Biocontrol News and Information 1996 ***Vol.17 No. 4*** 67N – 78N

Review Article

Honey bees and epiphytic bacteria to control fire blight, a bacterial disease of apple and pear

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ABSTRACT

Incidence of fire blight (*Erwinia amylovora*) can be reduced by spraying apple and pear flowers with some strains of *E. herbicola* or *Pseudomonas fluorescens*. Most of these beneficial bacteria were selected in the laboratory using an assay on immature pear fruit, which has also proved useful to study their mode of action. Recently, an assay on crab apple flowers has also been developed. Pre-emptive colonisation by beneficial bacteria of the stigmas, where *E. amylovora* usually multiplies, can be enough to prevent the pathogen from multiplying and infecting the plant. In addition, most strains of *E. herbicola* produce compounds inhibitory to *E. amylovora*, some of which have been shown to play a role in reducing incidence of fire blight. To prevent selection of strains of *E. amylovora* resistant to these inhibitory compounds and to ensure control over a wide range of climatic conditions, the possibility of using mixtures of beneficial bacteria has been investigated. Taking advantage of the fact that the interaction between the pathogen and biological control agents has to occur on the stigmas and that beehives are already present in orchards for pollination, honey bees have been used to bring the biological control agents to the flowers. Biological control agents can be integrated with antibiotics currently used to control fire blight and some of these agents might also reduce frost injury and russeting. Future strategies for control of fire blight will take advantage of our increased understanding of the mechanisms involved in biological control and of the technological advances in fields such as plant biotechnology.

INTRODUCTION

Never before has so much progress been made so rapidly on biological control of fire blight and a review on this topic is extremely timely. The increasing number of presentations on biological control submitted at the International Workshop on Fire Blight held every 3 years since 1977 reflects the increased interest in this topic over the last 20 years (Fig. 1). Some major developments have resulted from these studies: registration of the first biological control agent, which is for sale in the USA for the first time this spring; understanding the mode of action of some biological control agents and cloning some of the genes involved in these mechanisms; development of a delivery mechanism relying on honey bees; and development of a new assay to screen potential biological control agents, which might lead to identification of new strains and perhaps new species of bacteria to be used as biological control agents. In this paper I will review the progress made on biological control of fire blight, present how and why some of the most recent developments will allow rapid integration of biological control agents into existing strategies of control of fire blight and also what the future might offer.

THE FIRE BLIGHT PATHOGEN *ERWINIA AMYLOVORA*

Fire blight is a disease especially destructive to apple (*Malus pumila*) and pear (*Pyrus communis*) trees, but which also affects other plants such as hawthorn (*Crataegus*), *Cotoneaster* or *Pyracantha.* It destroys not only the crop of the year, but it can also kill mature trees within one season, resulting in huge losses. For example, an outbreak of fire blight in southwest Michigan in 1991 resulted in a US$3.8 million loss (Moses 1992). Fire blight also constitutes a market access barrier between countries where the disease has been reported and fire blight free countries.

Although it can use other natural openings, *Erwinia amylovora*, the causal agent of fire blight, uses flowers as the main port of entry (for a review see Vanneste 1995). Before entering the tissues, *E. amylovora* colonises the stigmatic surfaces of the flowers. For infection to occur, climatic conditions must be favourable: temperatures high enough to allow bacterial multiplication on the stigmas, and water present to allow migration of the bacteria to the nectaries, where they enter the tissues through the nectarhodes (the

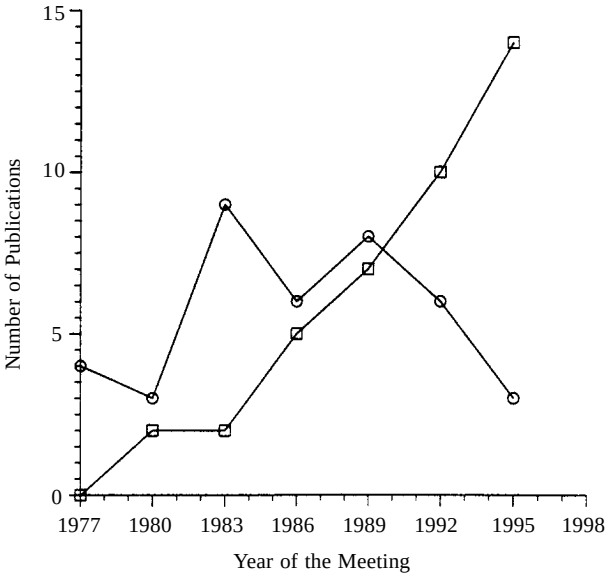

Figure 1. Number of publications on biological (open square) and chemical (open circle) control published in the proceedings of the International Workshops on Fire Blight.

orifices from where the nectar is secreted). When the bacterium is in the tissue it usually multiplies rapidly, leading to one of the first visible symptoms of fire blight: presence of drops of exudate. This exudate, made of bacteria embedded in exopolysaccharides, can be easily picked up and transferred to healthy flowers by insects, wind, rain or humans, resulting in the rapid and wide spread of the disease. If the host is sensitive and climatic conditions favourable, the bacteria move rapidly from the flower to the pedicel, then to the twig, reaching the main branch and sometimes getting to the trunk, killing the tree. The infected tissues suffer rapid necrosis and, ahead of these tissues, drops of exudate might be produced.

During summer the bacteria enter the tissue by either late flowers or, more generally, by insect and hail damage on leaves or young fruit, and wind break on young leaves. During autumn the bacterium forms a canker in which it overwinters. In early spring the bacteria multiply in the canker and are transferred to the flowers, starting a new cycle. The best stage to break this cycle is probably at flowering, because flowers constitute the main port of entry of the pathogen and it is flower infections that generate the inoculum indispensable for future infections.

Once the bacterium is in the tissue, progression of the disease cannot be controlled by spraying chemicals; all known treatments are preventative. Furthermore, there are only two treatments available: copper derivatives and antibiotics. Copper derivatives are phytotoxic on flowers at the dose required to kill the bacteria and can lead to russeting. Antibiotics have to be applied throughout the blooming period since their effectiveness decreases rapidly after they are sprayed (Fig. 2). In apple or pear growing regions where climatic conditions are usually not favourable for infection during bloom and where fire blight occurrence is erratic, growers often do not spray antibiotics because of the cost associated with their use. More importantly, strains of the pathogen resistant to streptomycin, which is the most effective and most used antibiotic against fire blight, have been isolated in several countries (Vanneste 1995). To avoid development of such resistance, which in some cases could be transmitted to animal and human pathogens, several countries in Europe prohibit the use of antibiotics to control plant pathogenic bacteria. This lack of available treatments for such an important disease prompted an increasing number of scientists to look at biological control as an alternative.

EPIPHYTIC BACTERIA AS BIOLOGICAL CONTROL AGENTS

Most studies on biological control of fire blight focused on two species of epiphytic bacteria: *Erwinia herbicola* and *Pseudomonas fluorescens*. Strains called *E. herbicola* belong to a diverse group of bacteria found in a variety of places, and include opportunistic pathogens of humans and animals also called *Enterobacter agglomerans* (Starr 1981). Recently, based on total DNA homology and electrophoretic protein pattern similarities, strains of *Erwinia herbicola* and *Enterobacter agglomerans* have been proposed to form a new genus called *Pantoea* (Gavini *et al.* 1989). In this review, I have used the most common name, *Erwinia herbicola*.

