Susceptibility of Apple Hypanthium to *Erwinia amylovora* in Relation to Flower Age and Cougarblight Model

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Abstract

Fire blight risk in the northwestern United States is widely determined with the Cougarblight model, involving a 4-day temperature evaluation developed on the assumption that flower stigmas support growth of *E. amylovora* for only a few days. Studies relating flower age to stigma receptiveness to bacteria did not offer a satisfactory explanation for the effectiveness of the model, and thus, the relation of flower age to hypanthial infection was investigated. Direct inoculations of flower hypanthia of various ages were performed with detached crab apple flowers in the laboratory and with Gala apple flowers in the orchard in 2005 and 2006. In both environments, disease incidence decreased with flower age. Rate of declining susceptibility was dependent on temperature in the laboratory. Regression analysis of orchard data indicated an inoculum dose effect, with steepest decline of susceptibility occurring during the initial days after petal expansion. In 2005, when conditions were most favorable for disease, the pathogen level of $10^4$ CFU (representing a maximum population due to wetting of a few hours according to laboratory tests) caused a disease incidence that decreased from 40 to 18% for flowers 0 to 4 days old and from 12 to 9% for flowers 5 to 8 days old. Cougarblight and other models now incorporating a blossom-age component (e.g., Maryblyt™) may be explained by multiple factors. Our general understanding is that high temperatures during bloom allow *E. amylovora* to reach maximal levels of population on stigmas within a time period when hypanthia are still highly susceptible to infection upon wetting. Further study of these interrelationships could lead to improvements in fire blight risk assessment.

INTRODUCTION

Fire blight of apple and pear is generally initiated when *Erwinia amylovora* colonizes the stigmas of blossoms at a temperature-dependent rate and, upon wetting, moves to the cup-shaped hypanthium where the pathogen enters through nectarthodes (Rosen, 1936; Thomson, 1986). Management systems have emphasized risk assessment (Billing, 2000) and suppression of *E. amylovora* on floral parts with antibiotics or other agents (van der Zwet, 1979).

The Cougarblight model was developed in the early 1990s (Smith, 1993, 1999) for assessing the risk of fire blight in pome fruit production areas of the northwestern United States. An important feature, which was distinctive at its inception, is a moving 4-day temperature evaluation that theoretically accounts for flower age; a running sum of degree hours above 15.5°C on 3 days before the day of a wetting event is added to those on the day of wetting. The parameters were established after applying different base temperatures (15.5 and 18.3°C) and periods (1, 2, 3, 4 and 6 days) to past weather data and actual infection events spanning more than 20 years. Other models at the time were based on temperatures of single days or the accumulation of degree hours throughout the bloom period (Billing, 2000). Maryblyt™, a model developed in the eastern US (van der Zwet et al., 1994), added a blossom-age component that became available with Version 4.3 in 1996 (G.W. Lightner, pers. commun.). According to this system, apple blossoms older than 44.4 degree days, using a base of 4.4°C, are unlikely to be infected upon wetting.
wetting; this equals approximately 4 d at an average temperature of 15.6°C.

An assumption of the Cougarblight model is that flower stigmas support bacterial growth only for a few days (Smith, 1999). The appropriateness of a 4-day temperature evaluation seemed at first to be confirmed by experiments of Thomson and Gouk (2003), in which apple stigmas inoculated at ages exceeding 3 or 4 days did not support growth of *E. amylovora*. In the same study, however, introduction of the pathogen within 4 days resulted in bacterial growth extending for up to 6 or 8 days from the time of flower expansion. A similar phenomenon occurred with *Pseudomonas fluorescens* strain A506 on pear blossoms (Lindow and Suslow, 2003). Populations of this antagonist increased during 12 or 13 days to approximately 10^6 CFU on flower stigmas inoculated within 5 days of opening and persisted at high levels for even longer periods. Eventual populations were shown to decrease with further increases in flower age at inoculation. The authors suggested possible competition with indigenous organisms because population sizes of strain A506 and indigenous bacteria had an inverse curvilinear relationship. In experiments with apple, enclosures surrounding trees were used to reduce insect dispersal of test bacteria and indigenous organisms, and to manipulate temperature (Pusey and Curry, 2004). Under low temperatures (average of 13.8°C), blossom stigmas supported growth of *E. amylovora* when inoculated at ages up to 12 days; under high temperatures (average of 22°C), stigmas supported pathogen growth when inoculated up to 5 or 6 days of age. Based on the above recent reports, apple and pear blossoms apparently have the capacity to support bacterial colonization and growth at ages much older than 4 days. Therefore, the effectiveness of Cougarblight in assessing disease risk must involve other factors than stigma receptiveness to *Erwinia amylovora*.

