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On-orchard management of *Pseudomonas syringae* pv. *actinidiae* infection and symptom expression: part C: Girdling - possible positive and negative effects on Psa

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On-orchard management of *Pseudomonas syringae* pv. *actinidiae* infection and symptom expression: part C: Girdling - possible positive and negative effects on Psa

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Girdling techniques are used extensively in the kiwifruit industry and are fundamental to achieving high productivity (yield and high dry matter) in both 'Hayward' and 'Hort16A' orchards. However, girdling results in significant wounds either on the trunk or on the canes. Based on knowledge of other *Pseudomonas* species infecting perennial fruit crops, it was anticipated that the wound sites created by girdling of kiwifruit vines may present a significant point of infection for *Pseudomonas syringae* pv. *actinidiae* (Psa).

Two trials were carried out with potted 'Hort16A' vines:

In the first trial two-year-old Bruno seedlings grafted with 'Hort16A' were grown in a greenhouse and girdles were applied to the 'Hort16A' scion, just above the graft. Some girdles were protected with Greenseal™ Ultra, Nordox® 75 WG (1.1 g/L) or Oxyspray® (15 mL/ L) and some vines were kept as ungirdled controls. Vines were inoculated by pipetting 50 µL of about 4 x 10^9 cfu/mL of the virulent strain of *Pseudomonas syringae* pv. *actinidiae* (Psa-V) directly into the girdling wound, or onto undamaged bark. Each treatment was replicated on 15 vines. Vines were destructively sampled on three occasions by cutting the trunks open and aseptically excising tissue above and below the girdles. We found:

- Although most vines did not show the typical symptoms of Psa-V infection, such as shoot die-back or leaf spotting, when vines were cut open virtually all the vines with an inoculated girdle had become infected with Psa-V.
- Vines that had not been inoculated did not have Psa infection, so we can assume that our vines were clean at the start of the trial, and no infection occurred while they were held in the greenhouse.
- Applying Psa to undamaged trunks did not result in vines becoming infected.
- Unprotected girdles could be infected with Psa for at least 15 days after the girdle had been applied.
- Callusing of the girdle on Psa-inoculated vines was much slower than on control vines. The slowing of callusing was especially obvious when vines were inoculated immediately after being girdled.
- Applying Greenseal™ Ultra, Nordox 75 WG or Oxyspray to girdles did not reduce Psa infection.
- In vines inoculated on the day of girdling, Psa had moved up to 7 cm from the girdle in 5½ weeks, at least 30 cm in 2½ months and up to 95 cm in 5 months.
- Psa moved up and down the trunks at a similar rate, and movement through the scion ('Hort16A') and the rootstock ('Bruno') were also similar. Psa was not inhibited by the graft.

In the second trial, potted 'Hort16A' vines were planted into an orchard block and were open to natural Psa-V inoculum from nearby mature vines. Vines were planted on two occasions during November 2011. Some vines were left as controls, some were girdled, and some girdles were protected with Nordox 75 WG or Eurogel. All girdles were applied in dry conditions. We found:

- All vines developed leaf spotting typical of Psa infection within eight weeks of being planted in the orchard.

- Earlier planting did not lead to earlier infection of vines. During the period 2-6 December, we received almost 40 mm of rain and it was 18 days after this that we observed heavy spotting on the vines. This suggests that infection followed specific weather patterns, rather than being related to the length of time vines had been in the orchard.

- All treatments developed leaf spotting at about the same rate. Application of trunk girdles did not increase the rate of leaf infection.

- Trunk girdles were applied about 0.4 m above ground level. Destructive sampling of some vines showed that in trunk-girdled vines 93% of the samples in the lower 0.6 m of trunk were strongly positive for Psa, while in control vines only 30% of the samples were strongly positive. This suggests that a large proportion of the infection in the lower part of the vine had entered via the girdle. However, Psa was also recovered from some of the trunks of control vines, suggesting that some systemic infection in the trunks had originated from leaf, shoot or other wound infections and had moved down into the trunk.

- Eighty days after being planted at Te Puke, virtually all vines were displaying secondary symptoms. This development of symptoms was far more rapid, and more obvious, than had occurred in the greenhouse trial.

- Visual assessment of vines indicated that trunk-girdled vines were more severely damaged than control vines. Protecting girdles with Nordox 75 WG or Eurogel did not reduce vine symptoms.

In the current work we have shown that Psa can infect girdles and none of the protectants tested was able to prevent this infection. Plant & Food Research are undertaking similar work on other pruning cuts and plant wounds to see if they are also likely to act as infection sites for Psa. The current work was carried out on 'Hort16A', which we know is highly susceptible to Psa. For the future, we need to understand how cultivars thought to be more tolerant, such as 'Hayward' and 'ZESY002' (commonly known as Gold3), respond when wounds are exposed to Psa.

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1 Introduction

Girdling techniques are used extensively in the kiwifruit industry and are fundamental to achieving high productivity (yield and high dry matter) in both 'Hayward' and 'Hort16A' orchards. Spring trunk girdling is commonly used to improve fruit size and summer trunk girdling is used to improve dry matter allocation to fruit. Summer and autumn girdling may also be beneficial in enhancing return bloom and in vigour control. However, girdling results in significant wounds either on the trunk (spring or summer trunk girdling), or on the canes (spring cane girdling).

Based on knowledge of other *Pseudomonas* species infecting perennial fruit crops, it is anticipated that the wound sites created by girdling of kiwifruit vines may present a significant point of infection for *Pseudomonas syringae* pv. *actinidiae* (Psa). In a comprehensive study on sweet cherry (Spotts et al. 2010) it was shown that up to seven types of wound sites were potential sites of infection for *Pseudomonas syringae* pv. *syringae* (Pss). One of the wound types investigated was scoring (similar in damage to girdling) and this was found to be a point of entry for Pss during spring and summer. The study also indicated that for sweet cherry heading (pruning) cuts became resistant to infection in about one week in summer and three weeks in winter.

In kiwifruit, girdles can heal rapidly (c. 3-4 weeks) during the growing season and we anticipate that the infection risk would decrease over this time period as callus forms and phloem bridges are re-established. As there are also likely to be seasonal influences on the infection process, it would be prudent to consider the risk of spring, summer, and autumn girdling as infection points.

2 Greenhouse trial

2.1 Methods

2.1.1 Vines

One hundred and eighty field-grown, two-year-old 'Bruno' seedlings grafted with 'Hort16A' were purchased from Te Puke Nursery in June 2011. At this time the nursery was outside the Psa-V Priority Zone, and the vines were thus expected to be free of Psa-V. Vines were transferred to a polythene greenhouse at the Te Puke Research Orchard on 27 June. Vines were spaced about 0.5 m apart and canes were trained up vertical strings. Each pot was supplied with drip irrigation.

Vines were forced to break bud early and develop shoots rapidly so that we were able to apply girdles some weeks before commercial growers would apply girdles to field-grown vines.

A 20 kW electric heater was turned on after the vines had been in the greenhouse for two days and the temperature controls were set to 12ºC night and 21ºC day. These settings were nominal since the heater was not always able to maintain the minimum temperature during cold nights, and even when the large roof vent was opened, temperatures often exceeded 21ºC during sunny days. During the first few days of operation the air temperature averaged 15ºC (Table 1).

Budbreak started on about 25 July, after vines had been heated for 28 days. This is about a month earlier than we would expect budbreak on field-grown vines.

On 1 August temperature settings were reduced to 8ºC night and 15ºC day to start hardening vines off. At this time most shoots were 50-100 mm long. The side vents on the greenhouse were manually opened on hot days to reduce maximum temperatures.

On 7 September the heater was turned off, but the roof vent was left to open automatically when temperatures were above 15ºC.

Table 1. Summary of air temperatures in the greenhouse during August and September 2011.

Month	Air temperature (°C)		
	Average	Minimum	Maximum
August	14.1	6.8	31.0
September	13.0	3.1	25.5

2.1.2 Experimental treatments & design

Girdles were applied to the 'Hort16A' scion just above the graft (Figure 2) over the period 8 September – 23 September 2011. Since the trunk was only 10-20 mm in diameter at this time, the 5-mm wide girdle was applied with the Vaca double-bladed girdling pliers that are normally used for cane girdling. We found that these girdles could hold 50 µL of fluid.

The potential of Psa-V to infect via trunk girdles was investigated by inoculating girdles with Psa-V on the same day, and 1, 2, 4, 8 and 15 days after the girdles were applied (Figure 1,

Table 2). We also tested the ability of Psa to infect directly through undamaged trunks; in addition, some girdles were protected with Greenseal™ Ultra, Nordox® 75 WG (1.1 g/L) or Oxyspray® (15 mL/L) before being inoculated. Greenseal was applied with the applicator brush provided, while Nordox and Oxyspray were sprayed on. Each treatment was replicated on 15 vines, and the vines were arranged in the greenhouse in a randomised block design.

Table 2 shows the schedule of treatments applied to greenhouse 'Hort16A' vines.

Table 2. Description of the treatments applied to 'Hort16A' kiwifruit vines in the greenhouse. TG = trunk girdle. Psa = *Pseudomonas syringae* pv. *actinidiae*.

Treatment number	Treatment	Description of treatment
1	Control	Vines were not girdled and Psa was not applied
2	Psa trunk no TG	Psa was applied to the bark of vines at the point where a girdle would normally be applied. A ring of Blu-Tack® was used to stop the inoculum from running down the trunk.
3	TG + BS	Vines TG on 22 Sept. and inoculated with bacteriological saline only (no Psa)
4	TG_Psa 1	Vines TG on 22 Sept. and Psa-V applied 1 day later
5	TG_Psa2	Vines TG on 21 Sept. and Psa-V applied 2 days later
6	TG_Psa4	Vines TG on 19 Sept. and Psa-V applied 4 days later
7	TG_Psa8	Vines TG on 15 Sept. and Psa-V applied 8 days later
8	TG _Psa15	Vines TG on 8 Sept. and Psa-V applied 15 day later
9	TG_Greenseal	Vines TG on 22 Sept., Greenseal™ Ultra applied immediately and Psa- V applied 1 day later
10	TG_Nordox	Vines TG on 22 Sept., Nordox® 75 WG applied immediately and Psa-V applied 1 day later
11	TG_Oxyspray	Vines TG on 22 Sept., Oxyspray® applied immediately and Psa- V applied 1 day later
12	TG_Psa0	Vines TG on 23 Sept. and Psa-V applied 5 hours later

Figure 1. Some girdles on 'Hort16A' kiwifruit vines were protected with Greenseal™ Ultra, Nordox® 75 WG or Oxyspray® before being inoculated with *Pseudomonas syringae* pv. *actinidiae* (Psa-V).

Figure 2. (A) The 5 mm-wide trunk girdle was able to hold 50 µl of inoculum. (B) Ungirdled trunks were inoculated above a ring of Blu-Tack®, which prevented the inoculum from running down the trunk. (C) Girdles were applied to the 'Hort16A' scion, just above the graft.

At the time of inoculation shoots were typically 0.6 – 1 m long and the bottom leaves were fully expanded (Figure 3). However, no vine carried any flowers. The lack of flowering was presumably due to the poor chilling experienced during May and June 2011.

Figure 3. At the time of inoculation (23 September) 'Hort16A' shoots were typically about 0.6 – 1 m long and the bottom leaves were fully expanded.

2.1.3 Meteorological data

Air temperature and humidity inside the greenhouse were monitored about 1.2 m above ground level. Temperature and relative humidity (RH) were recorded every 10 minutes from August 2011 until the termination of the trial on 21 February 2012.

2.1.4 Inoculation

The last trunk girdle was made at 1300 hours on 23 September 2011. The gravel floor of the greenhouse was hosed down at 1700 hours to increase humidity and the side and roof vents were closed. Inoculation started at 1700 hours, as the day was cooling, and finished by 1845 hours. During this period the temperature averaged 18.6ºC, the RH was 76% and the vapour pressure deficit was 0.5 kPa (Figure 4).

