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The presence and survival of *Pseudomonas syringae* pv. *actinidiae* on honey bees (*Apis mellifera*) that have been in infected kiwifruit (*Actinidia* sp.) orchards

Pattemore D, Goodwin M, Vanneste J, McBrydie H, Yorston W, Yu J, Felman C

June 2013

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Executive summary

The presence and survival of *Pseudomonas syringae* pv. *actinidiae* on honey bees (*Apis mellifera*) that have been in infected kiwifruit (*Actinidia* sp.) orchards

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A study in 2011 demonstrated the ability of *Pseudomonas syringae* pv. *actinidiae* (Psa), a serious pathogen of kiwifruit vines (*Actinidia sp.*), to survive for several days in a beehive (Zespri Contract No. V11255; Pattemore et al. 2011), but it was not known how much, if any, Psa-V (the virulent form of the disease) would be carried by honey bees (*Apis mellifera*) foraging in an infected kiwifruit orchard. The aim of this project was to determine this, and secondarily to assess for how long Psa-V could be detected in these colonies once the hives were removed from a source of inoculum. The purpose of this research is to provide Zespri, growers and beekeepers with the information they need to determine what risk the introduction of beehives into their orchard has in relation to Psa-V contamination of their vines.

Six beehives from the Waikato region were moved to PFR's Te Puke Research Orchard, Bay of Plenty on 16 October and left to forage on Psa-V-infected kiwifruit flowers for a week. On 24 October, anthers from staminate and pistillate kiwifruit flowers were sampled, along with honey bees foraging on flowers and foraging bees returning to the six trial hives. As a positive control, 10 samples of five bees were taken into a laboratory on site and inoculated with Psa-V and then treated in the same manner as our other sampled bees. To assess how long Psa-V could be detected in hives after being moved, c. 200 returning forager bees per hive were marked from each of the six hives. The hives were moved out of the orchard on the evening of 24 October and placed at a site in the Waikato region that was >5 km from any registered kiwifruit orchard (following Kiwifruit Vine Health recommended protocols). Samples of marked bees were taken from the hives on days 1, 2, 5, 7 and 9.

Pre-trial sampling did not detect any Psa-V on foraging bees in the six hives before they were moved to Te Puke. The positive controls exhibited decline in Psa-V from an initial population of approximately 1.6×10^3 CFU/bee to an average of 2.0×10^2 CFU/bee. Psa-V was detected on two out of 10 pistillate anther samples and three out of 10 staminate anther samples, with populations ranging from 10 to 2.9×10^5 CFU/flower. Psa-V was detected on one sample of bees foraging on flowers, with a mean population of 3.3×10^3 CFU/bee. Psa-V was also detected on two separate foraging bees returning to the hives, with populations of 1.8×10^2 and 1.8×10^4 CFU/bee. The results of the positive controls suggest that the initial populations on these bees could have been as high as 2.5×10^5 CFU/bee. Although Psa-V was not detected on marked bees from the hives on subsequent sampling days, the results from bees collected in the orchards confirm that the recommendations from the 2011 report are prudent.

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1 Introduction

1.1 Background

In November 2010, the bacterial disease *Pseudomonas syringae* pv. *actinidiae* (Psa) was confirmed in a New Zealand kiwifruit (*Actinidia* sp.) orchard, and has since led to significant losses of kiwifruit vines, especially 'Hort16A' (*Actinidia chinensis*). Over the last two and a half years, many research organisations including The New Zealand Institute for Plant & Food Research Limited (PFR), have focused on understanding the biology, epidemiology and management of Psa.

An important development in the identification of the disease-causing organism was the recognition that certain strains of Psa were more virulent than others (Vanneste et al. 2013). Specifically, it was shown that the virulent Psa-V strain (also known as Psa biovar 3), was the cause of the recent infection, and another less virulent strain, Psa-LV (or Psa biovar 4), was more broadly distributed throughout New Zealand and did not appear to result in similar disease (Vanneste et al. 2013).

Following discoveries of viable Psa in pollen taken directly from flowers (MAF 2011; Vanneste et al. 2011a), and initial surveys that failed to detect Psa-V in beehives in Psa-V-infected orchards or on honey bees (*Apis mellifera*) foraging on flowers on infected vines (Shane Max, Zespri, pers. comm.), Zespri Group Ltd (Zespri) commissioned an investigation into the survival of Psa-V in beehives as part of the process of determining how beehive management practices could be altered to minimise the risk of bees acting as vectors of the disease (Pattemore et al. 2011).

