

AN ABSTRACT OF THE THESIS OF

Andrew Robert Hubbard for the degree of Master of Science in Botany and Plant Pathology presented on Friday, May 13 2011.

Title: Evaluation of Kasugamycin-use strategies Designed to Delay Development of Resistance in *Erwinia amylovora*

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The antibiotics, streptomycin sulfate and oxytetracycline, are used widely for fire blight suppression in the Pacific Northwest region of the United States. The efficacy of streptomycin, however, is compromised by widespread streptomycin-resistance in *E. amylovora* populations, and oxytetracycline is only partially effective. Consequently, for several years, the aminoglycoside antibiotic, kasugamycin (Kasumin 2L and 10L), was evaluated as an alternative material for fire blight control. Research focused on development of kasugamycin-use strategies with potential to delay the development of pathogen-resistance compared to use of kasugamycin alone. The first strategy involved use limitation through integration with biological control. *Pantoea vagans* was applied at 30 and 70% bloom followed by Kasumin at full bloom. Kasugamycin-resistant strain, *P. vagans* C9-1^{Kr}, was obtained through step-wise selection from parental strain, *P. vagans* C9-1S. Comparison of establishment of these strains on flowers after overspray with Kasumin at 100 ppm showed C9-1^{Kr} with incidences of colonization averaging 69% and

63% on pear and apple, respectively, compared to 63% and 55% respectively, for C9-1S. Relative to the water-treated control, either strain followed by one application of Kasumin provided a similar level of blight control (84%), which was somewhat less than the 90- 92% control observed after two applications of Kasumin at 100 ppm. There was no apparent benefit obtained from use of strain C9-1^{Kr} compared to strain C9-1S. The second resistance management strategy involved mixing Kasumin with oxytetracycline. In particular, a mixture of Kasumin at 80 ppm with oxytetracycline at 80 ppm was as effective as Kasumin at 100 ppm. In pear, an integrated treatment comprised of C9-1^{Kr} at 30 and 70% bloom followed by the 80 plus 80 ppm mixture of Kasumin and oxytetracycline resulted in 93% control. Kasugamycin-use strategies involving a) use limitation through integration with biological control, and b) rate reduction and mitigation of selection pressure through antibiotic mixtures each can achieve effective blight control, and may potentially slow the development of pathogen-resistance to this material.

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Evaluation of Kasugamycin-use Strategies Designed to Delay Development of Resistance
in *Erwinia amylovora*

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Andrew Robert Hubbard

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Evaluation of kasugamycin-use strategies designed to delay development of resistance in *Erwinia amylovora*

Introduction

In the Pacific Northwest of the United States, fire blight, caused by the bacterial pathogen *Erwinia amylovora*, can be a lethal threat to the region's pear and apple crops. The standard control, the antibiotic streptomycin, has been losing effectiveness due to the emergence of streptomycin-resistant pathogen populations. An alternative antibiotic, kasugamycin (trade name: Kasumin), is currently in the process of evaluation and registration for use in the United States. To avoid the development of resistance in *E. amylovora* to kasugamycin, use strategies are required that will achieve a high level of disease control, but also mitigate the selection pressure placed on the pathogen population.

In addition to Kasumin, several biological control agents for fire blight suppression have been registered recently for use in the pear and apple industry. Biocontrol of fire blight has been found to be only partially suppressive, but this partial effect was shown to be additive if used in conjunction with chemical control.

This thesis reports on the evaluation of two resistance management strategies for the antibiotic, kasugamycin, when used to suppress fire blight of pear and apple. The two strategies evaluated were (a) integrated biological and chemical control, and (b) mixtures of kasugamycin with the previously registered but only partially effective antibiotic, oxytetracycline. With regard to integrated control, the strategy was evaluated with both a kasugamycin-sensitive and kasugamycin-resistant selection of the biocontrol agent, *Pantoea vagans* strain C9-1S.

Literature Review

Significance of fire blight: Fire blight, caused by the bacterial pathogen, *Erwinia amylovora* [(Burrill) Winslow et al], is a devastating disease of apple (*Malus*), pear (*Pyrus*) and other rosaceous hosts (van der Zwet and Kiel, 1979). The disease causes rapid necrosis of flowers, which leads to expanding, necrotic cankers on branches and limbs. Major outbreaks of fire blight can destroy entire orchards if left unchecked and if the environment is conducive to proliferation of the pathogen (Vanneste, 2000). Fire blight has been destructive to the pear and apple industry of the Pacific Northwest region of North America. For example, in Washington State, annual costs of the disease have been estimated to range from \$3-5 million, accounting for suppression efforts and losses to disease (T.J. Smith, *personal communication*).

Biology and epidemiology: The fire blight disease cycle begins with old ('holdover') cankers that developed from infections in the previous season. Cells of the pathogen released from these old cankers provide the new inoculum to flowers of apple and pear. On flowers, the pathogen grows epiphytically on the surfaces of stigmas, which are located on the tip of the floral styles. Epiphytic *E. amylovora* on a single stigma can grow to a population size that approaches 10^7 colony forming units (CFU) (i.e., bacterial cells) (Thomson, 1986). Pathogen colonies on styles are readily vectored to additional flowers by pollinating insects and/or rain. This epiphytic phase is accelerated by warmer temperatures, which accelerates the pathogen's growth rate (Thompson et al., 1982). The importance of temperature to the epiphytic phase of *E.*

amylovora on flowers has been captured by degree-hour based forecasting models (e.g., COUGARBLIGHT, <http://www.ncw.wsu.edu/treefruit/fireblight/CBuse.html>, and MARYBLYT, <http://www.caf.wvu.edu/kearneysville/maryblyt/>), which track the speed of epiphytic colonization of flowers and predict the likelihood that economically-destructive pathogen populations have developed on floral surfaces (Smith, 1996; Steiner, 1990). Consequently, the most serious fire blight epidemics occur after 3-5 days of warm temperatures that allow for large pathogen populations to develop in a large number of flowers (Thompson, 2000). The indices of infection risk provided by the models also capture flower-to-flower movement of the pathogen, which occurs as a result of temperature-dependent pollen and nectar gathering activities of bees and other insects (Johnson et al., 1993a; Johnson et al., 1993b; Nucló et al., 1998; Thomson et al., 1992)

Infection by *E. amylovora* is initiated when a wetting event (rain or heavy dew) washes epiphytic cells of *E. amylovora* from the stigmas onto the hypanthium (Thomson, 1986). The moisture has two purposes in infection: to move pathogen cells to the natural openings in the hypanthium (nectarthodes) and to dilute the concentration of the nectar to an osmotic potential more favorable for bacterial growth (Wilson et al., 1989; Pusey, 1999). Once entry has been made into the interior of the plant via the nectarthodes, *E. amylovora* multiplies rapidly and spreads through tissues intercellularly, and also through the xylem (Bogs et al., 1998). After an incubation period of 5 to 30 days, symptoms of blossom blight begin to develop. These symptoms are typified by a rapid wilting and necrosis of the flower cluster, and by the formation

of a sticky bacterial ooze on diseased host surfaces (van der Zwet and Kiel, 1979; Steiner, 2000). Once visual symptoms have developed, the only effective way to stop or slow the infection is to remove the diseased tissue by pruning 20-30 cm below the proximal edge of the expanding canker.

Prevention and suppression of fire blight

General strategies: Control of fire blight begins with the removal of disease within and around the orchard via pruning. The goal of sanitation is very simple: the removal of pathogen overwintering sites lessens the amount of primary inoculum available to initiate the floral epiphytic phase. Although pruning is done in the summer to remove active cankers, the most effective pruning for purposes of sanitation occurs in the winter when old cankers are easiest to see. Moreover, in winter, pruning crews carefully and thoroughly shape the trees horticulturally; thus diseased branches can be removed as they are encountered.

In spite of thorough sanitation of old cankers, the floral epiphytic phase can be initiated from small cankers that are missed by pruning crews, or by insects that carry the pathogen into the orchard from someplace else (e.g., other orchards or unmanaged hosts). Consequently, floral infections are commonly suppressed through treatments applied directly onto the flowers. Materials sprayed on flowers include antibiotics and biological control agents, with the type of agent sprayed onto the tree being partially dependent on whether or not the grower is practicing conventional or organic

production methods. Timings of these treatments are frequently guided by bloom stage and by the degree hour accumulation models.

The prevention of summer phases of fire blight is another important component of a management program. By the prompt removal of infected shoots and flower clusters, the orchardist can keep the pathogen from expanding within the tree. In dry areas where little summer rainfall occurs and orchards are irrigated, secondary tree-to-tree spread of the disease is usually of lesser importance, unless the orchard is in an area where thunderstorms occur or if overhead sprinklers are used.

Reliance on antibiotics in fire blight control.

Antibiotics are substances produced by a microorganism that inhibits growth of or kills another microorganism (Madigan et al., 1997). In regard to this study, this definition is satisfactory, although some antibiotic products, such as streptomycin, are produced and purified from a microbial fermentation. For fire blight control, the purpose of an antibiotic application during bloom is to suppress epiphytic growth and activity of the pathogen on stigmatic and hypanthial surfaces prior to infection. The materials are effective because they strongly inhibit growth of the pathogen over the 3-6 day period that a flower is susceptible to infection (Stockwell et al., 2008).

The antibiotic standards for fire blight control, streptomycin sulfate and oxytetracycline, have been in use since the 1950s. Streptomycin, an aminoglycoside antibiotic, was highly effective for many years. Strains of *E. amylovora* resistant to this antibiotic developed subsequently, first in California (Miller and Schroth, 1972)

followed by Washington and Oregon (Coyier and Covey, 1975; Loper et al., 1991; Thomson et al., 1993), and then in Michigan (McManus and Jones, 1994).

Oxytetracycline has been in use as a standalone product since the 1970s and is commonly applied in the western states and Michigan where streptomycin resistance is common. Studies have shown that oxytetracycline is considered inferior to streptomycin for fire blight control (Stockwell et al., 1996; Nucló et al., 1997; Jones et al., 2000), except where streptomycin-resistant strains of *E. amylovora* are present. Poorer control of fire blight by oxytetracycline is due, at least in part, to its bacteriostatic properties whereas streptomycin is bactericidal (McManus and Jones, 1994). Unlike streptomycin, no known resistance in *E. amylovora* to oxytetracycline has been documented.

Kasugamycin is an aminoglycoside antibiotic that is produced by fermentation of *Streptomyces kasugaensis*. Kasugamycin has been formulated both as Kasumin 2L (2% active ingredient) or 10L (10% active ingredient), and will be referred to subsequently as Kasumin (Arysta LifeScience North America, Cary, NC). As a pesticide, Kasumin was first used for rice blast control in Japan in the 1960's (Yamaguchi, 1998). Kasumin was tested extensively for fire blight control in the 1970s and 1980s with good results, although in the earlier research, phytotoxicity was a concern (Kooistra and de Gruyter, 1984; Aldwinckle and Norelli, 1990; Saygili and Ustun, 1996). Newer formulations of Kasumin seem to ameliorate this concern (Johnson et al., 2007; Adaskaveg et al., 2011). Kasumin, though not yet fully

registered in the United States, has been used on a Section 18 emergency status in Michigan where streptomycin resistance in *E. amylovora* occurs.

A national registration of Kasumin would mark the first time in 35 years that a new antibiotic would be available for fire blight control. The need to implement strategies to prevent resistance to Kasumin developing in *E. amylovora* is critical if the efficacy of the chemical is to be retained. Resistance development in the target organism to Kasumin has been a problem with previous registered uses of the product. The active ingredient, kasugamycin, was originally used as a fungicide to control *Pyricularia oryzae*, the causal pathogen of rice blast, but resistance in the pathogen became problematic (Miura et al., 1976). Consequently, the solitary use of Kasumin in intensive pome fruit culture poses the risk of material loss through the development of resistance in the target pathogen.

Moreover, concerns about resistance development in *E. amylovora* are elevated due to the multiple mechanisms by which it can arise in bacterial communities. One potential mechanism would be a spontaneous mutation that confers kasugamycin resistance in *E. amylovora*. A recent report by Sundin et al. (2010) placed the likelihood of this occurring in the field at intermediate (higher than oxytetracycline and lower than streptomycin) based on the observation that a spontaneous mutation to kasugamycin resistance in *E. amylovora* was unattainable in the laboratory when the bacterium was plated onto media containing the proposed label rate of Kasumin (100 µl/ml). Resistance to Kasumin, however, was induced in *E. amylovora* by a stepwise process where the initial selection of mutants occurred at 50 µl/ml followed by a

second selection at the proposed label rate of 100 µl/ml. A second mechanism is via horizontal transfer of DNA from another bacterium, which has been hypothesized for streptomycin-resistant *E. amylovora* in Michigan (McManus and Jones, 1994).

