

Effect of inoculum dose on infection of apple (*Malus domestica*) flowers by *Erwinia amylovora*

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Abstract The effects of inoculum dose, flower age, and climate (temperature, relative humidity, and rainfall) on the incidence of fire blight following inoculation of apple (*Malus domestica*) flowers with *Erwinia amylovora* were studied in an orchard. Disease symptoms were only observed when populations of *E. amylovora* exceeded 10^6 colony-forming units (cfu) on flowers 4 days after budburst. The rate at which these populations were reached depended on the climatic conditions over flowering. Ten cfu of *E. amylovora* inoculated on flower stigmas were capable of inciting fire blight in an orchard in which temperatures above 15°C and high rainfall provided ideal conditions for bacterial multiplication, infection, and subsequent expression of symptoms in flowers. The results from this study may lead to insights into improved management strategies and provide key information for phytosanitary risk assessments for exports of commercial apple fruit.

Keywords *Erwinia amylovora*; fire blight; inoculum threshold; infection

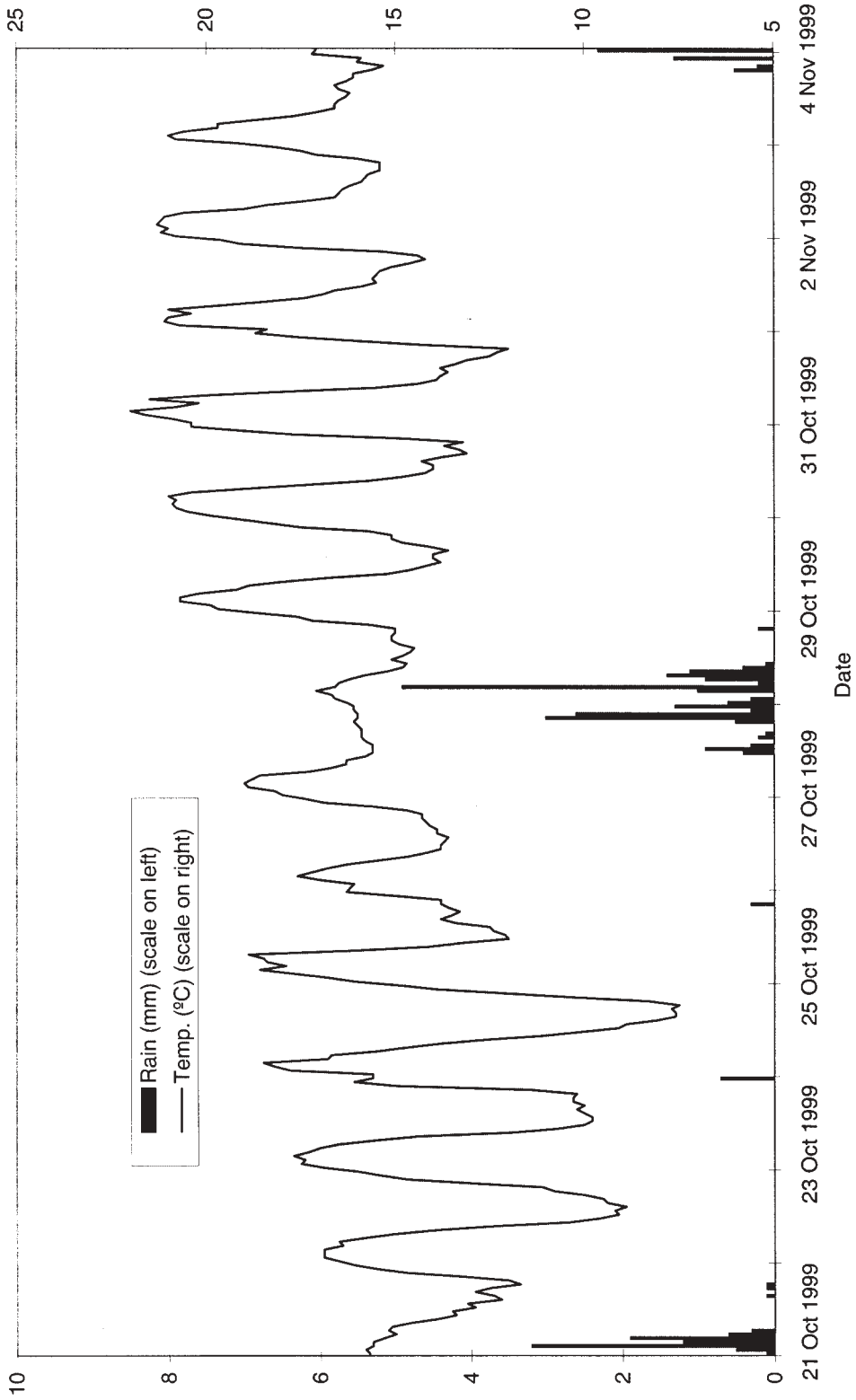
INTRODUCTION

Erwinia amylovora (Burr.) Winslow et al. 1920, causes fire blight of rosaceous plants and is an important bacterial disease of apple (*Malus domestica* Borkh.) and pear (*Pyrus communis* L.). Symptoms of fire blight on flowers include water-soaking and blackening of flowers and, under humid conditions, exudation of a