

FINAL PROJECT REPORT

Project Title: Survival of *Erwinia amylovora* on pear fruit

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Budget History:

Item	Year 1: 2003	Year 2&3: ***	Year 4: 2006
Salaries	19200		16200
Benefits	10176		11010
Wages	1000		
Benefits	50		
Equipment			
Supplies	1500		1290
Travel	2500		250
Miscellaneous	2000		250
Total	36426		29000

***In 2004 and 2005, the project was funded by USDA FAS via Northwest Horticultural Council: \$63K per year divided between OSU and USDA ARS Tree Fruit Res. Lab. Wenatchee.

Objectives:

Overall:

1. Estimate incidence of contamination of d'Anjou pear fruit cultivated in four growing districts in the Pacific Northwest with *Erwinia amylovora*.
2. Evaluate capacity of *Erwinia amylovora* to colonize or persist on pear fruit surfaces.
3. Evaluate internal fruit contamination by *Erwinia amylovora* on trees that were diseased in the spring and remained diseased through the summer until harvest.
4. Evaluate internal and external survival of *Erwinia amylovora* on wounded fruit in cold storage

In 2006:

- 1-06. Evaluate capacity of *Erwinia amylovora* to colonize and persist on the calyx of pear fruit.
- 2-06. Evaluate survival of *Erwinia amylovora* on in fruit wounds during cold storage.
- 3-06. Develop concise summary of 2003-06 data for peer and regulatory review.

Significant findings (overall):

- Over three years, we detected a few cells of *Erwinia amylovora* from only one fruit of ~5000 d'Anjou pear fruit sampled from commercial orchards in the Rogue, Hood River, Yakima, Wenatchee and Okanogan Valleys of the Pacific Northwest.
- *Erwinia amylovora* has a limited survival time on surfaces of healthy pear fruit, and that the survival rates are not different from those observed on mature, symptomless apple fruit.
- Over two years, we were unable to detect *Erwinia amylovora* inside mature symptomless pear fruit harvested from diseased pear trees located at Medford, OR and Wenatchee, WA.

Significant findings (2006):

- Calyx end survival of *Erwinia amylovora* on pear fruit is similar to that observed on apple with high populations detected near petal fall; these populations decline through the summer to nearly undetectable levels at harvest. The few cells of the pathogen that remain detectable at harvest become undetectable by the end of a 6-week cold storage period. In contrast to *Erwinia amylovora*, the non-pathogenic bacterial epiphyte, *Pantoea agglomerans* persists on calyx ends of most fruit through the summer and the period of cold storage.
- For a second year, we found that mature symptomless pear fruit contaminated with *Erwinia amylovora* and subsequently wounded requires an initial dose of >10,000 cells at the wound site to allow for persistence of the pathogen on the fruit through a 7 week cold storage period. By comparing the magnitude of this dose to its likelihood (as determined in our other studies), we conclude the risk of *Erwinia amylovora* establishing itself in small wounds on mature symptomless pear is very small.

Background. Export of winter pears grown in the Pacific Northwest into countries where fire blight does not occur is restricted by phytosanitary concerns over the possible contamination of fruit with the fire blight pathogen, *Erwinia amylovora*. Similar concerns have been applied to apples, but research, risk assessment analyses and trade resolution proceedings have concluded that introduction and successful establishment of *E. amylovora* into a new geographic region via commercial shipments of apple fruit is very unlikely. Reasons for this low likelihood include: 1) viable cells of *E. amylovora* are detected on mature apple fruit only rarely, 2) *E. amylovora* has a low pathogenic capacity on mature apple fruit, and 3) a pathway that demonstrates successful infection of susceptible host material via fruit borne inoculum has never been documented. The purpose of this study is to investigate if the reasons for the low likelihood of movement of *Erwinia amylovora* with apple fruit also hold true for mature, symptomless pear fruit.