Vines were inoculated with an isolate of *Pseudomonas syringae* pv. *actinidiae* taken from kiwifruit leaf spots at Te Puke Research Orchard in February 2011. The isolate used for inoculations was Psa 3.2.3 (cc691), previously determined to be haplotype NZ-V, and known to be pathogenic in leaf-disc assays and potted plant studies (Tyson & Curtis, unpub.).
The inoculum was suspended in bacteriological saline (0.85% NaCl in sterile distilled water) at a rate of approximately 4 x 10^9 colony forming units per mL (cfu/mL).

In each inoculated treatment, 50 µL of the inoculum suspension was pipetted directly into the girdling wound. In the girdled control (treatment 3), 50 µL of bacteriological saline was pipetted into the girdling wound.

The greenhouse was kept closed for 24 hours after inoculation.

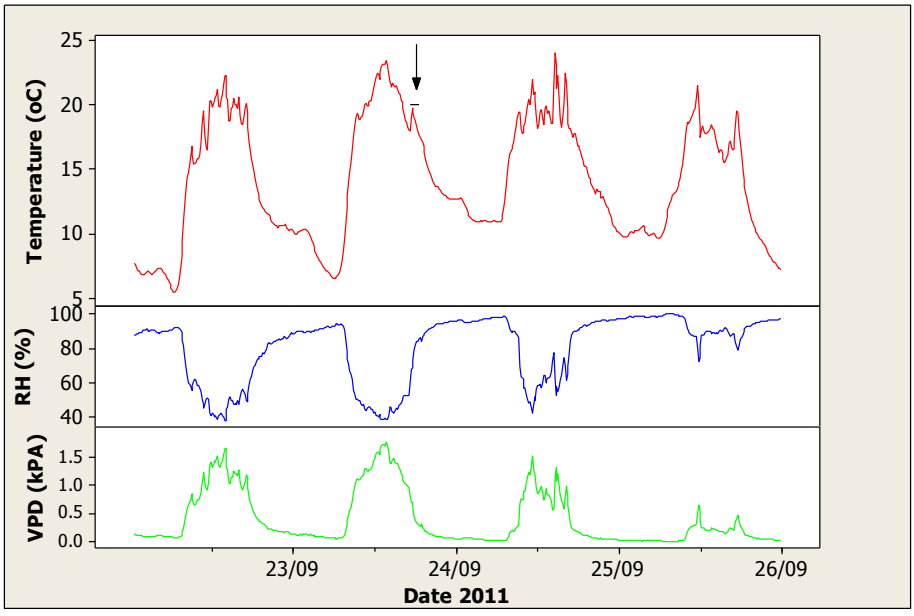

Figure 4. Girdles were inoculated with *Pseudomonas syringae* pv. *actinidiae* (Psa-V) on 23 September 2011 between 1700 and 1900 hours (arrowed). During this period the temperature averaged 18.6ºC, RH was 76% and the vapour pressure deficit was 0.5 kPa.

2.1.5 Vine observations

Development of leaf symptoms and expansion of callus into the girdle were monitored every few weeks. Callus growth was scored using the following ratings:

0 - no healing
1 - callus obvious
2 - callus bridges girdle in places
3 - completely callused.

2.1.6 Isolation and identification

Five vines from each treatment were destructively harvested on:

1. 31 October 2011 - 38 days after inoculation
2. 12 December 2011 - 80 days after inoculation
3. 21 February 2012 - 151 days after inoculation (treatment 12 only).

On the first two sample dates, stem sections were taken that included tissue from 30 cm above the girdle to 30 cm below the girdle and transported to Mt Albert Research Centre on ice.
For the last sample, the entire vine (above the soil) was taken to Mt Albert Research Centre.

Bacterial isolations were made as follows: small pieces of plant tissue were aseptically excised, macerated in 100 µL bacteriological saline (BS), and left for at least five minutes. A 100-µL aliquot of the resulting suspension was then spread onto a semi-selective agar medium (SNA++). Plates were incubated at room temperature (c. 20ºC) for 3-4 days, and then marked as growth/no growth. The two isolations farthest from the girdle showing bacterial growth were also identified using the method of (Rees-George et al. 2010), modified for use with qPCR.

Sample 1 (38 days after inoculation). Samples were taken from:

1. Girdle -2 cm (2 cm below the girdle, towards the graft)
2. Girdle -1 cm
3. Girdle
4. Girdle +1 cm
5. Girdle +2 cm (2 cm above the girdle).

Stems from treatment 4 (girdled one day before inoculation) were also isolated at 7 cm below and above the girdle.

Sample 2 (80 days after inoculation). Samples were taken from:

1. Girdle -14 cm (14 cm below the girdle)
2. Girdle -7 cm
3. Girdle -2 cm
4. Girdle +2 cm(2 cm above the girdle)
5. Girdle +7 cm
6. Girdle +14 cm.

Stems from treatment 12 (girdled immediately prior to inoculation) were also isolated at 5, 10, 15, 20, 25 and 30cm below and above the girdle.

Sample 3 (151 days after inoculation). Only vines from treatment 12 (inoculated the day they were girdled) were sampled. Samples were taken at 10-cm intervals from the base of the vine to the top of the first cane. Bacterial isolations were made from thin (1 mm) cross-sections of the stems, to compensate for the tendency of the bacterium to 'wind' through the plant; i.e. it is not found homogenously across a stem.

2.2 Results & Discussion

2.2.1 Meteorological data

After inoculation, the average temperatures in the greenhouse were always within the 15-20ºC range that suits Psa (Table 3, Figure 5). However, daily maximum temperatures typically reached about 25ºC, and on rare occasions, the temperature went as high as 30ºC, but this was generally for a very short time. Previous work with three-year-old potted 'Hayward' vines (Serizawa & Ichikawa 1993) has shown that growth of Psa is optimal at 15-18ºC, but at a constant 25ºC there was no bacterial oozing. When day-night temperatures fluctuated between 23 and 18ºC or 28-23ºC, some bacterial oozing was observed but this was localised near the infection site and did not develop into other tissues. At higher temperatures the bacterial population decreased compared with those observed at lower temperatures.

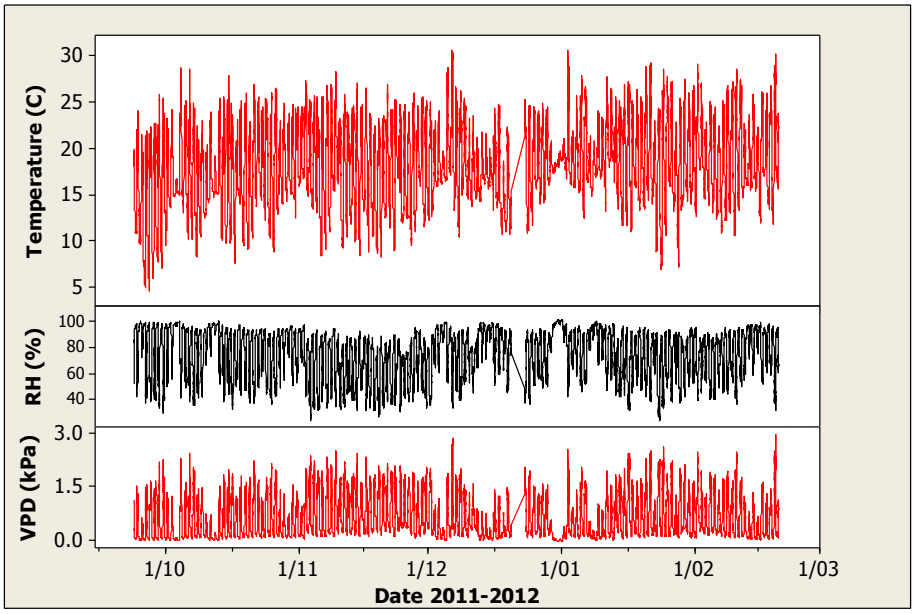

Figure 5. Temperature, humidity and vapour pressure deficit inside the greenhouse from the date that kiwifruit vines were inoculated with *Pseudomonas syringae* pv. *actinidiae* (Psa-V) to the end of the trial.

Table 3. Monthly average, minimum and maximum air temperatures (°C) 1.2 m above ground level in the greenhouse (September 2011 – February 2012).

Month	Average	Minimum	Maximum
September	13.9	4.5	25.7
October	16.6	7.5	28.6
November	17.5	8.3	28.3
December	17.7	10.4	30.6
January	18.9	6.9	30.5
February	18.6	10.5	30.2

2.2.2 Callus formation

During the first 38 days after inoculation, although some leaves developed brown necrotic spots, or large patches, these did not appear to be related to Psa infection. We did not observe any of the leaf spots with halos characteristically found on vines that have been infected with Psa in the field. Most of the vines appeared healthy, with only a few having leaf spots or dead patches on leaves.

The girdles treated with bacteriological saline had far better callusing after 26 days than girdles that had been inoculated with Psa suspended in bacteriological saline on the same day (Figure 6). This suggests that the presence of Psa reduces the rate of callus formation. Girdles treated with bacteriological saline were completely healed about 35 days after being girdled.

Treating girdles with Greenseal™ Ultra, Nordox 75 WG, or Oxyspray before being inoculated with Psa did not greatly affect callus formation. Although Greenseal™ Ultra may have delayed callusing for the first 25 days, by 50 days after girdling callusing was very similar to that on vines treated with Nordox, or Oxyspray, or left unprotected (TG_psa1).

For vines that had been inoculated with Psa, the amount of callus formation increased with increasing time elapsed between girdling and inoculation.

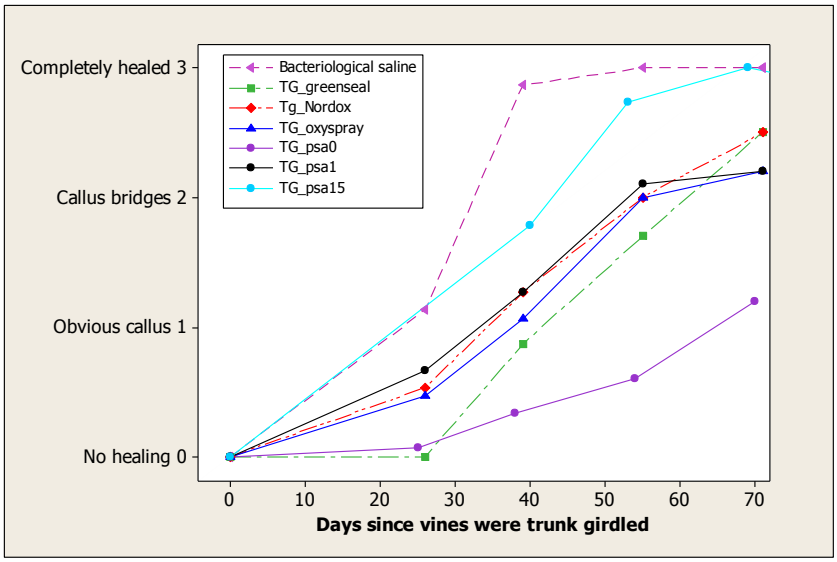

Figure 6. Rate of callusing after kiwifruit vines had been trunk girdled. Data for vines inoculated at 2-8 days after girdling has been excluded for clarity. TG = trunk girdle. Psa = *Pseudomonas syringae* pv. *actinidiae*.

The lowest rate of callus formation was observed on vines inoculated only hours after girdling. Most of these girdles were still not callused over 80 days after girdling (Figure 7), and there was an unusually gross expansion of the stem immediately above the girdle. The rate of callusing increased progressively as the time lapse between girdling and inoculation increased up to 15 days (Figure 6, data for some treatments have been excluded to improve the clarity of the figure). Callusing was most affected by inoculation during the first 24 hours after girdling.

When girdles were treated with Greenseal™ Ultra, callus formation was not evident for the first 25 days, but after this initial lag girdles callused at a similar rate to those that received only Psa and no protection.

Girdles treated with Nordox 75 WG or Oxyspray callused at a similar rate to those that received only Psa (on the same day) and no protection.

Figure 7. Callusing on 'Hort16A' vines inoculated 5 hours after girdling. These vines were destructively sampled on 12 December 2011, 80 days after vines were inoculated with *Pseudomonas syringae* pv. *actinidiae*.. All five vines show unusually gross expansion above the girdle, but the girdle has not healed over.