Psa-V was found to be able to survive on bees for at least six days within a beehive in the artificial environment of a containment facility (Pattemore et al. 2011). Because Psa-V could not be used in the field, an analogue for Psa-V, a strain of *Pseudomonas syringae* pv. *syringae* (Pss), was used to assess the likely survival and spread of plant pathogenic bacteria on bees and in beehives. The Pss survived for similar times in the field hives as Psa-V did in the hives in the containment facility. When Pss-contaminated pollen was introduced via pollen-covered worker bees to the hive, it was detected on control workers within 24 hours (Pattemore et al. 2011). On the basis of these results, it was recommended by Kiwifruit Vine Health that hives being removed from an infected region be placed at an apiary site >5 km from any registered kiwifruit orchard for two weeks before being brought back within 5 km of uninfected orchards.

However, this study did not address whether honey bees could became contaminated with Psa-V after foraging in infected orchards. Consequently this study was conducted to assess whether honey bees foraging in infected orchards could carry Psa-V and, if so, how long Psa-V could be detected in these hive after they had been removed from an infected region.

1.2 Objectives

The overall objective of this study was to provide growers and beekeepers with the information they need to determine what risk the introduction of beehives into their orchard has in relation to Psa-V infection of their vines.

The scientific objectives were to determine whether Psa-V could be detected on pollen-collecting bees that were foraging in a Psa-V-infected kiwifruit orchard and, if so, for how long the Psa-V could be detected in these colonies once the hives were removed from a source of inoculum. One limitation of previous studies to detect Psa-V on bees collected from infected orchards had been the lack of positive controls. This study corrects this by including positive controls consisting of known doses of Psa-V placed on honey bees and tested following the same protocol as used for samples collected from the infected orchard.

2 Methods

2.1 Study design

Six honey bee hives, each housed in two brood boxes from an apiary north of Hamilton (which had not been within 5 km of a Psa-V-infected orchard), were tested for the presence of Psa-V, and then moved into Te Puke Research orchard on 16 October 2012. The orchard contained vines infected with Psa-V. The colonies were left to forage in the orchard for seven days. Because of the foraging range of honey bees, the bees in these hives may have also been foraging in neighbouring orchards. On 24 October, samples of staminate and pistillate *A. chinensis* flowers, bees foraging on flowers, and foragers returning to the hive were collected. At the same time, a sample of bees was inoculated with a known concentration of Psa-V broth, and then subjected to the same collection and transfer protocols as the field samples. Following the collection of samples, c. 200 returning forager bees from each hive were individually marked with acetone-based paint on their thorax, and returned to the hives. The hives were then moved that evening to a Waikato apiary site that was >5 km from any registered kiwifruit orchard, based on Kiwifruit Vine Health's recommended protocols. Five marked bees were sampled from each hive on days 1, 2, 5, 7 and 9 after being removed from the orchard, and tested for the presence of Psa-V.

2.2 Identification of Psa-V

For all trials, the identity of colonies that had the same morphology as that of Psa-V was determined by polymerase chain reaction (PCR) initially using primers targeting the ITS region (PsaF1/R2 or PsaF3/R4) as described in Rees George et al. (2010). The identity of colonies that gave a 280 bp amplicon as expected for Psa was confirmed by PCR using the primers developed by Koh & Nou (2002), by Rikkerink et al. (2011) or Gallelli et al. (2011). The primers developed by Rikkerink and colleagues were used in a real time PCR machine. This real time PCR protocol and the duplex PCR developed by Gallelli et al. (2011) have the further advantage of distinguishing Psa-V from Psa LV (Rikkerink et al. 2011; Vanneste et al. 2013). Each suspect colony was tested using a minimum of three methods. A positive result was inferred only if no tests returned a negative result. This was necessary as we know that the primers targeting the ITS region (PsaF1/R2 and PsaF3/R4) give an amplicon of the size expected for Psa with DNA from strains other than Psa (false positive results; Vanneste et al. 2011b).

