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# VI1511: Winter inoculum on the vine – where is it hiding?

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**October 2015**

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# EXECUTIVE SUMMARY

## VI1511: Winter inoculum on the vine -- where is it hiding?

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Plant & Food Research: Te Puke

**October 2015**

Bacterial canker, caused by *Pseudomonas syringae* pv. *actinidiae* (Psa), does not usually express visible symptoms during winter months and therefore growers do not usually actively manage the disease during this time. However, information from overseas indicates that reducing the inoculum load during winter would be beneficial for disease control during the following summer. A reliable and rapid detection method of the causal organism of this disease in an asymptomatic tissue was needed. This trial was conducted on a grower's kiwifruit orchard in Te Puke during winter 2014 to determine whether it was possible to detect overwintering inoculum of *P. syringae* pv. *actinidiae* biovar 3 (Psa-V) on kiwifruit vines using molecular tools.

The grower applied copper at various times during the winter to manage bacterial canker. Samples were taken several times before and after application of copper and a detection and quantification protocol was applied to determine whether copper application over winter reduced the inoculum load of this disease.

Testing was conducted on Gold3 (*Actinidia chinensis* 'Zesy002'), Gold9 (*A. chinensis* 'Zesy003'), 'Hayward' (*A. deliciosa*) and Green14 (*A. deliciosa* 'Zesh004') female and male vines. Psa-V specific primers (hopZ2b) successfully detected Psa-V inoculum at overwintering. Although spraying copper did not seem to reduce the overall number of kiwifruit vines on which Psa-V was detected, it did have an effect on inoculum load (concentration of Psa cfu/mL). This indicated that applications of copper in winter reduced overwintering inoculum loads on vines.

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# 1 INTRODUCTION

Bacterial canker pathogen *Pseudomonas syringae* pv. *actinidiae* biovar 3 (Psa-V) causes significant economic losses in the kiwifruit industry all over the world (Scortichini et. al. 2012, Vanneste et. al. 2013). This pathogen can survive on kiwifruit vines during winter without showing any visible symptoms or signs of the disease. Molecular tools have been developed to detect this pathogen from cultures using strain specific primers (Rikkerink et al. 2011). This method has not previously been used at an orchard level to detect Psa-V on dormant vines in winter.

Application of copper fungicides is one of the most important practices to manage this disease (Vanneste et. al. 2011). Currently, regular applications of copper during winter (after winter pruning) is not widely practised in kiwifruit orchards in New Zealand probably due to lack of information on inoculum load during winter. In contrast, winter sprays in Italy reduced the amount of inoculum for the spring growing season (Scortichini et. al. 2014). Whilst there have been recommendations for winter copper applications, their benefits in New Zealand remain unproven.

This Psa epidemiological study aimed to quantify the inoculum present on vines over winter on established kiwifruit vines using molecular tools, targeting the areas of the scion that were bent onto the pergola support and any cankers visible on the trunk of the vine. In addition it aimed to measure the changes of inoculum load due to the application of copper.

# 2 MATERIALS AND METHODS

## 2.1 Field sampling and copper spray

This experiment was conducted in a commercial kiwifruit orchard at Te Puke in 2014. Female and male vines of four varieties: Gold3 (*Actinidia chinensis* 'Zesy002'), Gold9 (*A. chinensis* 'Zesy003'), 'Hayward' (*A. deliciosa*) and Green14 (*A. deliciosa* 'Zesh004') were chosen for the study. Samples were collected by swabbing vines following the schedule in Table 1. Each vine from each variety was treated as a biological replicate (Table 1). A sterile wet swab (cultiplast® SWAB, LP Italiana Spa) was wiped around the area that has been bent onto the pergola support (Figure 1), ensuring it was pushed into the crevices of the bark. In addition on September 9 2014, nine vines that had cankers (Gold3 vines 16, 43, 95, Gold9 vines 18, 20, 28, and male vines 14, 35, 38) had small core samples taken from the middle of the canker and at a regular spacing (3cm) above and below it to ascertain the presence of Psa-V within the plant material. Swabs were also taken from different positions around a distinct canker from male vine #35 without coring to detect the presence of Psa-V on the bark surface. Copper (NORDOX 75WG) was sprayed (@75g product/100L) on the orchard by the grower (Table 1) and sampling was conducted pre- and post-spraying to determine the effect of winter copper applications on overwintering inoculum load on vines.