cream-coloured bacterial ooze. *E. amylovora* predominantly invades the host via susceptible flowers through natural openings and has been reported to grow epiphytically on the stigma and infect via the nectaries (Miller & Schroth 1982; Thomson 1986). The significance of small bacterial populations, and their effect on infection and on the epidemiology of fire blight, is unclear. Hildebrand (1937) reported that a single bacterium was capable of causing infection on apple flowers incubated in growth chambers at 24°C. Ivanoff & Keitt (1941) also demonstrated that artificial inoculation with 10 colony-forming units (cfu) in the greenhouse could result in infection of pear flowers. However, in other greenhouse studies, Norelli & Beer (1984) found that pear flowers inoculated with 10^2 cfu did not develop symptoms. All of these studies were confined to *in vitro* or greenhouse conditions and are not necessarily indicative of what occurs in an orchard environment where ultra-violet (UV) radiation and fluctuating climatic conditions need to be taken into account.

In orchard studies naturally occurring bacterial populations of 10^3 – 10^7 cfu have been detected on stigmas of flowers of several rosaceous hosts (Thomson 2000). However, because bacterial multiplication may occur in flowers, the epiphytic population size present before infection occurred was not estimated. Previous reports on inoculum threshold levels required to cause infection have shown that there is a direct correlation between inoculum dose and symptom development (Norelli & Beer 1984;