The relation of flower age to infection has not been well established. Thomson and Gouk (2003), who focused on apple stigma age and bacterial colonization, also noted that incidence of infection was significantly higher in flowers 1 to 3 days old than in flowers 5 to 8 days old. About the same time, Taylor et al. (2003) inoculated stigmas of newly opened apple blossoms with various bacterial concentrations, enclosed them with polyethylene for 24 h and detected symptoms when total levels of *E. amylovora* per blossom (whole flowers were crushed in the procedure) exceeded 10^6 CFU within 4 days. In both of these studies, levels of *E. amylovora* in the hypanthium, and time and avenue of infection, were undetermined. The objective in the present report was to determine the susceptibility of apple hypanthia of various ages to pathogen levels resulting from typical wetting events and to assess how these factors may fit the Cougarblight disease risk assessment model (Smith, 1999) or other models incorporating a blossom-age component.

**MATERIALS AND METHODS**

**Pathogen and Host**

*Erwinia amylovora* strain Ea153N, resistant to nalidixic acid, was obtained from K. Johnson (Oregon State University, Corvallis).

Laboratory experiments were performed with detached blossoms from crab apple trees (*Malus* sp. 'Manchurian' on M26 rootstock) as previously described (Pusey, 1997). Flowers were maintained with cut pedicle in 10% sucrose and at 90% relative humidity.

In 2005 and 2006, a field experiment was conducted with a 1994 research apple planting (*Malus pumila* 'Gala' on M7 rootstock) at Columbia View near Wenatchee, WA.

**Laboratory Experiments**

Detached crab apple blossoms were held for various periods (0, 1, 2, 3, 4, 5, 6, 8 and 10 days) at different temperatures (10, 14, 18 and 22°C) prior to inoculation. A 2.5-μl volume of bacterial suspension (10^6 CFU/ml) was directed into the hypanthium of each flower with a micropipet. Flowers were incubated at 24°C for 5 days and rated for necrosis.

To evaluate the transfer of *E. amylovora* from stigmas to hypanthium during wetting, detached flowers were inoculated on stigmas as described previously (Pusey,
1997) before artificial wetting and incubation at 24°C and 90% relative humidity. Water was applied with a hand pump pressure sprayer delivering 0.1- to 2.0-μl droplets for 0.4, 2.0 or 4.0 min to simulate approximate rain (or dew) of 2, 10 or 20 mm, respectively. Flowers were held at 100% relative humidity and 14°C (near average during apple bloom in Washington) for 0, 3, 6, 12 or 24 h, then allowed to air-dry at room temperature (approx. 22°C) in a laminar flow hood for 45 min. This eliminated free moisture from more exposed flower parts (i.e., petals, calyx and anthers). Population sizes of *E. amylovora* on pre-wetting stigmas and hypanthia, and hypanthia of all other flowers after wetting, were estimated as described previously (Pusey, 2000).

**Field Experiment**

Fifteen ‘Gala’ apple trees with sufficient numbers of blossom clusters were selected within the orchard block. On each of nine successive days, six newly opened flowers per tree were tagged. Each tagged flower was in separate cluster, and all other flowers (or buds) in that cluster were removed; in 2006, one extra flower was tagged in two of the six designated clusters (per tree and date) for later estimation of bacterial population size. On the ninth day, all tagged flowers were inoculated by directing 10 μl of cell suspension into the hypanthium as described for laboratory experiments. Three suspension concentrations (10<sup>6</sup>, 10<sup>7</sup> and 10<sup>8</sup> CFU/ml) were used, and each concentration was applied to five trees selected randomly.

Irrigation was applied the day prior to inoculation to raise soil moisture and maximize water potential in trees. Inoculation was performed in 2005 during overcast conditions between 04:00 and 19:00. In 2006, inoculations were made between 17:30 and 20:30; flowers were inoculated on the shaded east side of trees while the sun was still above the horizon and on the west side of trees after the sun disappeared behind an eastern-facing slope. At between 16 and 18 h after inoculation in 2006, extra inoculated flowers were collected, and population size of *E. amylovora* in hypanthia estimated.

Each tagged flower was evaluated for disease symptoms only once, 12 or 13 days after inoculation, since prior experience indicated disease incidence from direct inoculations of hypanthia changes little after this period. Accumulated degree days >12.7°C from time of inoculation to disease evaluation was near 57, the value established in the MARYBLT model (van der Zwet et al., 1994) for predicting early symptom development.