2.2.3 Vine observations 151 days after inoculation

By 151 days after inoculation, many of the remaining vines were showing some degree of leaf spotting, leaf breakdown, or yellowing associated with nutritional deficiency. However, these symptoms were not characteristic of Psa. At this time, only 59 vines remained in the greenhouse; only five of these had secondary symptoms, such as shoot die-back, that were potentially caused by Psa.

Figure 8. By 151 days after inoculation, some 'Hort16A' leaves had necrotic spots and yellowing - these symptoms were not typical of *Pseudomonas syringae* pv. *actinidiae*.

Of the 50 remaining girdled vines, only three girdles had failed to heal and two of these were on vines that were dead above the girdle. Two of the three vines were from treatment TG_psa0 (girdled, then inoculated with Psa on the same day).

Callused girdles treated with Greenseal™ Ultra were very large, being typically twice the size of other calluses, but they were more symmetric than treatment TG_psa0, indicating that callusing was initiated both above and below the girdles (Figure 9).

All the TG_psa0 girdles still looked very odd. as all the callusing appeared to have originated above the girdles.

Figure 9. By 151 days after inoculation virtually all girdles were completely healed, but (a) callusing on 'Hort16A' vines treated with Greenseal™ Ultra was much larger than on most other vines, and (b) vines inoculated with *Pseudomonas syringae* pv. *actinidiae* five hours after being girdled had a distinctive callus that seemed to be formed entirely above the girdle.

2.2.4 Isolation and identification 38 days after inoculation

When vines were sampled 38 days after inoculation, none of the isolations from the control vines, or the non-girdled inoculated vines, resulted in bacterial growth on the semi-selective agar plates. This confirms that the vines were not infected with Psa prior to the trial or while they were being held in the greenhouse. Application of Psa to the undamaged trunks of vines did not result in infection.

In all other isolation plates, the bacterial growth was almost completely homogenous, indicating that one type of bacteria was being recovered from the stems. qPCR results from the two isolations on each stem farthest from the girdles were all strongly positive for Psa, confirming the identity of the recovered bacteria.

Despite the vines appearing outwardly healthy, all inoculated girdles were positive for Psa (Figure 10). In all treatments, the bacterium had moved several centimetres from the inoculation site, both upwards from the girdle and downwards. One of the stems from treatment 4 was positive for Psa at 7 cm below the girdle, showing that the bacterium had moved at least 7 cm in 5½ weeks. This isolation was from below the graft.

Figure 10 and Figure 11 (below) show the effect of girdle timing, and the effect of girdle treatments, respectively.

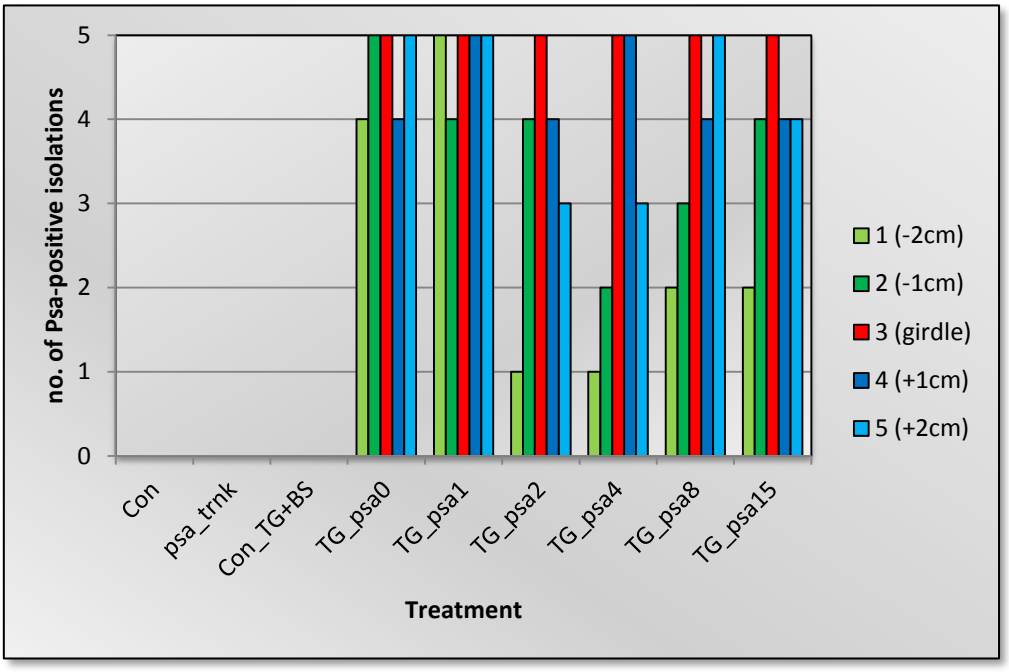

Figure 10. Isolation of *Pseudomonas syringae* pv. *actinidiae* (Psa) .from 'Hort16A' vines 38 days after inoculation. The control vines were neither girdled nor inoculated with Psa. The second set of vines were not girdled but had Psa added directly to the trunk, while the third set were girdled and had bacteriological saline added but there was no Psa in the saline. In the timing series, girdles were inoculated between 0 and 15 days after girdling.

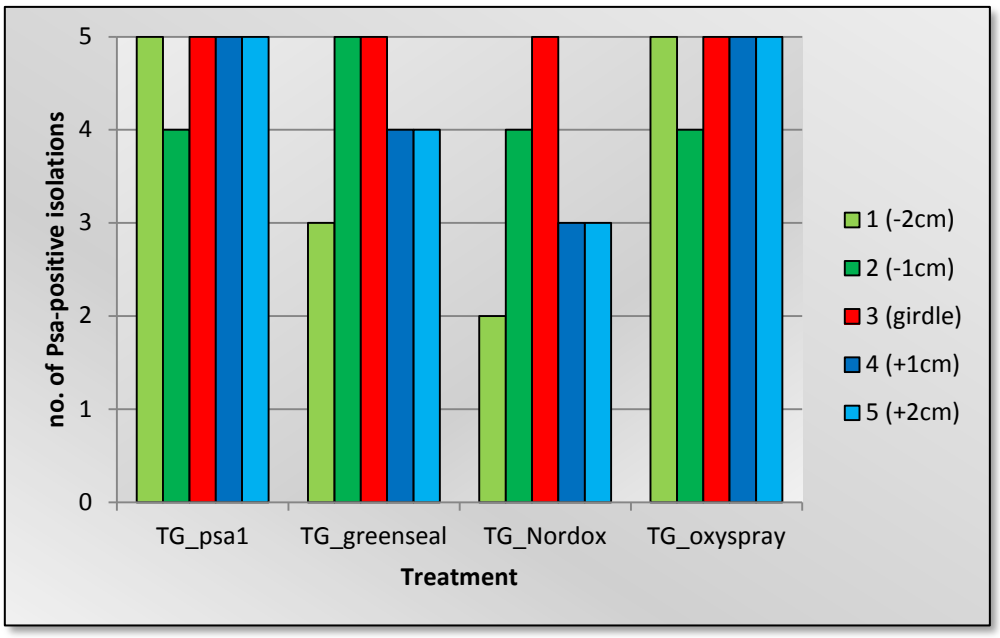

Figure 11. Isolation of *Pseudomonas syringae* pv. *actinidiae* (Psa) from 'Hort16A' vines 38 days after inoculation. All treatments were girdled on day 0 then some girdles were protected with Greenseal™, Nordox® 75 WG or Oxyspray® , while others were left as controls. All vines were inoculated with Psa one day after girdling.

2.2.5 Isolation and identification 80 days after inoculation

Eighty days after inoculation, none of the isolations from the control vines, or the non-girdled inoculated vines, resulted in bacterial growth typical of Psa on the semi-selective agar plates.

The bacterial growth on the isolation plates was almost completely homogeneous (all bacterial colonies morphologically similar), indicating that one type of bacteria was being recovered from the stems. qPCR reactions from representative isolations were all strongly positive for Psa.

Most, but not all, of the girdle-inoculated stems were positive for Psa (Figure 11). In all treatments, the bacterium had moved several centimetres from the inoculation site, in both directions. Sampling of Psa from any tissue is always problematic as although a positive result confirms that the bacteria are present, a negative result may indicate only that the presence of the bacterium through the vine is variable, and that this particular sample may have missed bacteria that are present in the vine. We suspect that the lower number of positive tests in the 80 day sample is due to the 'hit and miss' issues of sampling the bacterial population.

Figure 12 and Figure 13 (below) show the effect of girdle timing, and the effect of girdle treatments, respectively.

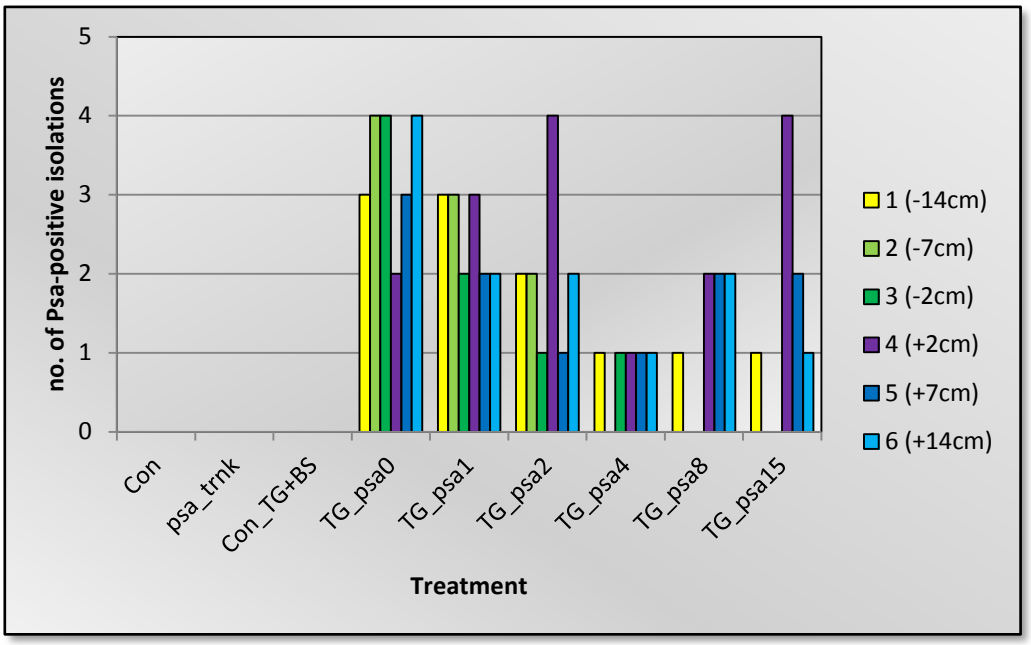

Figure 12. Isolation of *Pseudomonas syringae* pv. *actinidiae* (Psa) from 'Hort16A' vines 80 days after inoculation. The control vines were neither girdled nor inoculated with Psa. The second set of vines were not girdled but had Psa added directly to the trunk, while the third set were girdled and had bacteriological saline added but there was no Psa in the saline. In the timing series, girdles were inoculated between 0 and 15 days after being girdled.

