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Basis for a Predictive Model of *Xanthomonas arboricola* pv. *pruni* Growth and Infections in Host Plants

G. Morales[1], I. Llorente[1], E. Montesinos[1] and C. Moragrega[1]

Institute of Food and Agricultural Technology-XaRTA-CIDSAV, University of Girona. Girona, Spain

Abstract

***Xanthomonas arboricola* pv. *pruni* (*Xap*) is the causal agent of bacterial spot disease of stone fruits and almond. The bacterium is considered a quarantine pathogen in Europe and it has become a new and emerging threat for European crops. As the disease is strongly influenced by the weather, a forecasting model that predicts *Xap* infections based on climatic conditions could be implemented in stone fruit integrated pest management. The objective of this work was to constrain the basis for the development of a predictive model of *Xap* growth and infections, determining the effects of pathogen, host and climatic parameters on infection and disease development. A non-pathogenic specialization of *Xap* and cross-infection among host species was observed, although strains isolated from peach were the most virulent in peach leaves. *Xap* was able to infect unwounded leaves and it was observed that the presence of wounds on the leave surface did not favour *Xap* penetration in peach leaves. Otherwise, the water condition of plants played an important role in *Xap* infections and disease development in peach. The presence of water congestion and leaf wetness 48 h before inoculation favoured *Xap* infections and the duration of leaf wetness after inoculation was directly correlated to disease severity. Temperature and leaf age had a significant effect on *Xap* infections. Temperatures above 20°C favoured *Xap* infections, which were basically produced in young leaves; whereas severity was significantly lower at temperatures below 15°C and in mature leaves.**

Keywords: bacterial spot of stone fruits, peach, temperature, virulence, wetness period.

INTRODUCTION

Xanthomonas arboricola pv. *pruni* (*Xap*) is the causal agent of bacterial spot disease of stone fruits and almond (EPPO/CABI, 1997; Palacio-Bielsa et al., 2010). The bacterium is considered a quarantine pathogen in Europe (Anonymous, 2000) and it has become a new and emerging threat for European crops. Control methods are limited to preventive copper sprays early in the growing season, quarantine measures to avoid introduction and spread, and a rapid detection and eradication of the outbreaks (Boudon et al., 2005; Janse, 2012; Palacio-Bielsa et al., 2012).

The disease is strongly influenced by climatic conditions (Battilani et al., 1999; Bugiani et al., 2008), therefore *Xap* growth and infections could be predicted as function of different environmental parameters. Forecasting, decision support systems and risk mapping could be implemented in stone fruits integrated pest management. The objective of this work was to set up the basis for developing a prediction model of *Xap* growth and infection in host plants performed under greenhouse conditions that combine the effects of temperature and wetness duration. For this purpose, the effects of pathogen (virulence, ways of penetration in host), host (leaf age, water condition) and climatic parameters (wetness and temperature) on infection and disease development were determined.

MATERIALS AND METHODS

[a] E.mail: gerard.morales@udg.edu

Virulence of Strains

Seven strains of *Xap* isolated from different origins (host plant species and country) (Table 1) were evaluated. Bacterial suspensions were resuspended in sterile distilled water from LB agar cultures grown at 27°C for 24-48h and adjusted to 0,5 OD at 600 nm, corresponding to approximately 10^9 CFU ml^{-1}. Peach leaves (*Prunus persica* 'Big Top'), recovered from one-year-old potted plants grown in the greenhouse, were surface disinfected by immersion for 30s in a 5% solution of commercial sodium hypochlorite, rinsed three times with sterile distilled water and inoculated with each strain. For inoculation, a wound was done on the midvein by a single transverse incision using a scalpel and a 30 µL drop of 10^9 CFU ml^{-1} *Xap* suspension was placed onto the wound. Leaves inoculated with distilled water were used as controls. Three replicates of three leaves were inoculated with each strain, placed inside a plastic box that was covered with a plastic bag to maintain high relative humidity and incubated for 14 days at 25°C and 16-h light photoperiod in a controlled-environment chamber. Disease severity was assessed at the end of incubation time according to a 0-3 index scale (Moragrega et al., 1998; Ruz et al., 2008). The experiment was repeated twice. Additionally, the virulence of the strains originally isolated from peach was assessed on unwounded peach leaves. Five leaves were dipped into 10^9 CFU ml^{-1} suspensions of strains CFBP 5530, CFBP 5563 and CFBP 5725 respectively, placed inside a plastic box that was covered with a plastic bag and incubated at optimal conditions for disease development for 14 days, as described previously. Five leaves inoculated with sterile distilled water were used as controls. Disease severity was assessed at the end of incubation time following a 0-5 scale index corresponding to an area affected by 1, 3, 6, 12 and 24% or more, respectively (Battilani et al., 1999; Garcin et al., 2011).