Non-pathogenic yellow-pigmented bacteria, though not always formally identified as *E. herbicola*, have often been isolated from diseased plant tissues in association with *E. amylovora* (Isenbeck and Schulz 1985; Miller and Schroth 1972; Riggle and Klos 1972; Wrather *et al.* 1973; Erskine and Lopatecki 1975; Goodman 1967). It was reported that some of these non-pathogenic strains isolated from fire blight lesions could reduce the incidence of fire blight on plants in greenhouses and in orchards. Control of fire blight has been achieved by spraying suspensions of the antagonistic strain onto apple (Beer *et al.* 1984a; Goodman 1965; Vanneste and Yu 1990; Wrather *et al.* 1973), pear (McIntyre *et al.* 1973; Riggle and Klos 1972; Wrather *et al.* 1973), Asian pear (*Pyrus pyrifolia*) (Vanneste *et al.* 1995) or hawthorn blossoms (Wilson *et al.* 1990), before inoculating with *E. amylovora*. Isenbeck and Schulz (1985) also reported that injection of a suspension of *E. herbicola* in the stem of *Cotoneaster* before inoculation with *E. amylovora* reduced fire blight infection as effectively as injection of streptomycin. When *E. herbicola* is sprayed on healthy apple, pear or Asian pear flowers, it establishes easily and grows to reach populations of about 10^6 colony forming units (cfu) per flower. However, *E. herbicola* is not a major component of the microflora of healthy apple (Kearns and Hale 1995) or pear flowers (Manceau *et al.* 1990). A study involving four apple orchards in New Zealand revealed that natural populations of *E. herbicola* were below 50 cfu / flower until petal drop, when they multiplied to reach about 10^5 cfu / flower (Kearns and Hale 1995). In contrast to *E. herbicola*, large populations of *Pseudomonas fluorescens* can frequently be found on healthy apple and pear flowers (Manceau *et al.* 1990; Kearns and Hale 1995). Only a few strains of *Pseudomonas* have been studied in detail; paradoxically, the only control agent registered today against fire blight is a strain of *P. fluorescens* called A506.

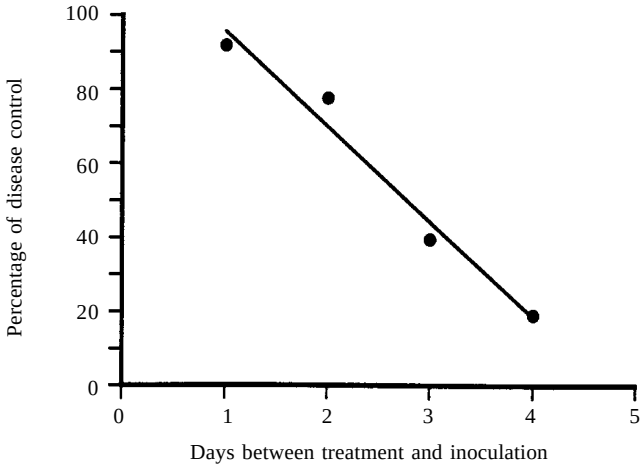

Figure 2. Influence of delayed inoculation on efficacy of streptomycin to control fire blight. Apple flowers were treated with streptomycin or water and then inoculated with *E. amylovora* at one day intervals. Data are from Vanneste *et al.* 1995.

Table 1. Comparison of percentage of infection of flowers treated with Eh252, streptomycin or water and inoculated with *E. amylovora*

Year	Location	Variety	Percentage of blossoms infected treated with			Reference
			Water	Eh252	Streptomycin 100ug / ml	
1982	NY	Apple cv. Idared	30	26	12	Beer *et al*., 1984a
1983	NY	Apple cv. Idared	42	21	16	Beer *et al*., 1984a
1984	NY	Apple cv. Idared	81	71	ND	Beer *et al*., 1984b
1985	NY	Apple cv. Idared	87	30	28	Beer *et al*., 1984b
1986	NY	Apple cv. Idared	26	12	4	Beer, Vanneste, Zumoff unpub.
1987	NY	Apple cv. Idared	39	21	8	Beer, Vanneste, Zumoff unpub.
1989	Fr.	Apple cv Golden Delicious	86	52	23	Vanneste & Yu 1990
1993	NZ	Apple cv. Granny Smith	58	31	9	Vanneste & Yu 1994
1994	NZ	Apple cv. Red Delicious	60	37	10	Vanneste Unpub. 1995
1994	NZ	Asian pear cv. Kosui	47	25	10	Vanneste *et al*.1995

A few laboratories also considered the use of non-virulent derivatives of *E. amylovora* as biological control agents. Such strains were shown to control fire blight on pear shoots (McIntyre *et al*. 1973), immature pear fruit (Wrather *et al*. 1973), apple trees (Goodman 1967) or apple seedlings (Tharaud *et al*. 1996), if present before inoculation. Only *E. amylovora* mutants affected in certain *hrp* genes could also confer some protection in coinoculation experiments (Tharaud *et al*. 1996).

Altogether, only a few strains have been studied in detail. However, over 10 years of data from field experiments are available for some of these strains, such as A506 or Eh252 (Table 1). As pointed out by Lindow *et al*. (1996), the artificially high levels of inoculum used in these field experiments do not allow an accurate estimation of the efficacy of biological control agents in reducing fire blight incidence. These high levels of inoculum result in a high percentage of infection, including on streptomycin treated flowers. To mimic natural infections of fire blight, Johnson *et al*. (1993a,b) used bees to disperse *E. amylovora*. Often, the level of infection is higher on flowers treated with a biological control agent than on flowers treated with streptomycin, but in most field experiments *E. amylovora* is applied 24 hr after treatment, when streptomycin is the most effective. When inoculation is delayed for two or three days, the decreased efficiency of streptomycin and the stable or increasing level of control obtained with the biological control agents erases any significant differences between the two treatments (Vanneste *et al*. 1995) (Fig. 3).

THE SEARCH FOR THE PERFECT LABORATORY ASSAY

To develop the best biological control strategy, we need to identify the most effective biological control agents and determine their mode of action. The development of an assay that can answer these two questions and can be performed in the laboratory all year round is therefore extremely important. If we want to compare the ability of different strains, including mutants, of biological control agents to control fire blight, this laboratory assay has to be reproducible. The standardised and controlled conditions necessary to make an assay reproducible contrast with the unpredictability and ever-changing conditions which rule every field experiment. These assays, therefore, might not mimic the field experiments, but they might help to understand what happens on flowers in the field.

Measuring fire blight incidence on immature pear fruits

One of the first screening assays to select potential biological control agents was production of a bacteriocin on plate (Beer *et al*. 1984a). However, it was rapidly noted that there was no correlation between inhibition on plate and ability to inhibit the pathogen in the field (Beer *et al*. 1984a), leading Beer and Rundle to develop an assay on immature pear fruit (Beer and Rundle 1983). Immature pear fruits were initially used to test the pathogenicity of suspected strains of *E. amylovora* in the laboratory (Billing *et al*. 1960). They have also been used to identify mutants altered in pathogenicity (Vanneste 1995). Immature pear fruits inoculated with pathogenic strains of *E. amylovora* and incubated in a humid chamber (usually a large Petri dish or a tray lined with wet paper towels) typically show production of exudate and necrosis. But if the fruits are first treated with bacteria antagonistic to *E. amylovora*, no symptoms are observed. Using half pears with a well bored in their cheek, in which the biological control agent and the pathogen were placed, Beer and Rundle (1983) found a correlation between the ability of 16 strains of *E. herbicola* to inhibit the development of *E. amylovora* in the laboratory and their ability to reduce fire blight incidence in apple orchards. Analysis of variance using orthogonal comparisons showed that strains with high or medium effectiveness on immature pear fruit were more effective in reducing infection on apple blossoms in the orchard than strains with low or no effectiveness. They concluded that this assay was useful for eliminating strains that are ineffective in the orchard. It was further noted that strains of *E. herbicola* that suppress *E. amylovora* on apple blossoms were also effective in immature pear fruit (Beer *et al*. 1984b), and *E. herbicola* Eh159, which is not effective in the orchard, is also not effective on immature pear fruit (Vanneste *et al*. 1996). Other laboratories using a modification of this assay came to similar conclusions. Nicholson *et al*. (1990) found