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Pukekohe



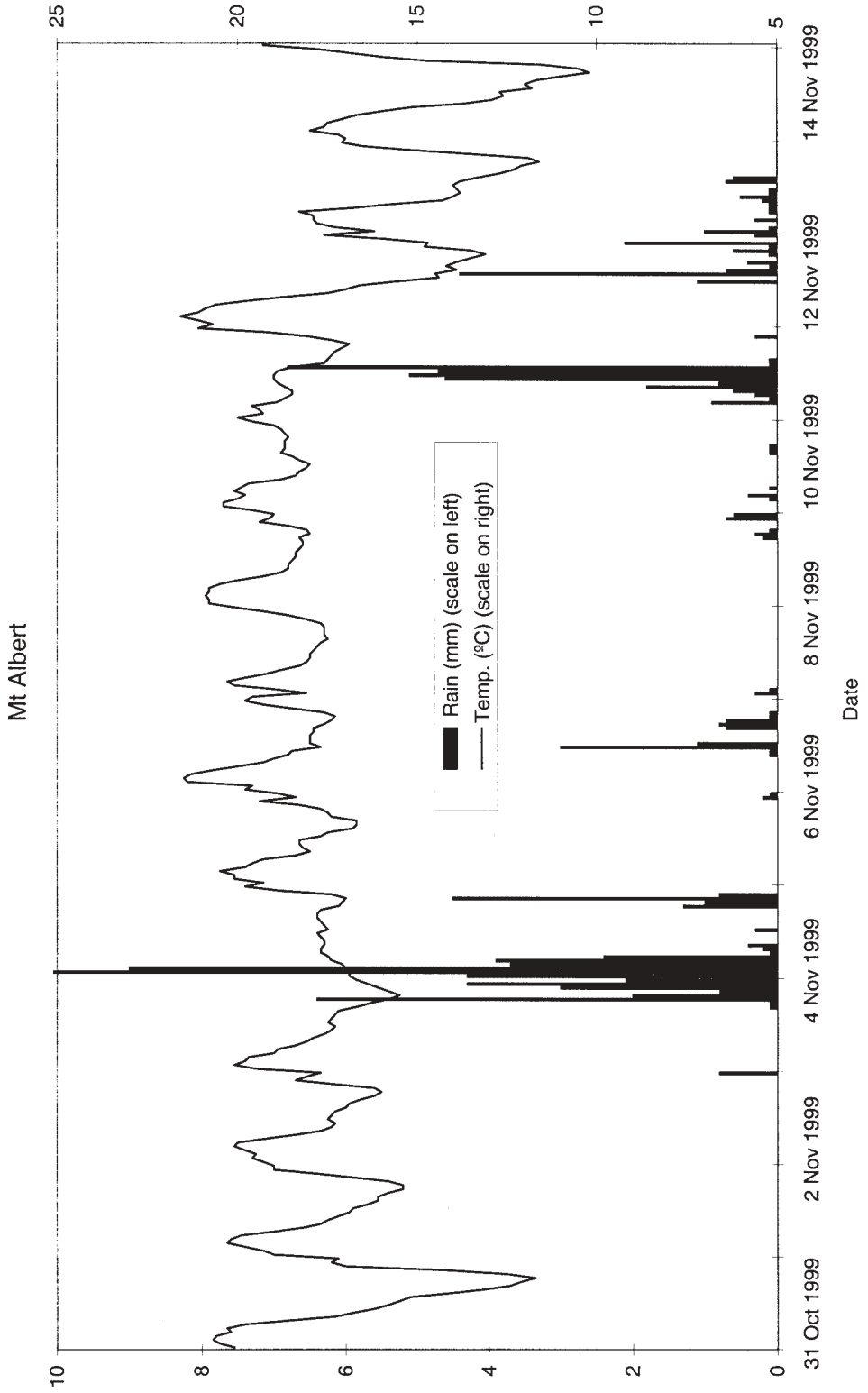


Fig. 1 Mean rainfall and temperatures recorded at the Pukekohe (PRS) and Mt Albert (MARC) orchards following inoculation with *Erwinia amylovora*.

Hale et al. 1996). These studies showed that inoculum concentrations of c. 10^2 – 10^3 cfu did not cause disease of flowers, whereas flowers inoculated with 10^7 cfu were likely to be diseased 5–8 days later. In New Zealand, Hale et al. (1996), in attempts to determine the inoculum threshold required to cause infection, showed that flowers inoculated with 10^0 – 10^4 cfu did not develop disease symptoms even though the pathogen was still detected by a PCR assay several days later. However, whether or not disease incidence was quantitatively related to the size of the epiphytic pathogen population in the orchard was not determined.

The minimum inoculum dose required to cause infection of apple flowers in New Zealand orchard conditions has not been studied. The objective of this study was to determine the quantitative relationship between flower-associated population levels of *E. amylovora* and subsequent disease severity in the orchard in order to determine the minimum inoculum threshold level necessary to cause fire blight. Other factors studied included the time between inoculation and infection, flower age and susceptibility, and the effect of climatic conditions. Elucidating the effects of these factors may enable the prediction of infection risk by *E. amylovora* and lead to improved management strategies. Key information can then be provided about the probability of small populations of *E. amylovora* inciting outbreaks of fire blight for phytosanitary risk assessments for exports of apple fruit.