**Statistical Analyses**

Single-flower replications (10 per treatment) were used in the laboratory; in the field, sub-samples of flowers per treatment and sampling date were averaged for each single-tree replication before obtaining means and standard errors. Data were subjected to nonlinear regression analysis using SigmaPlot 9.0 (SYSTAT Software, Inc., Point Richmond, CA). Data sets from two separate trials of laboratory experiments were tested for homogeneity using Hartley’s F-max test, and then pooled prior to further analysis.

**RESULTS**

**Laboratory Experiments**

Severity and incidence of infection initiated through the hypanthium decreased with flower age at a rate dependent on temperature. Inoculation of newly opened flowers resulted in maximal severity ratings and an infection incidence of 100%. At the low temperature of 10°C, incidence decreased by 40% when flowers were inoculated at 10 days of age. In contrast, flowers held at 22°C for 8 or 10 days prior to inoculation exhibited no disease symptoms.

In a second experiment, quantity of bacterial cells transferred from stigmas to hypanthium during wetting was evaluated. Prior to wetting, population size on the inoculated stigmas was near 10<sup>8</sup> CFU per flower and no bacteria were detected in hypanthia. Simulated rain at levels from 2 mm to 20 mm followed by incubation at 14°C
and wetness periods extending up to 12 h resulted in bacterial levels near 3.0 log CFU or less per flower hypanthium. After a 24-h wetting period, the population size of *E. amylovora* in the hypanthium ranged from 3.8 to 5.2 log CFU per flower, depending on amount of water applied.

**Field Experiment**

During the 8 days of tagging new flowers in the orchard experiment, temperatures averaged 13.0°C in 2005 and 14.5°C in 2006. Rate of flower development and senescence was similar in the two years. Petals began dropping from flowers 4 days of age and were absent by 7 days. In 2005, rain began near the end of inoculation and lasted 1.5 h, with an accumulation of 3.8 mm; no rain or surface wetness was recorded in 2006.

At 16 to 18 h after inoculation in 2006, population size of *E. amylovora* was similar for all flower ages, averaging >1.0 log unit below inoculum levels. With the detection limit at 10^2 CFU per flower, 4.8±0.22 (standard error) log CFU were recovered per flower inoculated with 10^6 CFU (applied as 10 μl of 10^8 CFU/ml), and 2.9±0.13 log CFU were recovered per flower inoculated with 10^4 CFU. Bacteria were detected in only 19% of flowers inoculated with 10^2 CFU.

In 2005, newly opened blossoms (day 0) inoculated with 10^6 CFU of *E. amylovora* per flower showed a disease incidence of 88%. Based on a curve (R^2=0.93), the incidence decreased to about 58% and 34% when flowers were inoculated at 4 and 8 days old, respectively. Inoculation with 10^5 CFU per flower resulted in a disease incidence of 38% in flowers inoculated at day 0, decreasing on a curve (R^2=0.73) to 16% and 8% in flowers inoculated at day 4 and 8, respectively. Incidence for inoculations with 10^4 CFU per flower were negligible, not exceeding 6.7%.

In 2006, inoculation with 10^5 CFU per flower at day 0 resulted in a disease incidence of 72%, based on curve (R^2=0.93), decreasing to 36% at day 4 and 28% at 8 days. For inoculations with 10^4 CFU, fit of data with quadratic equation was marginal (R^2=0.63); actual mean disease incidence in flowers inoculated at day 0 was 36%, and incidence for flowers of all other ages ranged from 0 to 15%. None of the flowers inoculated with 10^2 CFU exhibited disease symptoms.

**DISCUSSION**

This study demonstrated that pomaceous blossoms become less susceptible to invasion of hypanthial tissues by *E. amylovora* as flowers age. Temperature and dose effects were also significant. One cell of *E. amylovora* can potentially infect pomaceous flowers through the hypanthium (Hildebrand, 1937); however, the minimum infective dose generally depends on environmental conditions (Pusey, 2000), pathogen aggressiveness (Cabrefiga and Montesinos, 2005) and likely also cultivar susceptibility (van der Zwet and Keil, 1979). In our field experiment, the inoculum level of 10^2 CFU per flower hypanthium caused a very low disease incidence in 2005 and resulted in no detectible disease symptoms in 2006.

