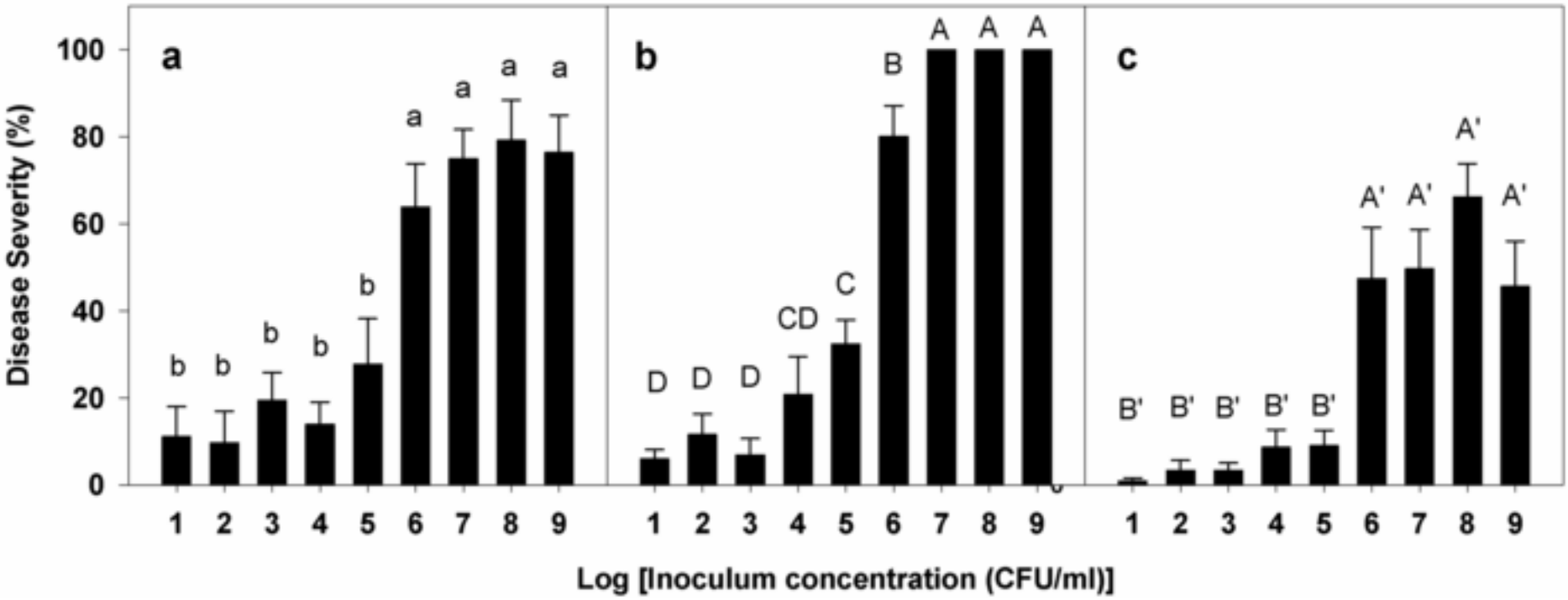

a
b
c
Disease Severity (%)
100
80
60
40
20
0
b b b b b a a a a
D D D CD C B A A A
B' B' B' B' B' A' A' A' A'
1 2 3 4 5 6 7 8 9
Log [Inoculum concentration (CFU/ml)]