MATERIALS AND METHODS

Research site and climate conditions

The experimental work was conducted in 1999 in apple orchards at the New Zealand Institute for Crop and Food Research, Pukekohe Research Station (PRS), Pukekohe, New Zealand and The Horticulture and Food Research Institute of New Zealand, Mt Albert Research Centre (MARC), Auckland, New Zealand. The population development of *E. amylovora* was monitored on the apple 'Granny Smith' (slightly resistant to fire blight) and 'Royal Gala' (susceptible to fire blight) at PRS, and on the 'Royal Gala' at MARC, after inoculation with different concentrations of the fire blight bacterium. Climate data, including maximum, minimum, and average temperatures, humidity, and rainfall were recorded hourly throughout flowering using Campbell scientific weather stations located adjacent to the orchards (Fig. 1). Climate data were analysed using the Cougarblight Fire Blight Risk Assessment Model (Smith 1996) to determine if conditions were conducive for the establishment of *E. amylovora* and the development of the disease.

Inoculum preparation

A marked strain of *E. amylovora* (ICMP 8865^{NR}), resistant to rifampicin and nalidixic acid, obtained from Dr S. C. Gouk, was used as inoculum. The strain used was a stable, spontaneously derived mutant, which had been shown to be as pathogenic

Table 1 Severity of fire blight symptoms and population levels (colony-forming units (cfu)/flower) of *Erwinia amylovora* present on apple (*Malus domestica*) flowers after inoculation with different inoculum concentrations of bacteria. (MARC, Mt Albert Research Centre, The Horticulture and Food Research Institute of New Zealand, Auckland, New Zealand; PRS, Pukekohe Research Station, New Zealand Institute for Crop and Food Research, Pukekohe, New Zealand; FB, fire blight infection; PBS, phosphate buffered saline.)

Inoculum conc.*	MARC orchard cv. Royal Gala		PRS orchard cv. Royal Gala		PRS orchard cv. Granny Smith	
	FB	cfu [†]	FB	cfu [†]	FB	cfu [†]
10^7	100 [‡]	3.74×10^8	100	2.06×10^{11}	100	3.41×10^{10}
10^5	100	2.26×10^8	100	1.41×10^9	100	6.06×10^8
10^4	100	1.66×10^8	75	4.44×10^9	75	1.06×10^8
10^3	100	2.38×10^8	50	3.96×10^8	50	6.78×10^7
10^2	100	2.70×10^8	0	6.15×10^5	0	1.88×10^7
10^1	100	1.11×10^8	0	1.54×10^6	0	3.41×10^3
PBS	0	0	0	0	0	0

*Initial concentration of *E. amylovora* inoculated onto the flower stigma.

[†]Mean cfu/flower 10 days after inoculation. Population levels are averaged over 10 individual flowers per sampling time. Mean cfu refer to all sampled flowers.

as other strains of *E. amylovora* in infection studies (Taylor et al. 2002). The use of this strain allowed the presence of inoculated *E. amylovora* on apple flowers to be monitored and populations levels on inoculated flowers over the flowering period to be determined. Rifampicin and nalidixic acid resistance are stable and selective markers used to study the ecology and epidemiology of bacteria in soils and other environments (Glandorf et al. 1992). Inoculum was prepared from cultures incubated at 26°C for 16 h on CCT medium (Ishimaru & Klos 1984) supplemented with 100 µg/ml of rifampicin and 100 µg/ml nalidixic acid. Bacterial cells were harvested and suspended in sterile phosphate buffered saline (PBS) (0.1M, pH 7.00). Initial inoculum concentration was adjusted spectrophotometrically ($\lambda = 600$ nm) to provide 10^9 cfu per ml and verified by dilution plating and counting *E. amylovora* colonies after incubation at 26°C for 20 h on CCT medium.

Inoculations

Individual flowers in clusters were inoculated as they opened in the orchard by pipetting 10 µl droplets of bacterial suspension onto the stigmas. The 10 µl droplet was calibrated to deliver a final concentration of 10^7 , 10^5 , 10^4 , 10^3 , 10^2 , or 10^1 cfu on individual apple flowers. Apple flowers, similarly inoculated with PBS + 0.05% Tween 20, served as controls. For each variety 250 apple flowers were inoculated with each inoculum concentration and with PBS + 0.05% Tween 20. Inoculated flower clusters were enclosed in polyethylene chambers that were then covered with aluminum foil to prevent heat build up. The temperature inside the chambers was monitored for 24 h after inoculation by measuring wet and dry bulb temperatures with YSI 44005 thermistors inserted in the chambers. Hourly mean temperatures were recorded on a LiCor LI-1000 data logger. The chambers were removed from the flower clusters 24 h after inoculation.

