Effects of Antibiotic Applications on Epiphytic Bacteria in the Apple Phyllosphere

Kiersten Tancos and Kerik Cox
Department of Plant Pathology and Plant-Microbe Biology
New York State Agricultural Experiment Station, Cornell University, Geneva

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_**Erwinia amylovora**, the causal agent of fire blight, is a destructive bacterial pathogen capable of causing disease in numerous rosaceous plant species throughout the world (Bonn 2000). This pathogen is especially devastating in apple and pear and leads to considerable production losses in the United States annually. **E. amylovora** causes blossom blight during bloom which, in turn, often leads to a more devastating infection of young shoots and rootstocks (Vanneste 2000). Blighted tissues typically become blackened and seemingly burnt, resulting in the appearance for which the disease is named (Figure 1A). The loss of blossoms and fruit-bearing shoots leads to a decrease in fruit production and potentially loss of bearing wood and entire trees. This is especially the case for moderate to highly susceptible apple cultivars, which are widely planted and in high demand (Bonn 2000). While there are several horticultural management practices and chemical management options, the most effective management practices for controlling infection at bloom are the aminoglycoside antibiotics streptomycin and kasugamycin. While use of kasugamycin is relatively new to the management of fire blight, streptomycin has been used to control fire blight in the eastern United States for over 50 years. Aside from these aminoglycoside antibiotics, there are no viable chemical management alternatives that provide a comparable and acceptable level of blossom blight control in the temperate production conditions of the eastern United States.

The reliance of the apple industry on streptomycin in particular has become an issue of concern in recent years, due to the emergence of streptomycin resistance in populations of *E. amylovora* the United States (Moller 1981). The first reports of streptomycin resistance occurred in California in 1972 and shortly afterwards in Washington (Miller 1972; Coyier 1975). Currently, streptomycin resistance is found in several western and midwestern states such as Missouri and Michigan (McManus 1994). The most recent reports of streptomycin-resistant (SmR) *E. amylovora* occurred in New York in 2002 (Russo 2008). These SmR isolates originated from a single orchard site, which prompted immediate eradication efforts in order to prevent the spread of SmR *E. amylovora* to other apple growing regions. Subsequent fire blight surveys in 2004 and 2006 did not result in the detection of SmR *E. amylovora*, leading to the assumption that eradication efforts were successful in containing the outbreak (Russo 2008). However, in recent investigations, SmR *E. amylovora* was recovered from apple production operations in several western New York counties spanning three of the state’s six apple growing regions (Bekoscke 2014).

The development of streptomycin resistance has been attributed to streptomycin overuse after bloom to control the shoot blight phase of the disease. Several survey studies have correlated the history of streptomycin use in production orchards with the recovery of streptomycin-resistant isolates of *E. amylovora* (Loper 1991; Yashiro 2012). Aside from the aforementioned studies, the majority of the evidence for the development of streptomycin resistance remains anecdotal. To bridge the knowledge gap between antibiotic applications and resistance development, we wished to monitor the presence of streptomycin resistance in epiphytic bacterial populations following applications of streptomycin and kasugamycin after bloom.

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Materials and Methods

To determine the effects of streptomycin and kasugamycin application schedules on the selection of populations of *E. amylovora* and other epiphytic non-pathogenic bacteria with streptomycin resistance, field trials were conducted in two plantings of 'Idared' apples at the New York State Agricultural Experiment Station. Both plantings consisted of tall spindle 'Idared' apples on B.9 rootstocks approximately 15 (Orchard 1) and 7 (Orchard 2) years in age. At 80% bloom in both orchard plantings, treatment programs consisting of varying applications of either streptomycin or kasugamycin were implemented as follows:

1. No applications of streptomycin or kasugamycin during full bloom or terminal elongation
2. A total of three applications of streptomycin or kasugamycin with one application during bloom, and two applications during early terminal elongation (1–3 inches)
3. A total of five applications of streptomycin or kasugamycin with one application during bloom, and four more applications through terminal elongation (15 inches)
4. A total of ten applications of streptomycin or kasugamycin with one application during bloom, and nine more applications through terminal elongation and beyond

Applications of streptomycin were made using Firewall 17WP (AgroSource, Inc., Cranford, NJ) at a rate of 24 oz/A and applications of kasugamycin were made using Kasumin 2L. (Arysta LifeScience, Cary, NC) at rate of 64 fl oz/A. All applications were made at weekly intervals using a gas-powered backpack sprayer (200 PSI) to four replicate single tree plots. Streptomycin programs were implemented in both the 7- and 15-year old 'Idared' planting (Orchards 1 and 2), but the kasugamycin program was only implemented in the 15-year old planting (Orchard 1). Calendar-based applications of protectant fungicides, insecticides, and herbicides were applied to all trees for plot maintenance throughout the duration of this trial.