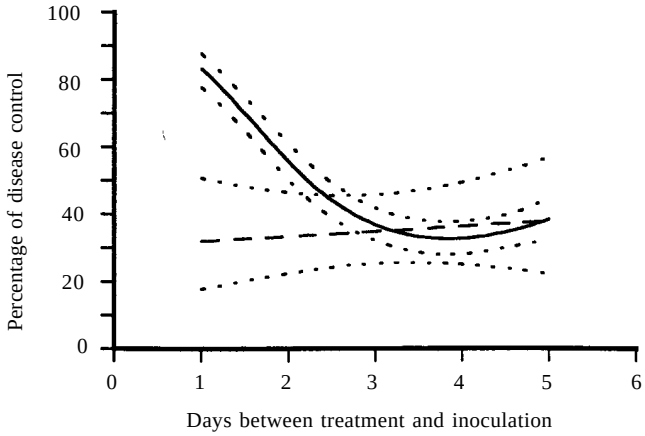

Figure 3. Influence of delayed inoculation on efficacy of *E. herbicola* Eh252 (dashed line) and streptomycin (solid line) to control fire blight. Curves were fitted to the data using a Bayesian smoother (Upsdell 1994). Treatments are significantly different at 95% confidence level if confidence bans do not overlap. Data are from Vanneste *et al.* 1995.

that this assay gave an accurate prediction of the performance of 4 strains of *E. herbicola* in the field on perry pear blossoms, and Wilson *et al.* (1990) found that even as a quantitative assay (see below) it was a useful assay to screen for antagonistic activity.

Every laboratory which used this assay modified it slightly to meet their own requirements or to satisfy their own beliefs about the control agent / pathogen interaction. As a consequence, we now have different assays carrying the same name. Some laboratories used slices with (Wilson *et al.* 1990) or without (Isenbeck and Schulz 1985) a well bored in them, others used a quarter of slices (Ishimaru *et al.* 1988) and more recently plugs of about 4 mm diameter (Vanneste *et al.* 1996). The slices of pear are either dipped in a suspension of biological control agents (Isenbeck and Schulz 1985), their upper surface is covered by the bacterial suspension (Wrather *et al.* 1973; Vanneste *et al.* 1996) or a drop of bacterial suspension is placed on their surface or in the well (Beer and Rundle 1983). *E. amylovora* was applied 24 hr (Isenbeck and Schulz 1985), 3 hr (Wilson *et al.* 1990), 2 hr (Vanneste *et al.* 1992; Beer and Rundle 1983), 15 minutes after treatment (Ishimaru *et al.* 1988) or simultaneously (Kearns and Mahanty 1993).

To add to the confusion, the assay can be read in two different ways. Each piece of fruit can be counted as infected or non-infected (qualitative assay), the relative number of non-infected fruit representing the relative ability of a biological control agent to protect against fire blight (Beer and Rundle 1983; Vanneste *et al.* 1992, 1996; Kearns and Mahanty 1993), or the amount of exudate produced per fruit can be used as a measure of the level of inhibition (quantitative assay) (Isenbeck and Schulz 1985; Ishimaru *et al.* 1988; Wilson *et al.* 1990; Pusey 1996). When used as a quantitative assay no correlation was found between the ability of different strains of *E. herbicola* to inhibit *E. amylovora* in fruits and their ability to inhibit *E. amylovora* in flowers (Wilson *et al.* 1990; Pusey 1996). This might indicate that the amount of necrosis and exudate produced on a pear slice does not depend only on the ability of a biological control agent to inhibit the pathogen; just as in the field the length of necrosis also depends on other factors than effectiveness of a biological control agent to prevent infection. The immature pear fruit assay should therefore be used as a qualitative assay.

When using immature pear fruit as a qualitative assay to compare different strains or derivatives of biological control agents, the number of fruits or pieces of fruit per treatment should be as large as possible. Furthermore, they should be incubated in the same humid chamber, since the time needed for appearance of exudate on an infected fruit depends on the humidity in the experimental box, which is difficult to control. The physiological state of the pears also influences the time needed for appearance of exudate. Therefore, the less fruit we need for the experiment, the less variation we will have. Caution should also be taken when applying the suspension of biological control agents and pathogen. In contrast to *E. amylovora*, *E. herbicola* does not multiply outside the area where it has been introduced (Beer *et al.* 1984b; Erskine and Lopatecki 1975). Thus, it is important to treat the entire fruit or to limit the evaluation to the site where both the biological control agent and the pathogen were deposited. When wells are made in half immature pear fruit (Beer and Rundle 1983) or on slices of fruit (Wilson *et al.* 1990), it might sometimes be difficult to ensure that all the surface exposed to the pathogen was protected by the biological control agent, which might lead to high levels of variation (Wilson *et al.* 1990). To address these different and seemingly contradictory requirements, Vanneste *et al.* (1996) developed an assay on plugs of pears. Since a large number of plugs can be made from one fruit, this assay requires only a small number of fruits. A large number of plugs can be incubated in the same humid chamber and it is easy to ensure that all of the inoculated surfaces were previously treated.

Immature pear fruit represents only imperfectly the way biological control agents interact with *E. amylovora* on flowers, but if some precautions are taken to ensure that all the inoculated surface is treated and the assay is interpreted qualitatively, it gives consistent results. This makes it useful to discriminate the good biological control agent from the bad and to compare strains when studying the mode of action.

Measuring population levels of the pathogen on flowers

Recently, Pusey (1996) developed a test on crab apple flowers that can be performed in the laboratory or in the greenhouse 11 months of the year. This assay consists of measuring the decrease in population of *E. amylovora* on the stigmas of flowers treated with biological control agents 24 or 48 hr before inoculation. This assay is based on the assumption that disease incidence is directly related to population level of the pathogen on stigmas. Although this seems a reasonable assumption and has been verified once (Johnson *et al.* 1993b), the relationship between population level and percentage of infection is not perfect. Thomson and Gouk (1992) reported that, in the field, the population of *E. amylovora* on pistils of apple flowers was not significantly affected by Eh318, while the percentage of infected flowers treated with this biological control agent was lower than that of flowers treated with water. On pear blossoms, Wilson and Lindow (1993) reported that coinoculation of A506 and *E. amylovora* had no significant effect on the population level of *E. amylovora.* However, the percentage of coinoculated flowers infected (50%) was significantly lower than that of the control flowers (71%). Johnson *et al.* (1993b) also reported that in two experiments on pear blossoms, the proportion of blossoms with more than 10^5 cfu / blossom was not affected by treatment of a mixture of A506 and C9-1, although the proportion of flowers with *E. amylovora* was slightly lower after treatments with these antagonists and that they significantly reduced incidence of fire blight. We also observed the opposite phenomenon in a controlled environment room on apple blossoms: different population levels of *E. amylovora* resulted in the same percentage of infection (Table 2).