Methods:

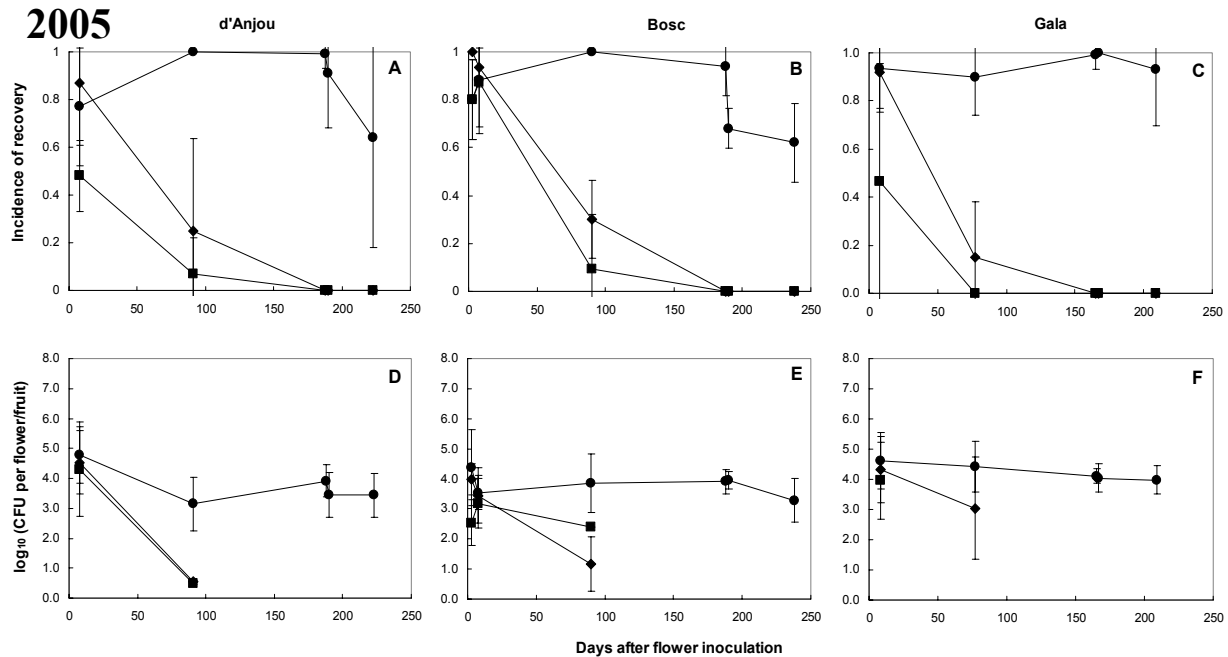
Objective 1-06. Calyx end survival. Field trials were conducted in d'Anjou and Bosc pear and Gala apple orchards to evaluate survival of *E. amylovora* on calyx-end of fruit. Freeze-dried cells of *E. amylovora* strain 153N, a non-pathogenic mutant of Ea153 (Ea153 HrpL-), and *Pantoea agglomerans* C9-1 were resuspended in water were sprayed onto flowers at full bloom. Fluorescent microspheres (1 µm in diameter) were co-inoculated with the pathogen to track flowers that received the inoculum spray. Flowers and immature fruit were sampled over the summer and processed for recovery of the inoculated strains. At each sample time, 8 flowers or 5 fruit from each of 3 replicate trees were placed individually into a plastic bag, and transported to the lab chilled on ice. Sterile washing buffer (50 ml of 10 mM phosphate buffer, pH 7) was added to each bagged fruit followed by sonication for 2 minutes to dislodge bacteria. The wash buffer was passed through a 0.2 µm filter membrane to capture the bacteria; the filter was placed onto Miller-Schroth medium and incubated for 7 days at room temperature. At harvest, 300 fruit per treatment per cultivar (2700 fruit total) were processed through a SOPP (sodium ortho-phenylphenate) dump tank followed by 6 weeks of cold storage. Periodically, fruit were sampled to measure residual bacterial population in association with calyx tissues as described above.