**Table 1. Sampling and copper spray schedule in trial orchard at Te Puke in 2014.**

| Variety | Activities | 1st | 2nd | 3rd | 4th | Total sample (biological replicate) |
|---|---|---|---|---|---|---|
| Gold3 | Sampling | 8 July | 22 July | 7 August | 19 August | 100 vines |
| | Spraying | 5 July | 10 August | | | |
| Gold9 | Sampling | 8 July | 22 July | 7 August | 19 August | 50 vines |
| | Spraying | 14 July | 10 August | | | |
| 'Hayward' | Sampling | 8 July | 22 July | 7 August | 19 August | 100 vines |
| | Spraying | 10 August | | | | |
| Green14 | Sampling | 8 July | 22 July | 7 August | 19 August | 53 vines |
| Gold3, Gold9, 'Hayward', Green14 male | Sampling | 8 July | 22 July | 7 August | 19 August | 40 vines |

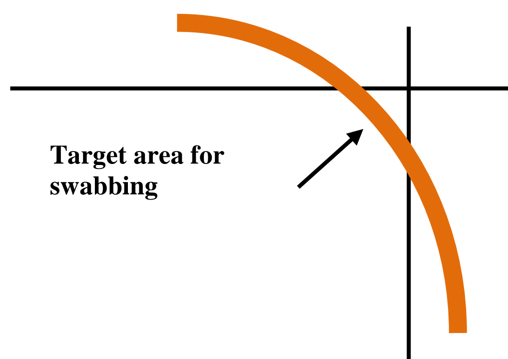



**Figure 1. Sampling area (arrow) of swab on kiwifruit vine (yellow).**

## 2.2 Bacterial viability

Bacterial viability was tested from cores of cankers as per Casonato et al. (2014). Samples were plated onto Kings B-C medium and colonies that had similar morphological features to a known Psa control plate were counted.

## 2.3 Psa-V detection and quantification

### 2.3.1 Swab extraction

For DNA analysis from swabs, each frozen swab (kept in -20°C) was thawed and the end of the swab (15 mm, diameter 4 mm) was cut off and placed directly into a 1.5 mL Eppendorf tube containing 200 µL of 10% Chelex (BioRad Chelex 100). The Chelex extraction process was as follows: the sample was vortexed vigorously for 30-60s. The tube was heated to 100°C for 10 minutes, again vortexed vigorously then heated for a further 10 minutes at 100°C. The sample was centrifuged at 13,500 rpm for 10 minutes and the supernatant was removed and placed in a clean, new tube for further processing (Casonato et. al. 2014).

### 2.3.2 Quantitative polymerase chain reaction (qPCR)

Quantitative PCR (qPCR) and data analysis were performed on a Rotor Gene 2-plex. The qPCR was performed using the Rotor-Gene® SYBR Green® PCR kit (Qiagen). The primers (Psa-V specific) used were hopZ2bF2-L: 5’- ACAACTTCAGGCTACAATACTTACGC-3’ and hopZ2bR2: 5’- CTCAGGATGCGTTTCGGTTAC’ (Rikkerink et al. 2011). Each 10 µL reaction contained 2.5 µL DNA templates, 5 µL 2x Rotor-Gene SYBR Green, 1 µL 5 µM hopZ2bF2-L, 1 µL 5 µM hopZ2bR2, and 0.5 µL sterile water. qPCR conditions were as follows: 10 min at 95°C to activate the hot-start *Taq* polymerase, 35 cycles of 95°C for 5 s, 64°C for 10 s and 72°C for 15 s, with fluorescence acquisition following each 72°C step. Following this a melt was performed during which the temperature was increased at 1°C per 5 seconds from 72°C to 95°C, with continual fluorescence acquisition. Each run consisted of 72 samples, which included a six-point standard curve and a non-template reagent control. Data were analysed using QIAGEN Rotor-Gene software version 2.1.0. For quantification analysis a threshold of 0.05 was applied to each run.