Table 2. Incidence of fire blight on apple blossoms treated with Eh252 or a non-antibiotic producing mutant

Treatments	Percentage of flowers infected		Population of *E. amylovora**	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2
Buffer	20	45	nd**	2×10^7
Eh252	3	24	nd	6×10^5
10:12***	12	28	nd	5×10^6

*: populations of *E. amylovora* on flowers three days after treatment
**: nd: non-determined
***: 10:12 is a non-antibiotic producing mutant of Eh252

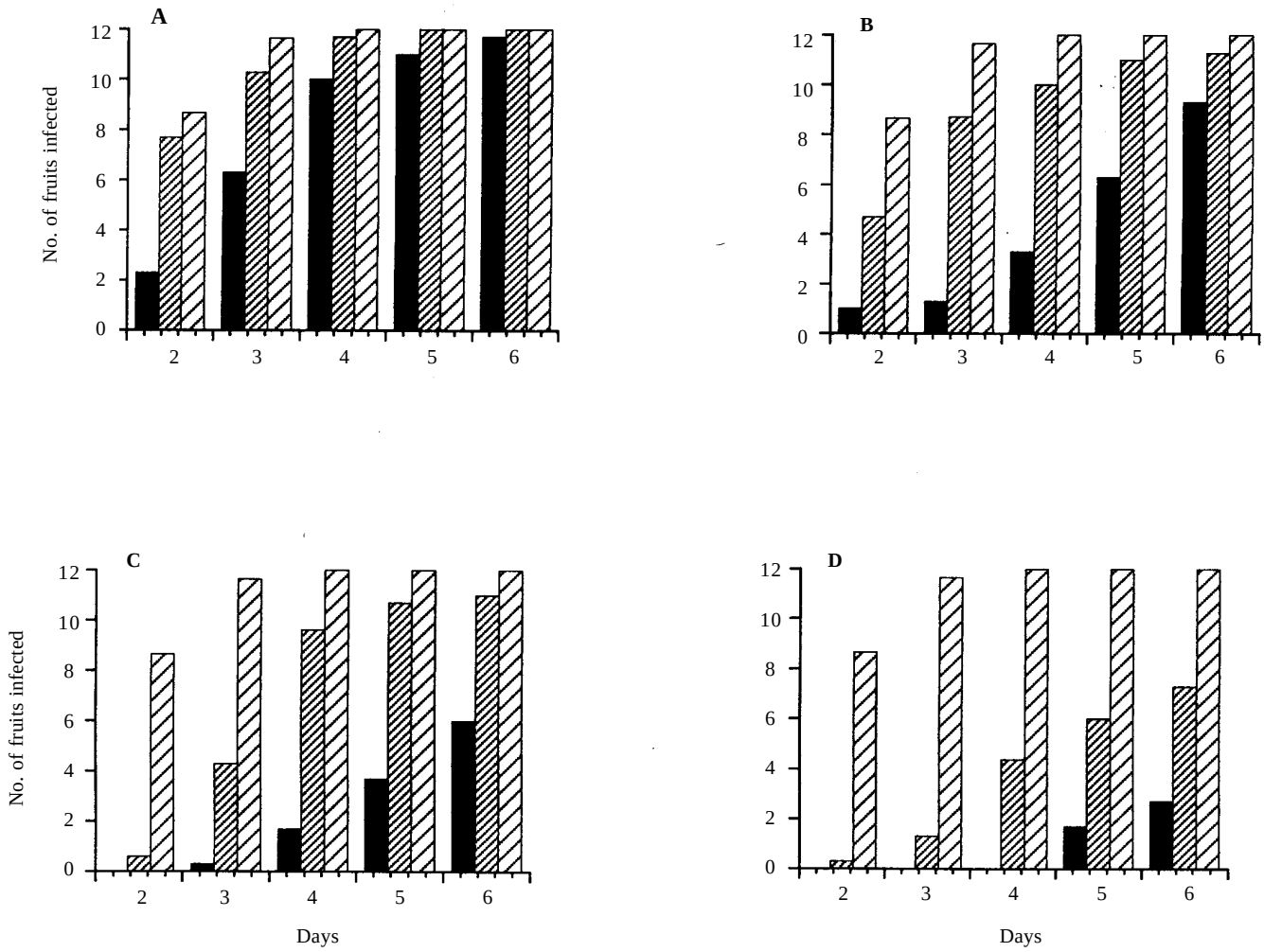

Figure 4. Number of immature pear fruits infected that were treated with *E. herbicola* Eh252 (dense hash marking), with the Ant- mutant 10:12 (medium hash marking), or with buffer (light hash marking) and inoculated with *E. amylovora*. The bars represent the average numbers of fruits infected from three independent experiments. Concentrations of *E. herbicola*: A, 5 x 10^3 cfu / ml; B, 5 x 10^4 cfu / ml; C, 5 x 10^5 cfu / ml; D, 5 x 10^6 cfu / ml. The concentration of *E. amylovora* was 3 x 10^3 to 4 x 10^5 cfu / ml. Published by Vanneste *et al*. (1992), reproduced with permission of the publisher of *Journal of Bacteriology*.

All of this clearly indicates that there is more to the level of infection than the population level of the pathogen. Nevertheless, after the first series of experiments, Pusey (1996) found a very good correlation between inhibition of *E. amylovora* on stigmas in the laboratory, in the greenhouse and in the field. What is exciting is probably not whether this assay in the laboratory mimics perfectly what happens in the orchards, but the fact that Pusey developed a new approach which might allow the identification of new biological control agents and perhaps new mechanisms. Furthermore, this is the only alternative to immature pear fruit. We do not have the perfect assay yet, but we do have two assays, with their own limitations and strengths, which can both help to identify biological control agents and to study their mode of action.

MODE OF ACTION

Several mechanisms have been proposed to explain the inhibition of *E. amylovora* by different strains of biological control agents. This includes production of toxic aglycones by hydrolysis of arbutin or phloridzin (Chatterjee and Gibbins 1969; Chatterjee *et al*. 1969; Hildebrand and Schroth 1964), induction of a phytoalexin-like compound (McIntyre *et al*. 1973), increase in acidity of the medium due to the growth of the epiphyte (Goodman 1965; Riggle and Klos 1972; Beer *et al*. 1984b) and competition for nitrogen (Beer *et al*. 1984b; Riggle and Klos 1972). However, the only mechanisms that have been shown to be involved in biological control of fire blight are competition for nutrients and space, and production of an antibiotic type molecule.

Antibiosis

Production of a substance inhibitory to *E. amylovora in vitro* has been reported for most strains of *E. herbicola* and yellow epiphytic bacteria isolated from fire blight lesions (Beer *et al*. 1984a; Erskine and Lopatecki 1975; Isenbeck and Schulz 1985; Ishimaru *et al*. 1988). In three studies involving respectively 301, 346 and 900 strains of *E. herbicola*, 12%, 42% and 45% of the strains tested inhibited *E. amylovora* on minimal medium (El-Goorani and Beer 1991; Wodzinski and Paulin 1994; Ophir and Beer 1993, respectively). Considering all 1500 strains together, 38% of the *E. herbicola* strains tested produce an antibiotic.