The quantity of epiphytic bacteria transferred to the flower hypanthium during wetting is limited by populations on the stigma, which reach maximal levels between 10^8 and 10^9 CFU per flower (Johnson and Stockwell, 1998). When crab apple stigmas bearing nearly 10^9 CFU per flower were subjected to simulated rain (or dew) and a wetness period of up to 12 h, bacterial numbers in the hypanthium were generally 3 to 4 log units below those of pre-wetting stigmas. In a pear field study involving natural wetness periods not exceeding 5 h (Thomson, 1986), populations of *E. amylovora* in hypanthia were only 1 to 2 log units smaller than populations on stigmas. These different findings may be explained by the fact that hypanthia of pear are more open compared to apple and crab apple, and thus, likely to receive and accumulate more bacterial cells moving via water from stigmas. When wetness on crab apple flowers was extended for 24 h, bacterial populations in crab apple hypanthia increased to within 1 or 2 log units of stigma populations, perhaps due more to cell multiplication than further migration. Given the above laboratory results with crab apple, and that bacterial populations on apple stigmas
can be as large as $10^7$ CFU per flower (Pusey and Curry, 2004), the inoculum dose of $10^4$ CFU per flower hypanthium in our field test appears to be near the upper limit of bacterial numbers transferred from apple stigmas to hypanthia during a few hours of wetness. The dose of $10^6$ CFU may represent a level that can be reached or exceeded when wetting is prolonged and temperatures favor significant multiplication of bacteria in the hypanthium (Pusey, 1997; Wilson et al., 1990). A return to dry conditions will generally lead to increased nectar concentration and osmotic pressure in young hypanthia, causing a slowing or cessation of bacterial growth and eventual decline in viable cell numbers (Ivanoff and Keitt, 1941; Pusey, 1999).

In general, our field results were consistent with those of others who used different methods to examine the relationship between flower age and infection by *Erwinia amylovora*. Thomson and Gouk (2003) observed a greater incidence of disease in flowers 1 to 3 days old than in flowers 5 to 8 days, and Taylor et al. (2003) detected symptoms when total levels of *E. amylovora* per blossom (epiphytic and endophytic) exceeded $10^6$ CFU within 4 d. In our 2005 field experiment, when moisture levels were high, a pathogen level of $10^4$ CFU caused a disease incidence that decreased along a quadratic curve ($R^2=0.73$) from 38 to 16% for flowers 0 to 4 d old and from 13 to 8% for flowers 5 to 8 days old.

To assess the role and importance of hypanthial susceptibility within Cougarblight and other models incorporating a blossom-age component, we must examine it in the context of other factors known to be important for infection to occur. Growth rate of *E. amylovora* on flowers, as dependent on temperature, is a primary basis of fire blight risk assessment (Billing, 2000). Also, longevity of floral parts (Soltesz et al., 1996) and duration of their receptivity to bacterial colonization (Pusey and Curry, 2004; Thomson and Gouk, 2003) is temperature dependent. Based on laboratory results presented here, duration of hypanthial susceptibility to infection is likewise affected by temperature. How pathogen and host change in relation to each other in response to temperature has not been addressed, but may be critical. As temperatures increase within the range supporting multiplication of *E. amylovora*, the rate of bacterial growth may increase more dramatically relative to the rate at which flowers senesce and become less vulnerable to colonization and infection. When newly-opened detached flowers are routinely inoculated and incubated at a constant of 24°C (which exceeds typical temperature averages in the orchard), *E. amylovora* increases from $10^5$ CFU to $10^6$ or $10^7$ CFU per flower within 24 h (Pusey, 1997; Pusey and Curry, 2004), and yet flowers appear only slightly changed (Pusey, unpublished). Petals are still attached and stigmas are light green with papillae mostly turgid. Thus, high temperatures can potentially lead to high epiphytic populations of the pathogen before significant flower senescence occurs. A conceptual diagram of how disease susceptibility of the hypanthium may relate to other factors and disease risk is presented in Fig. 1. The dynamics of host and pathogen are further complicated by the nonsynchronous emergence of flowers and overall increase of inoculum as bacteria spread from old to new flowers. Nevertheless, while flowers are still emerging, it seems reasonable that higher temperatures will result in higher percentages of blossoms that can potentially be colonized within a period of a few days and then infected when wetting occurs. Cougarblight and other models with similar blossom-age components may be explained by the critical effect of temperature on multiplication of *E. amylovora* within a flower-age window that is highly conducive both to growth on stigmas, and upon wetting, to invasion of hypanthia through nectariods. Additional research establishing with greater accuracy the above interrelationships could lead to advancements in fire blight risk assessment.

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Literature Cited


Fig. 1. Model of how hypanthial susceptibility to *Erwinia amylovora* for a single flower may relate to other factors as flower ages. Panels represent high-temperature (A) and low-temperature (B) scenarios. Solid line represents decreasing susceptibility of hypanthium, long-dashed curve represents growth of *E. amylovora* on the stigma, and short-dashed curve represents population of *E. amylovora* in the hypanthium due to wetting. The shaded area represents the disease risk, which peaks early under high temperature; by contrast, risk is delayed and reduced under low temperature.