Although no transferrable genes for kasugamycin resistance have been found, it has been speculated that they could occur in soil environments in which *Streptomyces kasugaensis* is a member of the microbial community (Sundin et al., 2010).

Biological Control

Biological control of fire blight was first reported in 1930, when an isolated yellow bacterium was shown to control the disease; the agent used to achieve this control was a strain of the saprophytic bacterium, *Erwinia herbicola* (Beer and Rundle, 1984), now reclassified as *Pantoea agglomerans* or *vagans* (Gavini et al. 1989 Rezzonico, et al., 2010). The use of *Pantoea* spp. (*P. agglomerans* and *P. vagans*) as biological control agents of fire blight has been studied intensively since the since the 1980s (Ishimaru et al., 1988; Wodzinski et al., 1994a; Wodzinski et al., 1994b; Vanneste, 1996; Kearns et al., 1998; Johnson and Stockwell, 2000; Wright et al., 2001; Pusey et al., 2008; Sundin et al., 2009). Significantly, *Pantoea* spp. have the potential to colonize flowers rapidly (Johnson et al., 2000; Pusey et al., 2004; Spinelli et al., 2005; Stockwell et al., 2008; Stockwell et al., 2010), occupying niches on the stigmatic surfaces and using the available nutrients for its own growth (Wilson et al., 1992). It has also been shown that the various *Pantoea* strains antagonize *E. amylovora* by mechanisms of competitive exclusion and of antibiosis (Ishimaru et al.,

1988; Johnson and Stockwell, 2000; Wilson et al., 1992; Johnson et al., 1993b; Kearns and Hale, 1996; Lindow, 1985; Stockwell et al., 2001; Stockwell et al., 2002b; Vanneste et al., 1992; Wright et al., 2001).

Competitive exclusion: When two organisms reside in the same ecological niche, the potential for one to exclude the other is possible. For this to take place, the organisms must compete for spaces and share nutrients required for growth (Johnson et al., 1993b; Lindow et al., 1996; Wilson and Lindow, 1994; Vanneste et al. 1992). A prior study by Hattingh (1986) demonstrated that the saprophytic bacterium, *Pantoea agglomerans*, if applied to pear or apple stigmas a day before *E. amylovora* could effectively inhibit the pathogen's ability to colonize the surface. Wilson et al. (1992) observed that *P. agglomerans* and *E. amylovora* both occupy the similar sites on a stigma and that if *P. agglomerans* is applied prior to *E. amylovora*, it will inhibit the growth of the latter; co-inoculation will also reduce the populations of *E. amylovora*, but not to the same degree. Similarly, Wilson et al. (1993) also conducted experiments with an alternative saprophyte, *Pseudomonas fluorescens* strain A506. This strain suppressed *E. amylovora* populations when applied to stigmas 72 h in advance of the pathogen, but not when co-inoculated.

Antibiosis: In addition to competition, the mechanism of antibiosis can contribute to the effectiveness of a biocontrol agent. Antibiosis is classically defined as production of a chemical (i.e., an antibiotic) by a microorganism that inhibits growth or kills another microorganism (Thomashow and Weller, 1995). Antibiosis is a common phenotype in phyllosphere bacteria, in particular with *Pantoea* spp. (Lindow,

1985; Ishimaru et al., 1988; Vanneste et al., 1992; Johnson et al., 1993b; Kearns and Hale, 1996; Stockwell et al., 2001; Wright et al., 2001; Stockwell et al., 2002b). In general, most strains of *P. agglomerans* produces two growth inhibiting chemicals that inhibit *E. amylovora* in defined culture media (Ishimaru et al., 1988; Kearns and Hale, 1998; Stockwell et al., 2002b; Vanneste et al., 1992; Wright et al., 2001). Also, antibiosis by *P. agglomerans* has been shown to contribute to control of fire blight. For example, Vanneste et al. (1992) demonstrated that mutants of *P. agglomerans* deficient in their ability to produce the growth-inhibiting chemicals did not provide the same degree of biocontrol of fire blight as compared to the wild type parental strain (Vanneste et al., 1992).

In a field study, Stockwell et al. (2002b) confirmed findings of Vanneste, which tested the importance of antibiosis of *P. agglomerans* strain Eh252 for suppression of fire blight. When comparing the wild type strain Eh252 and an antibiotic-deficient derivative of Eh252, they found that both strains applied to pear and apple flowers provided a significant level of biological control, but that control achieved by the wild-type, antibiotic-producing strain Eh252 provided a level of control significantly superior to the antibiotic-deficient mutant (Stockwell et al., 2002b). They concluded that *P. agglomerans* strain Eh252 was an effective biological antagonist of *E. amylovora* and that it and similar strains could be provide effective control of fire blight, especially in areas of where antibiotic resistance was prevalent.

***Pantoea* spp. registered for fire blight:** Various strains of *Pantoea agglomerans* have been tested for fire blight suppression, and several are now

registered for use in commercial production. The products are known as BlightBan C9-1S (*P. vagans* strain C9-1S), Blossom Bless (*P. agglomerans* strain P10c), and Bloomtime Biological (*P. agglomerans* strain E325). BlightBan C9-1S and Bloomtime Biological are both registered in the United States but only Bloomtime Biological is marketed and available to growers; Blossom Bless is marketed for fire blight control in New Zealand.

Similarities between *Pantoea* strains have been found. Each strain produces one to two Pantocin-type antibiotics that inhibit the growth of *E. amylovora* (Ishimaru et al., 1988; Vanneste et al., 1992; Wodzinski et al., 1994; Wright et al., 2001; Stockwell et al., 2002b; Pusey et al., 2008b). In multiple studies, strains of *Pantoea* readily colonize stigmas on pear, apple and hawthorne flowers (Wilson et al., 1992; Stockwell et al., 1998; Sundin et al., 2009; Stockwell et al., 2010). The ability of these strains to move tree-to-tree in the orchard via pollinators has also been documented (Johnson et al., 1993a; Nucló et al., 1998; Pusey, 2002). In inoculated field trials, reports of fire blight suppression with *Pantoea* spp. have typically averaged a 50-60% reduction of disease compared to a water-treated control (Sundin et al., 2009; Stockwell et al., 2010).

Prevention of antibiotic resistance by integration of control tactics:

Resistance management strategies: Resistance management is the use of strategies to delay pathogens from becoming resistant to chemical materials. The need for resistance management is very important in bacterial disease control because

pathogen strains tolerant to the chemicals can arise quickly. Moreover, the number of effective control materials for bacterial disease suppression is also limited. Because new antibiotic materials are registered only rarely, it is essential that effective strategies to delay pathogen resistance be implemented at the time the antibiotic comes available for commercial use.

For Kasumin, the idea of resistance management is to limit the exposure of the pathogen to the chemical agent; reduced exposure lessens the chance of selecting a fit mutant. On the proposed label for fire blight control, the manufacturer of Kasumin (Arysta Life Sciences, Tokyo, Japan) has incorporated resistance management in the allowable rates and pattern of product use. For example, they have limited the consecutive applications of Kasumin to two, which may force the alternation of other bactericides, and they have prohibited alternate row spraying to help ensure that an effective dose is applied to all parts of the tree. Nonetheless, additional resistance management strategies have been largely neglected. These include limiting application of chemical agents through the integration with a biological control agent and utilizing two mixtures of antibiotics with differing modes of action.

Integrated management: Integration of pest suppression tactics is frequently advocated as a means to achieve acceptable, economic levels of control without over reliance on any one method of control. With chemical control, in particular, its integration with other control methods typically results in lessened chemical use, a hallmark of pesticide resistance management (Steiner, 2000). With regard to fire blight, combining sanitation with bloom sprays is a form of integrated control. For

this thesis, however, integrated control will be more narrowly defined as the integration of biological control agents (*P. vagans* C9-1S) with conventional antibiotics (Kasumin).

Beginning in the 1980's, large scale fire blight control studies conducted by Lindow and colleagues (Lindow et al., 1996) demonstrated the potential for integration of biological control with antibiotics. In these studies, *P. fluorescens* strain A506 was applied to plot areas in several commercial orchards that were then subjected to the grower's usual antibiotic control program, which was comprised of mixtures and/or alternation of streptomycin and oxytetracycline. It was found that areas of the orchards treated with the biological agent plus antibiotics had significantly less fire blight than the treatments receiving antibiotics alone. The trial also suggested that population sizes of *P. fluorescens* strain A506 were little affected by application of antibiotics; in culture, this bacterium is resistant to streptomycin but sensitive to oxytetracycline. Lindow et al.'s (1996) multi-year study also demonstrated the significance of evaluation of biological control of fire blight under the conditions of challenge by natural inoculum; frequently, in plots inoculated artificially with *E. amylovora*, biological control is ineffective because a high pathogen dose is sufficient to infect the flower directly, bypassing the need for it to increase its population size via epiphytic colonization of the stigma (Sundin et al., 2009).

In Oregon, results similar to Lindow et al.'s (1996) were obtained when *P. vagans* C9-1S and *P. fluorescens* strain A506 were applied to trees and then followed with a single overspray of oxytetracycline (Stockwell et al., 2007). This integrated

strategy (treating with the biological followed by oxytetracycline) resulted in significantly better disease control than treating with either the biological control agent twice or with oxytetracycline twice. Under conditions of a relatively low dose inoculation, the author's speculated that the integrated approach results in a broader interference of the pathogen's two-stage infection process (i.e., the suppression of epiphytic phase on the stigma and infection through the nectarhodes) than either material was capable of when used alone.

A study in Michigan (Sundin et al., 2009) incorporated an integrated approach where biologicals were applied first followed by streptomycin. The results showed that the number of streptomycin applications required for satisfactory control could be reduced when the trees were first treated with a biological agent. This led the researchers to support the idea that the combination of both technologies could work in an additive fashion with one another and provide better control when combined than if either was used on their own. At the level of individual flowers, a working hypothesis for enhanced disease suppression with integrated biological and chemical control is that the biocontrol organism mostly suppresses the epiphytic build-up of the pathogen population on the stigmatic surfaces. In contrast, the chemical agent effectively inhibits infection in the nectary. This working hypothesis will guide the research presented in this thesis.

The goal of this study is to determine if the integrated biological and chemical control concept can be adapted to the antibiotic, Kasumin. If successful, the benefits of such a strategy would reduce use of antibiotics, and in all likelihood, enhance the

longevity of the chemical while maintaining excellent fire blight control. The specific tools to be used in this study are the biological control agent, *Pantoea vagans* strain C9-1S, and the antibiotic product, Kasumin.

In addition to the use of a biological control agent that is currently sensitive to labeled rates of Kasumin, a kasugamycin-resistant selection of *P. vagans* strain C9-1S will be evaluated in field studies to determine if a mutant strain with a kasugamycin-resistant phenotype would improve control beyond that observed with the kasugamycin-sensitive parental strain.

Research Objectives

With regard to fire blight control, the principal hypotheses being investigated in this study are: a) an integrated control strategy consisting of a biological control agent followed by an antibiotic can be adapted for Kasumin as a potential resistance management strategy for *E. amylovora*, and b) the selection and use of a kasugamycin-resistant mutant of a biocontrol agent will enhance this integrated control strategy compared to the use of a kasugamycin-sensitive parental strain.

A spontaneous kasugamycin-resistant mutant of *P. vagans* strain C9-1S will be generated using stepwise selection. Growth of *P. vagans* and its mutant derivative on pear and apple flowers oversprayed with Kasumin will be evaluated initially in growth chamber experiments. Fire blight control resulting from an integrated strategy involving *P. vagans* and Kasumin will be evaluated in field experiments. In addition, a

second resistance management strategy, mixtures of Kasumin & oxytetracycline, also will be evaluated in the field

Materials and Methods

Bacterial Strains: *Pantoea vagans* strain C9-1S (synonyms *Erwinia herbicola*, *Pantoea agglomerans*) was chosen as the biological control agent to evaluate integrated fire blight control strategies in combination with Kasumin. This strain was originally isolated by C. Ishimaru from ‘Jonathan’ apple fruit in Michigan (Ishimaru, et al., 1988; Rezzonico et al., 2009). *P. vagans* strain C9-1S is registered as a biopesticide with the U.S. Environmental Protection Agency (Blightban C9-1, NuFarm Americas, Burr Ridge, IL); in addition to streptomycin-resistance (100µl/ml) it is also resistant to rifampicin (50 µg/ml) but sensitive to kasugamycin. Strain C9-1^{Kr} was a spontaneous kasugamycin-resistant (150 µl/ml) derivative of C9-1S obtained through a two-step selection procedure (method follows). *E. amylovora* 153N (Ea153N) was the strain of the fire blight pathogen used in field experiments to evaluate integrated control; it is a spontaneous nalidixic acid-resistant (100 µl/ml) mutant of strain 153 that was originally isolated in 1989 from a canker on ‘Gala’ apple in Milton Freewater, Oregon. The pathogenicity of Ea153N has been verified in prior field experiments (Johnson et al., 1993; Nucllo et al., 1997; Pusey et al., 1997; Stockwell et al., 1998; Pusey et al., 1999; Pusey et al., 2002; Stockwell et al., 2002; Pusey et al., 2008; Johnson et al., 2009; Stockwell et al., 2010). Ea153N is sensitive to kasugamycin, streptomycin, and oxytetracycline. All strains were stored in nutrient broth amended with 15% glycerol at -80°C until use.