Objective 2-06. Postharvest survival in wounds on fruit. At harvest, mature symptomless fruit of d'Anjou pear and Braeburn apple were harvested and transported to the lab. Fruit were surface disinfested in 10% bleach, rinsed in sterile water, and air dried. A 10 µl drop of re-suspended, freeze dried cells of *E. amylovora* was placed onto a marked location on the surface of each fruit. The number of pathogen cells in a drop was zero, 1000 or 10,000 cells. Once the inoculum was air dry (~ 1 hour), a wound was introduced at the inoculation site with a small finishing nail secured to a wooden block. After wounding, fruit were incubated at room temperature for 24 hours, followed by a dump tank treatment in 1.5% SOPP for pear or 100 ppm bleach for apple, and then placed in 0-2°C cold room for up to 49 days. Surviving pathogen populations were enumerated on day 0 (pre and post dump tank), 7, 14, 28, and 49 days. For each sample, the tissue surrounding a wound site was removed from 15 fruit per cultivar per inoculum treatment. This tissue was macerated in 4 ml of sterile phosphate buffer (pH 7.0). The maceration buffer was passed through a 0.2 µm micropore filter. The filter was incubated on the surface of Miller-Schroth medium.

Results:

Objective 1-06. Calyx end survival:

For the calyx end survival experiment, by mid summer, immature fruit sprayed with Ea153N (pathogenic strain) had a recovery incidence of 6% of fruit with populations that averaged 100 cells per fruit; for Ea153 HrpL- (non-pathogenic strain applied a higher dose than the pathogenic strain), the recovery incidence was 23% of fruit with populations that averaged 100 cells per fruit; for *P. agglomerans* C9-1, the recovery incidence was 97% of fruit populations that also averaged 10,000 cells per fruit. No *E. amylovora* (pathogenic or non-pathogenic) was detected on calyx tissue after immersion treatment or during storage. *P. agglomerans*, however, persisted on 97% of fruit assayed at harvest or during the storage period. Fluorescent microspheres were observed on 100% of blossom and midsummer samples, but declined somewhat for fruit sampled at maturity (80, 74, and 80% for d'Anjou, Bosc and Gala, respectively).



