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Detection of the fire blight biocontrol agent *Bacillus subtilis* BD170 (Biopro®) in a Swiss apple orchard

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Abstract

Fire blight, caused by *Erwinia amylovora*, is a major disease threat to apple, pear and other pome fruit worldwide. The disease is widespread in Europe and has recently become established in Switzerland. Antibiotics are the most effective controls used in North America but these are not permitted for agricultural use in most European countries. A newly registered biological control product Biopro®, based on the antagonist *Bacillus subtilis* strain BD170, is being used as an alternative strategy for fire blight management. A specific molecular marker was developed for monitoring the spread of this agent on blossoms after Biopro® spray application in a Swiss apple orchard throughout the bloom period for 2 years. Direct spraying resulted in efficient primary colonisation of pistils in flowers that were open at the time of treatment. Subsequent bacterial dissemination (secondary colonisation) of flowers that were closed or at bud stage at the time of treatment was observed but was found to be dependent on the timing of treatments relative to bloom stage in the orchard. Foraging honeybees were shown to be disseminators of Biopro®. We also report detection of the biocontrol agent in honey collected from hives where bees were exposed by placing Biopro® at the entrance or in the hatching nest and from hives that were simply placed in sprayed orchards.

Introduction

Fire blight, caused by *Erwinia amylovora*, is a major economic threat to apple production worldwide. The disease is endemic to North America and was introduced into Europe during the late 1950s (Jock et al., 2002). It has since spread throughout Europe and the Mediterranean region, being first reported in Switzerland in 1989 (Holliger et al., 2003). Within an orchard, fire blight is easily spread via aerosols, insect vectors and contaminated farm equipment. Fire blight can occur via shoot infections, particularly after wounding (e.g., from hail damage), but the most problematic phase of disease in Switzerland is blossom blight. Blossom blight phase occurs when *E. amylovora* is introduced on the floral surface by insects, establishes in the hypanthium and enters the plant via nectarthodes. Once in the plant, disease progresses rapidly downward, often resulting in blight of the entire flower-cluster, shoot and dieback of the branches. Severe infections can kill a tree within a single season. Moreover, because the pathogen is listed as a quarantine organism in Europe, fire blight greatly restricts trade in propagative material between countries and between cantons within Switzerland and restricts the movement of beehives (Holliger et al., 2003).

Fire blight management options in Europe are limited. In North America, effective control can be achieved through application of the antibiotics streptomycin sulphate and to a lesser extent

oxytetracycline. However, this approach remains questionable because of development of antibiotic resistant strains (McManus et al., 2002). Some control can also be achieved with copper-based bactericides and oxolinic acid, but again with the risk of pathogen resistance development (Sholberg et al., 2001; Manulis et al., 2003). Optimised application timing is determined based on forecasting models such as Maryblyte (Johnson and Stockwell, 1998). In Switzerland, and most other European countries antibiotics are banned, and copper-containing pesticides are being phased out because of the low efficacy and of the possible phytotoxicity when used on flowers or at cold temperatures, necessitating the development of alternative control strategies to complement forecasting services.

Biocontrol has been studied and a few commercial bacterial products are currently available. By and large, the focus to date has been on use of non-clinical strains of *Pantoea agglomerans* (syn. *Erwinia herbicola*) and *Pseudomonas fluorescens* (Johnson and Stockwell, 1998). *Pantoea agglomerans*, being an organism on a list of organisms with restricted use, can currently not be used in field test in the European Community. Recently two products based on *Bacillus subtilis* strains have been registered for fire blight control in Europe: Serenade®, based on strain QST 713 and Biopro®, based on the strain BD170. *Bacillus subtilis* offers advantages for biocontrol because of its formulation and long-term survival characteristics (Emmert and Handelsman, 1999). Other than an early study demonstrating *in vitro* antagonism of *E. amylovora* by *Bacillus* strains (Elgoorani and Hassanein, 1991), few studies have examined the biocontrol activity and ecology of this group of potential antagonists against fire blight. Biopro® was recently shown to have promise for reducing blossom blight caused by *E. amylovora* in several field trials on several apple cultivars (Golden Delicious, Gloster, Idared and James Grieve) in Germany between 1998 and 2000 under both natural and artificial pathogen inoculation conditions, but the level of control achieved was found to be erratic providing anywhere between 43% and 71% disease reduction (Laux et al., 2003). Similar erratic results were found for the control of blossom blight on Idared apple trees by Serenade® in field trials in 2000 and 2001 in the USA, providing 64% control in the first year and 0% in the second (Aldwinckle et al., 2002).