Sampling and recovery of *E. amylovora* from apple flowers

Apple flowers were visually assessed for the presence of fire blight. At 1, 2, 4, 6, 8, 10, and 12 days after inoculation 10 flowers were sampled for each inoculum concentration from 'Granny Smith' and 'Royal Gala' at the PRS orchard and from 'Royal Gala' at the MARC orchard.

To recover *E. amylovora*, individual flowers were each placed in 1 ml of sterile PBS in 1.5 ml Eppendorf microcentrifuge tubes. Flowers were crushed using a sterilised stainless steel rod and then

mixed using a vortex mixer (Chiltern™) for 30 s to elute *E. amylovora* from the flower material. Appropriate dilutions of the flower suspensions were made in PBS and were duplicate-plated on CCT medium supplemented with 100 µg/ml rifampicin and 100 µg/ml nalidixic acid. Plates were incubated at 26°C for 48 h after which *E. amylovora* (ICMP 8865^{NR}) colonies were counted.

Statistical analyses

Population levels on the apple flowers were monitored over the sampling period, based on *E. amylovora* cfu per flower. The mean number of *E. amylovora* per flower was calculated for each inoculum concentration. The population data were log-transformed before analysis and presented graphically as a plot against time after inoculation (Fig. 2, 3). Analysis of variance (ANOVA) and least significant difference tests (LSD) were performed on the data to determine the effect of inoculum concentration on flower-associated populations of *E. amylovora*, and to compare disease progress curves resulting from the different inoculum concentrations. One-way analysis of variance was performed using the GENSTAT statistical package. Fisher's tests were used to calculate LSDs.

RESULTS

Effect of inoculum concentration on disease severity

Severe fire blight symptoms were visible on apple flowers in both orchards by 8 days after inoculation. The severity of disease symptoms in the inoculated flowers, and the populations associated with these flowers, are presented in Table 1. At the PRS orchard fire blight symptoms on apple flowers of 'Royal Gala' and 'Granny Smith' ranged from 0% with symptoms after inoculation with 10^1 and 10^2 cfu to 100% with symptoms after inoculation with 10^4 , 10^5 , 10^7 cfu. At the MARC orchard all the 'Royal Gala' flowers inoculated with 10^7 , 10^5 , 10^4 , 10^3 , 10^2 , and 10^1 cfu developed symptoms. No symptoms were observed on flowers at either orchard when inoculated with PBS + 0.05% Tween 20.

Population dynamics of *Erwinia amylovora* on apple flowers

Details of the changes in populations associated with flowers over the sampling period following inoculation with different concentrations of *E. amylovora*