2006

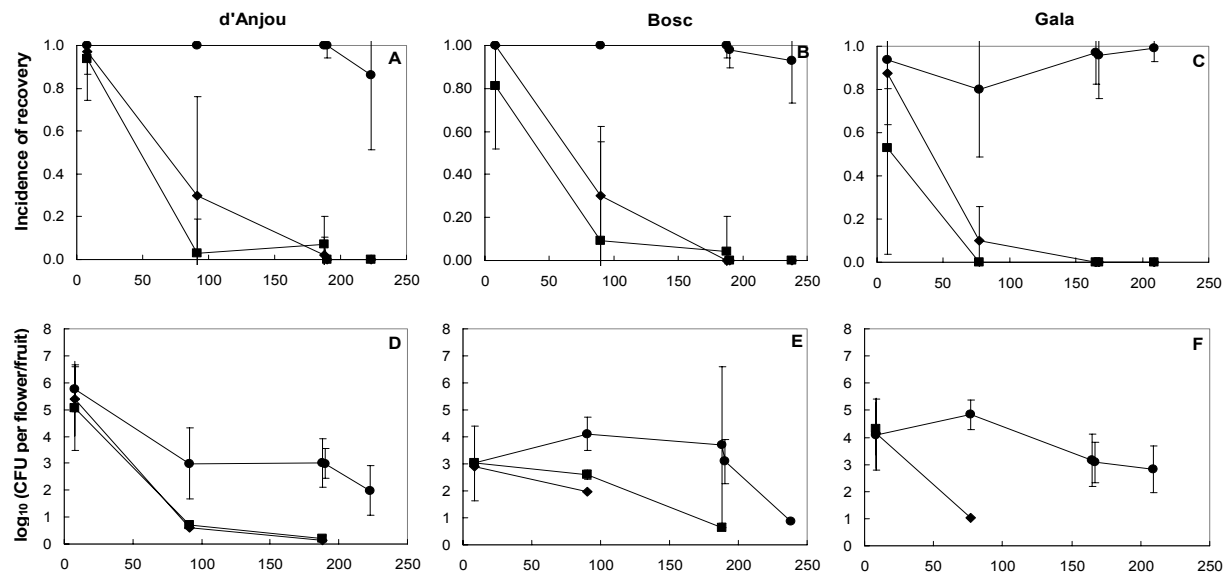


Fig 1. A-C, Incidence of recovery and, **D-F** days after flower inoculation, detectable populations (expressed as \log_{10} (cfu) per fruit) of *Erwinia amylovora* strains Ea153N (■) and Ea153HrpL (◆), and *Pantoea agglomerans* C9-1 (●) on floral and calyx tissue after inoculation in orchards located near Medford ('d'Anjou' pear) and Corvallis, OR ('Bosc' pear and 'Gala' apple) during summers of 2005 and 2006. Inoculum (1×10^7 cfu/ml for Ea153HrpL and C9-1; 1×10^5 cfu/ml for Ea153N) was misted onto flowers at full bloom. For each sample time during the growing season, 8 flowers or 5 fruit were sampled from each of three replicate trees per experiment, washed individually, and dilution plated and/or filter assayed onto Miller-Schroth medium. For sample dates at harvest and at pre-and post-storage, 90 fruit per bacterial strain (bulk harvested for treated trees) were individually assayed in each season. Vertical bars drawn through each point represent \pm one standard deviation of the mean. **Objective 2-06 Postharvest survival in wounds on fruit.**

Zero, 1000 or 10000 cells of the pathogen were applied to the fruit skin, and then a small puncture wound was made at the site of cell placement. Fruit were processed through an SOPP or bleach dump tanks and stored at 0-2°C.

The recovery of Ea153N introduced to mature fruit near small skin punctures prior to storage was dependent on the size of inoculum dose. Of the doses of inoculum evaluated in 2004 (water control, and 1×10^1 , 1×10^2 and 1×10^3 CFU per wound site), only the highest dose resulted in pathogen recovery from fruit after the dump tank treatment (Table 1). At this dose, Ea153N was detected on 27% of d'Anjou pear after 7 and 14 days of cold storage (0 to 2°C, regular atmosphere) and on 20% of Braeburn apple after 7 days storage; the pathogen was not detected on any fruit ($n = 480$) stored longer than 14 days. When detected, the mean recovered population size of Ea153N was $<1 \times 10^2$ CFU per fruit (Table 1). A dose of 1×10^3 CFU per wound site yielded similar results in the 2005 and 2006 experiments with no detection of the pathogen beyond 7 or 14 days from apple and pear, respectively.

The dose of 1×10^4 CFU per wound site, evaluated in 2005 and 2006 experiments, increased persistence of the pathogen on fruit relative to a dose of 1×10^3 CFU per wound site, which was evaluated in all years of the study (Table 1). For pear, at the highest dose, populations of the pathogen between 1×10^2 and 1×10^3 CFU per wound site were detectable on 13 to 37% of fruit at 49 days after inoculation; on apple, smaller populations (between 1×10^1 and 1×10^2 CFU per wound site) were detectable on 13 to 20% of apple fruit up to 28 days after inoculation. For both fruit types, the incidence of detection of Ea153N was highest at the pre-dump tank assessment (except for pear 2006 with NaOCl as the disinfectant) followed by consistently smaller rates of recovery as storage time progressed. In contrast, estimated population sizes for Ea 153N in the vicinity of wound sites were consistently an order of magnitude higher after 7 days of cold storage compared to samples taken at shortly after inoculation ('pre-dump tank' in Table 4). In pear, incubation of fruit at room temperature for 30 days after storage resulted in detection of Ea153N on 2 of 15 fruit in 2005 (average of 5 CFU per wound site), but no detection of the pathogen on similarly treated fruit in 2006.