Studies performed with strains *P. agglomerans* C9-1 and *P. fluorescens* A506 in orchards have demonstrated that the establishment of populations of these bacteria in blossoms is a critical step for early biocontrol of fire blight. Key factors affecting the establishment of these agents on floral surfaces are the inoculum preparation, the temperature at treatment time, and the bloom stage at treatment time (Johnson and Stockwell, 1998).

For *B. subtilis* similar information is needed on timing and colonisation dynamics after application. The objective of this study was to examine the population dynamics of *B. subtilis* after application in a Swiss orchard. We monitored the colonisation of blossoms that were open at the time of treatment and thus directly exposed to the biocontrol agent (primary colonisation). We also examined apple flowers that were not open at the time of treatment but became colonised during subsequent spread of the biocontrol agent from colonised surfaces (secondary colonisation). We developed a sensitive and selective molecular marker to facilitate field monitoring of *B. subtilis* on apple and honeybee vectors, and used it to evaluate the contamination of honey.

Materials and methods

Bacterial strains

Bacillus subtilis, strain BD170, was available as a commercial formulation (Biopro®) provided by BioSystem GmbH (Konstanz, Germany). Phytopathogenic *E. amylovora* strains 22,770, 23,482 and 158,000, non-pathogenic *P. agglomerans* strains 348 and 351, and a collection of apple flower epiphytic bacteria were isolated in Switzerland at the Agroscope FAW Wädenswil diagnosis clinic (http://www.feuerbrand.ch). Bacteria were grown in Luria–Bertani (LB) broth or on 10% tryptic soy agar (TSA) (Difco, Detroit, Michigan) at 27 °C. *B. subtilis* was easily distinguished from other bacteria based on its unique star-shaped colony morphology after several days growth on 10% TSA.

Molecular detection of B. subtilis

Bacillus subtilis specific primers were designed using available sequence data (http://genolist.pasteur.fr/SubtiList/). Primers COT1F (5′-TCAT-

CAGCATCGAGCATTTC-3′) and COT1R (5′-CCGAGTTTCGCAAGTCCTAC-3′) were used to amplify the region between cotB and cotH sporulation genes (Naclerio et al., 1996). Polymerase chain reactions (PCR) amplifications were carried out in Geneamp 9600 programmable thermocycler (Perkin-Elmer, Foster City, California). The PCR mixtures consisted of a final volume of 10 µl with the following reagents: 0.1 mM dNTP, 1.5 mM Mg^{2+}, 0.2 mM of each primer, 0.7 U *Taq* polymerase in 1 × PCR buffer (Amersham/Pharmacia Biotech, Uppsala, Sweden). The reaction conditions were a denaturing step of 94 °C for 5 min followed by 38 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min, finished with 72 °C for 10 min and held at 4 °C. The PCR products were visualised by staining with ethidium bromide after electrophoresis on 1% agarose gels in 0.5× TBE. PCR amplification tests were performed to assess the specificity of the molecular marker for *B. subtilis* DB170 by using the developed primers pair on DNA from pure cultures of all the bacteria described in the previous section.

Bacillus subtilis *BD170 application in apple orchards and weather data collection*

Orchard trials were conducted in 2001 and 2002 at Seegräben, canton Zürich, Switzerland. Four 200 m long rows of 3-year-old cv. Golden Delicious apple trees (90 cm between plants, 3.5 m between rows) were used. *Bacillus subtilis* BD170 was applied as 0.1% Biopro® (2×10^6 spores ml^{-1}) water suspension following manufacturer recommendations (1.6 kg Biopro® ha^{-1}) using a conventional orchard sprayer to all but the first 15 plants of the rows. In 2001, two sprays were made at 5% (3 May) and 85% bloom (10 May). In 2002, sprays were made at 25% (25 April) and 95% bloom (30 April, after sample collection).