Figure 3

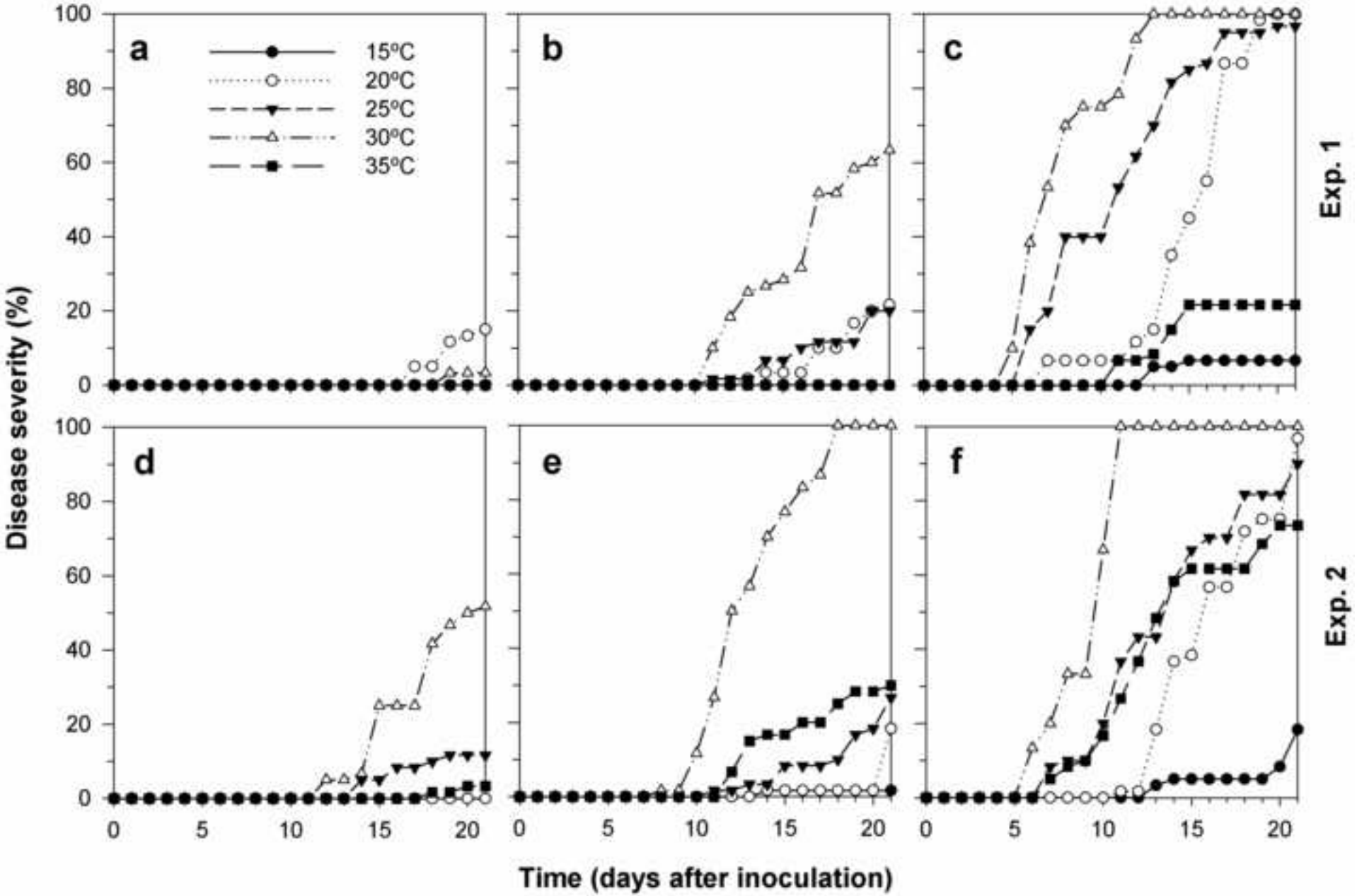

a
b
c
d
e
f
15°C
20°C
25°C
30°C
35°C
Disease severity (%)
Time (days after inoculation)
Exp. 1
Exp. 2
0
20
40
60
80
100
0
5
10
15
20