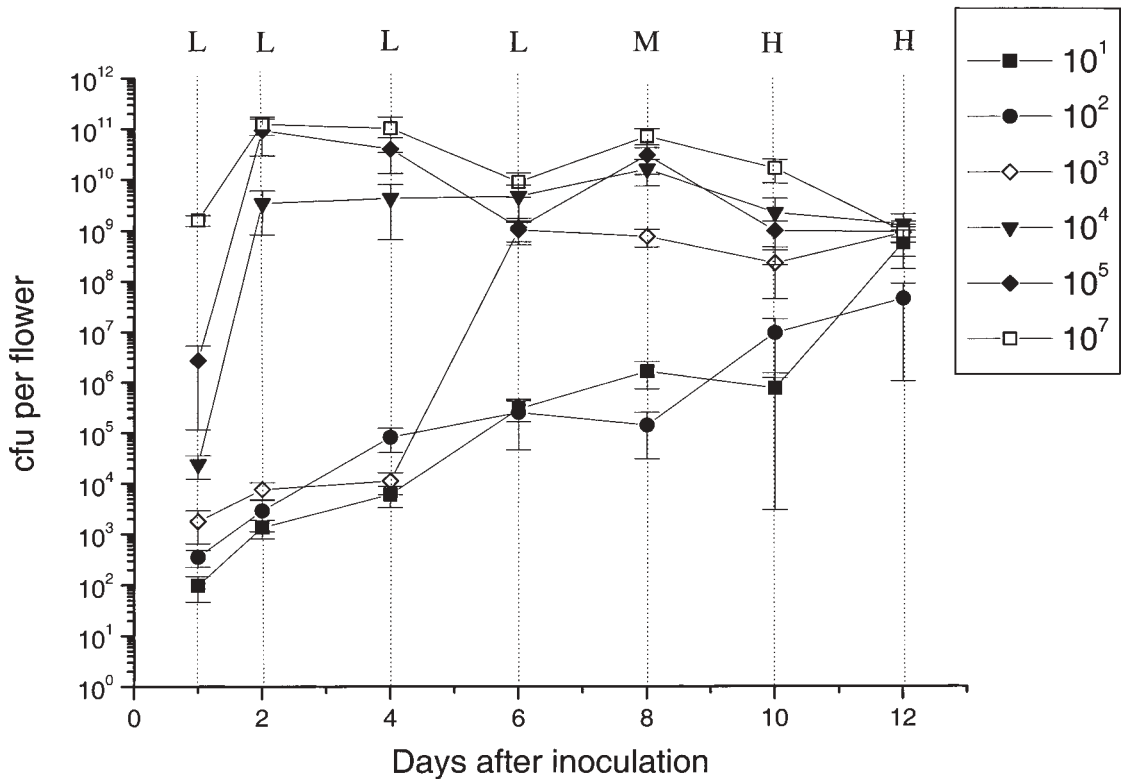


Fig. 2 Populations (colony-forming units (cfu)/flower) of *Erwinia amylovora* detected on apple (*Malus domestica*) flowers at the Pukekohe (PRS) orchard. Population levels are averaged over 20 individual flowers (pooled from 'Granny Smith' and 'Royal Gala') per sampling time and error bars represent standard errors calculated from the standard deviation. Fire blight infection risk: L, low; M, moderate; H, high.

are presented in Fig. 2 (PRS orchard 'Royal Gala' and 'Granny Smith') and Fig. 3 (MARC orchard 'Royal Gala').

At the PRS orchard, populations of *E. amylovora* on 'Royal Gala' flowers increased to $>10^8$ cfu in the first 2 days after inoculation with 10^7 , 10^5 , or 10^4 cfu. Symptoms were seen in most of the flowers at 8 days after inoculation and severe symptoms developed by 10 days after inoculation. Populations of *E. amylovora* on flowers inoculated with 10^3 cfu slowly increased over the 6 days after inoculation to reach 10^9 cfu, resulting in 50% of the flowers with disease symptoms. Populations of *E. amylovora* on flowers inoculated with either 10^2 or 10^1 cfu increased very slowly over the flowering period reaching 10^6 cfu at 10 days after inoculation. No symptoms were seen on these flowers and healthy fruit developed from the inoculated flowers not sampled.

Populations of *E. amylovora* on 'Granny Smith' flowers increased to $>10^{10}$ cfu in the first 2 days after inoculation with 10^7 , 10^5 , or 10^4 cfu. Symptoms were

seen in $>50\%$ of the flowers at 8 days after inoculation and in 100% of flowers inoculated with 10^7 cfu after 10 days. Populations of *E. amylovora* on flowers inoculated with 10^3 cfu increased very little until 6–8 days after inoculation when they reached 10^8 cfu. Symptoms were seen in 30% of these flowers 10 days after inoculation. Populations of *E. amylovora* on flowers inoculated with either 10^2 or 10^1 cfu increased only slowly throughout flowering to reach 10^7 – 10^9 cfu towards the end of the sampling period and no symptoms developed on these flowers. Population data for *E. amylovora* at the PRS orchard were pooled as no significant differences were detected between populations of *E. amylovora* on 'Royal Gala' and 'Granny Smith' flowers (Fig. 2).