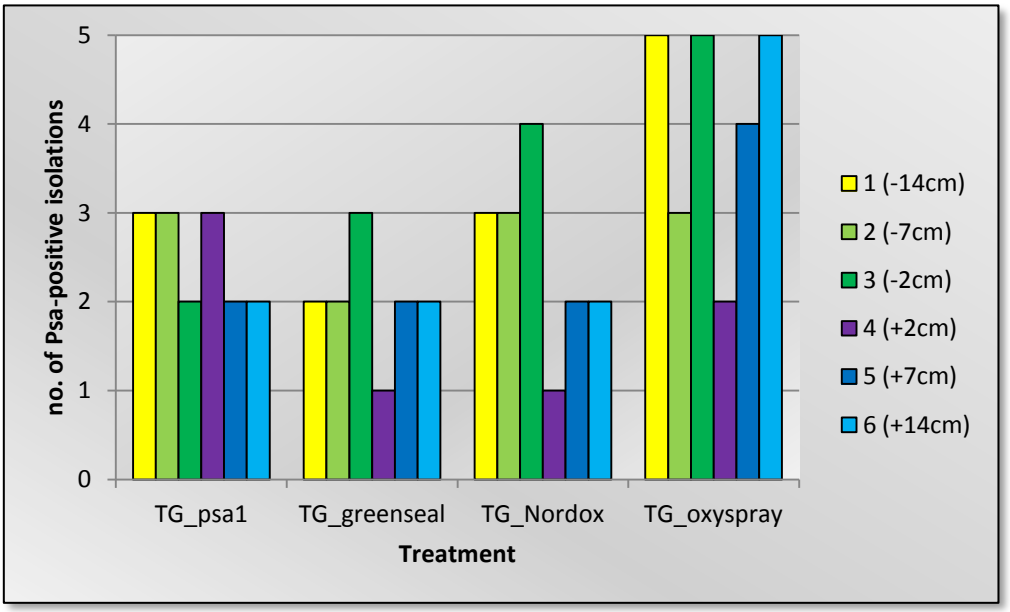

Figure 13. Isolation of *Pseudomonas syringae* pv. *actinidiae* (Psa) from 'Hort16A' vines 80 days after inoculation. All treatments were girdled on day 0 then some girdles were protected with Greenseal™ Ultra, Nordox® 75 WG or Oxyspray® , while others were left as controls. All vines were inoculated with Psa one day after being girdled.

Table 4. Trunk girdling (TG) treatments applied to 'Hort16A' kiwifruit vines in the greenhouse trial and the percentage of trunks that tested positive for *Pseudomonas syringae* pv. *actinidiae* (Psa). Infection data are from a combination of five vines tested 38 days after inoculation, and five more at 80 days after inoculation.

Treatment ID	Description of treatment	Vines infected with Psa-V (%)
Control	Vines were not girdled and Psa was not applied.	0
Psa trunk no TG	Psa was applied to the bark of vines at the point where a girdle would normally be applied.	0
TG + BS	Vines TG and inoculated with bacteriological saline only (no Psa)	0
TG_Psa0	Vines TG and Psa-V applied 5 hours later	100
TG_Psa 1	Vines TG and Psa-V applied 1 day later	90
TG_Psa 2	Vines TG and Psa-V applied 2 days later	100
TG_Psa 4	Vines TG and Psa-V applied 4 days later	60
TG_Psa 8	Vines TG and Psa-V applied 8 days later	90
TG_Psa15	Vines TG and Psa-V applied 15 days later	90
TG_Greenseal	Vines TG and Greenseal™ Ultra applied immediately. Psa-V applied 1 day later	90
TG_Nordox	Vines TG and Nordox® 75 WG applied immediately. Psa-V applied 1 day later	100
TG_Oxyspray	Vines TG and Oxyspray® applied immediately. Psa-V applied 1 day later	100

When the data from the first two sampling times are combined, it is clear that none of the control vines was infected with Psa, but virtually all vines that were girdled and inoculated became infected (Table 4). Only the infection rate at day 4 is lower than 90% and this is probably an anomaly associated with the difficulty in sampling vines reliably for Psa, as there is no indication that infection rates were trending downwards after this date.

Figure 14 shows the results of comprehensive isolations throughout 60-cm lengths of stems from treatment TG_psa0 (inoculated immediately after being girdled). Several of the stems from these vines were positive for Psa at 30 cm below the girdle, and all were Psa-positive 30 cm above the girdle, showing that the bacterium had moved at least 30 cm in 11½ weeks.
A number of the negative samples (e.g. above the girdle in vine 5) may have been due to the 'hit and miss' issues of sampling as the bacterial population moves through the vine, since Psa was present at 5 and 30 cm above the girdle.

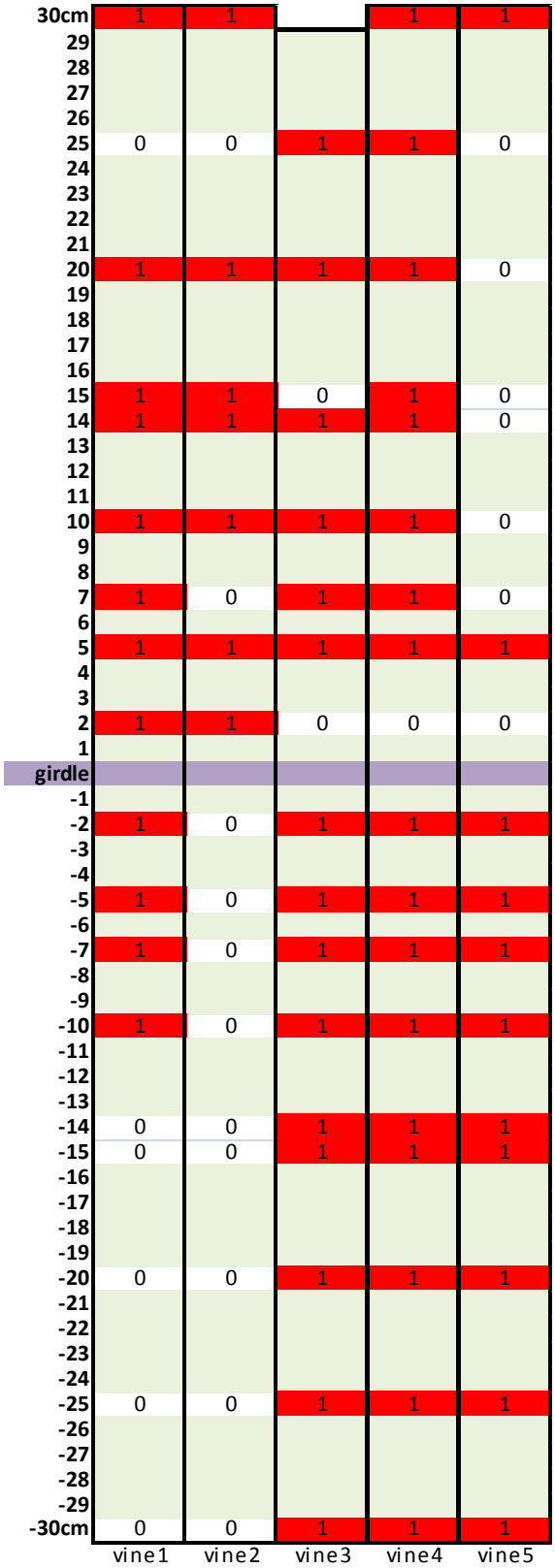

Figure 14. Recovery of *Pseudomonas syringae* pv. *actinidiae* (Psa) from 60-cm stem lengths of five 'Hort16A' kiwifruit vines 80 days after inoculation. All vines were inoculated with Psa five hours after girdling (purple line). 0 = Psa not detected, 1 = Psa-positive.

2.2.6 Isolation and identification 151 days after inoculation

Figure 15 shows the results of comprehensive isolations throughout five entire vines from treatment TG_psa0 (inoculated immediately after being girdled). Samples were taken at 10 cm intervals throughout the vines, 11½ weeks after inoculation. In this sampling set, making isolations from an entire cross-section of the stem compensated for the tendency of the bacterium to 'wind' through the plant.

After 11½ weeks all vines were positive for Psa at the inoculation site (girdling point), and all vines were Psa-positive at least 55 cm above the girdle and 35 cm below the girdle. Vine 5 was Psa-positive at 95 cm above the girdle and three of the vines were positive down to the soil.

From these isolations, it can be seen that Psa moved up and down the trunks at a similar rate, and that movement through the scion ('Hort16A') and the rootstock ('Bruno') were also similar. Psa was not inhibited by the graft.

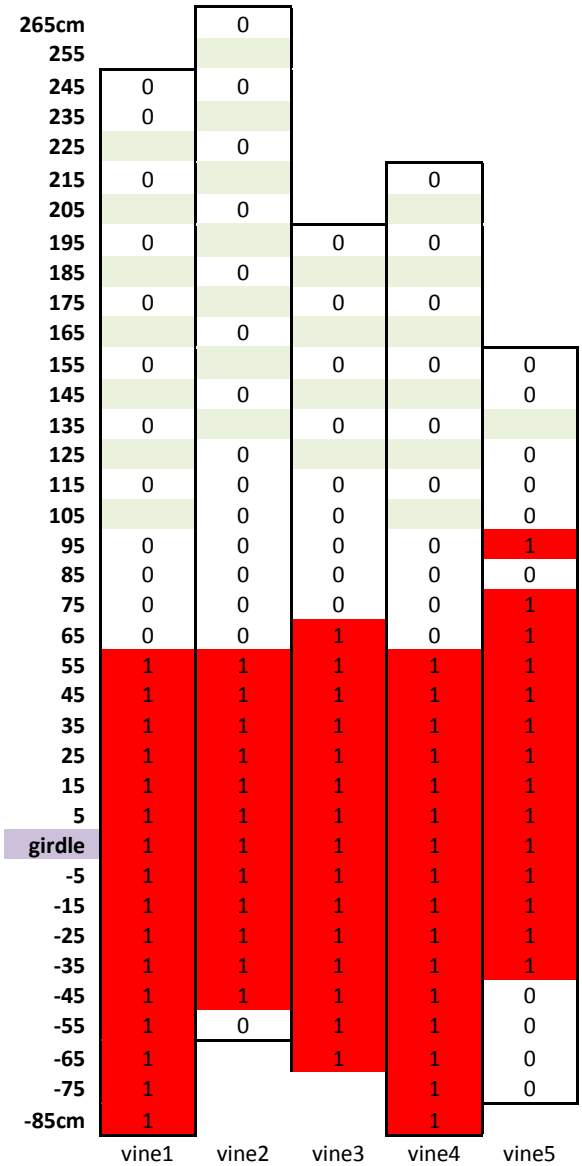

Figure 15. Recovery of *Pseudomonas syringae* pv. *actinidiae* (Psa) from five entire 'Hort16A' kiwifruit vines 151 days after inoculation. All vines were inoculated with Psa five hours after girdling. 0 = Psa not detected, 1 = Psa-positive.

3 Field trial

3.1 Background

The greenhouse trial showed clearly that girdles can become infected with Psa when a high inoculum load is applied, and none of the protectants we used reduced the rate of infection. Since we had not at that time observed major problems of girdles developing symptoms on commercial properties, we wondered if girdles might be less prone to infection under the natural inoculum loads we might expect to find on commercial orchards. To test this theory, we obtained more potted, clean 'Hort16A' vines and these were transplanted into a kiwifruit block at Te Puke Research Orchard during November 2011. The other vines in the block were mature 'Hort16A' vines that were progressively being removed as they developed Psa infection, so we expected our vines to be exposed to a high natural inoculum load.

Vines were planted at Te Puke on two dates (Table 5), and on each occasion some vines were left as ungirdled controls and some vines were trunk girdled with Vaca girdling pliers, then protected with a spray of Nordox 75 WG (1.1 kg Nordox/1000 L water). Girdles were imposed on the 'Bruno' rootstock, as the 'Hort16A' scions were sometimes too small to girdle (Figure 16). At the second planting, we also protected some girdles by painting on Eurogel (Eurogel is a fungicide for the postharvest control of European Canker on pipfruit and contains octhilinone).

All vines subjected to natural inoculation only.

Table 5. Summary of treatments applied to 'Hort16A' kiwifruit vines in the field trial at Te Puke Research Orchard in 2011. Each treatment was replicated on 12 vines. Vines were not inoculated with *Pseudomonas syringae* pv. *actinidiae* (Psa), but they were planted near mature 'Hort16A' vines that were already infected with Psa.

Planted at Te Puke	Girdled	Treatment	Treatment ID
16 November	n/a	Controls , not girdled	Control $_{16\ Nov}$
	22 Nov.	Girdled then protected with Nordox® 75 WG	Girdle $_{16\ Nov}$
24 November	n/a	Controls , not girdled	Control $_{24\ Nov}$
	8 Dec.	Girdled and left unprotected	Girdle $_{24\ Nov}$
	8 Dec.	Girdled then protected with Nordox® 75 WG	Girdle+Nordox® $_{24\ Nov}$
	8 Dec.	Girdled then protected with Eurogel	Girdle+Eurogel $_{24\ Nov}$

Figure 16. (a) The 'Hort16A' kiwifruit vines were about 2 m high and transplanted well; (b) Girdles were applied with Vaca girdling pliers; (c) some girdles were protected with Nordox® 75 WG or Eurogel.