Pathogen Penetration in Host

In order to determine whether *Xap* is able to penetrate host tissues through natural openings or wounds are required, detached 'Big Top' peach leaves were inoculated by pulverization with a 10^9 CFU ml^{-1} *Xap* CFBP 5563 suspension until runoff using an airbrush. Diatomaceous earth (0.2 mg ml^{-1}) was added to bacterial suspensions as an abrasive agent to produce wounds and three spraying pressures (50, 100 and 200 kPa) were tested. Treatments consisted of inoculation of bacterial suspensions with or without the abrasive agent (wound and unwounded leaves) and three spraying pressures. Three replicates of three leaves per replicate were used for each treatment. Leaves inoculated with distilled water at 200 kPa with and without the abrasive agent were used as controls. Inoculated leaves were placed in humid chambers and incubated for 16 days at 25°C and 16-h light photoperiod in a growth cabinet. Disease severity was assessed 13 and 16 days after pathogen inoculation following a 0-5 scale index, as described previously.

Water Condition of Peach Plants

The effect of water congestion and leaf wetness of peach plants before and after pathogen inoculation on *Xap* infections was assessed. Two sets of 'Big Top' potted peach plants were separated in the greenhouse before inoculation for different water treatments. One set of plants was maintained under sprinklers that were activated when leaf surface became dry using wetness sensors, and were covered with a plastic canvas that ensure 100 % relative humidity (RH) for 48 h. The second set of plants was subjected to a 60-80% RH without wetness under greenhouse conditions. Plants were inoculated with a 10^9 CFU ml^{-1} *Xap* CFBP 5563 suspension by leaf pulverisation at 100 kPa until runoff, and placed into plastic bags to ensure leaf wetness persistence for 24 or 48 h. After 24 or 48 h plants were transferred to a quarantine greenhouse where plastic bags were removed and plants were incubated at 20°C, 60-80% humidity and 12-h light photoperiod for 21 days. Three combinations of water treatments were assessed: 48 h wetness before and 24 h wetness after inoculation; 48 h wetness before and 48 h wetness after inoculation; no wetness before

and 48 h wetness after inoculation. Plants inoculated with distilled water and subjected to 48 h wetness before and 48 h wetness after inoculation were used as controls. Three plants were used for each water condition. Disease severity in the seven youngest completely formed leaves at the moment of inoculation in a plant was assessed 14 and 21 days after pathogen inoculation following a 0-5 scale index, as described previously.

Effect of Temperature on *Xap* Infection in Peach

The effect of temperature (5, 10, 15, 20, 30 and 35°C) on bacterial infection was determined. 'Big Top' potted peach plants were inoculated with a 10^9 CFU ml^{-1} *Xap* CFBP 5563 suspension (0.2 mg ml^{-1} diatomaceous earth) by pulverisation of leaves at 100 kPa until runoff. Before inoculation, plants were maintained in the greenhouse for 48 hours under sprinklers and a plastic canvas to assure leaf wetness and 100% RH (as described previously). After inoculation, plants were introduced into transparent plastic bags to maintain leaf wetness and incubated at different temperatures for 24 hours in a controlled-environment chamber. Plants were then transferred to a quarantine greenhouse where the plastic bags were removed and plants were incubated at 20°C, 60-80% humidity and 12-h light photoperiod for 21 days. Five plants were used per condition. Plants inoculated with distilled water (0.2 mg ml^{-1} diatomaceous earth) were used as controls. Disease severity was assessed in the five youngest leaves in a plant 14 and 21 days after pathogen inoculation, following a 0-5 scale index, as described previously. The experiment was performed two times.