Lyophilized preparations of C9-1^{Kr} were obtained by culturing for 4 days at 27°C followed by 4 days at 8°C on nutrient agar (Difco) amended with 0.4% w/v

glycerol. Cultured cells were harvested by scraping bacterial lawns into a sterile beaker, then weighed and amended with a glycerol/milk mixture at a 2:1 ratio (Stockwell et al., 1998). The homogenized slurry was poured into a frozen lyophilizing flask and placed into a -80°C freezer. After 1 h, the flask was placed onto a lyophilizer and was freeze dried for 18-30 h, depending on moisture content. Freeze-dried cultures were mortared, and passed through a 0.3 mm-mesh sieve for uniformity of granules. The same method was also used for *Ea153N*; use of lyophilized preparations of phyllosphere bacteria reduces variability in establishment of bacteria sprayed onto flowers (Stockwell et al., 1998).

Determination of minimum inhibitory concentration (MIC) to Kasumin. Cells of *P. vagans* strain C9-1S were grown overnight in Lysogeny broth (LB, formerly Luria broth) and then diluted to 1×10^8 CFU/ml (Bertani, 2004). Ten microliter aliquots of this suspension were transferred to test tubes containing 5 ml of LB amended with concentrations of Kasumin ranging from 0 to 100 µg/ml in 20 µg/ml intervals (Bertani, 2004). Cultures were placed on a rotary shaker (200 rpm) then allowed to grow for 24 h at 27°C. Turbidity of cultures was observed and recorded after the 24-h growth period.

Selection of mutant. Cells of *P. vagans* strain C9-1S were cultured overnight in 5ml of LB broth on a rotary shaker at 27°C. One hundred microliter aliquots of this culture were then spread onto fifty 9-cm petri plates containing half-strength Tryptic

Soy Agar (TSA, Difco, Detroit, MI) amended with Kasumin (50 µl/ml). After 4 days incubation, colonies of resistant mutants on the kasumin-amended TSA medium were obtained and subjected to a second iteration of selection at 150 µl/ml.

Characterization of mutant selection

A mutant of *P. vagans* strain C9-1S (designated strain C9-1^{Kr}) selected for its ability to grow on TSA amended with 150 µl/ml kasugamycin was subjected to stability and growth curve analyses. The purposes of these analyses were to determine if the mutation to kasugamycin resistant would remain stable in the absence of selection and to determine if the ability of the mutant to grow in broth was still comparable to the parental, kasugamycin-sensitive strain.

Stability assay. Cells of *P. vagans* strain C9-1S and strain C9-1^{Kr} were cultured overnight in test tubes containing 5 ml of King's medium B broth. After 24 h, 100 µl of each culture were transferred to a new test tube of King's medium B broth; this process was repeated for 5 days. At the end of the 5-day period, 100-fold dilutions were made of the cultures; dilutions were plated onto half-strength TSA amended with Kasumin concentrations of 0, 100 and 200 µg/ml. This experiment was conducted in triplicate.

Growth rates. Cells of *P. vagans* strain C9-1S and strain C9-1^{Kr} were cultured in 25 ml of King's medium B broth in a 125 ml side-arm flask with three replicate flasks per strain. Initial cell densities were adjusted to a value of 1×10^6 CFU/ml (i.e., 0.01 OD_{600nm}) with aid of a spectrophotometer (Spectronic 20, Bausch and Lomb, Rochester, NY) for measuring optical density. Cultures in side arm flasks were placed

on a rotary shaker (200 rpm) and incubated at 27°C. Optical density of the cultures was measured every 30 to 60 min over a 26 h-period.

Antibiosis assay. Cells of *P. vagans* strain C9-1S and strain C9-1^{Kr} were grown in an overnight culture of LB, then centrifuged and the pellet was washed twice with sterile distilled water. Initial cell densities were adjusted to 2×10^8 CFU/ml (i.e., 0.2 OD_{600nm}) with aid of a Spectronic 20. Fifteen microliter drops of each strain culture were placed in the centers of 9-cm petri plates containing 925 medium (Langley and Kado, 1972) amended with 100 mg/liter of nicotinic acid to support the growth of *E. amylovora*. Plates with spotted *P. vagans* cultures were grown for 3 days at 27°C. After incubation, each culture plate was scraped clean with a sterile cotton swap and then placed open in an exhaust hood. Glass Petri dishes containing chloroform also were placed in the exhaust hood. The culture plates were placed open side down onto the chloroform-containing glass dishes and exposed for 1 h, which is sufficient time for the chloroform to kill the remaining cells of *P. vagans* (Ishimaru et al., 1988). Plates with killed *P. vagans* cultures were then overlaid with 4 ml of molten 925 media (50°C) into which 100 µl of a 2×10^8 CFU/ml suspension of *E. amylovora* had been vortexed previously; this suspension had been prepared from an overnight culture of strain Ea153N. The plates with killed *P. vagans* and overlaid *E. amylovora* were then incubated for 2 days at 27°C. The diameters of the rings of inhibition, i.e., the areas of the plate where growth of *E. amylovora* was inhibited, were then measured.

Dilution series for loss of colonies. One tenth of a gram of freeze dried cells of *P. vagans* strain C9-1^{Kr} were placed into 100 ml distilled water spun with a stir bar. After the freeze-dried bacteria were resuspended, a 10-fold dilution series (10 µl transfers) was made with an endpoint dilution of 10¹¹. One hundred microliter aliquots of the suspensions were then spread onto plates that contained TSA media and one of four amendments: 50 µg/ml Cyclohexamide, 50 µg/ml cyclohexamide & 50 µg/ml Kasumin, 50 µg/ml cyclohexamide & 100 µg/ml Kasumin, and 50 µg/ml cyclohexamide & 200 µg/ml Kasumin. Plates were incubated at 20°C with colonies of *P. vagans* C9-1^{Kr} being counted after three days. Each Kasumin concentration was replicated three times

Growth Chamber Experiments

Flowers of ‘Bartlett’ pear and of ‘Gala’ apple were forced in the lab from branches collected in the field on 1 April and 10 April 2010, respectively. Flowers with pedicel intact were placed in 2-ml micro-centrifuge tubes (Qiagen, Valencia, CA) which were evenly spaced on a 96-count tube holder (24 flowers per holder). The tube holders were placed inside of 5.5-liter plastic crisper boxes (Sterilite Corporation, Townsend, MA); the boxes were filled with water to a depth of 1 cm to maintain humidity during incubation. Freeze-dried cultures of *P. vagans* C9-1S and C9-1^{Kr} were resuspended to a concentration of 1 x 10⁸ CFU/ml. Suspensions of each strain and a 1:1 mixture of the strains were misted onto the flowers held in the tube holders; a hand-held pump sprayer was used to apply the strain treatments to near runoff. After spraying, the flowers were allowed to dry in open air.

After treatment, lids were placed on the crisper boxes and the flowers were incubated at 16°C (a typical temperature during bloom) for up to 96 h. At 0, 48 and 96 h post-inoculation, 8 flowers were sampled from each treatment and subjected to dilution plating to determine the population size of each strain. Flowers were prepared for dilution plating by excising the pistil and nectary of each pear flower and only the pistil of each apple flower, and placing the excised tissues into test tubes containing 1 ml sterile 10 mM potassium phosphate buffer (pH 7.0). Tubes with pistils were sonicated (Sonix IV, Inglewood, CA) for 3 min. After sonication, a 10 µl sample of the wash and two 100-fold serial dilutions were spread onto half-strength TSA amended with 50 µg/ml of rifampicin, streptomycin and cycloheximide for recovery of C9-1S or onto half-strength TSA amended with 50 µg/ml of Kasumin and cycloheximide for selective recovery of C9-1^{Kr}. In the strain mixture treatment, the floral washed also were plated on half-strength TSA amended with 200 µg/ml of Kasumin and cycloheximide as a further control on selective recovery of strain C9-1^{Kr}.

In a second growth chamber experiment, the experimental design and protocols were the same as in the first detached flower experiment except that oversprays of Kasumin at 100µg/ml were applied to all detached flowers at 48 h after inoculation. Flowers were again sampled at 0, 48 and 96 h after inoculation; oversprayed Kasumin was allowed to dry on the flowers prior to processing the 48-h samples for dilution plating.

For each sampling date, the mean population size of C9-1S and C9-1^{Kr} on individual flowers were calculated by first transforming colony forming units (CFU) (obtained from serial dilution plates) to \log_{10} (CFU per flower) based on the observation that bacterial populations are usually distributed log normally (Hirano, 1982; Loper, 1984). Prior to transformation, the few flowers with no recovery of either C9-1S or C9-1^{Kr} were set to the detection limit of 99 CFU per flower. Analysis of variance (ANOVA) (PROC GLM, Statistical Analysis System, SAS Institute, Cary, NC) and Fischer's protected least significant difference test (LSD) at $P = 0.05$ was used to determine significance differences among the mean populations sizes measured for each bacterial strain.

Field Experiments.

Integrated fire blight control strategies involving *P. vagans* C9-1S and C9-1^{Kr} followed by Kasumin were evaluated in 2009 and 2010 in 0.5-ha blocks of 50-year-old pear (*Pyrus communis* L. cvs. 'Bartlett') and 0.4-ha plots of 11-year-old and 30-year-old apple (*Malus X domestica* Borrk. cvs. 'Gala' and 'Golden Delicious') located at the Oregon State University, Botany and Plant Pathology Field Laboratory located near Corvallis, OR.

Bacterial and chemical treatments were assigned to individual trees in randomized complete block designs with four replications. Trees were grouped into blocks based on the number of flower clusters per tree and the location in the orchard. An exception to this was the apple experiment in 2009 when low flowers densities

restricted the number of trees available for the experiment. As consequence, treatments in this experiment were replicated only three times, with two of the experimental blocks located in the ‘Gala’ orchard, and the third block located in the ‘Golden Delicious’ orchard. All cultivars used are moderately to highly susceptible to the fire blight.

The evaluated integrated control strategy called for treatments with *P. vagans* C9-1S and C9-1^{Kr} at 30 and 70% bloom in order to establish antagonist populations on the trees prior to inoculation with the pathogen (Table 2.1). Lyophilized cells of *P. vagans* C9-1S and C9-1^{Kr} were added to water to achieve a final concentration of 1×10^8 CFU/ml in the spray tank. Lyophilized bacteria were premixed in 1-liter Nalgene bottles filled partially with water and shaken vigorously, and then suspended in a 12-liter volume of water in backpack sprayer (Solo, Newport News, VA). Bacterial suspensions were applied under pressure to near run-off (3-4 liters per tree) with hand wands attached to the sprayers. Sprays were made in early morning (6 to 8 am) with still winds.

In each experiment, inoculation with pathogen, *E. amylovora* strain 153N, occurred at full bloom (Table 2.1). As with the antagonist treatments, freeze-dried inoculum of the pathogen was first resuspended in a 1-liter Nalgene bottle, then poured into a 100-liter volume of water in the tank of a motorized sprayer to achieve a final concentration of 5×10^5 CFU/ml. The pathogen suspension was misted under pressure onto the trees to near runoff with a hand wand attached to the motorized sprayer. The dates of inoculation were 19 April (pear) and 30 April (apple) in 2009

(evenings between 8 and 9 pm) and 25 April (apple) in 2010 (morning between 6 and 7 am) under still wind conditions; approximately 3 liters of the pathogen suspension were misted onto each tree.