3.2 Vine assessments

Vines were assessed visually for Psa using the simple Plant & Food Research scoring system during December and January:

0 no symptoms
1 0-33% of leaves have spots
2 34-66% of leaves have spots
3 67-100% of leaves have spots.

In addition, the callusing on each girdle was assessed using the same 0-3 scoring system used in the greenhouse trial:

0 - no healing
1 - callus obvious
2 - callus bridges girdle in places
3 - completely callused.

Infection of all vines progressed rapidly so that by mid-January shoot die-back was evident on many vines. On 23 January we destructively harvested five control vines and five trunk-girdled vines from the set planted on 24 November. These were taken to the Mt Albert Research Centre laboratory and bacterial isolates were made every 10 cm along each vine, from the base of the vine to the top of the first cane.

By 8 February it was clear that infection in all vines was so widespread it was impossible to tell if vines had been infected through the girdle, or through leaves and pruning cuts in the upper canopy. This made it impracticable to take further bacterial isolations from vines. Instead we carried out a final field assessment using the visual scores shown in Figure 17.

Figure 17. By 8 February (76 or 84 days after being planted at Te Puke) secondary symptoms had developed further and vine health was scored on a scale of 0 to 5, with 0 being no shoot die-back, and 5 being a vine with no uninfected shoots.

3.3 Results

3.3.1 Meteorological data

Data were obtained from the standard meteorological station on the orchard, which is about 250 m from where the trial vines were planted. Temperatures in the month after girdling (December 2011) averaged 16.6ºC (Figure 18). This is similar to the average temperature in the greenhouse for the month after vines were girdled (November 2011, Table 6).

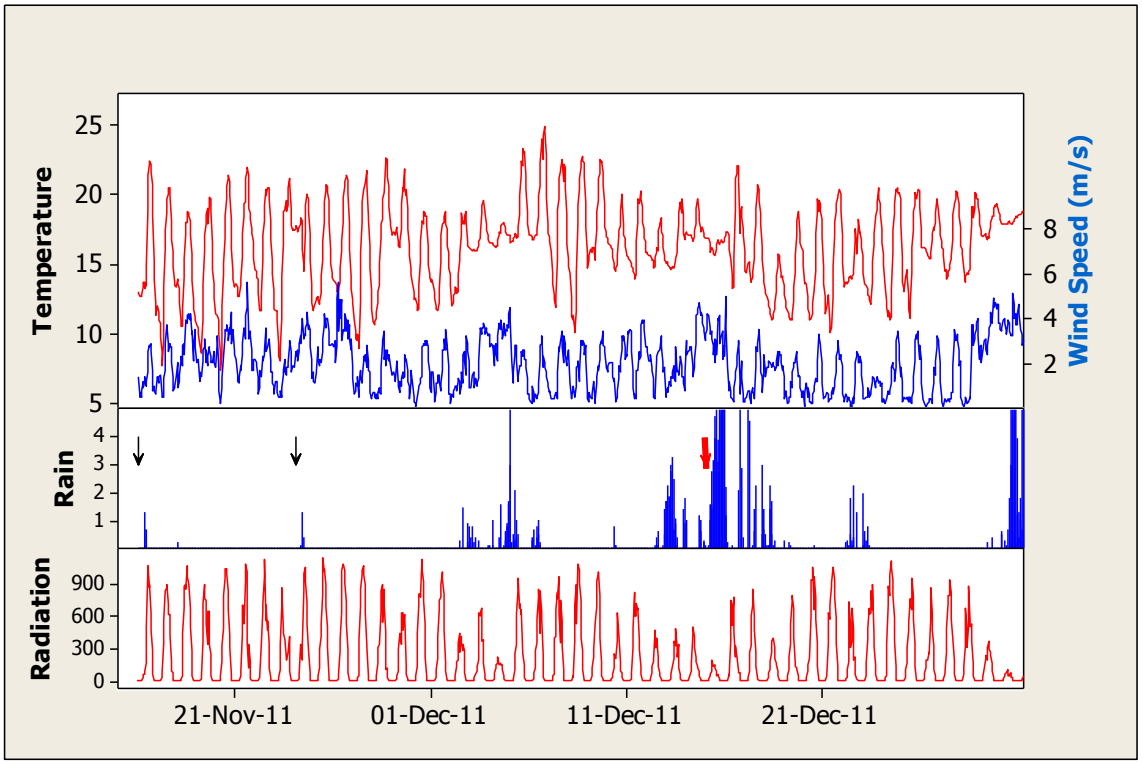

Figure 18. Temperature, windspeed, rainfall, and solar radiation at Te Puke Research Orchard. The black arrows indicate when kiwifruit vines were transplanted to Te Puke, and the red arrow indicates when leaf spotting was first scored.

Table 6. Mean air temperatures at Te Puke Research Station meteorological site during the 'Hort16A' field trial (November 2011 – January 2012).

Month	Average temperature (^{o}C)
November	15.7
December	16.6
January	18.3

3.3.2 Callus formation

Vines in the field, girdled on either 22 November or 8 December, healed quickly and at similar rates. After about 23 days the average callusing score was 1, indicating all vines had started callusing, and by 33 days the average score was 2, indicating that the callus had bridged the girdle at some points (Figure 19).

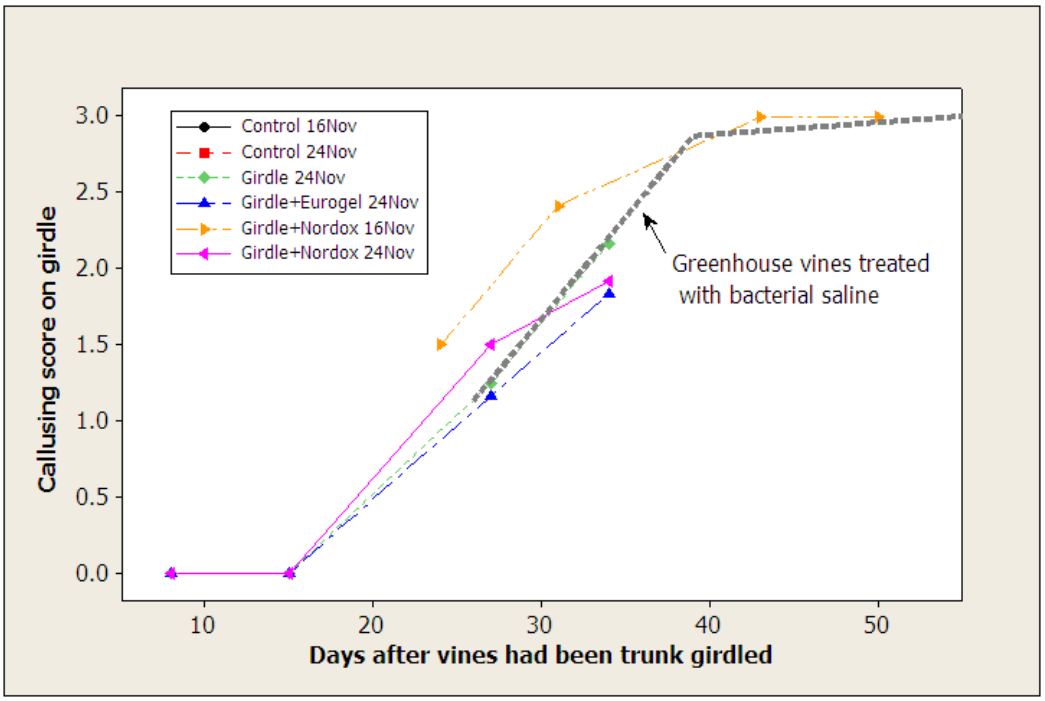

Figure 19. Rate of callusing in trunk girdles on 'Hort16A' kiwifruit vines. Note that for the field-grown vines the girdle was made on the 'Bruno' rootstock, while in the greenhouse girdles were applied to the 'Hort16A' scion.

Girdles treated with either Nordox 75 WG or Eurogel healed well and at a similar rate to the untreated girdles. However, we did observe that the Eurogel-treated girdles looked different, with less flaring of the stem above the girdle.

In the greenhouse trial we found that girdles that had not been inoculated with Psa (i.e. the bacteriological saline treatment) healed much more quickly than vines that had been inoculated with Psa, especially if they were inoculated shortly after vines were girdled. When we overlay the rate of healing observed in the greenhouse trial, we see that the rate of callusing is similar to that observed in the field. The average temperatures in the greenhouse (Table 3) and in the field (Table 6) were similar, at about 16.6ºC.

We noted in the greenhouse trial that inoculating girdles with Psa seemed to reduce callusing, so the rapid healing seen in the field trial may suggest that the girdles were not heavily infected soon after being girdled. However, it should be noted that in the greenhouse trial the 'Hort16A' scion was girdled, while in the field trial the 'Bruno' rootstock was girdled.

3.3.3 Psa infection of leaves from natural inoculum

At the first leaf assessment on 16 December, the vines planted out on 16 or 24 November all showed similar amounts of leaf spots (Figure 20). It is thought that infection for both sets of

vines may have been linked to the heavy rainfall between 2 and 6 December (39 mm; Figure 18).

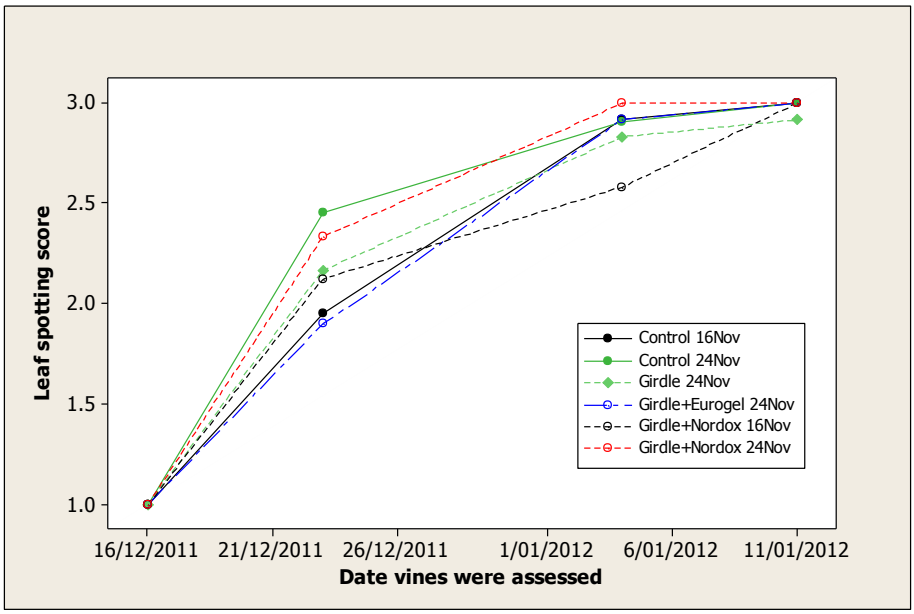

Figure 20. Development of leaf spotting after 'Hort16A' kiwifruit vines were planted out at Te Puke. Despite the difference of 10 days in planting dates, it is thought that all vines were infected over the same period, probably during the 38 mm of rain that fell between 2-6 December.

Approximately 80 days after being planted at Te Puke, virtually all vines were displaying secondary symptoms. Vine health was scored on a scale of 0 to 5, with 0 being no shoot die-back, and 5 being a vine with every shoot infected (Figure 17).

Although all vines were severely infected by this stage, vines that had been trunk girdled were clearly in worse condition than control vines (Figure 21). The protectants applied to girdles did not reduce disease symptoms.