At the MARC orchard, populations of *E. amylovora* on 'Royal Gala' flowers increased rapidly to 10^8 cfu immediately after inoculation with 10^7 , 10^5 , and 10^4 cfu and remained high throughout the 10-day sampling period. Populations of

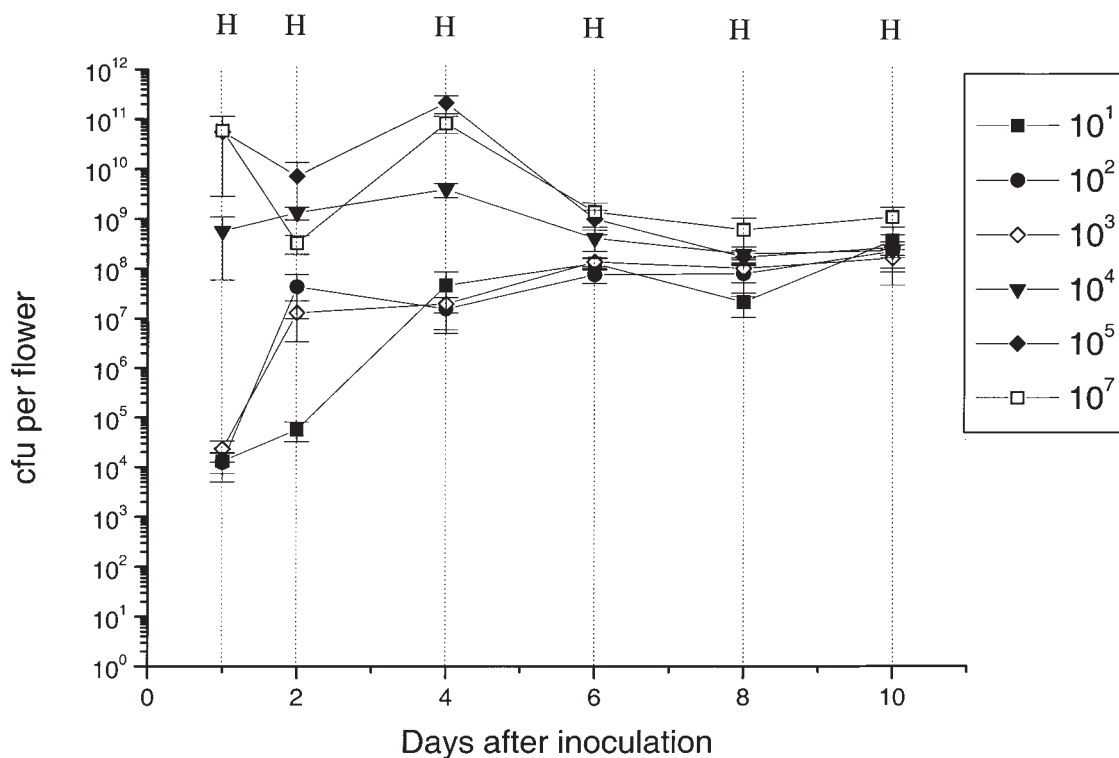


Fig. 3 Populations (colony-forming units (cfu)/flower) of *Erwinia amylovora* detected on apple flowers at the Mt Albert (MARC) orchard. Population levels are averaged over 10 individual flowers per sampling time and error bars represent standard errors calculated from the standard deviation. Fire blight infection risk: L, low; M, moderate; H, high.

E. amylovora on flowers inoculated with 10^3 , 10^2 , and 10^1 cfu increased steadily to 10^7 – 10^8 cfu 4 days after inoculation and remained at that level throughout the 10-day sampling period. Severe symptoms were observed in all the inoculated flowers by 8 days after inoculation. No symptoms were seen in flowers inoculated with PBS + 0.05% Tween 20.

Effect of orchard conditions on infection of apple flowers

In both orchards, temperature and rainfall conditions were conducive for infection. Relative humidity in both orchards was similar. However, the MARC orchard experienced higher daily temperatures and more rainfall than the PRS orchard. Analyses of meteorological data using the Cougarblight risk prediction model showed that there were 12 more infection periods during flowering at the MARC orchard but only four at the PRS orchard (Fig. 2, 3). In both orchards symptoms were only seen on flowers where populations of *E. amylovora* reached

$>10^6$ cfu before 4 days after inoculation. At the MARC orchard population levels of *E. amylovora* on flowers inoculated with 10^2 cfu increased to 10^6 cfu before 4 days resulting in disease symptoms. However, at the PRS orchard, symptoms were not observed on flowers inoculated with 10^2 cfu of *E. amylovora* and populations only increased to $>10^6$ cfu 8 days after inoculation even though conditions were conducive for infection (Fig. 2).