As part of integrated control treatments, a single application of Kasumin was timed to occur at full bloom, 1 to 4 days after the pathogen inoculation (Table 2.1). Kasumin 2L (kasugamycin 2% a.i., Arysta LifeScience North America, Cary, NC) was applied in the 2009 experiments, and Kasumin 10L (kasugamycin 10% a.i.) was applied in 2010. As with the *P. vagans* treatments, Kasumin treatments were applied with 12-liter backpack sprayers equipped with hand-wands. Three to four liters of spray volume was applied to each experimental tree.

In addition to the integrated control treatments, several antibiotic only treatments and a water treatment were included in the experiment to serve as comparative controls (Table 2.2). These treatments included streptomycin sulfate (2009: Agri-mycin 17, Nufarm, Burr Ridge, IL; 2010: Firewall 17, Sipcam Advan, Research Triangle Park, NC) and oxytetracycline (2009: Mycoshield, NuFarm, and 2010: Fireline, Sipcam Advan). In addition, several mixtures of kasugamycin and oxytetracycline, (2009: Kasumin 2L and Mycoshield; 2010: Kasumin 10L and Fireline in 2010,) also were evaluated as an additional resistance management strategy for Kasumin. In 2009, all antibiotic only treatments were applied twice with the first treatment occurring at 70% bloom and the second at full bloom. In 2010, two additional antibiotic treatments were Kasumin 10L, applied once at full bloom, and the mixture Kasumin 10L plus Fireline once at full bloom (Table 2.2).

TABLE 2.1. Dates of integrated biological and chemical control treatments applied to pear and apple trees located near Corvallis, OR in 2009 and 2010.

Year	Crop/cultivar	Bloom stage of bacterial treatments		Full bloom treatments		First symptom appearance
		30%	70%	Pathogen ^a	Antibiotic	
2009	Pear/'Bartlett'	16 APR	18 APR	19 APR	20 APR	9 MAY
	Apple/'Gala' & 'Golden Delicious'	25 APR	29 APR	30 APR	1 MAY	22 MAY
2010	Apple/'Gala'	17 APR	19 APR	21 APR	22 APR	18 MAY

^a *Erwinia amylovora* 153N inoculation.

TABLE 2.2. Experimental design used to evaluate integrated biological and chemical control of fire blight in pear and apple orchards near Corvallis, Oregon in 2009 and 2010.

Treatment	Concentration of active ingredient	Crop/Year*		
		'Bartlett' Pear 2009*= =	'Gala'/Golden Delicious' Apple 2009	'Gala' Apple 2010
Water control	-----	X	X	X
<i>Integrated treatments</i>				
<i>P. vagans</i> C9-1 ^{Kr} then oxytetracycline	1×10^8 CFU/ml 200 µg/ml	X	---	X
<i>P. vagans</i> C9-1S then oxytetracycline	1×10^8 CFU/ml 200 µg/ml	X	X	X
<i>P. vagans</i> C9-1 ^{Kr} then Kasugamycin	1×10^8 CFU/ml 100 µg/ml	X	X	X
<i>P. vagans</i> C9-1S then Kasugamycin	1×10^8 CFU/ml 100 µg/ml	X	X	X
<i>P. vagans</i> C9-1 ^{Kr} then Kasugamycin & oxytetracycline	1×10^8 CFU/ml 80 µg/ml 80 µg/ml	X	X	X
<i>Antibiotic standards</i>				
Agri-mycin	100 µg/ml	X	X	X
Oxytetracycline	200 µg/ml	X	X	X
Kasugamycin	100 µg/ml	X	X	X
<i>Antibiotic mixtures</i>				
Kasugamycin & oxytetracycline	80 µg/ml 80 µg/ml	X	X	X
Kasugamycin & oxytetracycline	80 µg/ml 100 µg/ml	X	X	X
Kasugamycin & oxytetracycline	100 µg/ml 100 µg/ml	X	X	X

* See Table 2.1 for dates of treatment application.

Evaluation of field treatments

Recovery of bacterial strains from flowers. After the pathogen inoculation at full bloom, mature flowers (dehiscent anthers) were sampled from the experimental trees to measure population sizes of the applied *Pantoea* strains and of *E. amylovora*. In 2009, the dates of sampling in Bartlett pear were 22, 26, and 30 April, which corresponded to 4, 8 and 12 days after inoculation. The 2009 apple experiment in cultivars ‘Gala’ and ‘Golden Delicious’ was sampled on 11 and 15 April, 5 and 9 days after inoculation. In 2010, flowers from the ‘Gala’ apple experiment were sampled on 25, 27 April, and 3 May, which was 3, 5 and 8 days after inoculation. On each sampling date, eight mature flowers were sampled haphazardly from each replicate tree, and each flower was placed individually into a sterile well within a 24-well, micro-titer plates (Corning Inc., Corning, NY) for transport to the laboratory. Flowers were sampled early to midmorning and processed the same day, with the exception of the sample taken on 27 April 2010 where the sample was taken in the afternoon and processed 28 April. In the lab, flowers were processed and dilution plated as described above for the growth chamber experiment.

To evaluate potential differences in measured population sizes of C9-1S and C9-1^{Kr} on flowers, two summary statistics termed the ‘relative area under the incidence’ curve’ (RAUIC) and ‘relative area under the population curve’ (RAUPC) was calculated for each treatment in each trial. The following formula was used to calculate RAU_C for each experimental replication:

$$\text{RAU_C} = \sum_{i=1}^n \{[(y_i + y_{i-1})/2] \bullet (t_i - t_{i-1})\} / t_{\text{total}}$$

where y and t are the values of the response variable and hours after inoculation, respectively, for the i th sample date, and n is the total number of sample dates.

Estimates of the bacterial incidence on flowers within a replication were summarized as RAUIC; estimates of population size on flowers were \log_{10} transformed, averaged within replicates, and summarized as RAUPC. Field replicate values of RAUIC and RAUPC were subjected to analysis of variance (ANOVA) and Fischer's t-test protected least significant difference (LSD) at ($P = 0.05$) (PROC GLM, Statistical Analysis System, SAS Institute, Cary, NC).

Disease incidence. Beginning approximately 4 weeks after inoculation, flower clusters with fire blight symptoms (necrosis, wilting, and/or bacterial ooze) were assessed by closely inspecting each replicate tree and removing (cutting) diseased clusters (i.e., those with typical necrosis and/or bacterial ooze) as they were identified. In general, each replicate tree was inspected weekly, and the period of assessment extended over a four-week period. The counts of diseased flower cluster numbers (strikes) on each replicate tree were summed to provide total strikes per tree, which was then converted to disease incidence by dividing total strikes by the number of flower cluster on each tree. Total number of flower cluster on each tree was determined in counts made prior to bloom. Disease incidence data were arcsine square root-transformed prior to ANOVA. Fisher's protected least significant difference (LSD) test at $P = 0.05$ was used to separate the mean relative values calculated for each treatment in each trial.

RESULTS

Determination of minimum inhibitory concentration (MIC) of Kasumin: The minimum inhibitory concentration of kasugamycin to *P. vagans* strain C9-1S was determined by growing this bacterial strain in test tubes containing broth of King's B medium amended with the antibiotic at the following concentrations: 0, 20, 30, 40, 60, 80 and 100 µg/ml. Tubes with ≤ 30 µg/ml kasugamycin became turbid after 24 h of incubation; no turbidity was observed in tubes with ≥ 40 µg/ml of the antibiotic. Consequently, 40 µg/ml was considered the minimum inhibitory concentration; 50, 100 and 150 µg/ml were chosen as the initial concentrations to attempt to generate spontaneous, kasugamycin-resistant mutants of *P. vagans* strain C9-1S.

Selection of Mutant: For cells of *P. vagans* strain C9-1S spread onto half strength TSA medium containing 50 µg/ml of kasugamycin, an average of 15 mutants was obtained from a total of 6×10^{11} CFU plated onto this concentration; thus, the mutation rate was approximately 4×10^{-10} . No mutants, however, were obtained by plating strain C9-1S on the higher kasugamycin concentrations (100 and 150 µg/ml). Subsequently, a mutant obtained at 50 µg/ml was plated at 150 µg/ml. In this plating, a total of one colony was recovered after 8×10^{10} were spread over 40 plates. This mutant was named 'C9-1^{Kr}' and subjected to further characterization.

Characterization of Mutant Selection:

Stability assay and growth rates of C9-1^{Kr}: Stability of kasugamycin-resistance in C9-1^{Kr} was evaluated by culture in non-antibiotic amended King's

medium B broth with a transfer of 100 μ l of the culture every 24 h to fresh medium over a period of 5 days. Dilution plating of the culture at the end of the fifth day resulted in population sizes of C9-1^{Kr} and C9-1S on non-kasugamycin amended medium that averaged 4.4×10^9 and 4.5×10^9 CFU/ml, respectfully. C9-1S was not recovered on half-strength TSA amended with 100 μ g/ml or 200 μ g/ml Kasumin. C9-1^{Kr} was, however, recovered with population sizes that averaged 2.5×10^9 and 2.6×10^8 CFU/ml, respectfully (Table 3.1).

In the growth curve experiment, both C9-1S and C9-1^{Kr} exhibited similar rates of growth in non-antibiotic-amended King's medium B broth when incubated at 27°C (Fig. 3.1). Growth curves began at an optical density of 0.01 which corresponded to 1×10^6 CFU/ml. For both strains, the doubling time was observed to be 120 min (data not shown).

Table 3.1. Population sizes and standard error of wild type *Pantoea vagans* C9-1S and the kasugamycin-resistant derivative, *P. vagans* C9-1^{Kr}, at the end of the fifth day after daily transfer and growth in non-antibiotic-amended King's medium B broth.

Strain	No antibiotic	Standard error	100 μ g/ml Kasumin*	Standard error	200 μ g/ml Kasumin**	Standard error
	CFU/ml		CFU/ml		CFU/ml	
C9-1S	4.4×10^9	0.63	---***		---	
C9-1 ^{Kr}	4.5×10^9	1.87	2.5×10^9	1.04	2.6×10^8	0.67

* Dilution plated onto medium amended with 100 μ g/ml Kasumin

** Dilution plated onto medium amended with 200 μ g/ml Kasumin

*** No growth was detected

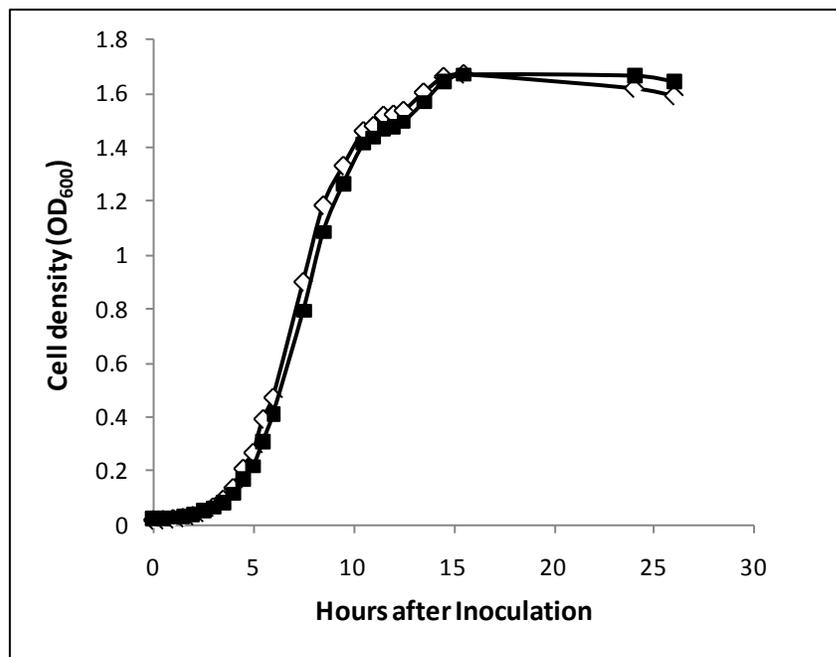


Figure 3.1. Growth of *Pantoea vagans* strains C9-1S (◇) and C9-1^{Kr} (■) in non-kasugamycin amended Lysogeny Broth over a 26-h period at 27°C. Initial cell density was adjusted to 1×10^6 CFU/ml (i.e., 0.01 OD_{600nm}).