NEXT PAGE: Table 1. Effect cold storage on percent of fruit with detectable populations of *Erwinia amylovora* strain Ea153N, and the mean recovered population (CFU) after small wounds^u were created near inoculation sites on mature symptomless pear and apple fruit near harvest

Table 1. Effect cold storage on percent of fruit with detectable populations of *Erwinia amylovora* strain Ea153N, and the mean recovered population (CFU) after small wounds^u were created near inoculation sites on mature symptomless pear and apple fruit near harvest.

Host/ Year ^u	Inoculum dose/Dump tank ^v disinfectant	Pre- dump tank	Post- dump tank	Weeks in cold storage ^w				Post-cold storage ^x
				7	14	28	49	
Pear								
2004	1 x 10 ³ SOPP	100% ^y 96 cfu	53% 43 cfu	27 % 50 cfu	7% 80 cfu	0%	0%	n.d. ^z
2005	1 x 10 ³ SOPP	20% 3 cfu	0%	0%	0%	0%	0%	0%
	1 x 10 ⁴ SOPP	67% 43 cfu	23% 10 cfu	43% 220 cfu	47% 204 cfu	57% 261 cfu	13% 90 cfu	13% 5 cfu
2006	1 x 10 ³ SOPP	34% 5 cfu	10% 29 cfu	10% 3 cfu	7% 239 cfu	0%	0%	0%
	1 x 10 ⁴ SOPP	90% 137 cfu	47% 163 cfu	67% 1376 cfu	47% 793 cfu	54% 263 cfu	37% 222 cfu	0%
	1 x 10 ⁴ NaOCl	64% 40 cfu	30% 101 cfu	67% 272 cfu	47% 317 cfu	54% 428 cfu	27% 230 cfu	0%
Apple								
2004	1 x 10 ³ SOPP	100% 88 cfu	13% 1 cfu	20% 11 cfu	0%	0%	0%	n.d.
2005	1 x 10 ³ NaOCl	13% 4	0%	0%	0%	0%	0%	n.d.
	1 x 10 ⁴ NaOCl	50% 70 cfu	7% 96 cfu	3% 115 cfu	3% 86 cfu	0%	0%	n.d.
2006	1 x 10 ³ NaOCl	47% 6 cfu	27% 7 cfu	20% 29 cfu	0%	0%	0%	0%
	1 x 10 ⁴ SOPP	87% 18 cfu	27% 5 cfu	53% 153 cfu	13% 83 cfu	13% 13 cfu	0%	0%
	1 x 10 ⁴ NaOCl	73% 18 cfu	20% 2 cfu	53% 232 cfu	27% 68 cfu	20% 31 cfu	0%	0%

Key to table:

^tFruits were inoculated by placing a 10 µl drop containing the indicated number (dose) of colony forming units on the fruit surface. After the drops were dry, a 1 x 3 mm deep skin puncture was introduced at the site of inoculation with a finishing nail.

^uPear experiments were conducted with cv. 'd'Anjou'. Apple cv. 'Braeburn' was used in Corvallis experiments and in Wenatchee in 2006; cv. 'Gala' was used in Wenatchee in 2005.

^vSOPP = 1.5% sodium ortho-phenylphenate; NaOCL = 100 ppm sodium hypochloride, which are the standard disinfectants used in commercial flotation systems for pear and apple, respectively. After inoculation, fruit were incubated at room temperature (20-22°C) for 24 hours prior dump tank immersion.

^wStorage temperatures averaged 2 and 0°C in Corvallis and Wenatchee, respectively.