Most of the antibiotics produced by these strains of *E. herbicola* are non-toxic in the presence of certain amino acids. Using this characteristic as a basis of classification, Wodzinski and Paulin (1994) could distinguish 13 classes of antibiotic after analysis of 90 strains and Ophir and Beer (1993) grouped 405 strains into 15 distinct classes. All antibiotics belonging to one of these classes are not identical. For example, the antibiotic from Eh252 and that

from C9-1 (called herbicolin O) are both non-toxic in the presence of histidine, but in contrast to the antibiotic produced by Eh252, herbicolin O is resistant to proteolytic enzymes (Ishimaru *et al*. 1988; Vanneste *et al*. 1992). The ability of 12 strains of *E. herbicola* to produce antibiotic on minimal medium was found to correlate with effectiveness in a field test on apple flowers (Wodzinski *et al*. 1987b). However, no general rule can be drawn and the role of the antibiotic has to be determined for each strain.

E. herbicola strain Eh252 produces an inhibitory compound which is not toxic in the presence of histidine and proteolytic enzymes (Wodzinski *et al*. 1987; Vanneste *et al*. 1992). The class of *E. herbicola* strains which produce an antibiotic non-toxic in the presence of histidine might be the largest one. In two studies mentioned earlier, 51% and 70% of the antibiotic producing strains examined were no longer inhibitory to *E. amylovora* in the presence of histidine (El-Goorani and Beer 1991; Wodzinski and Paulin 1994, respectively). However, as pointed out earlier, these compounds might not all be equivalent and their role in the reduction of fire blight incidence might not be identical. In the case of Eh252, several lines of evidence indicate that production of this antibiotic is one of the mechanisms involved in inhibition of fire blight. When independent spontaneous mutants of *E. amylovora* resistant on plate to the antibiotic produced by Eh252 were used to inoculate immature pear fruit, the level of protection conferred by Eh252 was significantly reduced (Vanneste *et al*. 1990; D.A. Cornish and J.L. Vanneste, unpublished data). Furthermore, transposon induced mutants which lost ability to produce the antibiotic, called Ant$^-$ mutants, were not as effective as Eh252 in reducing fire blight incidence on immature pear fruits (Vanneste *et al*. 1992) (Fig 4).

The genes necessary for antibiotic production have been cloned and expressed in Ant$^-$ mutants. These complemented mutants protected immature pear fruit as well as the wild type strain (Vanneste and Yu 1996a). Two experiments were also performed on apple flowers in a climatised growth chamber (J.L. Vanneste, S.V. Beer and C.H. Zumoff, unpublished data). In the first experiment the percentage of infected flowers treated with Eh252 was significantly lower than that of flowers treated with an Ant$^-$ mutant (Table 2). In the second experiment, though no difference in the percentage of infection could be observed, population levels of *E. amylovora* were lower on Eh252 treated flowers than that on flowers treated with an Ant$^-$ mutant. So, on immature pear fruit and on apple blossom, production of an antibiotic by Eh252 is, under some circumstances, a major mechanism leading to reduction of fire blight incidence. However, under other circumstances, such as very high population of the biological control agent on immature pear fruit, this antibiotic has only a minor role.

E. herbicola strain Eh318 produces two antibiotics, one of which has been identified as an inhibitor of a transaminase competing for the substrate N-acetylornithine (Wodzinski *et al*. 1989). On immature pear fruit, Eh318 reduces incidence of fire blight, but to a lesser extent when a derivative of *E. amylovora* resistant to the antibiotics produced by Eh318 is used for inoculation rather than a wild type strain (Wodzinski *et al*. 1994). Furthermore, a boiled supernatant of Eh318 culture adjusted to pH 7 delayed the development of disease on immature pear fruit when inoculated with a sensitive strain of *E. amylovora*, but not when inoculated with a resistant derivative (Wodzinski *et al*. 1994). All these experiments suggest that production of antibiotic by Eh318 is one of the mechanisms involved in the inhibition of *E. amylovora*. However, using the same test on immature pear fruit (wells bored in a half fruit), Wright and Beer (1996) found that mutants of Eh318 which do not produce either one of the inhibitory compounds or both of them, did protect the fruit as well as the wild type strain. When the same authors used apple flowers in a controlled environment room, they found that the mutant which did not produce any inhibitory compound did not protect as effectively as the wild type strain. They concluded that the antibiotics are most probably involved in control of fire blight, though to a small extent. The discrepancies between their results on immature pear fruit and on apple flower and Wodzinski's results may be due to a high level of variation using the half fruit assay.

E. herbicola strain Eh112Y produces an antibiotic which is inactivated by histidine and seven other amino acids (Wodzinski *et al*. 1987a), and is not sensitive to proteases. Tn5-induced mutants of Eh112Y that lost the ability to produce herbicolacin 112Y were as effective as the wild-type strain in protecting immature pear fruits from fire blight (Beer *et al*. 1984a). Perhaps as shown for Eh252, the role of this antibiotic is masked by other mechanism(s) when high populations of bacteria are used. Alternatively, Eh112Y might produce an inhibitory compound other than herbicolacin 112Y that has not yet been detected. Both hypotheses would be in agreement with the report that culture filtrates of Eh112Y reduce fire blight incidence on ornamentals (Isenbeck and Schulz 1986).

E. herbicola strain C9-1, isolated from Jonathan stem tissue, produces at least two inhibitory compounds called herbicolins O and I, which are resistant to proteolytic enzymes (Ishimaru *et al*. 1988). Herbicolin O is inactivated by the presence of histidine (Ishimaru *et al.* 1988). Bacterial suspension of C9-1 or purified antibiotics limited the development of fire blight on immature pear fruit inoculated with a sensitive strain of *E. amylovora* but not, or to a lesser extent, when inoculated with a derivative resistant to either one or both antibiotics (Ishimaru *et al.* 1988). This suggests that these antibiotics are involved in the control of fire blight. The same conclusion was reached for the antibiotic produced by *E. herbicola* strain Eh1087. This strain of *E. herbicola* produces a β-lactam type antibotic (Kearns and Hale 1996). Transposon induced mutants of Eh1087 which lost ability to produce this β-lactam did not protect immature pear fruit against fire blight (Kearns and Mahanty 1993).

E. herbicola HL9N13 produces an antibiotic like compound which has been indirectly involved in control of fire blight (Wilson *et al.* 1992). When HL9N13 was sprayed on hawthorn blossoms the rate of growth of *E. amylovora* was reduced before the amounts of nutrients available on the stigma became limiting, which is consistent with inhibition of *E. amylovora* by production of an antibiotic. *P. fluorescens* A 506 is the only biological control agent which does not exhibit any antibiosis to *E. amylovora* when tested on different media, including minimal medium (Lindow 1985).

It is important to note that every time antibiosis has been shown to be involved in reduction of incidence of fire blight, it is always only one of the mechanisms involved in the control. The non-antibiotic producing mutants of Eh252 (Vanneste *et al*. 1992) and Eh318 (Wright and Beer 1996) still retained some ability to suppress fire blight development, especially at high concentrations, and derivatives of *E. amylovora* resistant to one or several antibiotics produced by Eh252 (D.A. Cornish and J.L. Vanneste, unpublished data), Eh318 (Wodzinski *et al*. 1994), or C9-1 (Ishimaru *et al*. 1988) are still inhibited by the respective biological control agent. A similar situation has been described in other systems where production of an antibiotic has been involved in biological control. Mutants of *Agrobacterium radiobacter* K84, that do not produce agrocin 84, still reduce disease caused by the pathogen *A. tumefaciens* (Cooksey and Moore 1982). Similarly, Tn5-induced mutants of *P. fluorescens* strain 2-79 that do not produce phenazine, an antibiotic involved in the inhibition of some pathogenic fungi, still suppress take-all caused by *Gaeumannomyces graminis* var *tritici* (Thomashaw and Weller 1988).