Figure 4

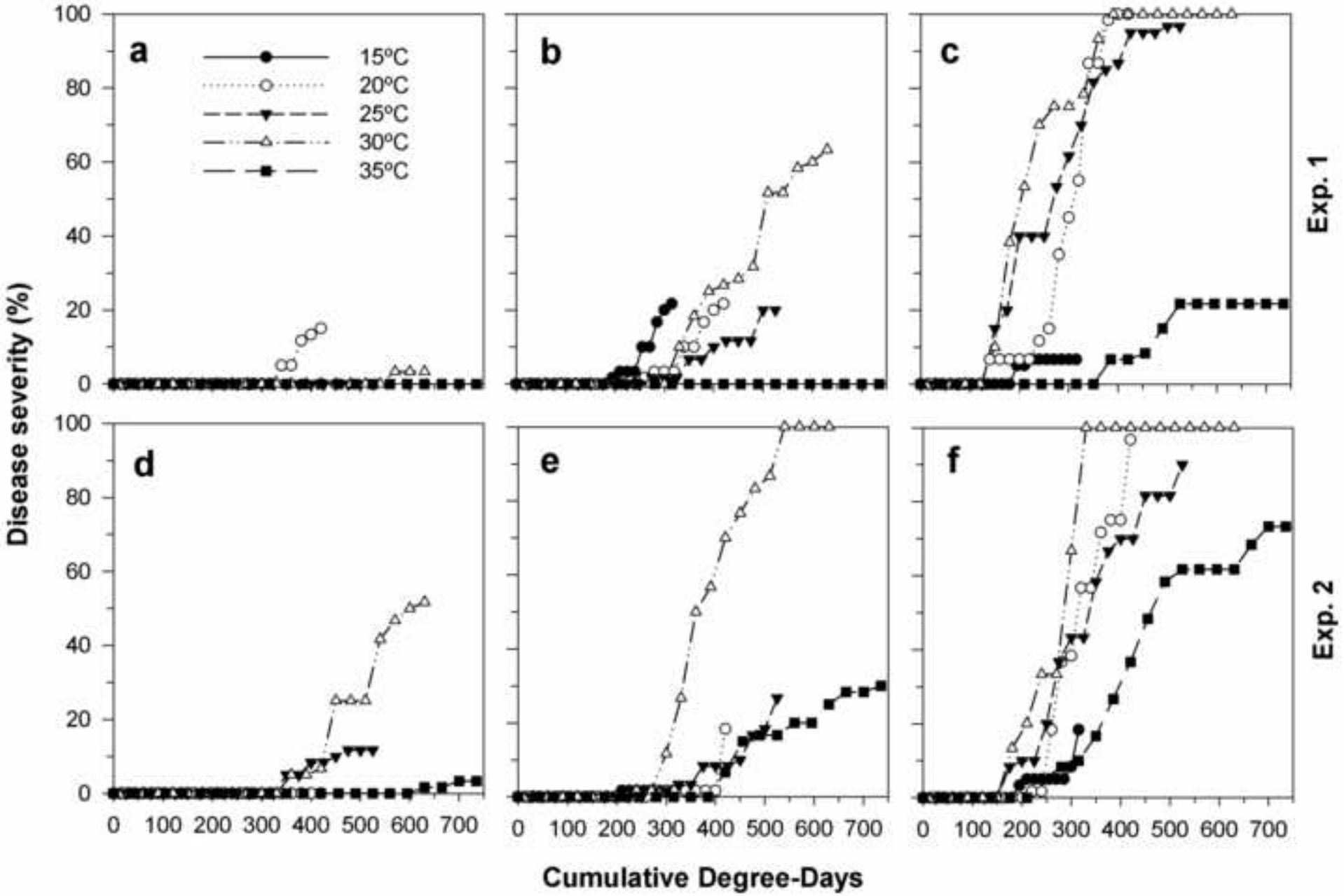

a
b
c
d
e
f
15°C
20°C
25°C
30°C
35°C
Disease severity (%)
Cumulative Degree-Days
Exp. 1
Exp. 2
0
20
40
60
80
100
0 100 200 300 400 500 600 700