2.3 Negative controls

The entrances to the six hives situated at the Waikato apiary site were closed and returning workers that gathered on the outside of the hive taken as samples in five groups of five bees from each hive.
Each group of five bees was placed in an Eppendorf tube containing 2.5 ml water with 0.05% Tween® 80. The samples were taken to a PC2 laboratory, vortexed and serially diluted before plating on King's B agar. Developing colonies were counted after 48 hours of growth and assessed for Psa-V following the methods outlined in Section 2.2 above.

2.4 Positive controls

Positive controls consisted of returning worker bees collected from hives in the infected orchard, and then taken to a PC2 laboratory on site. Ten groups of five bees each were placed in plastic queen bee cages, euthanized by chilling and then inoculated with a 2 μl drop of a suspension containing 8.1 x 10^5 colony forming units (CFU)/ml of the strain Psa10627. After 30 minutes of drying, each sample of five bees was placed in 2.5 ml 0.05% Tween 80 solution, and placed into the same chilled transport container as the other samples collected from the orchard.

All samples were transferred to a PC2 containment laboratory at Ruakura and were plated in the order in which they were collected. They were tested within 3 hours of collection. Samples were vortexed for 30 seconds, and then serially diluted five times and plated on King's B agar plates in three replicates per dilution. The average CFU count for each sample was determined.

2.5 Psa-V on flowers and bees in an infected orchard

Ten samples of recently dehisced staminate *A. chinensis* anthers cut from five flowers (one sample from each of ten vines in a Psa-V-infected block), were collected in 2.5 ml water and 0.05% Tween 80 solution. Ten samples from pistillate flowers from ten vines in the same block were collected in the same manner.

Ten samples of five bees (one from each of ten pistillate vines), were collected while foraging on flowers at the same time as the above samples were collected. These samples were also placed in 2.5 ml water and 0.05% Tween 80.

After the collection of these samples, the entrances were closed on the six trial hives to allow returning foragers to collect on the outside. Insufficient numbers of bees had been foraging on kiwifruit flowers (discernable by the colour of the pollen) to proceed with the initial plan to collect ten samples of five bees from each hive, with their collected pollen sampled separately. The pellets of kiwifruit pollen were removed from the legs of five foragers from each hive, and the pollen and the bees were placed separately into 2.5 ml water and 0.05% Tween 80 as composite samples. A further five foraging bees that had collected kiwifruit pollen from each hive were individually placed in 2.5 ml water and 0.05% Tween 80.

All samples were stored in a chilled container along with the positive control samples and driven to a PC2 laboratory at Ruakura for testing. Samples were vortexed, serially diluted and plated following the methods described in Section 2.3. For the orchard samples, growing colonies that had the same morphology as colonies of Psa-V were counted and assessed for Psa-V following the methods outlined in Section 2.2 above.

2.6 Survival of Psa-V on bees following movement away from infected orchard

Because of the low numbers of returning foragers that had collected kiwifruit pollen, it was not possible to follow the initial plan to separate and test the pollen loads from each bee to be marked. Instead, a sample of c. 200 returning foragers was collected from the entrance of each hive, anesthetised with CO_2, individually marked with a spot of acetone-based paint on the thorax, and then returned to the entrance of the hive and allowed to recover.

The hives were then closed that evening, and moved to an apiary site in the Waikato that was >5 km from any commercial kiwifruit orchard. Five marked bees were taken from each hive on days 1, 2, 5, 7 and 9 after being moved to the new sites, and individually placed in 1 ml water and 0.05% Tween 80.
The samples were then tested using the same methods described in Section 2.5.

2.7 Estimation of initial Psa-V load

For the positive controls, a reduction factor was calculated for each sample to describe the decline in Psa-V from the initial inoculation to the time of sampling. The reduction factor was calculated by dividing the initial CFU placed on each bee by the measured CFU per bee from the sample.

Two different methods were used to estimate the initial amount of Psa-V in the bee and flower samples based on the reduction factor observed in the positive controls. Samples that did not return a positive result for Psa-V were excluded from this analysis.

For the first estimate, each CFU count per bee or flower was multiplied by the median reduction factor, and the mean of these estimated initial values was calculated for each sample type along with the standard error. The median reduction factor was used instead of the mean due to the large variance in reduction factors.