Colonisation of the stigmatic surfaces

The ability to grow in the same ecological niche or on the same tissues as the pathogen is an indispensable characteristic of any biological control agent of fire blight. Without this ability, the biological control agent cannot interact with the pathogen and even production of an efficient antibiotic become useless. This was illustrated on immature pear fruit using a strain of *Escherichia coli* harbouring a multicopy plasmid carrying the genes necessary for production of the antibiotic from Eh252. When immature pear fruit were treated with this *E. coli* derivative, which on plate produces huge amounts of the same antibiotic as Eh252, no inhibition of fire blight could be detected. This is most probably due to the inability of this *E. coli* strain to colonise immature pear fruit tissues (Vanneste *et al*. 1996). It is therefore not surprising that all biological control agents studied (Eh252 on apple flowers (Hattingh *et al*. 1986), C9-1 and A506 on apple flowers (A. Harris and J.L. Vanneste, unpublished data), A506 and Eh252 on pear flowers (A. Harris and J.L. Vanneste, unpublished data), and HL9N13 on hawthorn flowers (Wilson *et al*. 1992)) were found by scanning electron microscope to colonise, like *Erwina amylovora*, the intercellular space between the stigmatic papillae. As Wilson *et al*. (1992) pointed out, stigmatic surfaces have a limiting carrying capacity for epiphytic bacteria. This means that because of either a lack of sites, or more probably a lack of food, there is a population level above which increase of epiphytic bacterial population is not possible. Therefore, when biological control agents and the pathogen multiply and colonise the stigma, they compete with each other for the limited amounts of nutrients available.

When A506 is coinoculated with *E. amylovora* on pear blossoms, both strains grow at their normal rate until the carrying capacity of the stigma is reached. A506 has no significant effect on the pathogen population. But if A506 is applied 72 hr before inoculation, colonisation of the stigma by *E. amylovora* is significantly reduced, leading to a dramatic reduction of incidence of fire blight (Wilson and Lindow 1993). This indicates that when A506 pre-emptively colonises the stigmatic surfaces it depletes them of some growth factors necessary for the growth of the pathogen, which results in reduction of fire blight incidence. When *E. herbicola* HL9N13 is applied on hawthorn blossoms 24 hr before inoculation, the rate of growth and the final population of the pathogen are significantly reduced indicating that, as with A506, this strain of *E. herbicola* reduces fire blight incidence by pre-emptive colonisation of the stigmatic surfaces. But, in contrast to A506, when HL9N13 is coinoculated with *E. amylovora*, the rate of growth and the population level of the pathogen are also significantly reduced. This indicates that HL9N13 is also able to colonise competitively the stigmatic surfaces (Wilson *et al*. 1992).

Colonisation of the stigma by biological control agents seems to be absolutely essential. When applied before the pathogen, this colonisation results in the depletion of essential nutrients from the stigmas. Inhibition of fire blight is then the result of the pre-emptive colonisation. Strains which produce an antibiotic are not only depleting the stigma of essential nutrients but are also able to compete with the pathogen for these nutrients. Inhibition of fire blight results then from pre-emptive and competitive colonisation of the stigmas. If environmental conditions are extremely favourable for multiplication of the biological control agents or if the initial inoculum is extremely high, pre-emptive colonisation becomes the major factor responsible for disease control. Competitive colonisation can only complement the effect of pre-emptive colonisation. This could explain why sometimes antibiotic production seems to be only a minor component of the control of fire blight.

PREDICTABLE LONG TERM CONTROL

Inconsistent performance of biological control agents in the field might be the main obstacle for commercialisation of these agents. Inconsistent performance can be attributed to variability in production or effectiveness of antibiotics and / or to variability in colonisation of stigmatic surfaces. Using mixtures of biological control agents, which might have different climatic requirements, can limit the variability in colonisation, resulting in a more predictable level of control. Mixtures can also prevent the selection of strains of the pathogen resistant to the antibiotics produced by the biological control agents if the strains used in the mixture inhibit the pathogen by different antibiotics or different mechanisms. This includes production of different antibiotics, as long as *E. amylovora* cannot develop cross resistance to these antibiotics. Therefore, the best strains to use in mixtures are strains which inhibit the pathogen by different modes of action and have different climatic requirements for colonisation of the stigmatic surfaces. Based on these criteria, it is not surprising that today most of the combinations tested include *P. fluorescens* A506, which does not produce an antibiotic, and a strain of *E. herbicola* (Eh252 or C9-1) which produces such a compound. Only one study involved a combination of two strains of *E. herbicola*, Eh252 and C9-1 (Voyle *et al*. 1996).

To be effective, mixtures have to be composed of bacterial strains that do not inhibit each other when applied together on stigma. Only once, a strain of *E. herbicola* C9-1 was reported to reduce flower colonisation by *P. fluorescens* A506 (McLaughlin and Roberts 1992). In all the other experiments (two on pears involving C9-1 and A506 (Stockwell *et al* 1992), four on three different cultivars of apple involving Eh252 and A506, and one experiment on Asian pear involving Eh252 and A506 (Vanneste and Yu 1996b; Voyle *et al* 1996))

Table 3. Incidence of fire blight on flowers treated with Eh252 or A506 separately or together

Treatment	Experiment on Asian pear		Experiment on apple			
	Percent infection*	Angular trans. % infected**	Percent infection	Angular trans. % infected	Percent infection	Angular trans. % infected
Water	47.2	43.4 a***	70.1	57.0 ab	58.2	49.9 a
Eh252	25.2	30.0 bc	48.8	44.3 b	31.1	32.2 b
A506	32.8	34.5 ab	74.8	60.0 a	29.1	32.2 b
A506+Eh252	28.2	32.0 ab	59.0	50.2 ab	33.0	34.6 b
Streptomycin	10.0	17.1 c	18.8	24.8 c	8.8	17.0 c

* Weighted mean of the percentage of infection per tree.
** The angular transformation used in this study, is the arcsine of the square root of the percentage of infection divided by 100
*** Means followed by the same letter are not significantly different (Fisher's protected Least Significant Difference, P = 0.05)

treatments with the mixture of strains did not significantly affect the population level of either strain. When Eh252 and C9-1 were applied together, the population of C9-1 was ten times lower than when it was applied separately (Voyle *et al* 1996). This could reflect that competition for nutrients is more intense between strains of the same species than strains of different species.

Although the proportion of blossoms colonised by beneficial bacteria was greater when a mixture of C9-1 and A506 was applied rather than only one of these strains, incidence of fire blight was not significantly different when the flowers were treated with mixtures of *E. herbicola* (Eh252 or C9-1) and *P. fluorescens* A506 than when they were treated with each strain separately (Stockwell *et al* 1992; Vanneste and Yu 1996b) (Table 3). This lack of synergy between A506 and the two *E. herbicola* strains tested is disappointing; however, the main goal of using mixtures was not to increase the level of control, but to provide a more consistent control over a wider range of climatic conditions and to prevent the development of strains of the pathogen resistant to the biological control agents. So far, there is no sign that these goals are not met.

DELIVERY OF BENEFICIAL BACTERIA

Spraying bacteria to control fire blight is labour intensive, most of the bacteria never reach the flowers and some flowers do not receive bacteria because of their location or orientation on the tree or because they were not open at the time. Honey bees (*Apis mellifera*) offer an alternative to spraying; they can pick up bacteria from the hive and deliver them directly onto the stigmas of apple and pear flowers. Bees can be made to carry biological control agents by fixing, at the exit of the hive, a pollen insert (Fig. 5) filled with either a powder of lyophilised biological control agents or with a carrier (often pollen) coated with the biological control agents. When leaving the hive the bees become covered with the pollen and the bacteria, which they deposit on the flowers they visit. Bacteria then colonise apple and pear stigmas rapidly, especially when brought to the near sterile environments that are the stigmatic surfaces of a newly open flower. Therefore, only a few bacteria brought early enough might be sufficient to protect flowers against fire blight.