Approximate colony forming unit concentrations per mL (cfu/mL) were calculated by comparing the crossing threshold (Ct) value obtained from the known bacterial standards of Psa-V that were made to $10^8$, $10^7$, $10^6$, $10^5$, $10^4$, $10^3$ cfu/mL. A calculation was made using the Rotor-Gene software that compared the known concentration of the standards against the Ct value derived from the qPCR run. The unknown sample Ct values were obtained from the run and the estimated cfu /mL of each sample was calculated.

# 3 RESULTS

## 3.1 Psa-V detection

It was possible to detect Psa-V during winter on the vines of all four cultivars kiwifruit using molecular tools. In this trial, the pathogen was detected and quantified on all four cultivars and male vines for all four sample dates (8 and 22 July 2014 and 7 and 19 August) except in male vines sampled on 8 July and on cultivar 'Hayward' sampled on 19 August (Figure 2). In this experiment the lowest detection ($2.80\times10^{1}$ cfu/ml) was on 'Hayward' on 8 July and highest on a male vine on 19 August ($1.82\times10^{5}$). Overall, it was noticed that inoculum load increased from 8 July to 7 August in all varieties.

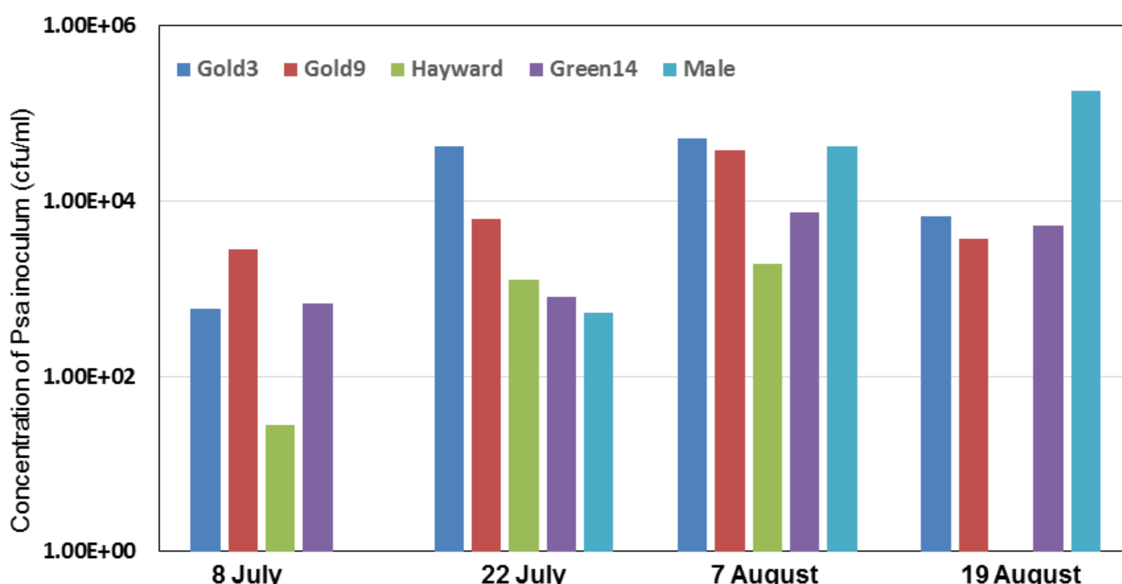
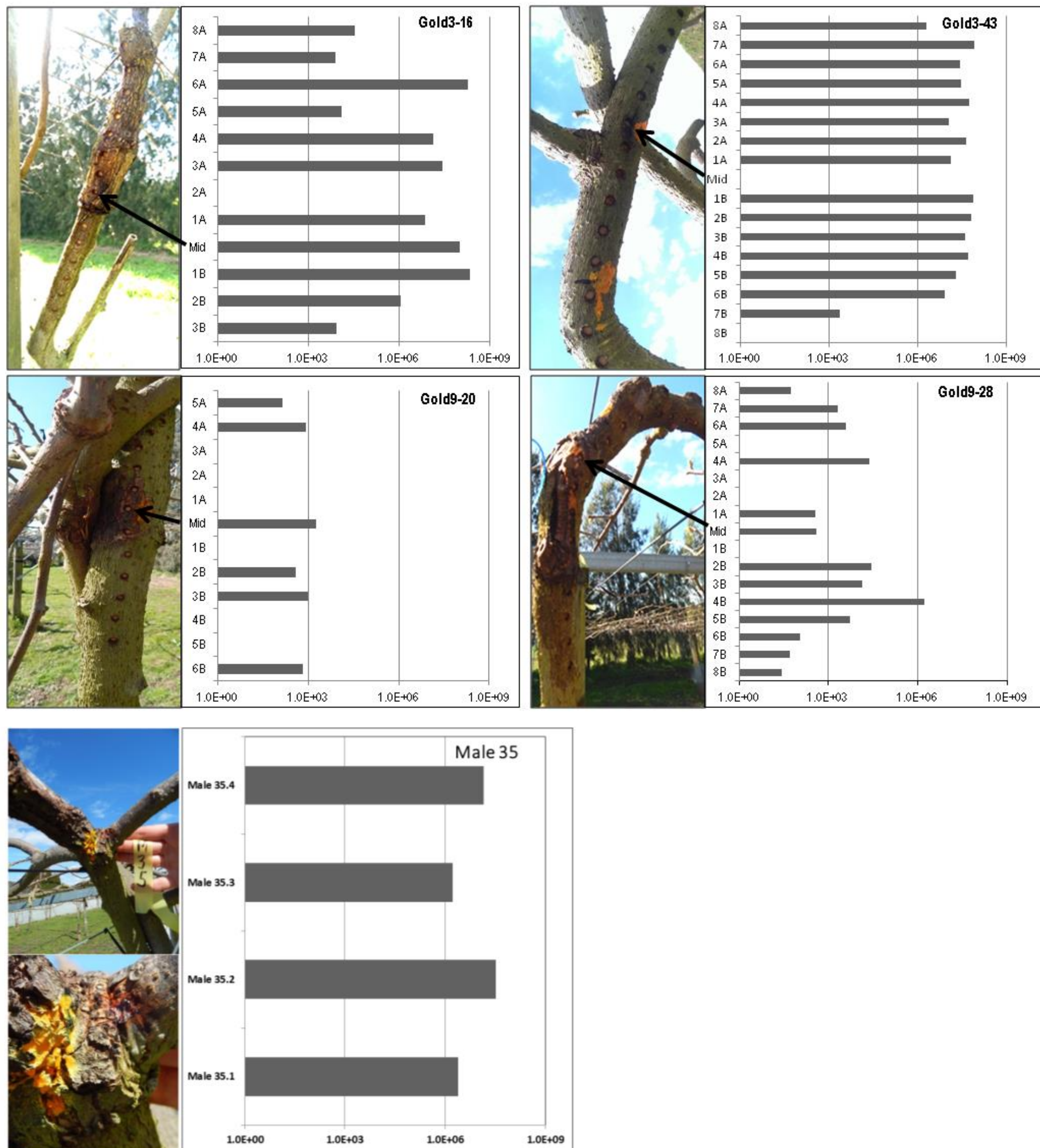