The blossom stage was estimated counting approximately the average number of open flowers per cluster, which is normally composed of five flowers, so that one open flower per cluster corresponded to 20% bloom. Weather parameters, including temperature and moisture, were monitored continuously with an HP100 field weather station (G. Lufft Mess- und Regeltechnik GMBH, Fellbach, Germany).

Sample collection and detection on apple flower pistils

Flower samples were taken at 2–4 day intervals in each trial in 2001 and 2002 (Table 1). Primary colonisation was assessed by analysing a set of randomly selected flowers 1 day after a treatment, selecting those that were open at treatment time. In order to evaluate secondary colonisation within two sampling dates, flowers at the balloon stage (BBCH 59) were tagged during the first sampling and collected at the following sampling. The

Table 1. Primary and secondary colonisation of apple blossoms by *B. subtilis* BD170 in 2001 and 2002

Sampling date	Samples analysed	Kind of colonisation measured	% Open flowers at sampling $Y(t)$	% Analysed flowers colonised $X(t)$ or $Z(t)$
Year 2001[a]				
2 May	135	Background	5	0.7
3 May	260	Primary	5	82.3
5 May	258	Secondary	5	97.3
9 May	283	Secondary	80	44.9
11 May	291	Primary and secondary	95	96.6
14 May	292	Secondary	100	88.4
Year 2002[a]				
25 April	142	Background	25	0
26 April	144	Primary	50	93.1
29 April	144	Secondary	90	72.2
30 April	116	Secondary	95	24.1
2 May	140	Primary and secondary	100	99.3

[a] Application of 0.1% Biopro® by spray, in 2001 on 2 May (5% bloom) and on 10 May (85% bloom) and in 2002 on 25 April (25% bloom) and on 30 April (95% bloom).

colonisation percentage of the pistils of these flowers corresponds to secondary colonisation during this interval.

Flowers were collected by cutting the stem, placing them in individual wells of a seedling tray (GVZ-Bolltec AG, Switzerland) and transporting the samples to the laboratory within approximately 2 h. There, pistils were excised with sterile forceps and transferred to a 96 wells PCR plates. During dissection, PCR plates were floating on a mixture of dry ice and 96% ethanol. Samples were stored in closed PCR plates at −20 or −80 °C until PCR analysis. Pistils were washed by adding 150 µl sterile distilled water and shaking vigorously for 30 s. Qualitative analysis was performed by transferring 20 µl of the resulting wash solution to 200 µl of LB-Broth (Difco, Detroit, MI) which was consecutively grown overnight (at least 16 h) at 27 °C. A small drop (less than 1 µl) of the culture was then transferred to 10 µl of PCR mix for selective amplification.

Data transformation

Since this study was focused on flower colonisation at the stage critical for blossom blight prevention, a transformation step of the measured values into a percentage of colonised flowers related to the total amount of flowers in the orchard was performed. The measured primary colonisation was related to the total amount of open flowers, whereas the secondary colonisation was related only to the tagged flowers that opened during a certain interval and thus is not giving any information about the total amount of colonised flowers in the orchard at a given sampling date. To calculate the total percentage of colonised flowers after each biocontrol application by spraying, the primary colonisation rate was multiplied by the percentage of open flowers. For the others sampling dates the secondary colonisation was determined only on the freshly opened flowers. It was thus necessary to assume that the frequency of colonisation of the older not colonised flowers was the same as that measured on newly opened flowers. To calculate the percentage of colonised flowers related to the total amount of flowers in the orchard the following formula has been used:

$$X(t_n) = [Y(t_n) - Y(t_{n-1})] \cdot Z(t_n) + W(t_{n-1})Z(t_n) + X(t_{n-1})$$

$X(t_n)$ = total % flowers colonised at t_n; $Y(t_n)$ = % blossoms at t_n; $Z(t_n)$ = % of analysed flowers that were colonised at t_n (secondary colonisation); and $W(t_{n-1})$ = total % flowers open and not colonised at $t_{n-1} = Y(t_n) - X(t_n)$.