The second more conservative method was based on the assumption that low CFU counts may be due to high rates of decline in Psa, and high counts may be due to low rates of decline. The first and third quartiles of each data set were calculated, and the observed CFU count was multiplied by its equivalent opposite reduction factor quartile. Sample values that lay between the first and third quartiles were multiplied by the median reduction factor. Samples values less than the first quartile were multiplied by the third quartile of the reduction factor and sample values greater than the third quartile were multiplied by the first quartile reduction factor. The means and standard error of each sample type were then calculated.

Because of the very small sample size of positive results, the sample and estimated initial CFU counts were then compared to the initial inoculum loads used in Pattemore et al. (2011) to assess whether the inoculum loads used in that previous study were comparable to those found in infected orchards.

3 Results

3.1 Negative controls

No Psa-V was found on the initial samples of bees from the six trial hives before they were moved into the infected orchard.

3.2 Positive controls

Based on the population of Psa-V in the broth used for inoculation, it was calculated that each bee was inoculated with 1.6×10^3 CFU. One plated sample was overgrown by another microorganism, preventing a count of colonies and was thus removed from further analysis.

The observed CFU count per bee from the remaining nine samples was 199 ± 81.3 (mean ± SEM), and ranged from 10 to 800. The number of Psa-V colonies had reduced between 2 to 162-fold since inoculation.

The detection limit was 42 CFU per sample, or 9 CFU per bee.

3.3 Psa-V on flowers and bees in an infected orchard

Two pistillate flower samples and three staminate flower samples returned a positive result for Psa-V. The mean CFU/flower for each sample was 10 and 1.5×10^3 for the pistillate flowers and 7×10^1, 8×10^2 and 2.9×10^5 for the staminate flowers (Table 1). Because of the small sample size and the large overlapping variation in CFU counts, both staminate and pistillate flower samples were considered one sample type for further analysis, with $5.7 \times 10^4 \pm 5.7 \times 10^4$ CFU/flower (mean ± SEM; Table 1). The standard error was very large because of the sample size of five and the wide range of values.

Twenty percent of pistillate flower samples and thirty percent of staminate flower samples tested positive for Psa-V, but the percentage of individual flowers carrying Psa-V could be as low as four and six percent (respectively) if only one flower in each positive sample originally contained Psa-V. However, if this lower rate is accurate, the actual Psa-V count per infected flower would have been five times greater than the amount stated above (i.e. a mean of 2.9×10^5 CFU/flower).

One of the ten samples of five bees foraging on flowers returned a positive result for Psa-V, with 3.3×10^3 CFU/bee (Table 2). Of the 30 individual returning foragers sampled from the entrance of the hives, two bees from two separate hives were positive for Psa-V, with 1.8×10^2 and 1.8×10^4 CFU/bee (Table 2). Two of these three positive bee samples had greater CFU counts than the positive control bees that were inoculated with Psa-V.

Ten percent of samples of foraging bees tested positive for Psa-V, but the percentage of individual bees carrying Psa-V could be as low as two percent if only one of the bees in the positive sample of five were carrying Psa-V. However, this would mean that the CFU count from this bee was five times greater than that reported above (i.e. 1.6×10^4 CFU/bee). Of the samples of bees returning to the hives, 5.6% were positive for Psa-V, or 3.3% of the total number of bees caught.

The detection limit was 42 CFU per sample, or 9 CFU per bee or flower for the samples of five, and 17 CFU per bee for the individual returning foragers collected from the hive entrance.

3.4 Survival of Psa-V on bees following movement away from infected orchard

Not more than 10% of returning forager bees were carrying kiwifruit pollen at any of the six hives. Over 200 returning foragers per hive were marked with acetone-based paint, and then five marked bees per hive were individually sampled on days 1, 2, 5, 7 and 9 after the hives were moved away from infected orchards. Positive results from PCR tests using PsaF1/R2 primers were found on day 1 (three bees from three hives) and day 2 (eight bees from four hives), and with PsaF3/R4 primers on day 5 (four bees from four hives). However, subsequent negative results from testing using real-time PCR and the duplex PCR demonstrated that these were false positives and not Psa-V.