Johnson *et al* (1993a) noted that bees show an avoidance to *P. fluorescens* A506. We also noted that sometimes bees preened themselves after being dusted with pollen coated with this *Pseudomonas* strain, but not with pollen coated with *E. herbicola* Eh252 (J.L. Vanneste and R.M. Goodwin, unpublished data). No other adverse or detrimental effect to the bees was observed during these experiments.

Thomson *et al*. (1992), who were the first to publish the use of bees to deliver biological control agents of fire blight, used cattail pollen coated with either Eh318 (10^8 cfu / g) or A506 (10^9 cfu / g). Bees leaving the hive carried about 10^5 cfu of A506 and about 10^4 cfu of Eh318. Using kiwifruit pollen coated with 10^7 cfu / g of pollen of A506, and 10^8 cfu / g of Eh252, bees carried between 10^3 and 10^4 cfu of A506 and the same number of cfu of Eh252 (J.L. Vanneste and R.M. Goodwin, unpublished data). Using freeze dried preparations containing more than 10^{10} cfu of A506 per gram, the number of bacteria carried by bees varied widely from less than 10^3 cfu / bee to more than 10^8 cfu / bee. However, the mean bacterial population per bee was quite high: 10^6 cfu (Johnson *et al*. 1993a). We do not know yet how much of this load the bee leaves behind when visiting a flower, nor how many flowers can be "treated" by one bee in one flight. Johnson *et al*. (1993a), by dividing the number of blossoms with bacteria by an estimate of the number of hours individual bees carrying these bacteria foraged on that tree, estimated that a bee could inoculate 20 blossoms per hour. This corresponds to one flower inoculated for every 20 flowers visited. These estimates would most probably vary between sites and seasons.

Bacteria used in these experiments are naturally resistant to several antibiotics, such as A506, or are spontaneous antibiotic resistant derivatives. No bacteria resistant to the same antibiotics were found in the orchards before the pollen inserts were attached; therefore the presence of such antibiotic resistant bacteria on flowers was entirely attributed to bees bringing them from the hive. The percentage of flowers which received beneficial bacteria varied between experiments. Thomson *et al*. (1992) found that two days after using pollen coated with Eh318, 92% of the apple flowers examined carried Eh318. The same authors found that 72% of pear flowers picked within 7.6 m from the hive eight days after filling the insert with pollen coated with A506 carried an average of 300 cfu of A506 / flower. Johnson *et al*. (1993a), reported a maximum of recovery of A506 from apple blossoms ranging from 23% to 81%. Finally, four days after the start of the experiment in

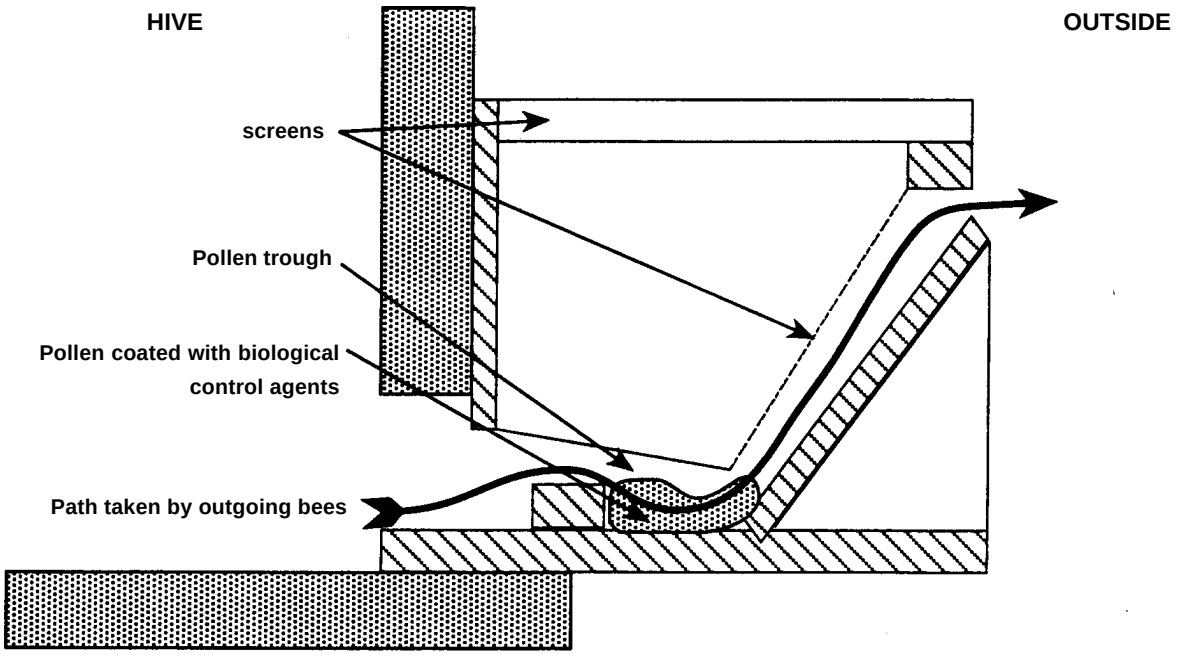

Figure 5. Cross section of a pollen insert. Other types of pollen dispensers have also been used to disperse a biological control agent, as in the case of *Gliocladium roseum* to strawberry flowers to control *Botrytis cinerea* (Peng *et al*. 1992).

an apple orchard, we found that all flowers examined were colonised either by Eh252 or by A506, the two bacteria used in that experiment, and that at least 70% were colonised by both Eh252 and A506 (J.L. Vanneste and R.M. Goodwin, unpublished data). Some of these flowers received the biological control agents from bees which picked them up when leaving the hive. Other flowers, however, could have received these biological control agents from bees or other insects who picked them up when visiting flowers already colonised by these agents. Secondary transmission of biological control agents has been suspected or documented by several laboratories (Nuclo *et al.* 1995; Stockwell *et al.* 1996; Lindow *et al.* 1996; Johnson *et al* 1993b; J.L. Vanneste and R.M. Goodwin, unpublished data).

Many variables influence the frequency of visits by bees, such as temperature, wind, rain, presence of other flowers, as well as population levels of other bees in the vicinity. These factors will have an impact on the ability of the bees to transmit the biological control agents. The strength, placement and manipulation of colonies will also contribute to the effectiveness of bees as vectors of biological control agents. The management practices that are usually taken to get good pollination will help bees to act as vectors.

IMPLEMENTATION OF BIOLOGICAL CONTROL OF FIRE BLIGHT

Working on biological control of fire blight is today extremely exciting, as there is a real prospect of seeing some of the results being used by growers in a relatively short period of time. This feeling has recently been strengthened by the fact that *P. fluorescens* A506 is on the market and that the same company is trying to register *E. herbicola* C9-1.