Antibiosis assay. Both *P. vagans* C9-1S and C9-1^{Kr} were evaluated for antibiotic production, which is considered the mechanism of antagonism exhibited by the parental strain toward *E. amylovora* (Ishimaru, 1988). Both strains produced zones of inhibition toward *E. amylovora* in 925 medium (Table 3.2). After 24 h, three types of growth of *E. amylovora* could be observed on the Petri plates: first, outside of a larger zone of inhibition (ring) where growth was completely uninhibited; second, within a zone intermediate to the edge of the large ring and to the edge of a second inner ring, where growth was partially inhibited; and third, inside of the inner ring

where growth was completely inhibited. The two apparent zones of pathogen inhibition are indicative of two antibiotics being produced by C9-1S, which has been reported previously (Ishimaru, 1988). Relative to the parental strain, the zones of inhibition produced by the mutant, C9-1^{Kr} were smaller when measured at 24 h (Table 3.2). By 48 h, however, rings of inhibition produced by C9-1^{Kr} were 95% the size of those produced by the parental strain. By 72 h, the size of the zone of inhibition was similar for both strains with no clear distinction between the inner and outer rings.

Table 3.2. Diameter of inner and outer zones of inhibition produced by *Pantoea vagans* strain C9-1S and the kasugamycin-resistant derivative, *P. vagans* C9-1^{Kr} in antibiosis assay against *Erwinia amylovora*.

Strain	24 h		48 h		72 h
	inner (cm)	outer (cm)	inner (cm)	outer (cm)	inner* (cm)
C9-1S	n.d.**	6.6 ±0.23 [#]	6.7 ±0.09	7.1 ±0.07	7.4 ±0.08
C9-1^{Kr}	4.9 ±0.05	5.5 ±0.05	6.3 ±0.03	6.8 ±0.02	7.5 ±0.13

* No difference between the inner and outer rings.

** n.d. = not detectable

[#] Standard error mean.

Dilution series for loss of colonies: After freeze-drying, the titer of freeze-dried C9-1^{Kr} measured on TSA was dependent on the amount of Kasumin in the medium. At 0 and 50 µg/ml of Kasumin, the titer of C9-1^{Kr} averaged 4×10^{12} and 1×10^{12} CFU/g,

respectfully (Fig. 3.2). In contrast, Kasumin at concentrations of 100 and 200 $\mu\text{g/ml}$ significantly reduced the estimated titers to 2×10^{11} and 5×10^9 CFU/g, respectively.

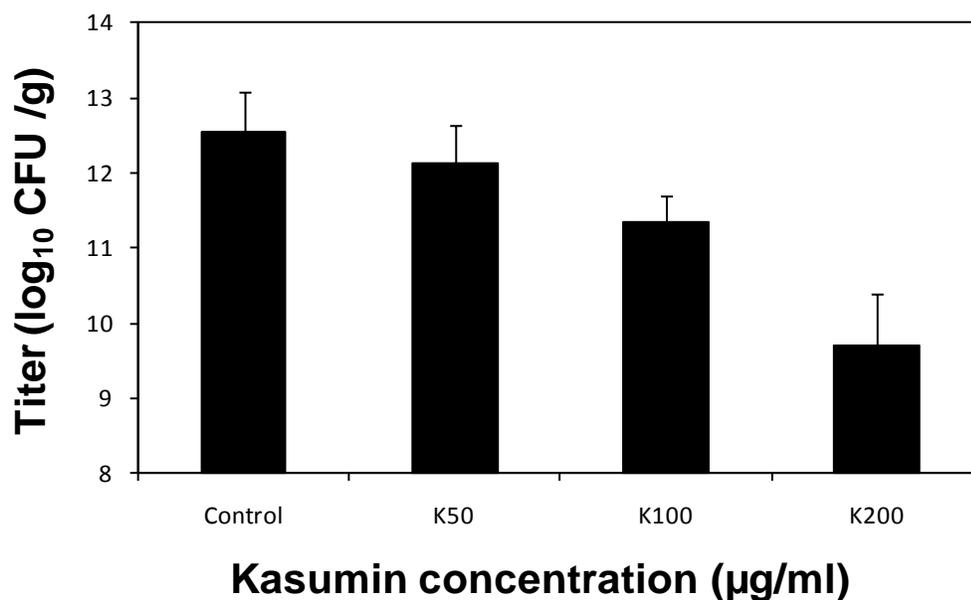


Figure 3.2. Titer of a freeze-dried preparation of *Pantoea vagans* strain C9-1^{Kr} as influenced by the concentration of Kasumin in the culture medium. Control is unamended Tryptic soy agar (TSA), K50 is Kasumin in TSA at 50 $\mu\text{g/ml}$, K100 is Kasumin in TSA at 100 $\mu\text{g/ml}$ and K200 is Kasumin in TSA as 200 $\mu\text{g/ml}$. Error bars represent the standard error.

Growth Chamber Experiments:

C9-1S and C9-1^{Kr} were inoculated in water and recovered from 100% of pear and apple flowers sampled over the time course of the experiment. After 96 h, mean population sizes of C9-1S and C9-1^{Kr} and the 1:1 mix of both strains ranged from 2×10^5 to 2×10^7 CFU/flower (Fig. 3.3A). After 96 hours of incubation, analysis of variance (ANOVA) of \log_{10} CFU per flower revealed no significant differences ($P > 0.05$) among populations on either apple or pear.

For the growth chamber experiment where the detached flowers were oversprayed with Kasumin, C9-1S and C9-1^{Kr} inoculated in water established on 100% of inoculated pear and apple flowers sampled over the time course of the experiment. Mean population sizes of C9-1S and C9-1^{Kr} and the 1:1 mix of both strains ranged from 2×10^5 to 8×10^6 per flower (Fig. 3.3B). For both the pear and apple ANOVA of \log_{10} CFU per flower, the Kasumin overspray ($100 \mu\text{g/ml}$) at 48 h after inoculation significantly ($P \leq 0.05$) reduced the 96-h size of the C9-1S population relative to the population size of C9-1^{Kr}.

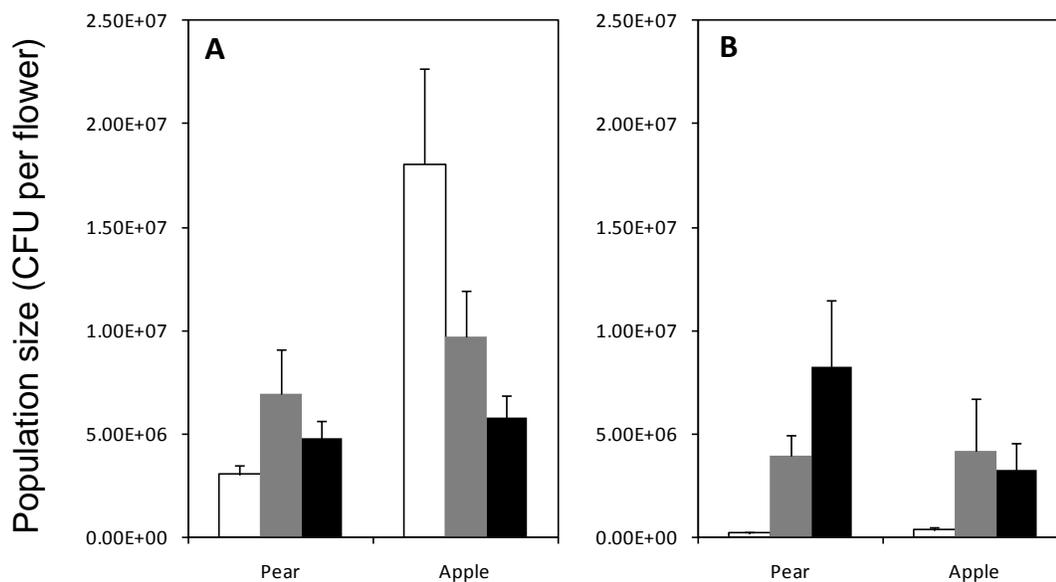


Figure 3.3. Population sizes of *Pantoea vagans* strain C9-1S (□), an initial 1:1 mix of *P. vagans* C9-1S and *P. vagans* C9-1^{Kr} (■), and *P. vagans* strain C9-1^{Kr} (■) on detached flowers pear or apple flowers after incubation at 16° C for 96 h. Flowers (24 per treatment) were inoculated by spraying a bacterial suspension to near runoff; sprayed flowers were allowed to air dry before being sealed in humidity box. **A.)** Flowers that did not receive any additional treatments. **B.)** Flowers oversprayed with Kasumin (100 ug/ml) at 48 h after inoculation. Vertical bars are standard error of the mean.

Field Experiments

Establishment of bacterial antagonists on flowers. For all field trials, C9-1S and C9-1^{Kr} inoculated in water established on flowers of pear and apple, and were recovered in flower washes over the sampling periods.

For ‘Bartlett’ pear in 2009, the overall incidence of recovery of C9-1S and C9-1^{Kr}, was initially 31% and 26%, respectively, on trees treated with these bacteria. These incidences increased to 81% and 90% by the third sampling made near petal fall (Fig. 3.4). Population size of C9-1S and C9-1^{Kr} on pear flowers with detectable populations of these strains averaged 3×10^5 and 2×10^5 CFU/flower, respectively (Fig. 3.4). ANOVA of RAUIC revealed that pear flowers treated with a particular strain had a significantly ($P \leq 0.05$) higher incidence of recovery of that strain relative to flowers treated with water (Table 3.3). Moreover, ANOVA of RAUIC revealed the incidence of recovery of C9-1^{Kr} was significantly ($P \leq 0.05$) higher on pear flowers oversprayed with Kasumin (100 µg/ml) compared to flowers treated with oxytetracycline (200 µg/ml). In contrast, overspray of kasugamycin-sensitive strain C9-1S with Kasumin or oxytetracycline resulted RAUIC values that were similar statistically to those obtained for overspray of C9-1^{Kr} with Kasumin or with a mixture of Kasumin (80 µg/ml) and oxytetracycline (80 µg/ml). With respect to relative area under population curves (RAUPC), C9-1^{Kr} oversprayed with Kasumin resulted in a higher population size than C9-1^{Kr} with oxytetracycline ($P \leq 0.05$) (Table 3.4).

For the first sample in ‘Gala’/‘Golden Delicious’ in 2009, the incidence of recovery of C9-1S and C9-1^{Kr} averaged 61% and 53%, respectively, for apple flowers

treated with these strains; these incidences increased to 66% and 60% by the second sampling date (Fig. 3.5). Population size of C9-1S and C9-1^{Kr} on individual flowers with a detectable population of these strains initially averaged 7×10^4 and 6×10^4 CFU/flower, respectively, and by the second sampling date populations were similar for both strains, averaging 3×10^5 CFU/flower (Fig. 3.5). ANOVA of RAUIC revealed that apple flowers treated with a particular strain had a significantly ($P \leq 0.05$) higher incidence of recovery of that strain compared to the incidence of recovery of a *Pantoea* strain from flowers of trees treated with water (Table 3.3). With respect to population size, values of RAUPC for C9-1S and C9-1^{Kr} did not differ significantly between treatments (Table 3.4).

In 'Gala' apple in 2010, incidence of recovery for C9-1S and C9-1^{Kr} each averaged 96% percent over the first two sampling dates; these incidences decreased to 44% and 55%, respectively, by the third sampling date (Fig. 3.6). The population size of C9-1S and C9-1^{Kr} on individual apple flowers with detectable populations initially averaged 9×10^5 and 7×10^5 CFU/flower, respectively, but declined to 3×10^5 and 4×10^5 CFU/flower, respectively, by the third sampling date (Fig. 3.6). ANOVA of RAUIC revealed that trees treated with a *P. vagans* strain had a significantly ($P \leq 0.05$) higher incidence of recovery of that strain relative to the incidence of recovery from flowers treated with water (Table 3.3). Moreover, the RAUIC for C9-1^{Kr} was significantly ($P \leq 0.05$) higher on apple flowers oversprayed with Kasumin compared those oversprayed with oxytetracycline. In contrast, the RAUIC for C9-1S oversprayed with Kasumin was lower than on trees oversprayed with oxytetracycline.

Also, overspray of strain C9-1S with Kasumin or oxytetracycline resulted in RAUC values that were similar statistically to those obtained for overspray of C9-1^{Kr} with Kasumin or with the Kasumin/oxytetracycline mixture. The ANOVA of RAUC revealed significantly higher ($P \leq 0.05$) values for both C9-1S oversprayed with oxytetracycline and C9-1^{Kr} oversprayed with Kasumin compared to C9-1^{Kr} with oxytetracycline (Table 3.4).