For example, from Table 1 on the 26 April 2002 (t_{n-1}): $Y = 50\%$, $X = 46\%$ and on 29 April 2002 (t_n): $Y = 90\%$, $Z = 72\%$. Then: $(90–50\%) * 72\% + 4\% * 72\% + 46\% = 78\%$ of the flowers were colonised.

Detection of B. subtilis *on honeybees* (Apis mellea)

In 2001, to evaluate the potential role of honeybees as vectors for dispersal of the biocontrol agent, 10 foraging bees were randomly sampled from the treated rows in the orchard at Seegräben on 9 and 11 May 2001, 7 days after the first and 1 day after the second Biopro® application. In this orchard the beehives were not treated with a direct application of Biopro® at the beehive entrance. Another 16 bees were sampled in the orchard at Gottshalde, canton Zürich, on 10 May where Biopro® was not sprayed in the orchard, but where a 2×10^{11} spores g^{-1} Biopro® wettable powder was placed at the beehive entrance for 7 consecutive days.

The bees were placed in individual 2 ml microcentrifuge tubes and frozen at −20 °C. For analysis, samples were thawed and shaken vigorously with 1 ml sterile distilled water for 20 s. Aliquots of 300 µl were transferred to fresh tubes and pasteurised at 80 °C for 15 min, and 20 µl of the pasteurised suspension were used to inoculate 200 µl of LB-broth. The qualitative analysis was performed as described for the flower pistils.

Detection of B. subtilis *in honey*

The presence of *B. subtilis* BD170 in honey was determined in 2001. From three Swiss orchards in Neukirch-Egnach (canton Thurgau, three honey samples), Ehrbar Altnau, canton Thurgau (two honey samples), and Gottshalde, canton Zürich (one honey sample) a total of six honey samples from beehives were analysed. The beehives were treated directly by applying a Biopro® powder formulation (2×10^9 spores g^{-1}) and placed in orchards that were either treated by spraying 0.1% Biopro® or untreated. In Gottshalde, a concentrated *B. subtilis* BD170 formulation (2×10^{11} spores g^{-1}) was applied by spreading the

powder on a cardboard tray ($5 \times 25 \times 0.5$ cm) placed at the hive entrance. At one hive in Neukirch-Egnach, Biopro® was applied to the hatching nest. We analysed honey from two hives where no Biopro® was directly applied: one hive at Neukirch-Egnach was located beside an orchard treated by spraying (at 10 m from a treated row), one hive at Ehrbar-Altnau was placed in an untreated orchard at about 1 km distance from a second orchard where the biocontrol agent was applied placing 3 g Biopro® powder at beehive entrance for 3 days.

Bacillus subtilis contamination in the honey was quantified by diluting 0.5 g honey with 1 ml sterile phosphate buffer saline (PBS, pH 7.4), centrifuging for 45 min at 3000 *g* and discarding the upper two-third phase of the supernatant leaving *Bacillus* spores in the lower phase (Hornitzy and Clark, 1991). Resuspended spores were pasteurised at 80 °C for 15 min and 20 µl aliquots were plated onto 10% TSA. After 2 days incubation at 27 °C, characteristic colonies were enumerated and the kinship of part of these colonies to the *B. subtilis* was confirmed by PCR amplification using the developed molecular marker. The marker was also used on colonies not showing the typical morphology to verify that they do not show the typical *B. subtilis* PCR product. Each honey sample was tested eight times.