3.5 Estimation of initial Psa-V load

The reduction factors for the positive controls averaged 33.9 ± 16.8 (mean ± SEM) and ranged from 2 to 162 with a median of 14.9. As the data exhibited significant positive skew, it was considered that the median reduction factor was the more appropriate value to use to estimate initial Psa-V loads for the flower and bee samples.

Using the median reduction factor, the estimated initial Psa-V inoculum load for the flowers on infected vines was $8.1 \times 10^5 \pm 8 \times 10^5$ CFU/flower (mean ± SEM), and ranged from 1.4×10^2 to 4×10^6 CFU/flower (Table 1). Using the opposite equivalent quartiles of the reduction factor, the estimated initial Psa-V inoculum load for the flowers was $3.8 \times 10^5 \pm 3.7 \times 10^5$ CFU/flower (mean ± SEM; Table 1).

Table 1. Sample and estimates of initial Psa-V (*Pseudomonas syringae pv. actinidiae*) colony forming unit (CFU) counts per flower from anthers collected from staminate and pistillate kiwifruit flowers in an infected orchard.

Flower type	Sample CFU/flower	Estimate of initial CFU/flower	
		From median[a]	From quartiles[b]
Pistillate	10^1	1.4×10^2	4.1×10^2
Pistillate	1.5×10^3	2.1×10^4	2.1×10^4
Staminate	2.9×10^5	4.0×10^6	1.8×10^6
Staminate	7×10^1	9.9×10^2	9.9×10^2
Staminate	8×10^2	1.1×10^4	1.1×10^4
Mean (SEM)	5.7×10^4 (5.7×10^4)	8.1×10^5 (8.0×10^5)	3.8×10^5 (3.7×10^5)

[a]Estimates of initial CFU/flower based on median reduction factor observed in positive control samples.
[b]Estimates of initial CFU-flower based on opposite equivalent quartiles of reduction factors observed in positive control samples.

Using the median reduction factor, the estimated initial Psa-V inoculum load for the foraging bees collected from infected orchards was $1 \times 10^5 \pm 7.9 \times 10^4$ CFU/bee (mean ± SEM), and ranged from 2.5×10^3 to 2.6×10^5 CFU/bee (Table 2). Using the opposite equivalent quartiles of the reduction factor, the estimated initial Psa-V inoculum load for the bees was $5.7 \times 10^4 \pm 3.3 \times 10^4$ CFU/bee (mean ± SEM; Table 2).

Table 2. Sample and estimates of initial Psa-V (*Pseudomonas syringae pv. actinidiae*) colony forming unit (CFU) counts per bee from honey bees (*Apis mellifera*) collected while foraging on kiwifruit flowers of infected vines and while returning to their hives.

Bee sample	Sample CFU/bee	Estimate of initial CFU/bee	
		From median[a]	From quartiles[b]
Foraging on flower	3.3×10^3	4.6×10^4	4.6×10^4
Returning to hive A	1.8×10^4	2.6×10^5	1.2×10^5
Returning to hive D	1.8×10^2	2.5×10^3	7.1×10^3
Mean (SEM)	7.2×10^3 (5.6×10^3)	1.0×10^5 (7.9×10^4)	5.7×10^4 (3.3×10^4)

[a]Estimates of initial CFU/bee based on median reduction factor observed in positive control samples.
[b]Estimates of initial CFU/bee based on opposite equivalent quartiles of reduction factors observed in positive control samples.

4 Discussion

Viable Psa-V bacteria were found on honey bees foraging in a Psa-V-infected kiwifruit orchard. This is the first confirmation of Psa-V on honey bees in an orchard environment. This is an important finding, as the potential role of honey bees as vectors of the disease has previously been questioned because of the absence of evidence of Psa-V on foraging bees. However, this result is not surprising, as Psa-V was also detected on anthers of flowers collected in the same orchard in which these bees were foraging. Psa-V was not detected on any marked bees following the movement of the hives out of the infected orchard.

A major limitation of previous attempts to detect Psa-V on honey bees was the lack of positive controls to assess the likelihood of detecting Psa-V given the sampling methodology. Here we demonstrate that our method of immersion of bees in water and immediate chilling, followed by transportation to a laboratory and testing three hours following capture, resulted in a decline in CFU count of between 2- and 162-fold. It is not clear what caused this large variation in survival of Psa-V on the positive controls, but it demonstrates the importance of not treating a negative result as proof of absence. All the flower and bee samples that returned negative results (75% and 92.5% respectively) could have originally been carrying Psa-V up to a count of 15×10^3 CFU (the detection limit per bee or flower multiplied by the maximum reduction factor).