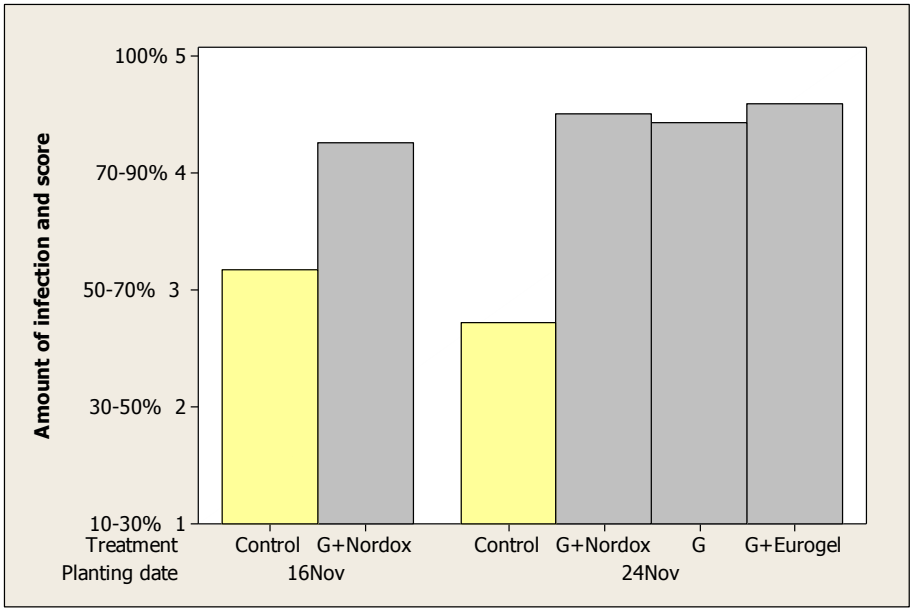

Figure 21. Infection scores for 'Hort16A' kiwifruit vines on 8 February 2012, when vines had been in the block for 76-84 days. Vines were subjected to natural infection of *Pseudomonas syringae* pv. *actinidiae* (Psa) from surrounding vines. One standard orchard protectant spray was applied to these vines in early December.

The bar graph clearly shows that although all vines had extensive secondary symptoms, the vines that had not been girdled did have lower scores. Non-parametric analysis of the scores (Kruskin-Wallis) confirmed that that both groups of control vines had significantly less infection than any of the girdled treatments (P=0.02).

3.3.4 Isolation and identification of Psa

The results of qPCR analysis of five non-girdled control vines and five trunk-girdled vines are presented in Figure 22 and Figure 23.

Trunk girdles were applied about 0.4 m above ground level, and when we summarised all trunk samples in the first 0.6 m of trunk, we found that on trunk-girdled vines 93% of the samples were strongly positive for Psa, while in non-girdled control vines only 30% of the samples were strongly positive (Table 7). This suggests that a large proportion of the infection in the lower part of the vine had entered via the girdle. However, because some of the trunks on control vines were also infected, the data also suggest that some systemic infection in the trunks originated from leaf or shoot infections and had moved down into the trunk.

Table 7. *Pseudomonas syringae* pv. *actinidiae* (Psa) scores for the bottom 60 cm of control vines and girdled kiwifruit vines sampled on 23 January 2012, 53 days after vines were planted at Te Puke. Five vines from each treatment were sampled.

	Psa	1	2	3	4	5	% all
Control plants	**+ve**	0	2	1	3	3	30%
	weak +ve	2	2	4	2	3	43%
	-ve	4	2	1	1	0	27%
Girdled plants	**+ve**	6	6	5	6	5	93%
	weak +ve	0	0	1	0	1	7%
	-ve	0	0	0	0	0	0%

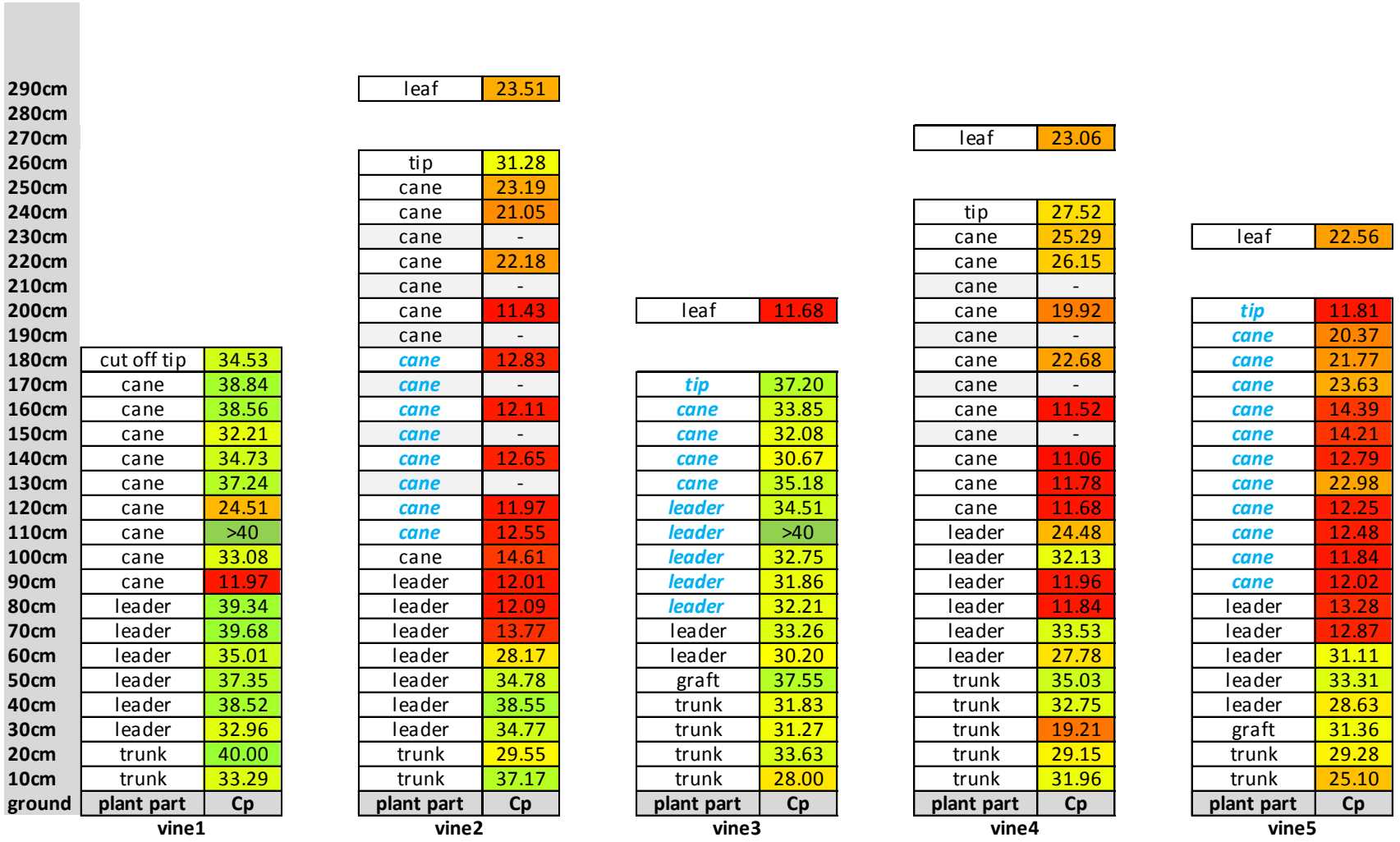

Figure 22.qPCR results from five non-girdled control 'Hort16A' field kiwifruit vines. In this study, a Cp (crossing point or threshold value) value below 30 was interpreted as a *Pseudomonas syringae* pv. *actinidiae* (Psa)-positive result and a Cp value above 35 as a negative result.

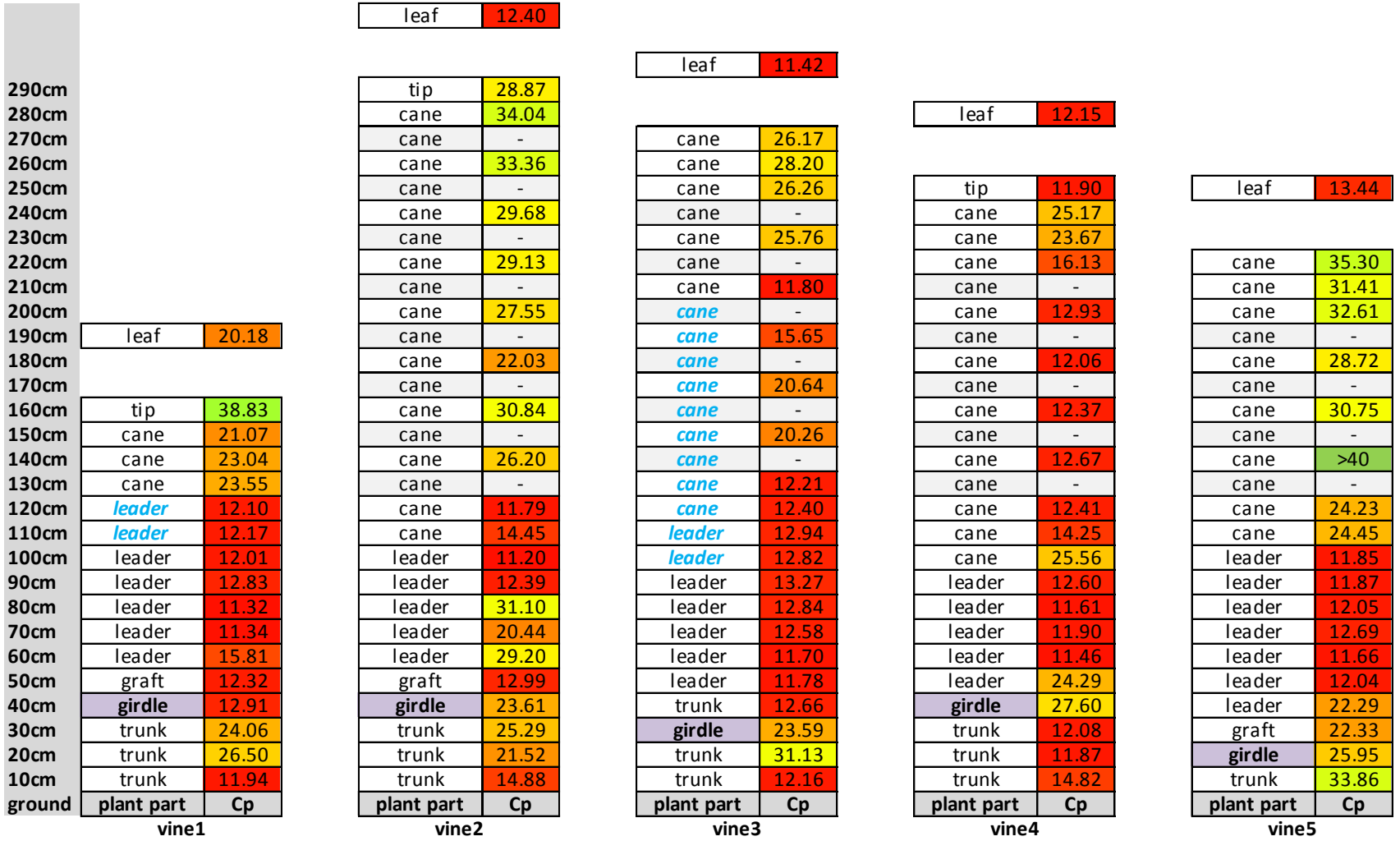

Figure 23.qPCR results from five girdled 'Hort16A' field kiwifruit vines. In this study, a Cp (crossing point or threshold value) value below 30 was interpreted as a *Pseudomonas syringae* pv. *actinidiae* (Psa)-positive result and a Cp value above 35 as a negative result.

4 Summary

4.1 Rapidity of disease development

The greenhouse trial, where potted 'Hort16A' vines were inoculated with Psa-V, demonstrated that trunk girdles are infection sites for Psa. This trial produced a particularly clear result because there was only one point of infection. In vines inoculated on the day of girdling, Psa had moved up to 7 cm from the girdle in 5½ weeks, at least 30 cm in 2½ months and up to 95 cm in 5 months.

However, despite the vines being inoculated with a high concentration of bacteria (10^9 cfu/mL), it was most noticeable that the development of visible disease symptoms in the vines took several weeks. Up to the time we took the first tissue sample, we were unsure that we had managed to infect the vines. Even by the end of the trial (after 151 days), there were a relatively small number of vines exhibiting severe symptoms such as cane die-back or bacterial ooze. This relatively slow disease development may have been due to:

- The high maximum temperatures in the greenhouse, although the average temperatures were within the optimum range for this pathovar
- Infection at only one site on each vine

In other trials, we have observed that ***expression*** of disease above a cane-girdle, where carbohydrates are expected to be more plentiful, is far more evident than below the girdle. This occurs even though our samples show that Psa was present both above and below the girdle. Plant & Food Research are currently analysing how carbohydrates above and below girdles change with time after girdling.