Results

Molecular detection of B. subtilis *on apple flowers*

Specific DNA amplification of the expected 629 bp product was observed with the newly developed marker only for the *B. subtilis* strains BD170, whereas all other bacteria tested did not show any PCR product amplification. PCR inhibition was observed when attempting direct detection from pistils washings. Consequently we established a culture enrichment step in LB broth prior to analysis. The detection limit (number of inoculated spores required to obtain a positive PCR result from the overnight culture) was determined by inoculating 200 µl enrichment broth with a dilution series of *B. subtilis* BD170 spores and growing overnight. Dense cultures were obtained with as few as five inoculated spores. The flower samples collected in 2001 and 2002 in Seegräben before any treatment were analysed with the molecular marker to assess its reliability in field experiments. The negligible *B. subtilis* background presence detected with the marker (0.74% on 2 May 2001, 0% on 25 April 2002, Table 1) indicated that the marker can be used in the orchard.

Primary and secondary colonisation of apple blossoms in orchard trials at Seegräben

Primary and secondary colonisation of apple blossoms was determined by sampling throughout the blossom period in 2001 and 2002 (Table 1).

In 2001 a total of 1519 flowers were sampled. Primary colonisation was found to be greater than 80% in samples taken 1 day after spraying (82% at 3 May, 96% at 11 May). Secondary colonisation was measured on the 5, 9 and 14 May, and found to be highly variable with over 97% on 5 May, 88% on 14 May and as low as 45% measured on 9 May. Analysis of random samples taken from not directly treated trees adjacent to our treated test plot (first 15 plants of the rows) revealed colonisation by *B. subtilis*. Colonisation values for these trees were between 20% and 81%.

In 2002, a total of 686 flowers were sampled. None of the samples collected before the first Biopro® treatment (25 April) were positive for *B. subtilis* indicating that the biocontrol agent applied in the previous year cannot recolonise the flowers (Table 1). Primary colonisation measured the day after a spray treatment was 93% and 99% on 26 April and on 2 May, respectively. Secondary colonisation rates were highly variable, ranging from 72% on 29 April to 24% on the 30 April.

Transformed colonisation data

The data from Table 1 were then transformed so that the resulting values represent the percentage of flowers in the orchard that were open and either colonised or not colonised by *B. subtilis* (Figure 1). This transformation of the data allows to identify bloom intervals with high or low colonisation ratios of all open flowers taking in consideration the evolution of the bloom, primary and secondary colonisation all at once.

One day after spraying Biopro® in the orchard in 2001 0.88% and in 2002 3.5% of all season flowers were open and not colonised (Figure 1). A more relevant difference between the 2 years is found when the bloom has reached about 80–90%.

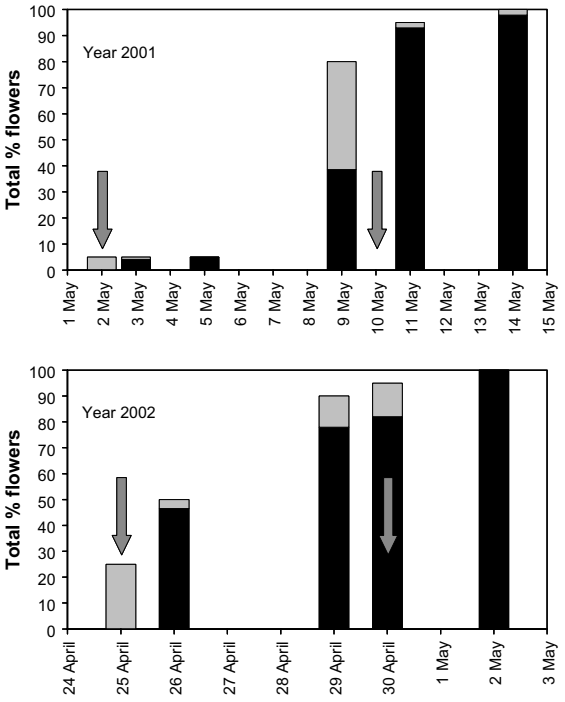

Figure 1. Evaluation of the colonisation of the pistils of open flowers during bloom in 2001 and 2002 in the Seegräben orchard. *Bacillus subtilis* colonisation values are extrapolated from Table 1. Black bars indicate the percentage of flowers that were open and colonised by *B. subtilis* at each sampling date. Gray bars indicate the percentage of flowers that were open but not colonised by *B. subtilis*. Gray arrows indicate applications of 0.1% Biopro®; treatments were done after the samplings.