Effect of Peach Leaf Age

Plants from previous assay on the effects of temperature were used in this experiment. Disease severity in plants exposed to temperatures of 20, 25, and 30°C was recovered individually for each leave in a plant. The position of each leaf in the twig was noted. Two leaf ages were established: young (leaves not fully expanded at the moment of inoculation) and old (mature leaves formed one-two weeks before inoculation). Five plants were used per temperature and leaf age. The experiment was performed two times.

Data Analysis

Disease severity (S) was calculated for each replicate or plant, depending on the experiment, according to the following formula:

$$S = \sum_{n=1}^{N} \frac{I_n}{N \times I_{max}} \times 100$$

where I_n is the severity index for an inoculation/leaf, N is the number of inoculations/leaves in a replicate/plant, and I_{max} is the maximum index value in the scale.

Treatment effects on disease severity were analysed by means of repeated measures analysis of variance (ANOVA) for parametric data or the Kruskal-Wallis test for non-parametric data using IBM SPSS Statistics Version 19. Differences among treatments were analysed using Tukey's LSD mean comparison test or pairwise comparisons for non-parametric analysis.

RESULTS

Virulence of Strains

The seven strains isolated from different hosts and countries were pathogenic on peach, since disease symptoms were observed for all strains on peach leaves at the end of incubation time. Significant differences in disease severity in peach leaves were observed among strains of *Xap* (p=0.045) (Figure 1). Strains isolated from peach (CFBP 5530, CFBP 5563 and CFBP 5725) were highly virulent with disease severity from 40 to 60% for wounded and unwounded peach leaves. Strains IVIA 33 and IVIA 3162-1 isolated from

almond and strain CFBP 3903 isolated from plum were moderately virulent (disease severity from 24 to 42.6%). The type strain CFBP 3894 isolated from Japanese plum was the less virulent on peach, with a mean disease severity of 18.5%.

Pathogen Penetration in Host

The disease severity in detached peach leaves inoculated with *Xap* CFBP 5563 increased through the incubation time, from 20-40% 13 days after inoculation to 80-90% at 16 days. The addition of diatomaceous earth as an abrasive agent in bacterial suspensions did not increase the disease severity in plant leaves. No differences were observed among treatments with the addition of an abrasive agent in disease severity values for each incubation time (p=0.422 and 0.951 for 13 and 16 days, respectively). Moreover, spraying pressures (50, 100 and 200 kPa) used for inoculation of pathogen did not have a significant effect on disease severity (p>0.7).

Water Condition of Peach Plants

First disease symptoms on leaves were observed 10 days after inoculation. Disease severity increased from 14 to 21 days after pathogen inoculation. Significant differences in final disease severity were observed depending on water condition of plants (p=0.002). Plants submitted to irrigation and continuous leaf wetness 48 h before inoculation and incubated with continuous leaf wetness for additional 48 h after inoculation displayed significantly higher disease severity (74.4%) at the end of incubation time. Plants maintained under irrigation and continuous leaf wetness for 48 h prior to inoculation but with a reduction to 24 h of leaf wetness after inoculation were less infected and final disease severity was reduced (41%). Similar results were obtained in plants not exposed to water congestion before pathogen inoculation and 48 h of leaf wetness post-inoculation (35.6%).

Effect of Temperature on *Xap* Infection in Peach

Differences among experiments were observed in final disease severity values in most temperatures tested. These differences could be attributed to the differences in plant material used. Experiment 1 was performed in spring, after bud break and experiment 2 was performed in autumn, six months later. Although a significant effect of temperature on disease severity was observed in both experiment replicates, the relationship between temperature and final disease severity was more evident in plants inoculated after bud break (experiment 1) than in autumn (experiment 2). In experiment 1, incubation at low temperatures (5 to 15°C) resulted in low disease severity, whereas incubation at higher temperatures (from 20 to 35°C) produced higher disease severity. In experiment 2, disease severity values in plants incubated at 10, 15, 20 and 25°C were lower than in experiment 1. However, in both experiments the maximum disease severity was observed at 30°C with values between 40 and 45% (Figure 2).