The 'Hort16A' vines in the field trial succumbed to disease far more rapidly than vines inoculated in the greenhouse. This may have been because of inoculum entering via many infection sites on leaves and shoots, or because of multiple ongoing inoculations from the environment. However, although we did not observe any vine stress, these vines were transplanted at the start of the trial and this may have weakened them.

4.2 Vine size

Both of the trials in this project used young potted vines. It is possible that these vines are more susceptible to infection since they have fewer carbohydrate resources than a mature vine. However, this possibility is entirely speculative, since we do not have any evidence that carbohydrate resources affect bacterial infection. Ideally, we need to understand how Psa infection affects mature cropping vines in a commercial situation. It is currently impossible to do this, since we need to start with vines that we know are not infected, and then inoculate them with Psa. The only way of ensuring vines are uninfected would be to move to a region without Psa. However, it would be unacceptable to infect vines in such a region.

4.3 Future work

4.3.1 Infection sites

The current project has shown that girdles are potential infection sites for Psa. Plant & Food Research are undertaking similar work on other pruning cuts and plant wounds to see if they are all equally likely to act as infection sites for Psa. We need to understand the relative importance of all infection sites before we can make sensible decisions about how we manage vines to minimise infection.

4.3.2 Cultivars

The current work was all carried out on 'Hort16A', which we know is highly susceptible to Psa. For the future, we need to understand how cultivars that are thought to be more tolerant, such as 'Hayward' and 'ZESY002', respond to Psa infection via wounds. With these cultivars, the issue of how vines become infected may be less important than how the vines cope with this infection. Trials with less susceptible cultivars may need to be much longer term than the work described in this report.

5 References

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Serizawa S, Ichikawa T 1993. Epidemiology of bacterial canker of kiwifruit. 4. Optimum temperature for disease development on new canes. Annals of the Phytopathological Society of Japan 59(6): 694-701.

Spotts RA, Wallis KM, Serdani M, Azarenko AN 2010. Bacterial Canker of Sweet Cherry in Oregon-Infection of Horticultural and Natural Wounds, and Resistance of Cultivar and Rootstock Combinations. Plant Disease 94(3): 345-350.

6 Appendix. Stem samples 80 days after inoculation

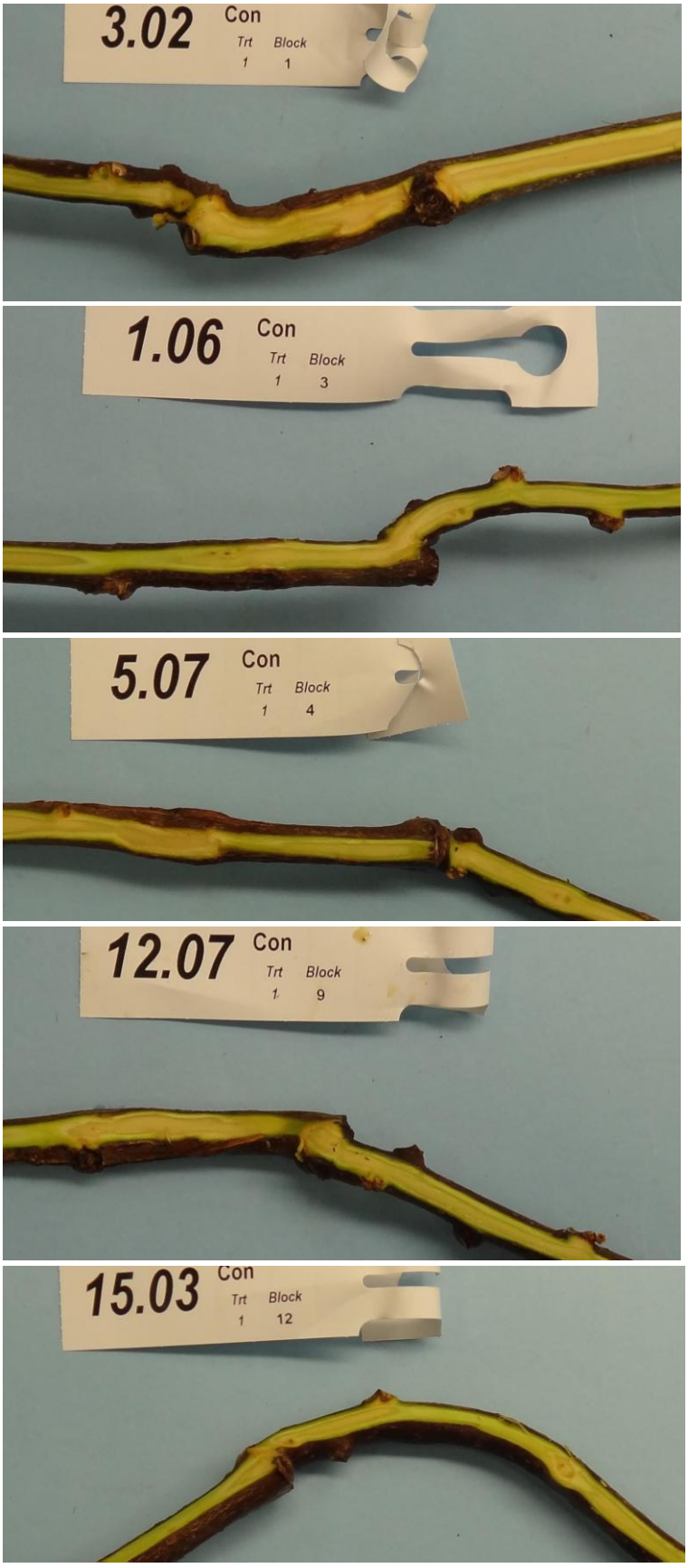

Figure A1. Stem sections from treatment 1 (Vines were not girdled and Psa was not applied). Vines were destructively sampled on 12 December 2011, 80 days after inoculation

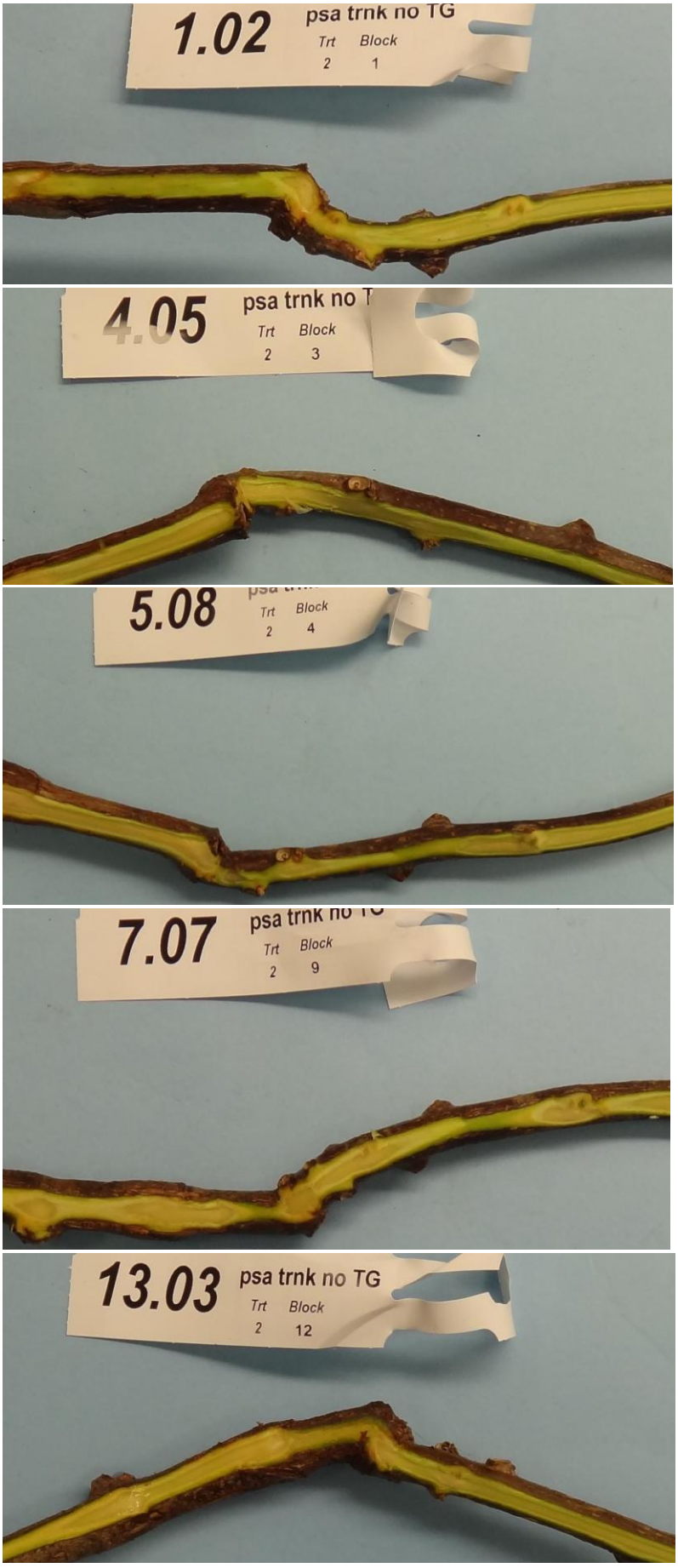

Figure A2. Stem sections from treatment 2 (Psa was applied to the bark of vines at the point where a girdle would normally be applied). Vines were destructively sampled on 12 December 2011, 80 days after inoculation.

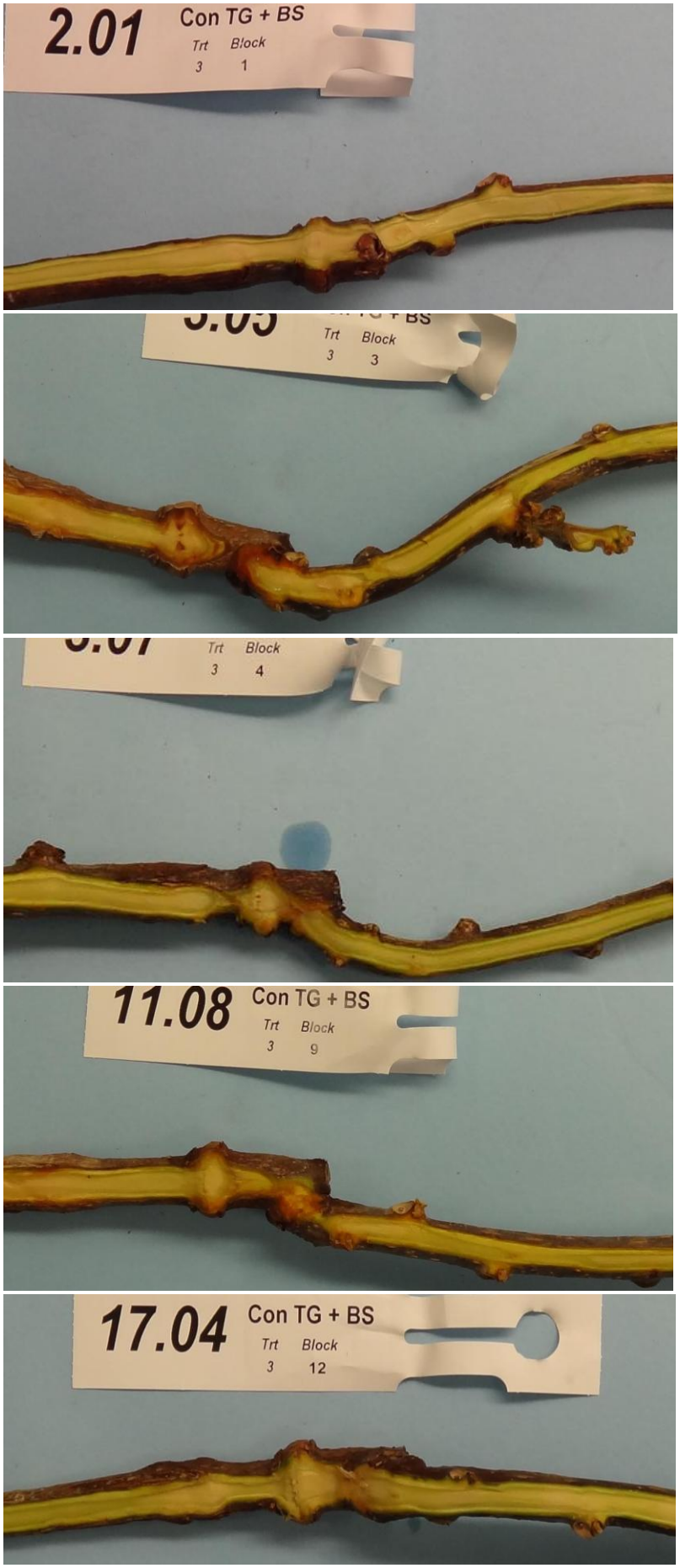

Figure A3. Stem sections from treatment 3 (Vines TG on 22 Sept. 2011 and inoculated with bacterial saline which did NOT contain Psa). Vines were destructively sampled on 12 December, 80 days after inoculation.