In 2001, where the first application occurred at 5% bloom, a great amount of flowers were open and not colonised (41.4% on 9 May), whereas in 2002, where the treatment was performed at 25% bloom, this value was smaller (12.8% on 29 April). At the end of the bloom period in both years, colonisation was 97.9% in 2001 and 99.9% in 2002.

Detection of B. subtilis *on honeybees*

The number of bees carrying the biocontrol agent was determined in 2001. In Seegräben, 3 of the 10 analysed bees carried *B. subtilis* on 9 May, and all of the 10 sampled bees carried *B. subtilis* spores on 11 May. In Gottshalde, 14 of the 16 sampled honeybees sampled on 10 May resulted positive to *B. subtilis*.

Detection of B. subtilis *in honey from orchards treated with Biopro®*

The average concentration for samples from Neukirch-Egnach (canton Thurgau) was 5910 cfu g^{-1} honey for the sample from the beehive where Biopro® was applied at the hive entrance in addition to the normal orchard spray procedure (Table 2). This value was of 4512 cfu g^{-1} honey if Biopro® was applied in the hatching nest under the same conditions. The average concentration of *B. subtilis* spores in the honey sample from the beehive placed near the orchard treated by spray amounted to 263 cfu g^{-1} honey. Analysis of honey from beehives placed in Altnau, canton Thurgau revealed that treatment with Biopro® only at the beehive entrance for 3 days resulted in a concentration of 2393 cfu g^{-1} honey, whereas a second honey sample from an untreated beehive placed about 1 km away from the previous beehive revealed a concentration of about 201 cfu g^{-1} honey. Honey sampled from beehives placed in an apple orchard in Gottshalde, canton Zürich, where the beehive

Table 2. Detection of *B. subtilis* BD170 in honey

Hive location	Beehive treated directly [a]	Orchard treated by spray [b]	*B. subtilis* cfu g^{-1} honey [c]
Neukirch-Egnach[d]	No	Yes	263 ± 150
Neukirch-Egnach	Yes	Yes	5910 ± 580
Neukirch-Egnach	Yes	Yes	4512 ± 902
Gottshalde	Yes[e]	No	13,081 ± 1511
Ehrbar Altnau[f]	No	No	201 ± 99
Ehrbar Altnau	Yes	No	2393 ± 362

[a] Beehives were directly exposed to *B. subtilis* by placing powder formulation of Biopro® (2×10^9 spores g^{-1}) at the beehive entrance.
[b] Alternatively, hives were indirectly exposed to *B. subtilis* by placing them in orchards after trees were sprayed with Biopro®
[c] Average number of *B. subtilis* spores (± standard deviation) detected per gram honey from eight samples per hive.
[d] Beehive was placed at 10 m from an orchard treated by spraying 0.1% Biopro®.
[e] A concentrated formulation of 2×10^{11} spores g^{-1} was used at Gottshalde.
[f] Beehive was placed at 1 km from the beehive treated at the entrance by Biopro® powder application.

entrance was covered with several grams of a more concentrated Biopro® powder formulation showed an average concentration of 13,081 cfu g^{-1} honey.

Discussion

A sensitive PCR method was designed to detect as few as five spores of the fire blight biocontrol strain *B. subtilis* BD170, recently registered in Germany as Biopro®. Employing a simple culture enrichment step prior to analysis increased sensitivity and reduced interference from plant compounds. Although *Bacillus* are common in field samples, the fact that we detected very few cross-reactions in non-treated samples further bolsters the use of this method specifically for monitoring the population dynamics of the introduced strain.

The initial establishment of the bacteria on the flowers 1 day after treatment ranged from 82% to 99%. These values are similar to these found 2 h after a similar treatment performed with two others biocontrol agents (*P. fluorescens* A506 and *P. agglomerans* C9-1S) on pears by Nuclo et al. (1998), whereas they found primary colonisation to vary between 94% and 100%. However they applied a more concentrated mixture of bacterial antagonists suspension. The lower primary colonisation found on 3 May 2001 can be explained by the fact that the treatment was performed at 5% bloom, when few flowers were open. Pears studied by Nuclo et al. (1998) bloomed before their leaf expansion, so that the flowers are completely exposed to the biocontrol treatment. In apple, this does not happen and flowers are within a more dense canopy and thus may not be completely exposed.