^xAfter cold storage, fruit were incubated at room temperature (20°C) for an additional 30 days before assay.

^yPercent of 15 (2004 and post-storage samples in 2005 and 2006) or 30 fruit (2005 and 2006; 15 from each location) with detectable populations of Ea153N. Mean population size (CFU per fruit) and standard deviation are computed only for fruit on which Ea153N was detected.

^zNot determined.

Discussion

It was our goal to have three to four location years to support the conclusions for each of the objectives outlined above in the 'significant findings' section of this report. Prior to the 2006 season, the two objectives of this the study where we considered the data incomplete concerned survival of the pathogen on the fruit calyxes, and understanding the potential for pathogen survival in wounds.

In the calyx end survival study, the data indicate that *E. amylovora* can survive on the calyxes for a short period after bloom, but that survival is characterized by small numbers of cells that are declining over time. Surviving populations of *E. amylovora* were detected only rarely at harvest, and in both 2005 and 2006, the pathogen could not be detected on any fruit by the end of a 6- to 7-week cold storage period. In contrast, populations of the non-pathogenic bacterial epiphyte, *Pantoea agglomerans* persisted on calyxes of most fruit through the summer and the period of cold storage, indicating that this organism is better adapted to epiphytic survival than is the fire blight pathogen. Moreover, fluorescent microspheres were recovered from nearly all sampled fruit from which we attempted to isolate *E. amylovora*; this indicates that a similar proportion flowers received an initial dose of the pathogen, and that lack of detection of the pathogen at harvest and during storage was not due to the flowers escaping the initial bacterial spray. With the inoculations of wild type Ea153N in pear in 2006 (and the Bosc pear and Gala apple inoculations in 2005), the incidences of blossom blight were very high (70 to 85% of blossom clusters), demonstrating virulence in the pathogen isolate. Similar patterns of survival resulted from the virulent and avirulent strains of *E. amylovora*, suggesting that virulence (the ability to cause disease) is not strongly associated with the ability to survive epiphytically. Based on data from both seasons, there were no apparent differences in the patterns of calyx end survival on pear compared to those observed on apple.

The microwound inoculation study, although realistic in its emulation of industrial fruit handling practices, represented an improbable scenario in that a small wound (mimicking a stem puncture or other small abrasion induced during handling) was placed on fruit at a position that coincided with a concentration of pathogen cells confined within an area that represented approximately one thousandths of the total fruit surface. With this experiment, our overall goal was to understand, in a dose response framework, how the reportedly greater susceptibility of pear fruit relative to apple would influence pathogen persistence in storage when intimately associated with wounded host cells. The results confirmed our expectation that wounded pear fruit provided a somewhat more conducive environment for persistence of *E. amylovora* compared to wounded apple fruit, but this result was dose-dependent, with the difference between the hosts only apparent at the highest concentration of pathogen inoculum (i.e., 1×10^4 CFU in close proximity to the wound site). For both pear and apple, the data also showed small but consistent increases in pathogen population size over the first 7 to 14 days of cold storage, followed by declining incidence of detection and population size in the latter portion of the storage period. These increases in recovered population size during the early part of the storage period, however, were considerably smaller than reported for growth of *E. amylovora* in a nutrient broth incubated at 2°C for 20 days (Taylor and Hale 2003). Macroscopically, both pear and apple fruit remained symptomless at the wound sites, although a very small, necrotic discoloration was apparent at the base of the wound of some fruit that were split with a knife. Pear fruit that received the highest dose of Ea153N also were used to monitor the ability of the pathogen to grow on fruit after the 49 day cold storage period. Pears incubated at room temperature for 30 days post-cold storage showed a low incidence of detection with an estimated population size that was two orders of magnitude smaller (i.e., 5 CFU per fruit) than observed on fruit sampled at the end of the cold storage period (Table 1). Consequently, winter pears are apparently a poor substrate for growth of *E. amylovora* when allowed to ripen at room temperature after cold storage.