Figure 5

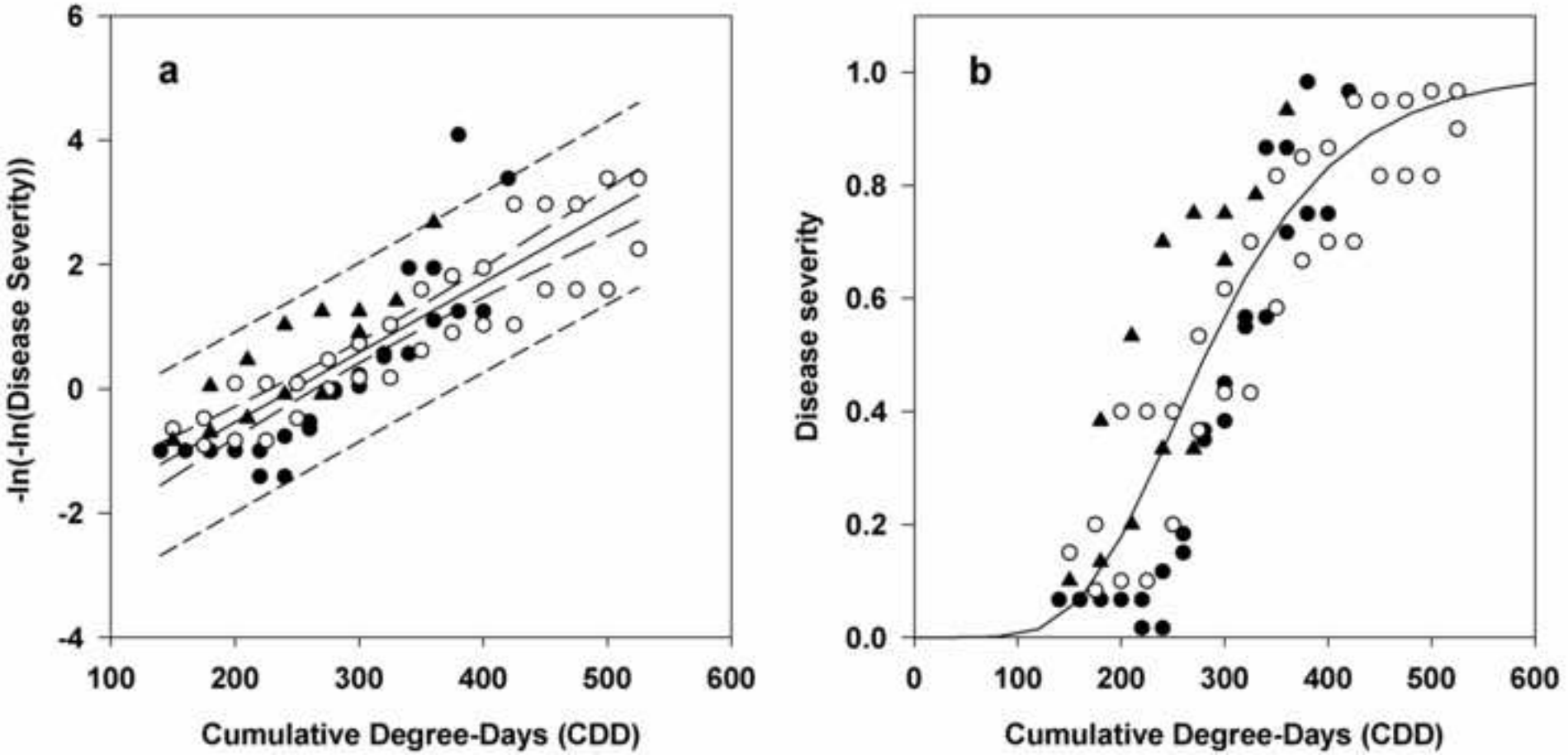
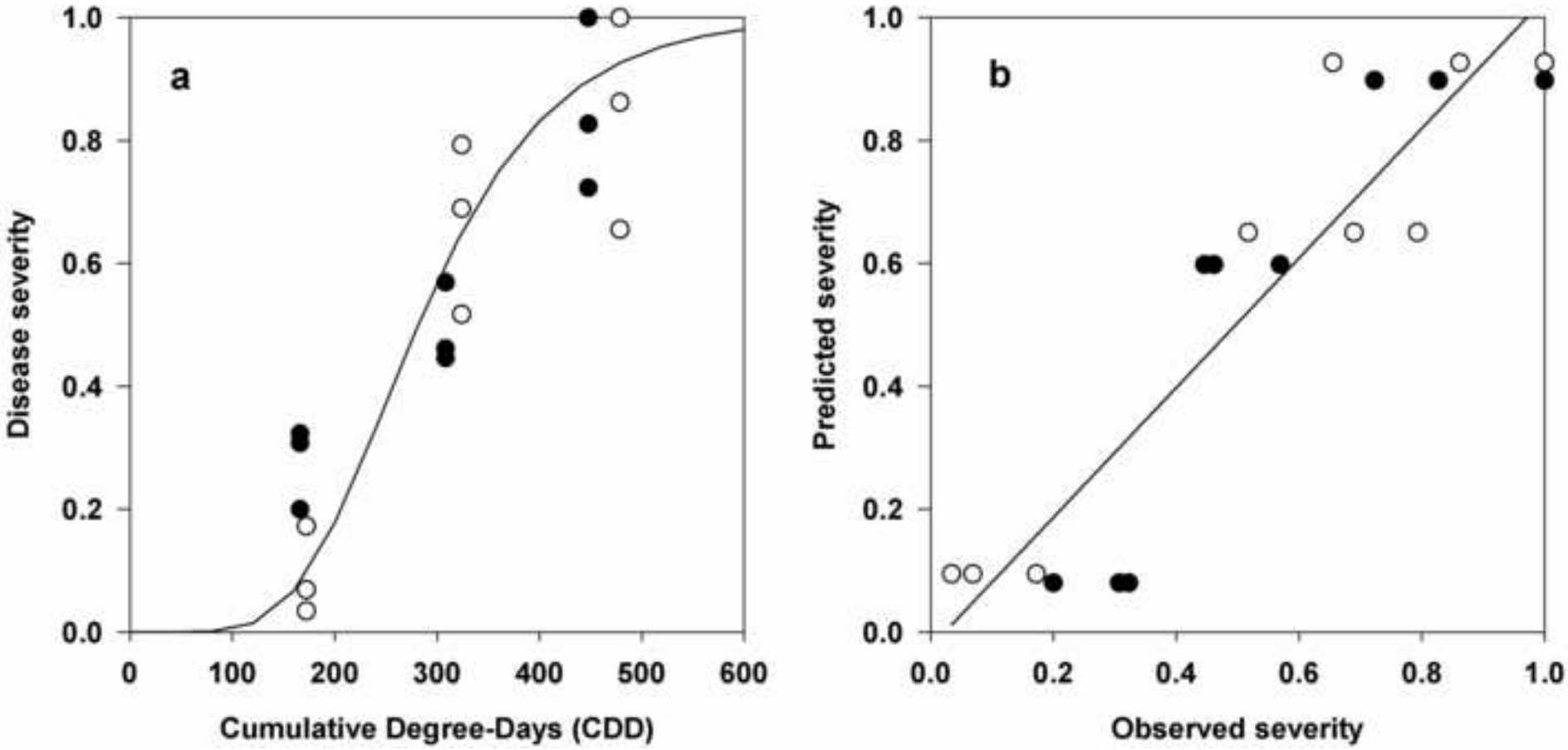

a
-ln(-ln(Disease Severity))
6
4
2
0
-2
-4
100
200
300
400
500
600
Cumulative Degree-Days (CDD)
b
Disease severity
1.0
0.8
0.6
0.4
0.2
0.0
0
100
200
300
400
500
600
Cumulative Degree-Days (CDD)

Figure 6


a
Disease severity
1.0
0.8
0.6
0.4
0.2
0.0
0
100
200
300
400
500
600
Cumulative Degree-Days (CDD)
b
Predicted severity
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.2
0.4
0.6
0.8
1.0
Observed severity