Secondary colonisation was found to be highly variable with the biocontrol agent successfully disseminated from 24.1% to 97.3% of flowers. Several factors have been found by others authors to play a key role in the secondary dispersion of biocontrol organism by with the most important being weather, insect activity and bloom stage at the time of treatment (Nuclo et al., 1998; Johnson et al., 2000). In our trials, secondary dispersion appeared to be affected by the number of colonised flowers and the time of exposure of freshly open flowers to the foraging bees. The drastic reduction in secondary colonisation that was measured between 29 April and 30 April 2002 (from 72.2% to 24.1%) can partly be explained by the short exposure time (less than 24 h) of the freshly opened flowers to the insect disseminators, under cold temperatures (average was 10 °C at 29 April). The reduction in secondary colonisation from 5 May to the 9 May 2001 (97% down to 45%) can be explained by the rapid flush in bloom. During this short period three quarters of the flowers opened fully, which caused a dilution of the inoculum source (i.e. colonised flowers). The low temperatures during this period may also have played a role. The average temperature between 5 and 8 May was about 10 °C. This led probably to a reduced insects flight and could have led to reduced population sizes on the blossom, which resulted again in a dilution of the inoculum source.

In 2001, where the first treatment was performed at an early bloom stage (5%), a lower colonisation of the flowers during the rapid flush in bloom was observed (more than 40% uncolonised flowers on 9 May). Early estabilishment of biocontrol agent populations in the orchard is desired because the populations of indigenous microorganisms that could influence negatively the establishment of the biocontrol organism on newly opened blossoms are low (Johnson and Stockwell, 1998). However this requires the dispersal from few open flowers inoculated by treatment to a larger number of flowers that open later. To reduce this effect it was thus decided to postpone the first treatment in 2002 at the 25% bloom. Indeed we found that the percentage of colonised open flowers was higher when the treatment was applied at a later stage of bloom. Timing of biocontrol applications relative to percent bloom in orchards was also found by Nuclo et al. (1998) as a factor influencing the overall efficacy of treatments.

Our data showing erratic secondary colonisation of blossoms dependent on timing of application by a *B. subtilis* product could offer an explanation for the erratic level of blossom blight control with Biopro® reported recently by Laux et al. (2003) as well as with Serenade® reported by Aldwinckle et al. (2002).

Honeybees, often from hives installed directly in orchards during bloom period, are the most important insect disseminator of both the fire blight pathogen and biocontrol organisms (Thomson et al., 1992; Johnson et al., 1993). We have shown for the first time that bees can also transport *B. subtilis* to apple blossoms. Together with previous studies

that have focused on two blight biocontrol strains *P. fluorescens* A506 and *P. agglomerans* C9-1 (Thomson et al., 1992; Johnson et al., 1993) our findings further demonstrate the non-specific nature of bacterial dissemination by bees. Our preliminary tests demonstrated that bees can pick up and transport freeze-dried preparations of *B. subtilis*, however, dew and moisture caused clumping of the inoculum and reduced acquisition by bees. Commercial pollen inserts and other autoinoculation devices are available that should improve carrying capacity of bees leaving hives (Johnson et al., 1993; Vega et al., 1995; Hatjina, 1998). A different formulation of the product could also increase the dissemination potential of bees.

In conclusion, spray application of Biopro® resulted in a good primary colonisation of floral surface, whereas secondary colonisation was affected by several climatic factors as well by the bloom stage at treatment time. Further investigation are necessary to assess factors influencing the biocontrol agent population sizes on floral surface and to relate these to the biocontrol activity. It was demonstrated that honeybees can disseminate *B. subtilis* either from already colonised surface and from inoculating devices placed in beehives. *B. subtilis* spores were found in different concentrations in the honey collected from beehives placed in treated orchards and, although no pathogenicity of *B. subtilis* has been reported, further research is needed.

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