One week after the completion of the final antibiotic application in each treatment program, 50 leaves were collected throughout the canopy from each of the four replicate trees. Leaves were subsequently sonicated in 10X phosphate-buffered saline solution to remove epiphytic bacteria. From each sonicated leaf rinse sample, ten replicate aliquots of 100 ul were dilution plated to an enumerable amount of bacterial colony forming units when necessary on Cross-Goodman (CG) media at 28°C for 2 days, to select for *Erwinia spp.* and closely-related epiphytic bacterial species. To detect the presence of antibiotic-resistant epiphytic bacteria, resulting colonies from the dilution plating were also spot-plated on CG media that was amended with 100 ppm analytical grade streptomycin sulfate, or 100 ppm analytical grade kasugamycin hydrochloride and grown at 28°C for 48 hrs. To detect the presence of epiphytic *Pseudomonas sp.*, colonies from the dilution plating were also spot-plated on King’s B medium. The resultant colony forming units were identified on the basis of characteristic morphology on CG media and fluorescence on King’s B media. To confirm scoring by morphology, a subset of colonies from each plate, selected on the basis of colony morphology, were subjected to confirmation by PCR using primers specific to the 16S rRNA of *Pseudomonas sp.* (Widmer, 1998), the pag2R gene of *Pantoea agglomerans* (syn. Erwinia herbicola) (Braun-Kiewnick 2012), and the pEA29 plasmid of *E. amylovora* (Russo 2008). Selected colonies were also tested for the presence of the gene pair strA/strB, which is commonly responsible for streptomycin resistance in *Pseudomonas sp.*, *Pantoea agglomerans*, and *E. amylovora* in NY (Russo 2008). A subset of the remaining miscellaneous epiphytic bacteria with distinctive colony morphology on the two selective media was identified through PCR and sequencing of the internal transcribed spacer region (ITS) in bacterial ribosomal DNA using the primers and procedures described previously (Jensen et al. 1993). The effect of antibiotic treatment program on bacterial count data was determined using Generalized Linear Mixed Models with the GLIMMIX procedure of SAS v9.3 (SAS Institute). Differences in the mean CFUs and mean percent of CFUs between antibiotic treatment programs were determined using the ‘lsmeans’ statement of GLIMMIX at the 5% level of significance.

Results and Discussion

In both orchard locations and for all antibiotic treatment programs, *Pantoea agglomerans* and fluorescent *Pseudomonas* species were the most frequently recovered epiphytic bacteria. This observation was not surprising given that these bacteria are commonly recovered from apple blossoms and leaves in similar studies on apple epiphytes (McGhee 2010; Yashiro 2012). Despite the fact that there was active shoot blight throughout the orchard planting, *Erwinia amylovora* was rarely recovered from apple leaves. Across all treatment programs, *E. amylovora* was present in less than 0.1% of leaf rinseate samples. Given that *E. amylovora* is a poor epiphyte (Bonn 1981), the
lack of *E. amylovora* in leaf rinsate samples was somewhat expected. In addition to the aforementioned bacteria, a variety of miscellaneous epiphytic bacteria were recovered from leaf rinsate. Across all treatment programs, they represented less than 20% of the total CFUs recovered from dilution plating (Figs. 2, 3). These included several *Pantoea* spp. (e.g., *P. ananatis*), and *Erwinia* spp. (e.g., *E. rhiphontici*), and *Sphingomonas* spp. Irrespective of the species, nearly all of the epiphytes recovered had streptomycin resistance. Across all treatment programs and orchards, less than 5–20% of the epiphytic bacteria recovered were streptomycin sensitive. In both orchards, the recovery of streptomycin-resistant epiphytes was significantly lower (*P* < 0.05) for the trees that received no applications of streptomycin than those receiving three, five, or ten applications. Although we believed that applications of kasugamycin would not exert equivalent selection for streptomycin resistance and sensitivity, increased application of kasugamycin resulted in a decrease in the percentage of streptomycin-resistant epiphytes recovered from the apple phyllosphere. While one might be tempted to suggest that there is a fitness cost that may be associated with streptomycin resistance, this observation may be an artifact of the decrease in total epiphytic bacteria following increased application of kasugamycin (Figure 3B). Further field and laboratory studies would be necessary to elucidate the effects of kasugamycin on total streptomycin-resistant bacteria.