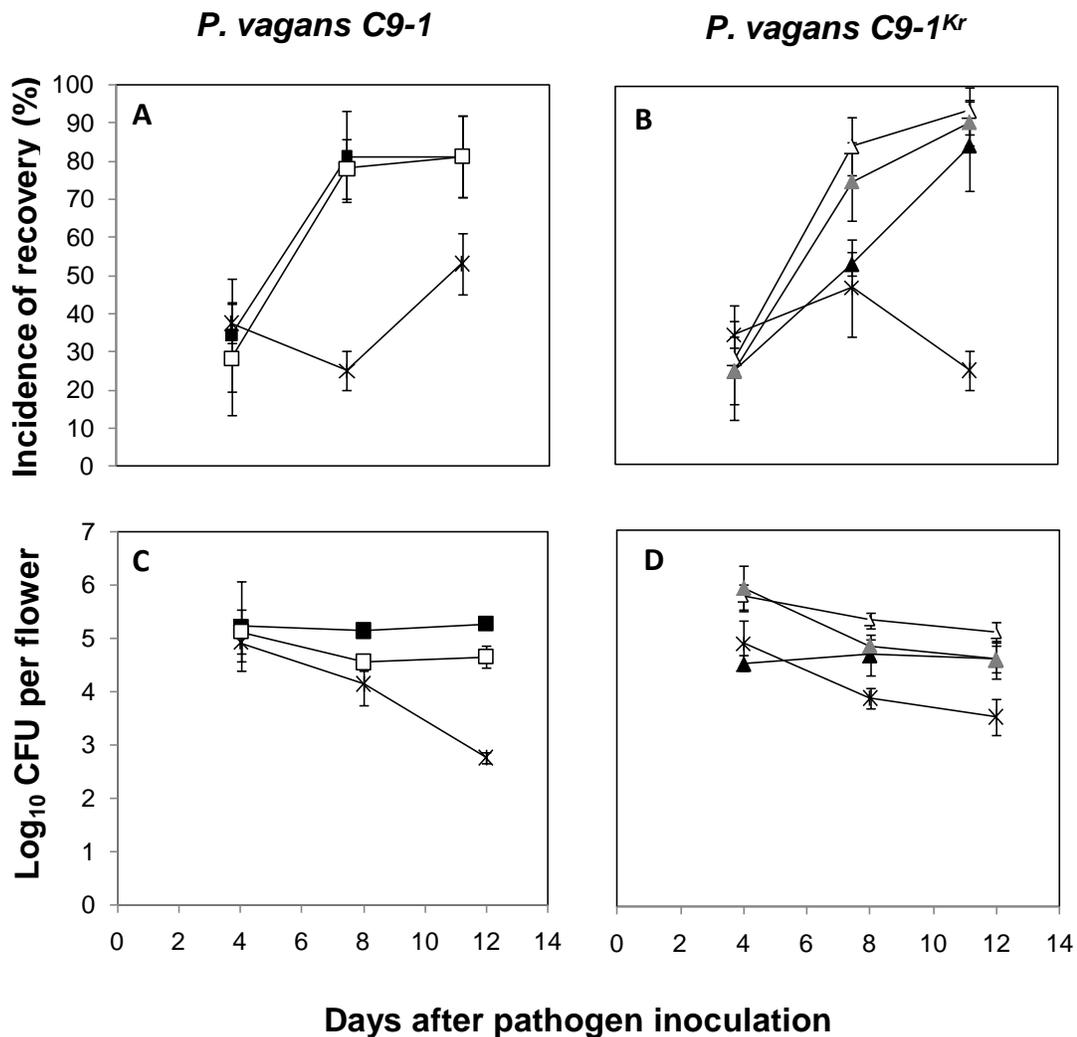


Figure 3.4. Incidence of recovery (A, B) and population size (C,D) of *Pantoea vagans* strains C9-1S (A,C) and strain C9-1^{Kr} (B,D) on flowers of Bartlett pear treated with these bacterial antagonists and then oversprayed with antibiotics. The antagonists and antibiotic treatments were made in the context of integrated biological and chemical fire blight control and occurred in 2009 in experimental orchards located near Corvallis, OR. Specific integrated antagonist strains and antibiotic treatments were: C9-1S with oxytetracycline (200 µg/ml, ■), C9-1S with Kasumin (100 µg/ml, □), C9-1^{Kr} with oxytetracycline (200 µg/ml ▲), C9-1^{Kr} with Kasumin 100 µg/ml (△) and C9-1^{Kr} with oxytetracycline (80 µg/ml) and Kasumin (80 µg/ml, ▲). (*) indicates each strain on water control.

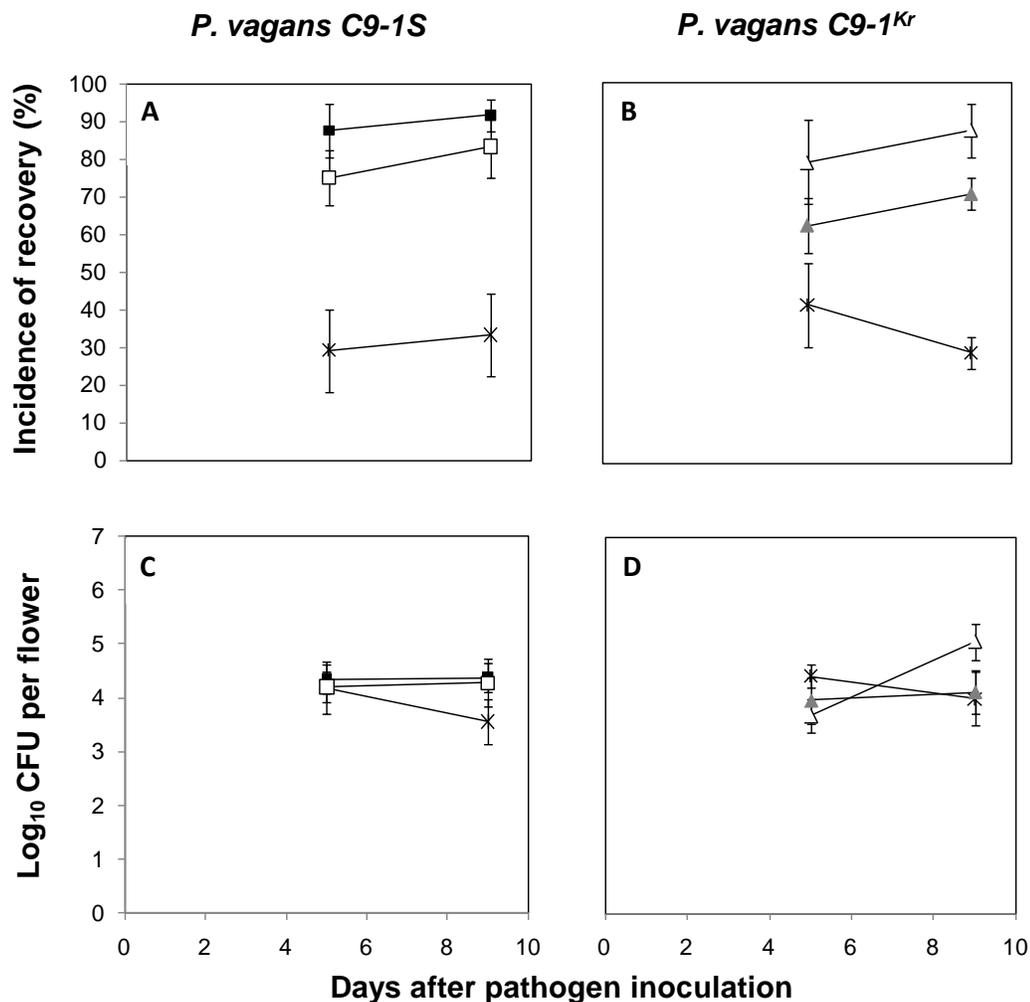


Figure 3.5. Incidence of recovery (A, B) and population size (C,D) of *Pantoea vagans* strain C9-1S (A,C) and strain C9-1^{Kr} (B,D) on flowers of 'Gala'/'Golden Delicious' apple treated with these bacterial antagonist and then oversprayed with antibiotics. The antagonist and antibiotic treatments were made in the context of integrated biological and chemical fire blight control and occurred in 2009 in experimental orchards located near Corvallis, OR. Specific integrated antagonist strain and antibiotic treatments were: C9-1S with oxytetracycline (200 µg/ml, ■), C9-1S with Kasumin (100 µg/ml, □), C9-1^{Kr} with oxytetracycline (200 µg/ml ▲), C9-1^{Kr} with Kasumin 100 µg/ml (△) and C9-1^{Kr} with oxytetracycline (80 µg/ml) and Kasumin (80 µg/ml, ▲). (*) indicates each strain on water control.

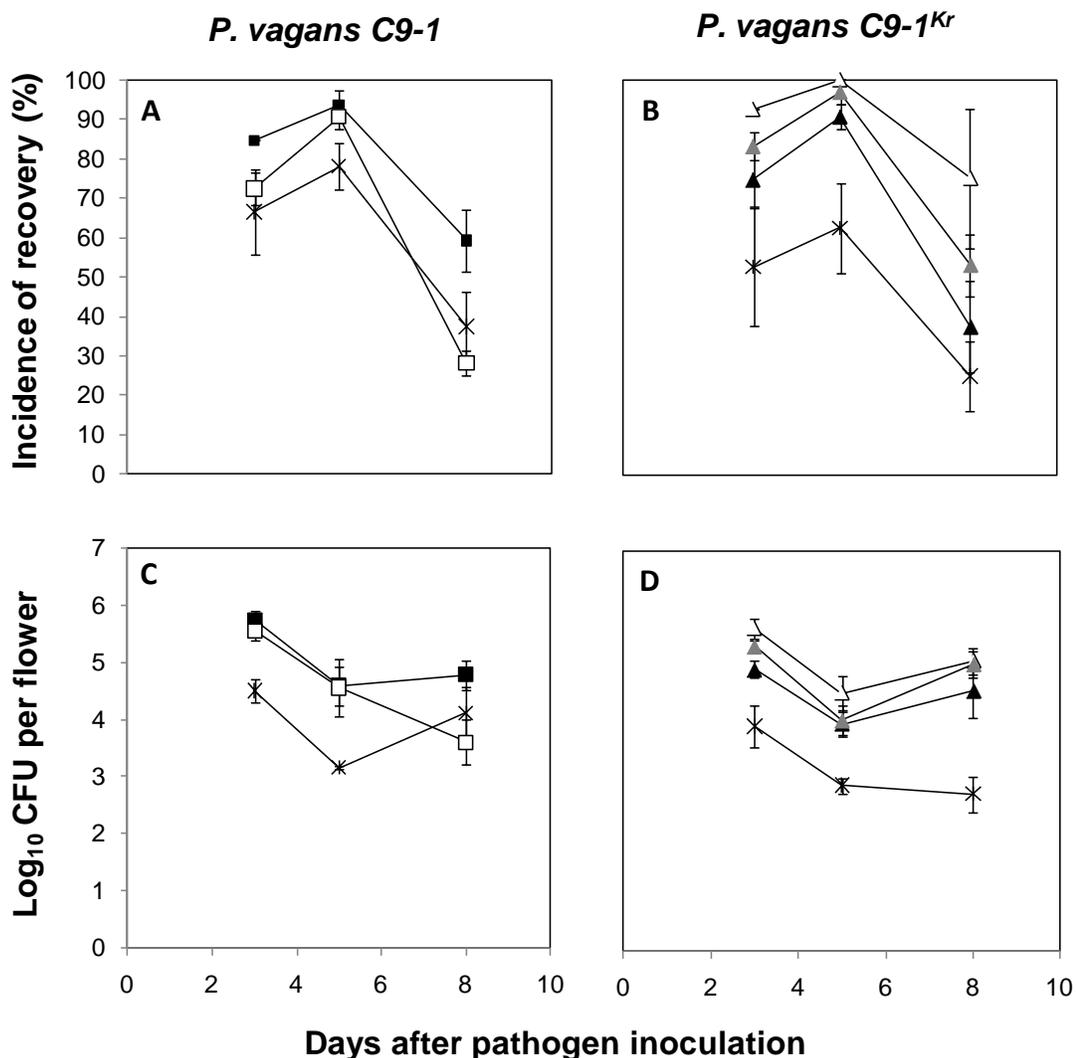


Figure 3.6 Incidence of recovery (A, B) and population size (C,D) of *Pantoea vagans* strain C9-1S (A,C) and strain C9-1^{Kr} (B,D) on flowers of Gala apple treated with these bacterial antagonist and then oversprayed with antibiotics. The antagonist and antibiotic treatments were made in the context of integrated biological and chemical fire blight control and occurred in 2010 in experimental orchards located near Corvallis, OR. Specific integrated antagonist strain and antibiotic treatments were: C9-1S with oxytetracycline (200 µg/ml, ■), C9-1S with Kasumin (100 µg/ml, □), C9-1^{Kr} with oxytetracycline (200 µg/ml ▲), C9-1^{Kr} with Kasumin 100 µg/ml (△) and C9-1^{Kr} with oxytetracycline (80 µg/ml) and Kasumin (80 µg/ml, ▲). (*) indicates each strain on water control.