Several factors might encourage growers to use A506. In addition to controlling fire blight, A506 also offers some control of frost injury and limits russeting on pears (Lindow 1992; Lindow *et al.* 1996). Furthermore, it is the only resource that can be used for orchards with streptomycin resistant strains of the pathogen. Integration of A506 in current strategies of control will be made easier thanks to the fact that A506 is naturally resistant to streptomycin. The percentage of apple flowers colonised by A506 and C9-1S, a streptomycin resistant derivative of C9-1, was similar whether flowers were treated two days after treatment with streptomycin or with water (Stockwell *et al.* 1996). However, oxytetracycline, if sprayed less than 7 days after treatment, reduced the percentage of flowers colonised with biological control agents and population levels of these biological control agents (Stockwell *et al.* 1996). Furthermore, there is some synergy between streptomycin and A506. Lindow *et al.* (1996), who studied A506 for over 16 years, recently reported that not only was the population of A506 unaffected by spraying streptomycin, but incidence of fire blight was lower on trees treated with both A506 and streptomycin than on trees sprayed with either one of these treatments.

The ability of biological control agents to spread to non-treated flowers is yet another advantage that distinguishes beneficial bacteria from chemicals. This secondary spread of bacteria throughout the orchard means that if beneficial bacteria are applied early enough in the season, they will not need to be reapplied (either by spraying or by using bees and coated pollen) as often as chemicals.

Growers might easily be tempted to use honey bees as vectors of biological control agents because they are already using honey bees for pollination. Pollination is important to get a good apple or pear crop. Flowers that are not pollinated do not set fruit and inadequately pollinated flowers develop into misshapen fruit with low market value. Seven of the 10 possible seeds in an apple for example are needed for good development of the fruit. Pollination of apples and pears relies heavily on insects delivering cultivar-compatible pollen and the most important pollen delivery agents are honey bees. Bees collect pollen and deposit it on the stigmas of other apple or pear flowers, so each spring growers routinely rent hives to help pollination. If there are no compatible cultivars in bloom in the orchard, compatible pollen can be provided to the bees by using a pollen dispenser and some growers are already using pollen inserts to dispense compatible pollen to their crops. If the compatible pollen could be coated with the beneficial bacteria, the flowers would not only be pollinated, but would also face a reduced risk of fire blight.

All these characteristics might result in the rapid integration of biological control agents into current strategies for control of fire blight. With further developments, such as registration of new biological control agents and development of cultivar compatible pollen coated with these bacteria, control strategies might be redesigned to get the most from biological control.

BEYOND BEES AND BENEFICIAL BACTERIA

Beneficial bacteria carried by honey bees might be the first strategy of biological control of fire blight. However, we might rapidly be able to move beyond beneficial bacteria as the main agent of control and beyond bees as the vectors of these agents.

We might soon be able to improve biological control agents and not have to rely only on wild type strains. The genes for antibiotic production from Eh1087 (Kearns and Mahanty 1993), Eh318 (Wright and Beer 1996) and Eh252 (Vanneste and Yu 1996a) have been cloned and could be expressed in strains better suited for control or strains with additional advantages. Other mechanisms than antibiosis could also be involved in inhibition of *E. amylovora* on flowers, such as competition for iron through siderophore production, as demonstrated in the biological control of soil pathogens (Handelsman and Parke 1989). Eh252, A506 and *E. amylovora* possess an iron uptake

Table 4. Control of blossom blight on *Cotoneaster salicifolius* var. *floccosus*

Treatment	27 Days		41 Days	
	% of Infected Clusters	% of Disease Control	% of Infected Clusters	% of Disease Control
Copper oxychloride	5.2 a*	85.5	7.2 a	80.2
Streptomycin sulfate	6.4 a	82.2	10.8 ab	70.2
Mahonia aquifolium	20.0 b	44.3	17.0 bc	53.2
Berberis vulgaris	23.5 b	34.5	17.9 c	50.7
Rhus typhina	21.4 b	40.4	26.3 d	27.5
Allium sativum	28.2 b	21.4	21.1 d	25.6
Water	0.0	0.3	0.0	0.0

* Percentages followed by the same letter are not significantly different (Duncan-test, $P < 0.05$)

Table 5. Inhibition of *E. amylovora* by essential oils and plant extracts

Essential oil	Distance (mm)
Thyme	14.88
Cinnamon	10.11
Clove	8.45
Pimento	8.37
Pine	8.28
Lemongrass	7.83
Spearmint	5.55
Jasmine	4.57
Melissa	4.18
Teatree	3.87
Sassafras	3.45
Peppermint	3.44
Lavender	2.65
Rosemary	2.65
Petigrain	1.93
Nutmeg	1.78
Water	0.00
Plant Extracts	**Distance (mm)**
alpha-terpineol	10.11
Linalol	7.43
p-Cymene	5.93
alpha-terpinene	0.43

system (Vanneste *et al*. 1992; Lindow 1985; Vanneste and Expert 1990, respectively) which in the case of *E. amylovora* could be involved in pathogenicity (Vanneste 1995).

Novel methods of disease control such as immunisation using bacteria or compounds which elicit the plant defence mechanisms could also prove useful to control fire blight. It has been suggested that some Hrp$^-$ mutants of *E. amylovora* could elicit plant defence mechanisms (Tharaud *et al*. 1996). We could also envisage the use of bacterial metabolites or plant extracts including essential oils to inhibit *E. amylovora.* Mitchell (1993) and Mitchell *et al*. (1996) have already identified some secondary metabolites produced by different *Pseudomonas* strains that can inhibit the development of fire blight on plate and on immature pear fruit. Some plant extracts (Mosch *et al.* 1990) and some essential oils (B.H. Rohitha and J.L. Vanneste, unpublished data) can also inhibit *E. amylovora* on plate (Tables 4 and 5). More importantly four plant extracts were also shown to reduce fire blight incidence on *Cotoneaster* (Mosch *et al*. 1990) (Table 4).

The next best vector after the bees might actually be the plant itself. The technology to produce transgenic apple and pear plants is already developed and several cultivars of apple and pears have been successfully transformed and regenerated. As mentioned earlier, genes that produce inhibitory compounds have been cloned from different *E. herbicola* strains. These genes could be introduced and expressed in apple or pear plants. Other genes coding for lytic proteins (attacin E, cecropin and lysozym) have already been introduced into the apple rootstock M26. Preliminary field trials with one transgenic line expressing the attacin E gene showed increased resistance to *E. amylovora* (Norelli *et al* 1996). More transgenic lines from M26 have been obtained and will soon be tested in the field for resistance to fire blight (Norelli *et al* 1996). Transgenic lines derived from the apple cultivar Royal Gala and containing different lytic protein genes have also been obtained and planted for future field experiments on resistance to fire blight (Aldwinckle *et al*. 1996).

Transgenic plants by themselves might not be the answer to all problems caused by fire blight. This technique would most probably be restricted for the first years to some of the most economically important cultivars, and will concern only new orchards. There are still too many unanswered questions about transgenic plants to predict what their role in the control of fire blight will be. But all these new tools and techniques are keeping the future of biological control of fire blight bright and exciting.

ACKNOWLEDGEMENTS

Work done in my laboratory is funded by grants from the Foundation of Research Science and Technology, ENZA New Zealand (International) and the Agriculture and Marketing Research and Development Trust (AGMARDT). I thank all the friends and colleagues who provided information or unpublished data and especially Steve Beer, Gordon Bonn, Ken Johnson, Leah Kearns, Steven Lindow, Robin Mitchell, Larry Pusey, Virginia Stockwell, Michel Tharaud, Sherman Thomson, Mark Wilson and Sandra Wright. I also thank Deirdre Cornish, Mark Goodwin, Darienne Voyle and Janet Yu for comments on the manuscript, Deirdre Cornish for helping with the references and the figures, and Janet Yu for her patience and support.

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