Effect of Peach Leaf Age

A significant effect of leaf age was observed on disease severity caused by *Xap* on peach plants at all temperatures tested. Young leaves (corresponding to the newly formed leaves) were significantly more susceptible to *Xap* infections (40-50%) than mature leaves with lower disease severity values (15-26.8%). When disease severity was assessed regarding the position of the leaf in the twig, a progressive decrease of severity was observed from the apical to the basal placement. Therefore, the younger the leaf the more susceptible was to *Xap* infections (Figure 3).

DISCUSSION

All *Xap* strains were able to infect peach leaves, although some of them were originally isolated from other host species. Nevertheless, *Xap* strains isolated from peach were the most virulent on peach leaves. Despite the fact that the virulence of strains varied, these

results confirmed the existence of non-pathogenic specialization and the possibility of cross-infection among hosts (Scortichini et al., 1996).

It is known that *Xap* infection may be produced via natural opening such as stomata, hydathodes or glands (EPPO/CABI, 1997). Nevertheless, under field conditions leaves may have wounds on surface caused by friction in windy days, hail, insect bites, *etc*., which could also be used by *Xap* to penetrate the host in addition to the natural openings. These wounds could be simulated at the laboratory adding an abrasive agent in the bacterial suspension, such as diatomaceous earth. It was expected that artificially wounded leaves would be more sensitive to *Xap* infections in agreement to the reported mechanical transmission of *Xap* during pruning in plum nurseries (Goodman and Hattingh, 1986) or the results obtained in the evaluation of inoculation techniques (Socquet-Juglard et al., 2012). However, there were no differences in disease severity produced by *Xap* infections in artificially injured peach leaves compared to unwounded leaves; in both cases the disease severity was similar. The reason for the similar severity values in both treatments (wounded and unwounded leaves) could be that all parameters were optimal for *Xap* infections succeed. Plants were grown at the greenhouse and they were well irrigated, thus stomata were hydrated and opened to transpire. *Xap* might have used those natural openings to produce infections and artificial wounds did not increase the disease severity. In addition, young leaves were selected to be inoculated with a high concentration of *Xap* applied with pressure by pulverization. Otherwise, under field conditions there are not such favourable conditions for infections. Firstly, stomata are not always open for the penetration and infection of *Xap*, because the stomata opening plays an important role in the hydric balance of the plant and it is conditioned by the water potential in soil and atmosphere. Secondly, the cuticle of plants under field conditions may be thicker than the cuticle of plants grown in the greenhouse, forming a physical barrier that resists the penetration of pathogens.

Similarly, the pressure at which *Xap* suspensions were inoculated on the surface of leaves did not had a significant effect on bacterial infection. Nevertheless, pulverization of pathogen at a pressure of 50 kPa resulted in large drops of bacterial suspension, not optimal for uniform distribution of pathogen on leaves. On the other hand, pulverization of pathogen at a pressure of 200 kPa produced a high flow of pathogen suspension and a low uniformity of pathogen distribution. The optimal pulverization pressure for uniform distribution of pathogen suspension on leaf surface was 100 kPa, which was selected for spraying the bacterial suspension in further assays.Previous studies concluded that water congestion before inoculation followed by a period of leaf wetness after inoculation was necessary for symptom development of *Xap* infections (Zehr and Shepard, 1996). Our study demonstrated that 48 h of leaf wetness combined with appropriate irrigation before inoculation caused water congestion in leaves that lead to a rapid entry of pathogen into the leaves through the congested stomata. Additionally, a wetness period of 48 h after inoculation was shown to increase the disease severity compared to 24 h of wetness. However, some infections were observed when neither water congestion nor wetness was present before inoculation, although the severity was significantly lower. To conclude, the presence of water congestion and leaf wetness before and after inoculation may favour *Xap* infections in peach.