TABLE 3.3. Relative area under incidence curve (RAUIC) of *Pantoea vagans* strains C9-1S and C9-1^{Kr} on pear and apple flowers as affected by antibiotic oversprays made in experimental orchards located near Corvallis, OR in 2009 and 2010*.

Treatment	Relative area under incidence curve		
	'Bartlett' Pear 2009	'Gala'/'Golden Delicious' Apple 2009	'Gala' Apple 2010
C9-1 ^{Kr} Water control	35.2 d	31.3 c	66.6 b
C9-1S Water control	38.3 cd	35.4 c	52.5 c
C9-1 ^{Kr} then Mycoshield 200 µg/ml	53.9 bc	---	74.7 b
C9-1S then Mycoshield 200 µg/ml	69.5 ab	89.5 a	84.7 ab
C9-1 ^{Kr} then Kasumin 100 µg/ml	72.7 a	83.3 ab	92.5 a
C9-1S then Kasumin 100 µg/ml	66.4 ab	79.2 ab	72.5 b
C9-1 ^{Kr} then Kasumin 80 µg/ml & Mycoshield 80 µg/ml	66.4 ab	66.7 b	83.1 ab

* Trees arranged in a complete randomized block design with 3 to 4 replications per treatment. Three liters of a suspension of C9-1S or C9-1^{Kr} (1×10^8 CFU/ml) were applied to trees at 30 and 70% bloom. Flowers were sampled from trees on two or three dates between full bloom and petal fall, from which the RAUIC values were determined and subjected to analysis of variance. Means within a column followed by the same letter do not differ significantly ($P \leq 0.05$) according to Fischer's protected least significant difference.

TABLE 3.4. Relative area under population curve (RAUPC) of *Pantoea vagans* strains C9-1S and C9-1^{Kr} on pear and apple flowers as affected by antibiotic oversprays made in experimental orchards located near Corvallis, OR in 2009 and 2010*.

Treatment	Relative area under population curve		
	Bartlett' Pear 2009	'Gala'/'Golden Delicious' Apple 2009	'Gala' Apple 2010
C9-1 ^{Kr} Water control	4.04 c	4.21 a	2.46 c
C9-1S Water control	3.99 c	3.88 a	2.26 c
C9-1 ^{Kr} then Mycoshield 200 µg/ml	4.33 c	---	3.24 b
C9-1S then Mycoshield 200 µg/ml	5.19 a	4.36 a	3.63 ab
C9-1 ^{Kr} then Kasumin 100 µg/ml	5.37 a	4.37 a	3.67 a
C9-1S then Kasumin 100 µg/ml	4.40 bc	4.24 a	3.31 ab
C9-1 ^{Kr} then Kasumin 80 µg/ml & Mycoshield 80 µg/ml	5.05 ab	4.05 a	3.44 ab

* Trees arranged in a complete randomized block design with 3 to 4 replications per treatment. Three liters of a suspension of C9-1S or C9-1^{Kr} (1×10^8 CFU/ml) were applied to trees at 30 and 70% bloom. Flowers were sampled from trees on two or three dates between full bloom and petal fall, from which the RAUPC values were determined and subjected to analysis of variance. Means within a column followed by the same letter do not differ significantly ($P \leq 0.05$) according to Fischer's protected least significant difference.

Establishment of E. amylovora on flowers. For each experiment, *E. amylovora* strain Ea153N was recovered from pear and apple flowers on all sampling dates in the range of 3 to 12 days after inoculation. Incidence of recovery of Ea153N was highest on trees treated with water compared to all other treatments (Fig. 3.7). Overall all sampling dates, the incidence of pathogen recovery on flowers from water treated trees averaged 70%, with a range among sampling dates of 54 to 88%. Over all sampling dates, population sizes of Ea153N on water treated trees averaged 2×10^6 CFU/flower and ranged between 1×10^2 and 5×10^7 CFU/flower.

Integrated treatments which had *P. vagans* C9-1S or C9-1^{Kr} followed by an antibiotic showed a significantly lower ($P \leq 0.05$) incidence of *E. amylovora* compared to the water treated trees (Fig. 3.7). For example, in 2009, 'Bartlett' pear trees treated with C9-1S or C9-1^{Kr} then Kasumin averaged an 11% incidence of recovery compared to 73% on the water-treated control. The apple experiments in 2009 and 2010 were similar: 8% incidence of recovery of the pathogen from flowers treated with C9-1S or C9-1^{Kr} then Kasumin compared to 68% for treated with water (Fig. 3.7). Population size of Ea153N on flowers on which it could be detected also was greatly reduced by the integrated treatment of C9-1S or C9-1^{Kr} followed by Kasumin (data not shown). There were, however, no significant differences among specific integrated programs in how these strains affected the detectable population size of Ea153N.

The antibiotic component of the integrated programs resulted in some differences in the size of the pathogen population. In 2009 in 'Bartlett' pear, the

treatment with C9-1^{Kr} followed by the Kasumin/ oxytetracycline mixture (both at 80µg/ml) resulted in a significantly ($P \leq 0.05$) smaller value of RAUPC compared to the other biological and overspray treatments (data not shown). Similarly, for all three experiments, AVOVA of RAUIC and RAUPC showed that overspray of C9-1S or C9-1^{Kr} followed by an overspray of Kasumin generally resulted in significantly ($P \leq 0.05$) greater pathogen suppression than overspray of the antagonists with oxytetracycline (Fig. 3.7).

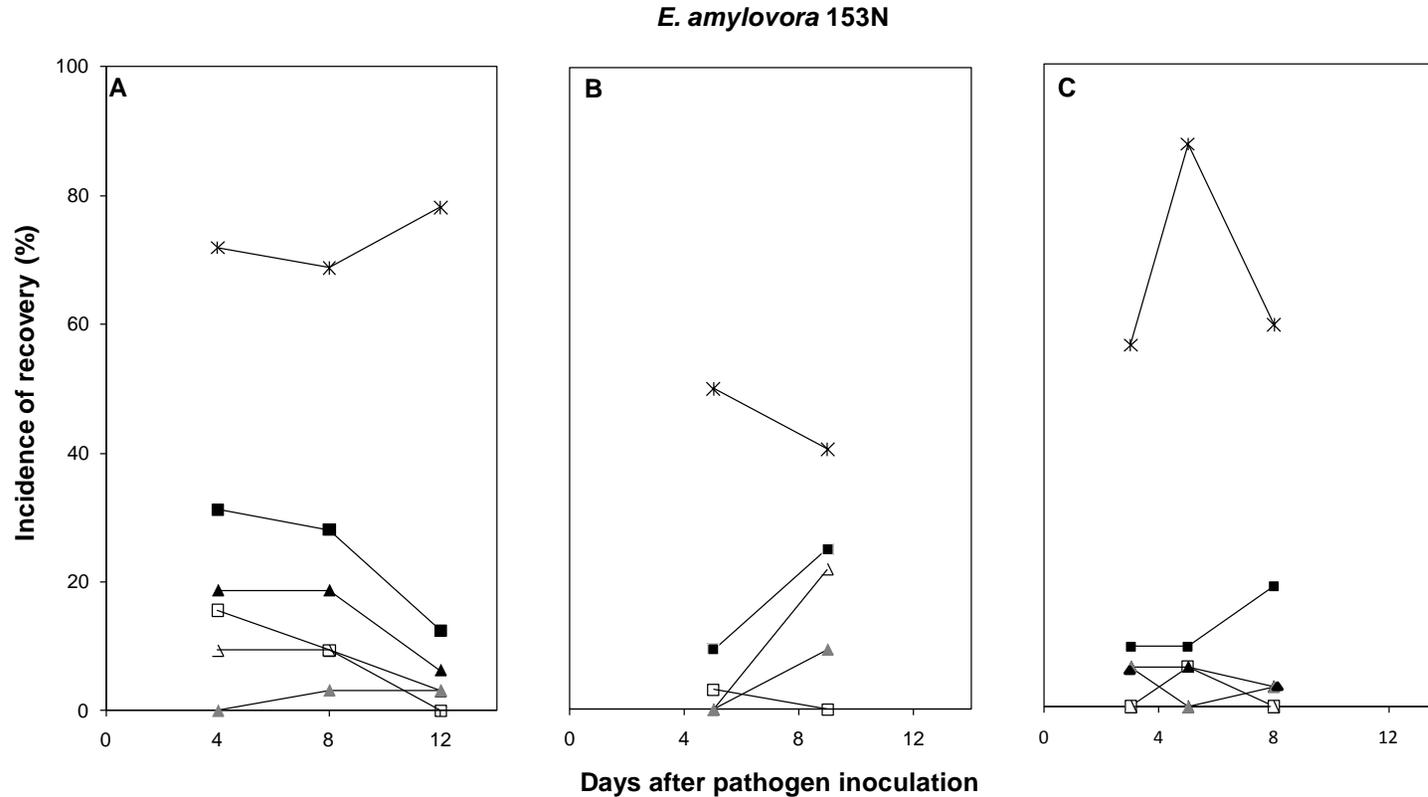


Figure 3.7. Incidence of recovery of *Erwinia amylovora* strain 153N from flowers of **A)** Bartlett pear in 2009, **B)** ‘Gala’/‘Golden Delicious’ apple in 2009 and **C)** ‘Gala’ apple in 2010 as influenced by treatments designed to evaluate integrated biological and chemical control of fire blight. Experimental orchards used in the study were located near Corvallis, OR. Treatments were *Pantoea vagans* strain C9-1S followed with oxytetracycline (200 µg/ml, ■) or Kasumin (100 µg/ml, □), *P. vagans* strain C9-1^{Kr} followed with oxytetracycline (200 µg/ml, ▲), Kasumin (100 µg/ml, Δ), or a mixture of oxytetracycline (80 µg/ml) and Kasumin (80 µg/ml, ▲), and a water-treated control (*). *P. vagans* strain C9-1^{Kr} followed by oxytetracycline (200 µg/ml) was not a treatment in the 2009 ‘Gala’/‘Golden Delicious’ apple experiment.

Disease incidence. Fire blight was observed in all experiments. In 2009, water-treated trees of Bartlett pear averaged 485 infected flower clusters (strikes); the same treatment in ‘Gala’/‘Golden Delicious’ apple experiment averaged 133 strikes per tree. In 2010, water-treated trees in ‘Gala’ apple averaged 236 strikes per tree. In 2009, the most effective treatment was streptomycin sulfate, which suppressed the incidence of blighted flower clusters by an average of 98% when targeted at the streptomycin-sensitive pathogen strain Ea153N (Table 3.5). In 2010, however, the most effective treatment was a single application of the tank mix of Kasumin (80 µg/ml) with oxytetracycline (80 µg/ml), which suppressed disease incidence by 99% (Table 3.5).

In all experiments, integrated treatments with biological control agent, C9-1S and its derivative C9-1^{Kr}, used in conjunction with an antibiotic significantly reduced ($P \leq 0.05$) the incidence of blighted flower clusters (Table 3.5). In 2009, the average disease suppression of integrated treatments was 95% and 92% in ‘Bartlett’ pear and ‘Gala’/‘Golden Delicious’ apple, respectively. In 2010, the average disease suppression from integrated treatments in Gala apple was 98%.

Integrated treatments with C9-1S and its derivative C9-1^{Kr}, for the most part, showed few differences when oversprayed with the same antibiotic. In those treatments oversprayed with Kasumin, no significant differences were found with regard to the *P. vagans* strain used in combination when compared to the water only control treatment. The same observation resulted from oversprays of oxytetracycline after application of either biological control strain. Furthermore, although integrated treatments involving Kasumin oversprays were always numerically superior to integrated treatments with

oxytetracycline oversprays, these differences within a specific experiment were not significant ($P > 0.05$).

Integrated treatments and antibiotic only programs showed statistically similar levels of disease suppression. Exceptions to this were in the 2009 'Bartlett' pear trial, where C9-1^{Kr} and the overspray of oxytetracycline (80 µg/ml) and Kasumin (80 µg/ml) suppressed disease significantly better than oxytetracycline alone. The performance of the Kasumin/oxytetracycline mixture was similar (and once significantly better) compared to Kasumin by itself (Table 3.5).

TABLE 3.5. Incidence of fire blight among clusters of pear or apple flowers as affected by integrated biological and chemical control treatments, and by antibiotic treatments made in experimental orchards located near Corvallis, OR in 2009 and 2010.