In contrast, in streptomycin-amended medium, no bacteria were recovered from leaf rinsate plated on kasugamycin-amended medium. Hence, excessive application of kasugamycin (i.e., 10 applications) after bloom had no impact on the selection or recovery of kasugamycin-resistant epiphytes. Certainly, additional years of field trials would be needed to validate such an observation.

Despite the fact that little to no *Erwinia amylovora* was recovered from shoot tissues, increasing applications of streptomycin (Fire Wall 17 WP) did have an impact on the overall populations of *P. agglomerans* and *Pseudomonas* in the phyllosphere. In Orchard 1, the percentage of the total epiphytic population represented by *P. agglomerans* decreased with increasing applications of streptomycin (Figure 2A). Similar results were obtained for Orchard 2 (Figure 2B). By comparison, the percentage of the total epiphytic population identified as *Pseudomonas* sp. increased as streptomycin applications increased in both Orchard 1 and Orchard 2 (Figure 2). No significant differences were observed within the percentage of the total epiphytic population represented by the miscellaneous epiphytic bacteria (Figure 2). In terms of total epiphytic bacteria recovered, the number of streptomycin applications had no bearing on the CFU/L for each of three types of epiphytic bacteria (data not shown).

Interestingly, increasing applications of kasugamycin (Kasumin 2L) had a slightly different impact on the overall populations of *P. agglomerans* and *Pseudomonas* in the phyllosphere. In Orchard 1, the percentage of the total epiphytic population represented by *P. agglomerans* increased with increasing applications of kasugamycin (Figure 3A). By comparison, the percentage of the total epiphytic population represented by *Pseudomonas* species and the remaining miscellaneous epiphytic bacteria decreased with increasing applications of kasugamycin (Figure 3A). In terms of total epiphytic bacteria recovered, trees receiving ten applications of kasugamycin had considerably lower CFUs/L for each of three types of epiphytic bacteria (Figure 3B). Moreover, all the bacteria colonies recovered for trees receiving ten applications of kasugamycin were identified as *P. agglomerans*.

Despite the fact the total number of epiphytic bacteria declined with increasing applications of kasugamycin, the increase in the proportion of *P. agglomerans* in the phyllosphere following applications of kasugamycin may be disconcerting. In the case of streptomycin resistance development in *E. amylovora*, it is believed that streptomycin resistance genes strA and strB were transferred to *E. amylovora* from *P. agglomerans* (Chiou 1993; McGhee 2010). Hence, this epiphytic bacterial species could be abundant following kasugamycin applications to serve as a source of horizontally transferred kasugamycin resistance genes should they arise in the future. Regardless, it’s important to note that no kasugamycin-resistant bacteria were recovered in these trials, and that the population of *P. agglomerans* was reduced by nearly 1000-fold after ten applications of kasugamycin (Figure 3A).

In summary, our results further confirm that *E. amylovora* is not present in the phyllosphere in high abundance, even if there is active fire blight in the planting. Along these lines, three, five, or ten applications of streptomycin after bloom did not result in an increased recovery of streptomycin-resistant *E. amylovora*. However, other common bacterial epiphytes of apple (*P. agglomerans* & *Pseudomonas*) with streptomycin resistance
did increase with increasing application of streptomycin, further suggesting that use of streptomycin after bloom should be minimal and reserved for trauma events. Finally, increasing application of kasugamycin appeared to reduce the overall number of bacterial epiphytes and the percentage of bacterial epiphytes with streptomycin resistance.

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Literature Cited


Kerik Cox is a research and extension professor who leads Cornell’s program in disease control of tree fruits and berries. Kiersten Tancos is a graduate student who works with Kerik Cox.