The temperature is an important factor for infection and development of plant diseases. *Xap* infections were reduced in plants submitted to low temperatures during the 24 h after the inoculation and increased as the temperature rise. The maximal disease severity was observed at 30°C, in agreement with the optimal temperature for *Xap* growth *in vitro* (Young et al., 1977). At 35°C, the mean value of disease severity decreased, but it had a large variability. The reason of this variability could be related to the fact that the temperature of 35°C is the upper limit for *Xap* growth, previously determined *in vitro* (data not shown).

Our results demonstrated an ontogenic resistance of peach leaves to *Xap*. Leaves completely formed at the moment of inoculation and monitored during the experiments were less infected than newly formed leaves. Therefore, under field conditions disease symptoms would probably be more important in growing shots than in old leaves.

A forecasting model for *Xap* infections in host plants is now in progress in order to improve the efficiency of management actions on stone fruit crops against the bacterial spot disease. The results obtained in the present study are taken into account to develop this forecasting model of *Xap* infections in host plants.

CONCLUSIONS

The following conclusions can be drawn from the study:

- There was a non-pathogenic specialization of *Xap*; consequently, cross-infection among host species could be produced.
- Wounds on leave surface were no necessary for *Xap* infections, which may be produced via natural opening such as stomata, hydathodes or glands.
- The presence of water congestion and leaf wetness 48 h before inoculation favoured *Xap* infections.
- The duration of leaf wetness after inoculation was directly correlated to disease severity.
- Temperatures above 20°C favoured *Xap* infections.
- There is an ontogenic resistance of peach leaves to *Xap* infections. Severity was higher in young leaves than in mature ones.

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Tables

Table 1. List of *Xanthomonas arboricola* pv. *pruni* strains used in this study.

Strain[a]	Host	Origin country
CFBP 3894[b]	Japanese plum	New Zealand
CFBP 3903	Plum	Italy
CFBP 5530	Peach	Italy
CFBP 5563	Peach	France
CFBP 5725	Peach	EUA
IVIA 33	Almond	Spain
IVIA 3162-1	Almond	Spain

[a] CFBP, Collection Française de Bactéries associées aux Plantes (Angers, France); IVIA, Instituto Valenciano de Investigaciones Agrarias (Moncada-Valencia, Spain)
[b] Pathotype strain

Figures

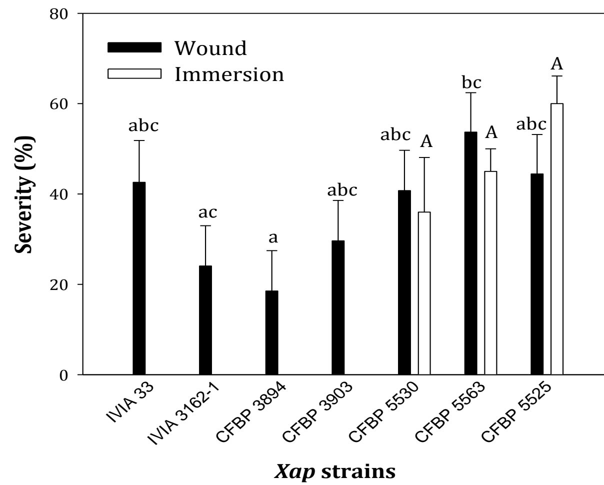

Figure 1. Virulence of *Xanthomonas arboricola* pv. *pruni* strains inoculated on peach cv. Big Top detached leaves, by deposition on a wound or by unwounded leaf immersion. Disease severity was recorded 14 days after inoculation. Values are the mean of five leaves. Error bars are the standard error. Different letters on a bar indicate significant (p=0.05) differences in virulence among strains inoculated on wounded leaves (lower case) or by leaf immersion (upper case).