Furthermore, the results of these positive controls allow us to estimate the initial Psa-V loads that the flowers and bees may have been carrying at the time that they were sampled. The mean reduction factor calculated from the positive controls was strongly influenced by one value that was 3.5 times greater than the next highest value. Applying this mean across all samples was thus likely to overestimate the initial value, so instead we used the median reduction factor. With a mean of 1×10^5 and a maximum of 2.6×10^5, these estimated values based on observations in the field are of the same order of magnitude as the results in an earlier report from inoculations of Psa-V placed on bees (Pattemore et al. 2011). A more conservative estimate based on an assumption that the low counts resulted from a high reduction factor and vice versa reduced these estimates only by about half. Even the raw observed value of 1.8×10^4 CFU/bee from one forager represents a significant inoculum load.

This study confirms that foraging bees can carry Psa-V, and the number of bacteria carried is similar to the inoculum rates used in a previous study (Pattemore et al. 2011). It is prudent, therefore, to pay attention to the conclusions of the previous study that Psa-V may be able to survive in hives for 6-9 days. If a high degree of certainty that bees are not carrying viable Psa-V is required, this study confirms that a stand-down period of more than nine days is advisable before moving hives from an infected zone into a new area within range of un-infected orchards. It is still as yet unknown what rate of inoculum carried on a bee could lead to a new infection.

In the earlier trial investigating the survival of Psa-V on bees, bees inoculated with Psa-V were returned to the hive in plastic cages so that subsequent sampling was conducted on bees that were known to have been carrying Psa-V (Pattemore et al. 2011). This current trial was limited because of the very low number of returning foragers that had been foraging on kiwifruit (discernable by the colour of pollen collected). Although the proportion of workers with kiwifruit pollen was not measured, observations on the day estimate the proportion to be less than 10%. This means that of the c. 200 worker bees per hive that were individually marked, it is possible that only 20 had been foraging on kiwifruit flowers. From our sampling of bees directly from kiwifruit flowers, the maximum percentage observed of workers carrying Psa would have been about 10%, thus further reducing the numbers of marked bees potentially carrying Psa-V to fewer than two individuals per hive. Of the c. 200 marked bees per hive, a maximum of 25 individuals per hive were re-caught and tested. Given these results, in hindsight it is unsurprising that all samples were negative.

The decision to capture and mark 200 bees per hive was based on our experience in recapturing marked bees on subsequent sampling dates (Pattemore et al. 2011). In the previous study we had experienced difficulty in recapturing sufficient numbers of marked bees when 100 foragers were marked. Based on our data from this trial, it is now apparent that 200 marked bees per hive is an insufficient number for this type of trial. However, painting marks on 1,200 individual bees is difficult and time-consuming, and it is unlikely that any more could be reasonably marked within the necessary time frame.

There are two different methods that could be trialled in future studies. One method would be to select one hive in an orchard that showed considerable kiwifruit foraging activity, and to focus the attention on this one hive rather than spreading the effort over six hives. However, replication in this case would be limited to bees from one hive, which may be problematic. An alternative solution would be to capture bees returning from kiwifruit flowers and place them in plastic cages similar to the earlier trial so that it was certain that sampled foragers were the same individuals that had been foraging on kiwifruit flowers. The limitation of this approach is that there may be some other factor such as grooming or contact with substances with anti-bacterial properties that may lead to a difference in survival of Psa on bees in cages and bees free to move about the hive. However, the results from this study and Pattemore et al. (2011), already provide enough evidence to warrant a cautious approach to moving hives from infected to uninfected orchards. Further research on this topic is unlikely to decrease these management recommendations.

In conclusion, the bacterial pathogen has now been confirmed on honey bees foraging on infected kiwifruit vines infected with the pathogen. Although a low proportion of honey bees had a detectible amount of Psa-V contamination, the rates of Psa-V are comparable to those used in an earlier trial to assess the survival of Psa-V on bees and bee hives. These results confirm that the Kiwifruit Vine Health recommendations about hive movement from infected to non-infected regions are advisable.

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