**Figure 2. Inoculum load of *Pseudomonas syringae* pv. *actinidiae* (Psa-V) in winter on kiwifruit vines.**

Inoculum load (concentration of Psa inoculum cfu/ml) per vine presented in Y axis is the average of inoculum where Psa were detected. Sampling dates are presented in X axis. n= 100, 50, 100, 53

Psa-V was detected in the cores that were taken from above and below cankers on some selected vines. There was no detectable difference in inoculum concentration between cores taken from above and below the canker. However, the concentration of Psa-V did vary with distance from the cankers with bacterial numbers tending to be lower with increasing distance from the canker. Psa-V was not detected in some of the core samples (Figure 3 and Appendix Figure A1), suggesting that Psa-V was not distributed evenly within the vines. In addition, a swab taken from four places around a canker on vine male35 had Psa-V.




## Figure 3. Detection of *Pseudomonas syringae* pv. *actinidiae* (Psa) on visible cankers and its adjacent areas.

In *Actinidia chinensis* 'Zesy002' Gold3 and *A. deliciosa* 'Zes004' Gold9, the middle position of the canker is indicated by a black arrow and concentration of Psa-V from above and below the middle position is indicated by A and B respectively on the Y-axis, and the number (with A or B) indicates the interval away from the middle position. In male vine 35, the swab was taken from four positions around a canker. All concentrations are graphically presented on the right side of the figures as colony forming units per mL.

## 3.2 Inoculum load and effect of copper on *Pseudomonas syringae* pv. *actinidiae*

Copper was applied by the grower and swabs were taken pre- and post-application to identify the effect of copper on inoculum load on vines. Overall, it was observed that copper application on 10 August reduced the inoculum load dramatically on both Gold3 and Gold9 and also slightly on 'Hayward' vines (Figure 4). Copper application on 5 July also showed a trace amount of Psa-V when sampling was carried out on 8 July in Gold3. However, copper did not reduce the overall number of kiwifruit vines on which Psa-V was detected.

Variation was observed when inoculum load was quantified from the Psa-detected vines. Inoculum load of Psa-V varied between the kiwifruit varieties at the same sampling date. In addition, standard deviation in one particular time point of one variety was found to be very high.