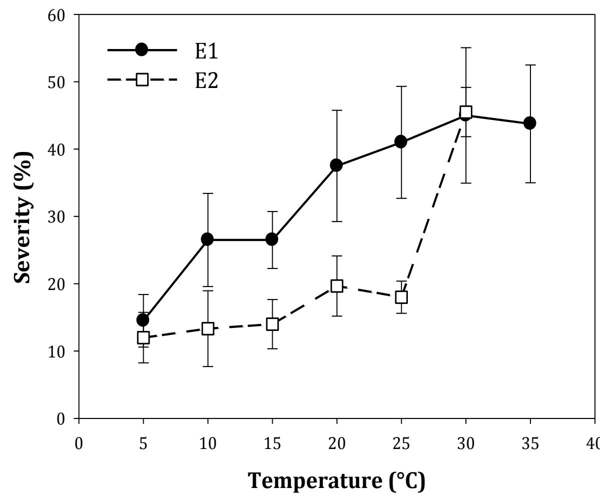

Figure 2. Temperature effect on *Xanthomonas arboricola* pv. *pruni* infection in peach plants cv. Big Top. Plants were exposed to different temperatures for 24 h after inoculation with *Xap* CFBP 5563 suspensions (10^9 CFU ml^{-1}), and then incubated at optimal conditions for disease development for 21 days. Values are the mean disease severity of five plants at the end of incubation period. Two independent experiments are presented (E1 and E2). Error bars are the standard error of mean.

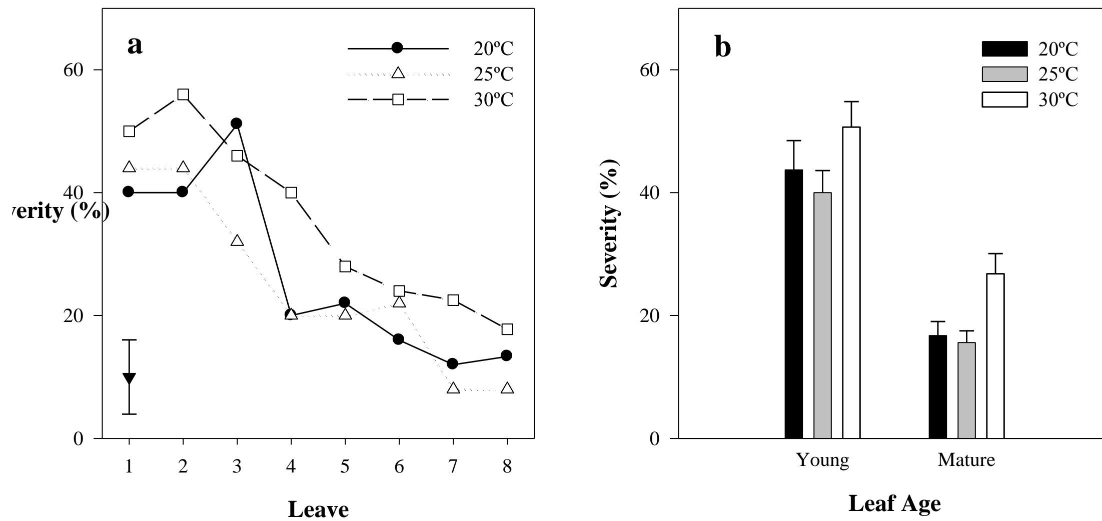

Figure 3. Leaf age effect on disease severity caused by *Xanthomonas arboricola* pv. *pruni* in peach plants cv. Big Top. Disease severity was assessed 14 days after inoculation with *Xap* CFBP 5563 suspensions (10^9 CFU ml^{-1}). Plants were incubated for 24 h after inoculation at different temperatures and then transferred to optimal conditions for disease development. Severity values are the mean of ten plants. Error bar (lower left corner) represents the mean standard error. (a) Leaf position is indicated for a total of eight leaves in a plant from the apical leaf (1) to the basal leaf (8). (b) Leaf age was considered as young for leaves not fully expanded at the moment of inoculation and mature for those completely formed before inoculation. Different letters indicate differences among treatments according to a least significant difference test (p=0.05).