DISCUSSION

The results of this study show that the initial threshold level of *E. amylovora* inoculum capable of inciting a fire blight outbreak is dependent on a number of factors. In the orchard, infection of flowers by *E. amylovora* only occurred when populations had exceeded 10^6 cfu per flower by 4 days after inoculation. In the PRS orchard, flowers inoculated with 10^1 – 10^2 cfu did not develop disease symptoms even

though populations multiplied to levels $>10^6$ cfu after 4 days and the climatic conditions were conducive to infection. The ability of the pathogen to multiply to levels that cause infection within this period was dependent on temperature, rainfall, and flower age. These results are similar to those of Pusey (2000), Shwartz et al. (2003), and Thomson (1986) who found that *E. amylovora* populations on apple and pear flowers increased whether relative humidity was high or low and that the main climatic factors governing flower infection were temperature and rainfall. In a recent study flower age has been implicated in the growth and development of *E. amylovora* on flowers (Thomson & Gouk 2003). Thomson & Gouk (2003) suggested that this might be due to the production of inhibitory compounds produced on the stigma during the degeneration of the papillae preventing colonisation and growth of *E. amylovora*. A population size of c. 10^6 cfu per flower is required, before disease development, for infection to establish. This phenomenon may be due to a function of density-dependent induction of factors influencing virulence or colonisation (Whitehead et al. 2001). Another possibility is that large populations of *E. amylovora* can overcome plant resistance mechanisms.

In the MARC orchard, flowers inoculated with only small inoculum doses of *E. amylovora* (10 cfu) developed fire blight symptoms where populations of the pathogen on flowers increased to $>10^6$ cfu by 4 days after inoculation. Higher temperatures and rainfall, and more high infection-risk periods were recorded at the MARC orchard than at the PRS orchard suggesting that climatic conditions in the orchard played a significant role in both the rate of population increase of *E. amylovora* and the susceptibility of flowers to infection. This study demonstrates that small populations of *E. amylovora* can increase rapidly and cause fire blight in the orchard. The lack of fire blight observed with lower inoculum doses in this study at the PRS orchard and previous studies (Norelli & Beer 1984; Hale et al. 1996) does not necessarily imply that there is a threshold level for infection but suggests that the efficiency of a small population to incite disease is much less than that of a large population. The greater the number of bacterial cells that arrive on the flower stigma, and the younger the flower, then the greater is the probability of rapid colonisation leading to infection and expression of symptoms. This study showed that 100% infection of apple flowers could result from inoculation with 10^5 – 10^8 cfu even when infection-risk periods were low. This result is consistent with the research of Shwartz et al. (2003) which suggested

that a large amount of inoculum might compensate for sub-optimum conditions for infection.

Thomson (1986) and van der Zwet et al. (1988) have suggested that the effects of fire blight could be minimised if flower-associated pathogen populations are kept at low levels. The relationship between population levels, flower age and susceptibility, and climate conditions provides an insight into potential control strategies. Disease control treatments applied within the first 4 days after budburst will restrict the ability of *E. amylovora* to survive and multiply on flower surfaces, delay development of symptoms, and reduce the subsequent disease severity.

The results of this study suggest that large populations ($>10^4$ cfu) of *E. amylovora* in the early stages of flowering are required for successful infection in the orchard. Small populations ($<10^3$ cfu) of *E. amylovora* have a low probability of causing disease as population levels influence the time required to cause infection and disease symptoms and consequently are considered of little epidemiological significance (Thomson 2000). The population levels of *E. amylovora* required to cause disease in this study far exceeded those found in previous studies on infested apples at harvest (Hale et al. 1987; van der Zwet et al. 1990; Clark et al. 1993), after cold storage (Taylor & Hale 2003) and apple discard in an orchard (Taylor et al. 2003). This study confirms that discarded fruit are unlikely to be sources of infection as the small populations associated with apple fruit will not be transferred to flowers on receptive hosts and incite fire blight outbreaks.

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