Collectively, the results of all experiments in this study can be compared to a peer-reviewed risk model developed to assess the probability that successful establishment of *E. amylovora* in a disease-free area could occur by importation of commercial apple fruit (Roberts et al. 1998). In that model, independent probabilities (P) were assigned to steps of the introduction pathway: P₁, the fruit is infested with *E. amylovora*; P₂, *E. amylovora* survives storage; P₃, contaminated fruit is discarded near host; P₄, host is receptive; and P₅, *E. amylovora* is transferred to the new host and infection occurs. Using these probabilities and an estimate of the potential number of fruit exported annually to the new area, Roberts et al. found the likelihood of successful introduction of fire blight to the new area ranged from once every 11,000 to 38,000 years, depending on the level of phytosanitary precaution taken prior to export. In this likelihood estimate, their derived values for P₁, depending on phytosanitary scenario, ranged from 0.001 (one in a thousand fruit) to 0.035 (one in 30 fruit), which are considerably higher than the 1 in 5600 fruit that we obtained in our surveys of commercial pear orchards. Similarly, Roberts et al. estimated P₂ to be 0.1 (one in ten fruit), which also was greater than we observed in the calyx survival and postharvest epiphytic survival experiments (L. Pusey, reported previously), where fruit were in cold storage for periods of 7 and 8 weeks, respectively. Published values of P₃ and P₄ estimated for the apple hold similar values for pear, although in absolute terms, the potential number of pear fruit exported would be considerably less than apple (fresh pear production in the Pacific Northwest is one eighth that of apple). P₅, the probability that '*E. amylovora* is transferred to new host and infection occurs' is largely dependent on how well this pathogen, starting from a relatively small number of cells on a mature symptomless fruit, could increase its population size on the discarded fruit to enable its transfer to the receptive host, most likely by a visiting insect. Although, the results of the microwound study indicate that mature pear, compared to apple, is somewhat more suitable for growth and persistence of *E. amylovora* in wounds during a storage event, this difference was not large, especially at levels of inoculum reasonably expected to occur under natural conditions. By the end of the storage (and the required chilling)

period, our data also indicated that mature pear fruit were an unsuitable substrate for continued reproduction of this pathogen.

In summary, we found that *E. amylovora* has a limited survival time on surfaces of healthy pear fruit, and that the survival rates are not different from those observed on mature, symptomless apple fruit. Calyx end survival of *E. amylovora* on pear fruit is similar to that observed on apple with significant populations detected near petal fall; these populations declined through the summer to low numbers at harvest, and become undetectable after a 7-week cold storage period. We were unable to detect *Erwinia amylovora* as an endophyte in mature symptomless pear fruit harvested from diseased pear trees. In three years of survey, we detected a few cells of *Erwinia amylovora* from only one fruit of 5600 d'Anjou pear fruit sampled at harvest from commercial orchards in Pacific Northwest region of the United States. Pear fruit contaminated with *E. amylovora* and subsequently wounded required an initial dose of >10,000 cells at the wound site to allow for persistence of the pathogen on the fruit through a 7 week cold storage period. By comparing the magnitude of this dose to its likelihood, we conclude that epiphytic survival of *E. amylovora* through a summer survival phase and a postharvest chilling period is unlikely given the unrealistically high population size required for persistence.

Literature Cited:

Roberts, R. G., Hale, C. N., van der Zwet, T., Miller, C. E., and Redlin, S. C. 1998. The potential for spread of *Erwinia amylovora* and fire blight via commercial apple fruit; a critical review and risk assessment. *Crop Protection* 17:19-27.

Taylor, R. K., and Hale, C. N. 2003. Cold storage affects survival and growth of *Erwinia amylovora* on the calyx of apple. *Letters in Applied Microbiology*. 37:340-343.