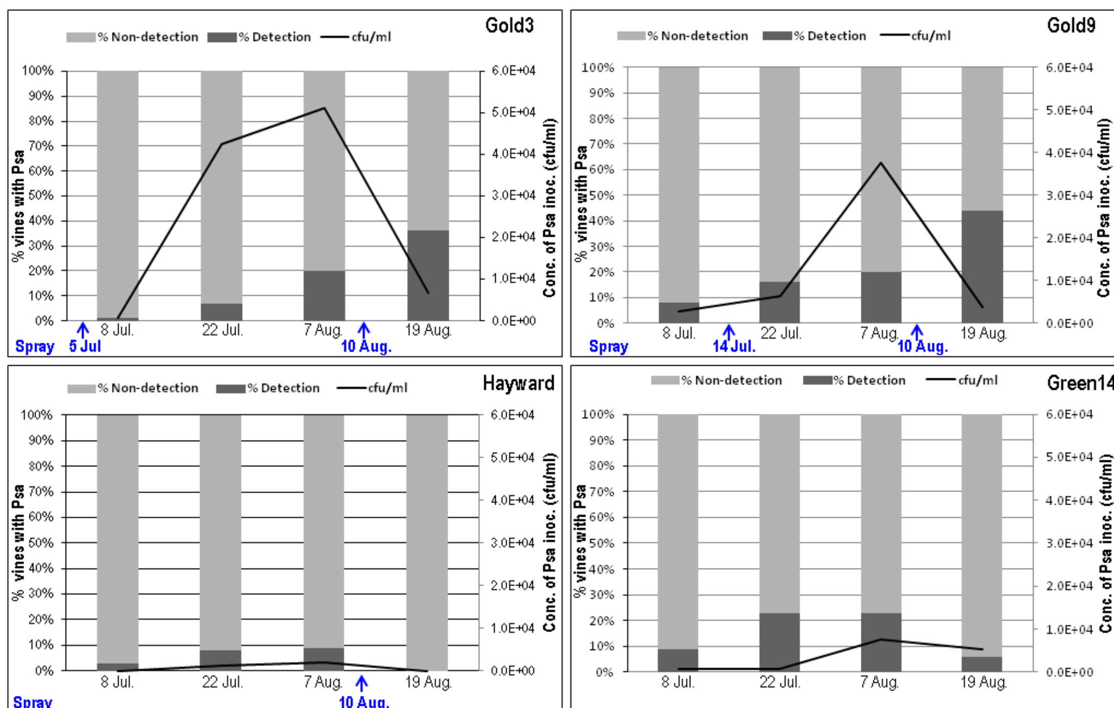



**Figure 4. Detection of *Pseudomonas syringae* pv. *actinidiae* (Psa-V) in winter on kiwifruit vines.**

Graph showing percentage of vines having Psa-V in left Y-axis of *Actinidia chinensis* 'Zesy002' Gold3, *A. chinensis* 'Zesy003' Gold9, *A. deliciosa* 'Hayward', and *A. deliciosa* 'Zesh004' Green14 kiwifruit varieties based on hopZ2b Psa-V specific primers at four swabbing dates in X-axis. Inoculum load per vine was shown in right Y-axis that is the average of inoculum of the detected vines only. Spray dates were indicated on X-axis in colour. n=100, 50, 100, 53 for Gold3, Gold9, 'Hayward', Green14 respectively.

# 4 DISCUSSION

Results from this experiment confirmed that it was possible to detect Psa-V on vines using molecular tools even when there were only trace amounts of inoculum present and there were no symptoms on vines. Although no observation was followed on spring and summer in this experiment, Scortichini's (2014) results showed that applying copper at winter time reduces spring leaf spot and winter exudates of kiwifruit in Italy. These results suggest that proper management practices against Psa is necessary from winter. These practices may include covering the orchard with breathable plastic cover (Casonato & Bent 2014), sanitation of pruning materials and applications of copper.

This experiment was conducted on four varieties and variation of Psa-V detection was observed among the varieties. Different mechanisms of host-pathogen interaction and physiology may account for these variations. For example, in Gold3 after having one spray on 5 July, inoculum load was above $5.0\times10^{4}$ cfu/mL by 7 August whereas in 'Hayward', without any copper spray the inoculum load remained below 0.2 cfu/mL at the same time point (Figure 2). Host physiology and age of vine could be the reasons to explain this phenomenon. It was noted that Psa-V concentration varied widely between cores taken from above and below the cankers, indicating that, internally, Psa-V was distributed within vine evenly. This event is well supported by the findings of Everett et al. (2014) and Tyson et al. (2014).

High variation was also noticed between the biological replicates within each variety in spite of similar management practice and microclimatic conditions. One possible explanation for this variation would that the swabbing was not done in same spot every time. Therefore, while Psa-V was detected at one time point, it was not necessarily detected in the next. Although this was a limitation of this experiment, sampling a large number of vines (50+ of each variety) in an orchard provided a useful overview of inoculum load at the orchard level.

This work has shown that the application of copper to kiwifruit vines during the winter will reduce the inoculum load in winter. It is necessary to continue disease assessments into spring and summer to determine whether winter copper applications will result in lower disease levels during the season.