Treatment	‘Bartlett’ Pear 2009	‘Gala’/‘Golden Delicious’ Apple 2009	‘Gala’ Apple 2010
	Percent blighted floral clusters*	Percent blighted floral clusters	Percent blighted floral clusters
Water control	44.0 a [#] (485) ^{##}	44.1 a (133)	26.0 a (236)
<i>Integrated treatments**</i>			
C9-1 ^{Kr} then Mycoshield 200 µg/ml ^{***}	7.0 bc	---	2.2 bcd
C9-1S then Mycoshield 200 µg/ml	5.1 bc	11.2 b	2.3 bc
C9-1 ^{Kr} then Kasumin 100 µg/ml	3.5 bcd	6.8 b	1.4 bcde
C9-1S then Kasumin 100 µg/ml	4.0 bcd	5.2 b	1.8 bcde
C9-1 ^{Kr} then Kasumin 80 µg/ml & Mycoshield 80 µg/ml	3.0 cde	7.2 b	1.9 bcde
<i>Antibiotic standards</i>			
Agri-mycin 100 µg/ml	1.1 e	2.3 b	1.4 bcde
Mycoshield 200 µg/ml	9.3 b	2.4 b	2.6 b
Kasumin 100 µg/ml	3.5 bcd	4.0 b	1.2 cde
Kasumin 100 µg/ml (once)	---	---	2.3 bc
<i>Antibiotic mixtures</i>			
Kasumin 80 µg/ml & Mycoshield 80 µg/ml	4.8 bc	3.9 b	1.8 bcde
Kasumin 80 µg/ml & Mycoshield 80 µg/ml (once)	---	---	1.0 e
Kasumin 80 µg/ml & Mycoshield 100 µg/ml	3.3 bcde	3.6 b	1.5 bcde
Kasumin 100 µg/ml & Mycoshield 100 µg/ml	2.5 de	---	---

* Data were transformed arcsine(\sqrt{x}) prior to analysis of variance; non-transformed means are shown.

Means within a column followed by the same letter are not significantly different according to Fischer’ protected least significance difference at $P = 0.05$.

Mean number of fire blight strikes on water-treated trees.

** Bacterial strains C9-1S and C9-1^{Kr} were applied at a rate of 1×10^8 CFU/ml.

*** All antibiotic treatments were applied once in integrated treatments and twice in all others unless noted.

Discussion

This study was concerned with an alternative antibiotic, Kasugamycin, for control of fire blight of pear and apple, and with the evaluation of two strategies designed to mitigate the risk of resistance to this antibiotic developing in the pathogen population. A recent report (McGhee and Sundin 2011) demonstrated a potential for the fire blight pathogen to become resistant to this antibiotic, but that achieving a level of resistance above the label rate (100 $\mu\text{l/ml}$) required a stepwise selection process. This stepwise process is unlike mutation to streptomycin-resistance, where a mutant with a high level of resistance can be achieved in a single selection event. Based on their results, McGhee and Sundin (2011) recommend maintaining an effective dose in the orchard (full rate, avoid alternate row spraying) as a resistance management practice, but they did not provide any additional recommendations.

Additional tenets of pesticide resistance management include limitation of fungicide use, integration with other control practices, and chemical mixtures (Staub, 1991), each of which are part of the specific resistance management practices examined in this thesis. The primary resistance management strategy evaluated was to integrate an alternative practice (biological control), which also results in the limitation of Kasumin use. By first treating trees with a biological agent followed by the antibiotic, this ‘integrated strategy’ reduces selection pressure on the pathogen population by limiting Kasumin to one application, as opposed to the two applications often typical in commercial production, and which has been proposed on the pending Kasumin label. In addition, by first treating with a biological agent, epiphytic populations of the pathogen

on stigmas are suppressed via the competition that occurs with the antagonist, *P. vagans* C9-1S (Johnson et al., 1993b; Nucló, et al., 1998; Johnson et al. 2000a; Johnson et al., 2000b; Pusey et al., 2002;). A smaller pathogen population size on flowers at the time of the antibiotic treatment should also lessen the likelihood of selection of a resistant strain.

In a previous study of integrated biological and chemical control of fire blight, Stockwell et al. (2007) oversprayed a biological treatment with oxytetracycline and obtained a level of disease control that was significantly superior to either two applications of the biological agent, or to two applications of oxytetracycline. The working hypothesis put forth to explain this result is that an integrated strategy first provides protection of the stigma with the antagonist treatment, then provides protection of the nectary (the pathogen point of entry to the flower) when the antibiotic is applied later in bloom. Consequently, this thesis was concerned with evaluation of the integrated strategy with Kasumin substituted for oxytetracycline. Overall, it was observed that integrated strategy represented by *P. vagans* strain C9-1S followed by an overspray of Kasumin provided outstanding fire blight control (average level of suppression was 96%). Nonetheless, results from the integrated strategy were only on par with the two applications of Kasumin, and did not provide control superior to antibiotics alone as was observed by Stockwell et al. (2007). In fact, in 2010, when Kasumin was applied once by itself, the level of control was statistically similar to that achieved by the biological agent, *P. vagans* C9-1S, oversprayed with Kasumin. Thus, while the integrated strategy forced a limitation of Kasumin use, further trials are needed to show the benefits of the biological agent within the program.

It was hypothesized that a kasugamycin-resistant strain of *Pantoea vagans* strain C9-1S would improve the efficacy of the biological component of the integrated strategy, but the evidence collected in the field experiment failed to support this idea. A rationale for this hypothesis was that the gram negative bacteria currently registered for fire blight control (*Pseudomonas fluorescens* strain A506, *Pantoea agglomerans* strain E325, and *Pantoea vagans* strain C9-1S) all were selected to be resistant to streptomycin (Stockwell et al., 1996). With resistance to streptomycin, these bacteria are able to continue to colonize pear and apple flowers when oversprayed with this antibiotic (Johnson et al., 2002; Lindow et al., 1996; Stockwell et al., 1996). In contrast, none of these bacterial agents are resistant to oxytetracycline (Johnson et al., 2002; Lindow et al., 1996; Stockwell et al., 1996), and the overspray of this bacteriostatic chemical onto established antagonist populations did not greatly impact their population dynamics (Stockwell et al., 1996). The results of this study demonstrated that the effect of Kasumin on a non-target bacterial inhabitant of the flower, in this case *P. vagans*, is more like that observed with oxytetracycline than observed with streptomycin. This conclusion was evidenced by the lack of differences in incidence and population size among the *P. vagans* strains C9-1S (kasugamycin-sensitive) and C9-1^{Kr} (kasugamycin-resistant) in integrated programs with Kasumin. Growth chamber studies of *P. vagans* strains in the presence of Kasumin overspray differentially reduced the population size of C9-1S relative to C9-1^{Kr} (Fig. 3.3). Perhaps in the field, the selection intensity imposed by Kasumin is not as great as in the growth chamber experiment with detached flowers. Alternatively, perhaps the stepwise mutation in C9-1^{Kr} to kasugamycin-resistant resulted in a cost to reproductive fitness that

reduced its ability to grow and disperse itself in the field environment relative to the sensitive, parental type. McGhee and Sundin (2011) reported reduced fitness in kasugamycin resistant strains of *E. amylovora*, which was manifested by reduced virulence in detached fruit. These researchers also noted that while Kasumin induced a 100-fold reduction in total cultural bacteria on field-treated trees, many different bacteria surveyed after overspray were present, suggesting that these organisms were insensitive to the antibiotic. They speculated that a lack of sensitivity in indigenous bacteria could be advantageous in that their presence on the flowers after Kasumin treatment would lessen the selection pressure on the pathogen and other bacterial that harbor a Kasumin-resistance gene. If true, the utilization of the kasugamycin-sensitive *P. vagans* strain C9-1S in an integrated control strategy could potentially enhance this effect.

The selection of kasugamycin resistance in *P. vagans* showed parallels to what is known about the development of resistance to kasugamycin in *E. amylovora*. As was observed by McGhee and Sundin (2011), it was not possible to achieve a mutant of *P. vagans* strain C9-1 when plated on kasugamycin at 100 $\mu\text{l/ml}$ (the proposed field rate for Kasumin), but it was possible to achieve this level of resistance by stepwise selection at 50 $\mu\text{l/ml}$ followed by 150 $\mu\text{l/ml}$. Base on laboratory characterization studies, the mutation in C9-1^{Kr} was apparently stable and did not appear to influence growth rate in the absence of kasugamycin. Nonetheless, replating of freeze-dried C9-1^{Kr} onto a kasugamycin-amended medium demonstrated that the concentration of antibiotic influenced the recovery of viable colony forming units of the antagonist. This is in contrast to streptomycin-resistant mutants, which are generally unaffected by high rates

(100 to 200 $\mu\text{l/ml}$) of the antibiotic. The reduced recovery of the mutant at high rates of kasugamycin showed that the chemical still has some effect of the cell as it transitions from the stationary phase to active growth.

The second resistance management strategy evaluated in the thesis was the use of mixtures of Kasumin and oxytetracycline. Mixtures of active chemicals has long been advocated in plant disease control (Jones, et al. 1985; Culbreath, et al. 1995; Reuveni, 2001), and in fact, the original formulations of streptomycin registered for fire blight control (late 1950s) were amended with 10% oxytetracycline for the purpose of resistance management (van der Zwet and Kiel 1972). The oxytetracycline formulated as a premix with streptomycin was later dropped in the mid-1960s; resistance in *E. amylovora* to streptomycin was first reported in California in the early 1970's, 12 years after first registration, but only a few years after removal of the mixture partner. In developing a mixture to evaluate in these studies, the assumption was that effective amounts of each material should be present in the mixture, but with concerns toward the cost of the mixture to the grower and also toward the previous observations that Kasumin applied multiple times at higher rates ($> 100 \mu\text{l/ml}$) has been phytotoxic to pear and apple (Adaskaveg et al., 2011; Aldwinckle and Norelli, 1990; Psallidas and Tsiantos, 2000). Thus, 80 $\mu\text{l/ml}$ Kasumin and 80 $\mu\text{l/ml}$ oxytetracycline was chosen for evaluation, and although many other mixtures could be evaluated, this particular mixture provided excellent fire blight control. Moreover, the 80:80 mixture was very effective when oversprayed in an integrated program *P. vagans* C9-1^{Kr}. A reduction, however, in the rate of Kasumin used in the mixture was unnecessary as phytotoxic effects of the

antibiotic on pear or apple were not observed for any of the rates in any of the experiments.

It would be interesting to take the control strategies developed in this thesis to a larger orchard setting as was done by Lindow et al. (1996) in the first paper concerned with integrated biological and chemical control. The advantage of this approach is that fire blight infections develop from natural inoculum of *E. amylovora*, which requires the prerequisite epiphytic phase on the stigma. The high doses pathogen inoculum used in artificially inoculated studies can place sufficient number cells of the pathogen on the nectary to achieve infection, which bypasses the need for an increase of cell number on the stigma. As was done by Lindow et al. (1996), the way to implement this experiment would be to allow the participating growers to use Kasumin as they would normally, and split the orchards block so the half receive pretreatment with the biological agent.

Overall, the orchard trials showed that there was no statistical difference between most integrated or biological and chemical control with Kasumin/oxytetracycline mixed treatments when compared to the standard, streptomycin. This antibiotic, when used with the streptomycin-sensitive strain of the pathogen, is considered to be the standard to which alternative strategies and materials for fire blight control should be compared. Importantly, if Kasumin obtains an EPA registration in the next year, the ability and flexibility of growers to successfully manage fire blight in their orchards should be improved. Results in this thesis demonstrate effective control can be achieved with strategies that limit Kasumin use.

Conclusion

The principal hypothesis addressed in this study concerned adaptation of integrated biological and chemical control of fire blight as a potential resistance management strategy for Kasumin when used against *E. amylovora*, and whether the use of a kasugamycin-resistant strain of *P. vagans* strain C9-1S within the context of integrated control would be more effective in overall suppression compared to its non-resistant parent strain. For the former, the answer is yes, Kasumin can be adapted to an integrated management strategy. The use of a biological control agent, *P. vagans* strain C9-1S, followed by an application of Kasumin at full bloom provided excellent disease control, which was comparable to two applications of Kasumin by itself and also to the streptomycin standard. The selection and field use of a kasugamycin-resistant strain, *P. vagans* strain C9-1^{Kr}, however, did not enhance fire blight suppression beyond that achieved by the non-resistant parent strain, C9-1S. Thus, the study showed that the integration of a currently available, kasugamycin-sensitive biological control agent with Kasumin is an acceptable approach to fire blight control. This approach achieves reduced selection pressure on the pathogen population through a limitation in Kasumin use.

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