# 5 ACKNOWLEDGEMENTS

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# APPENDIX

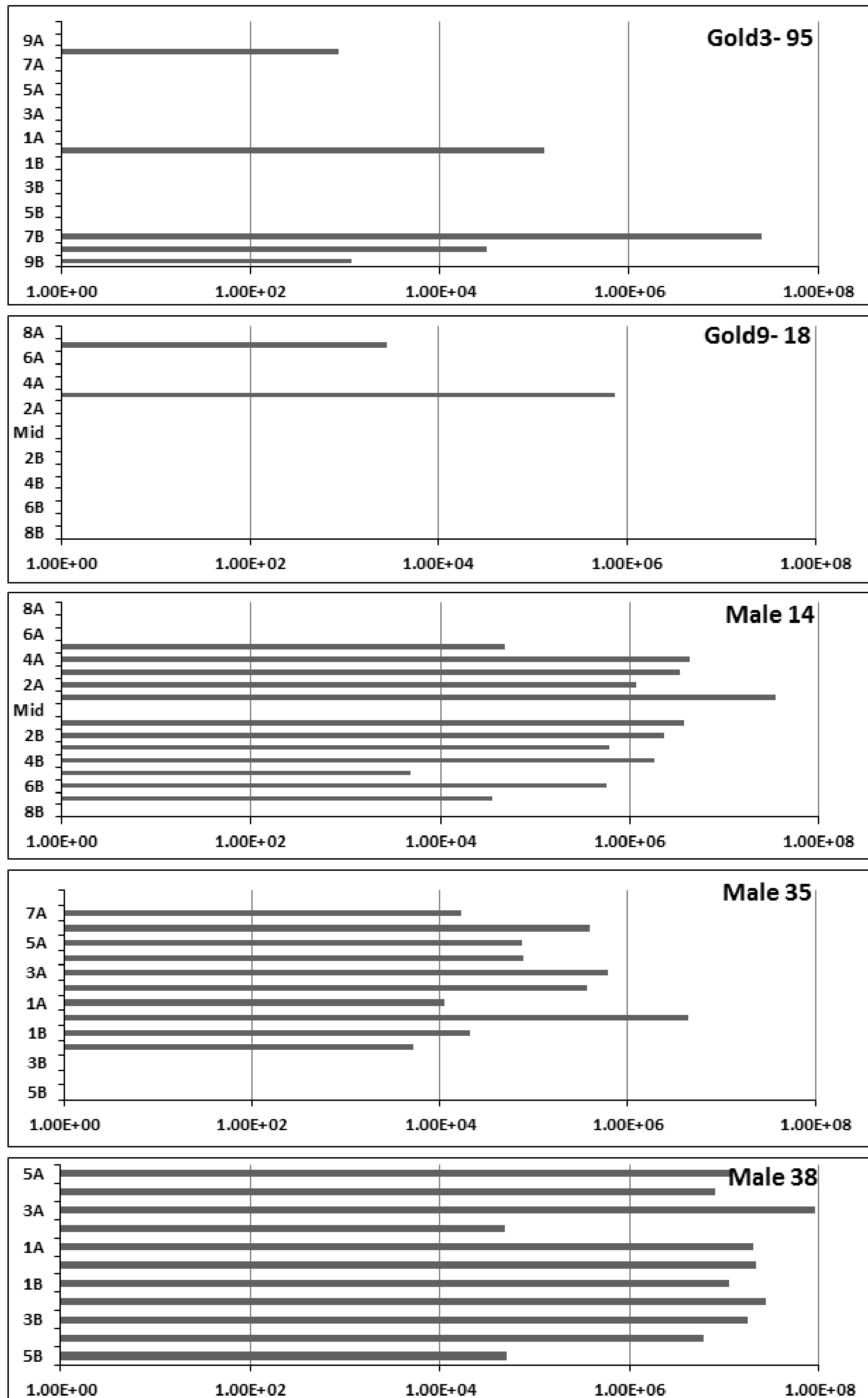


**Figure A1. Detection of *Pseudomonas syringae* pv. *actinidiae* (Psa) on cankers and adjacent areas.**

In *Actinidia chinensis* 'Zesy002' Gold3, *A. chinensis* 'Zesy003' Gold9 and male vine concentration of Psa-V on above and bottom position was indicated by A and B respectively on Y-axis, and the number indicates the regular interval away from the canker. Concentration was presented with a unit of cell forming unit (cfu) per mL.

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