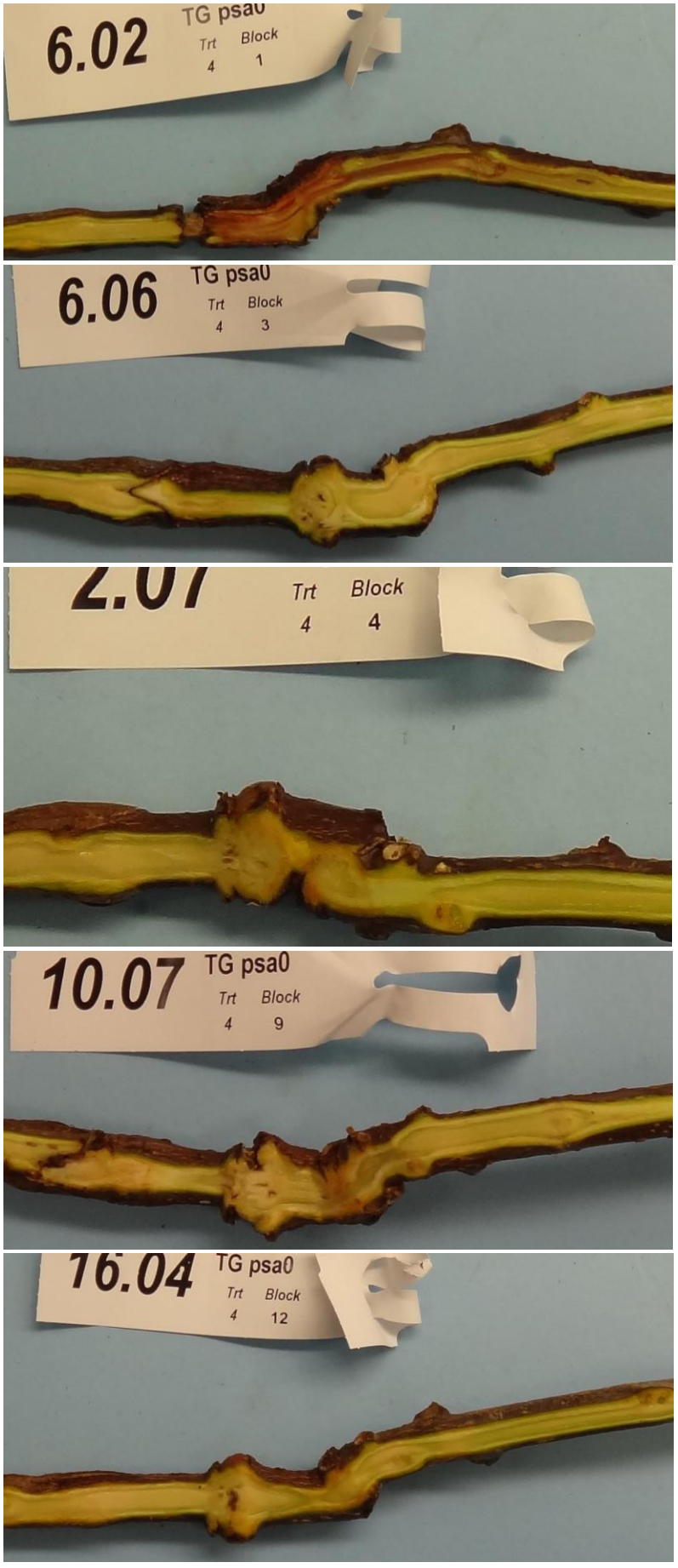

Figure A4. Stem sections from treatment 4 (Vines TG on 22 Sept. 2011 and Psa- V applied 1 day later). Vines were destructively sampled on 12 December, 80 days after inoculation.

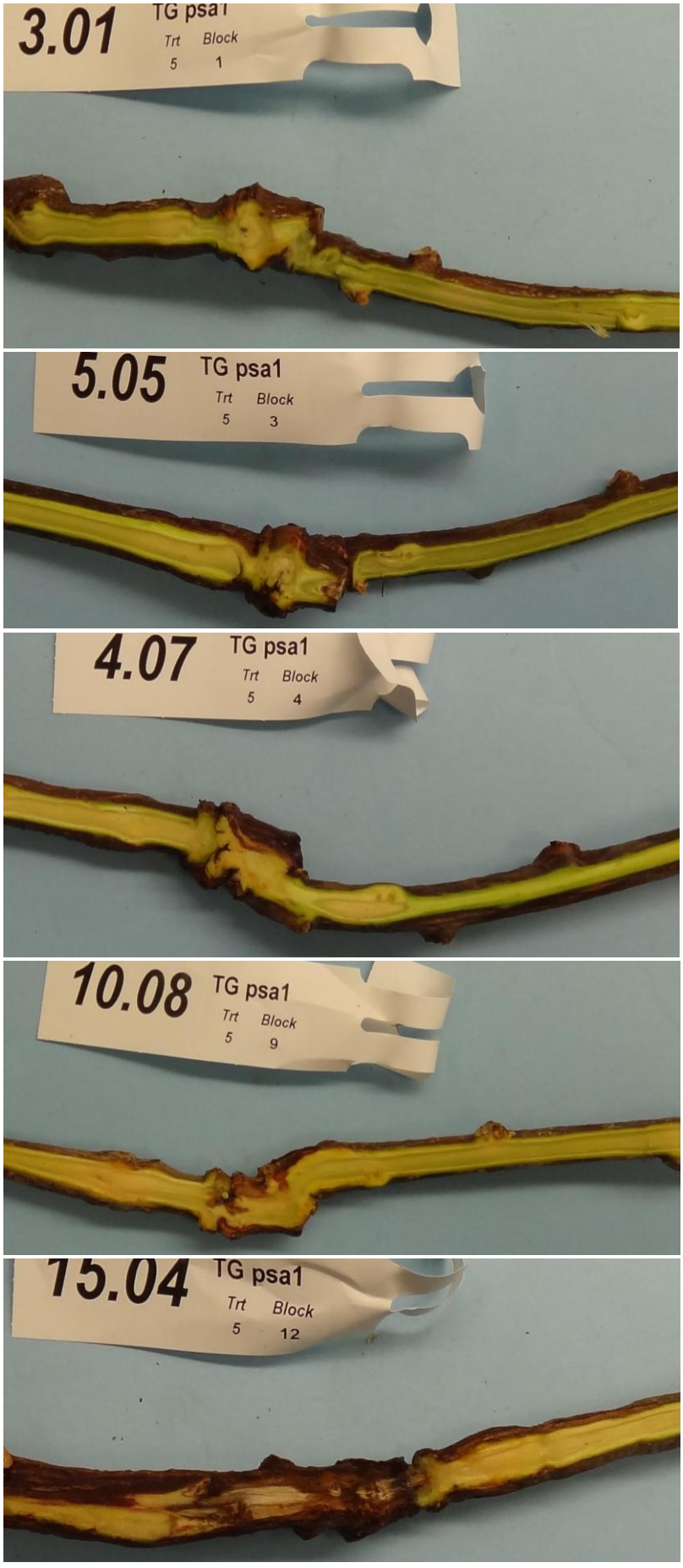

Figure A5 Stem sections from treatment 5 (Vines TG on 21 Sept. 2011 and Psa- V applied 2 days later). Vines were destructively sampled on 12 December, 80 days after inoculation.

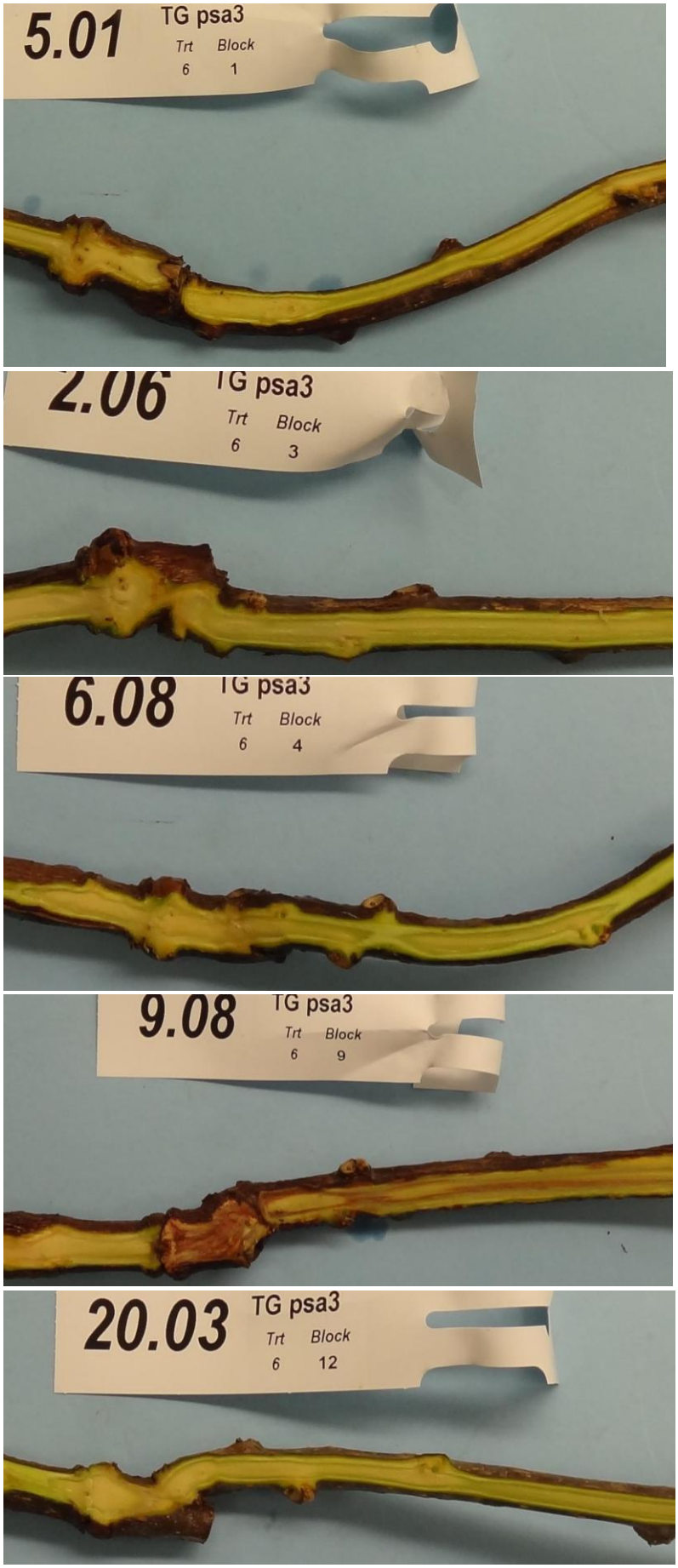

Figure A6. Stem sections from treatment 6 (Vines TG on 19 Sept. 2011 and Psa- V applied 4 days later) . Vines were destructively sampled on 12 December, 80 days after inoculation.

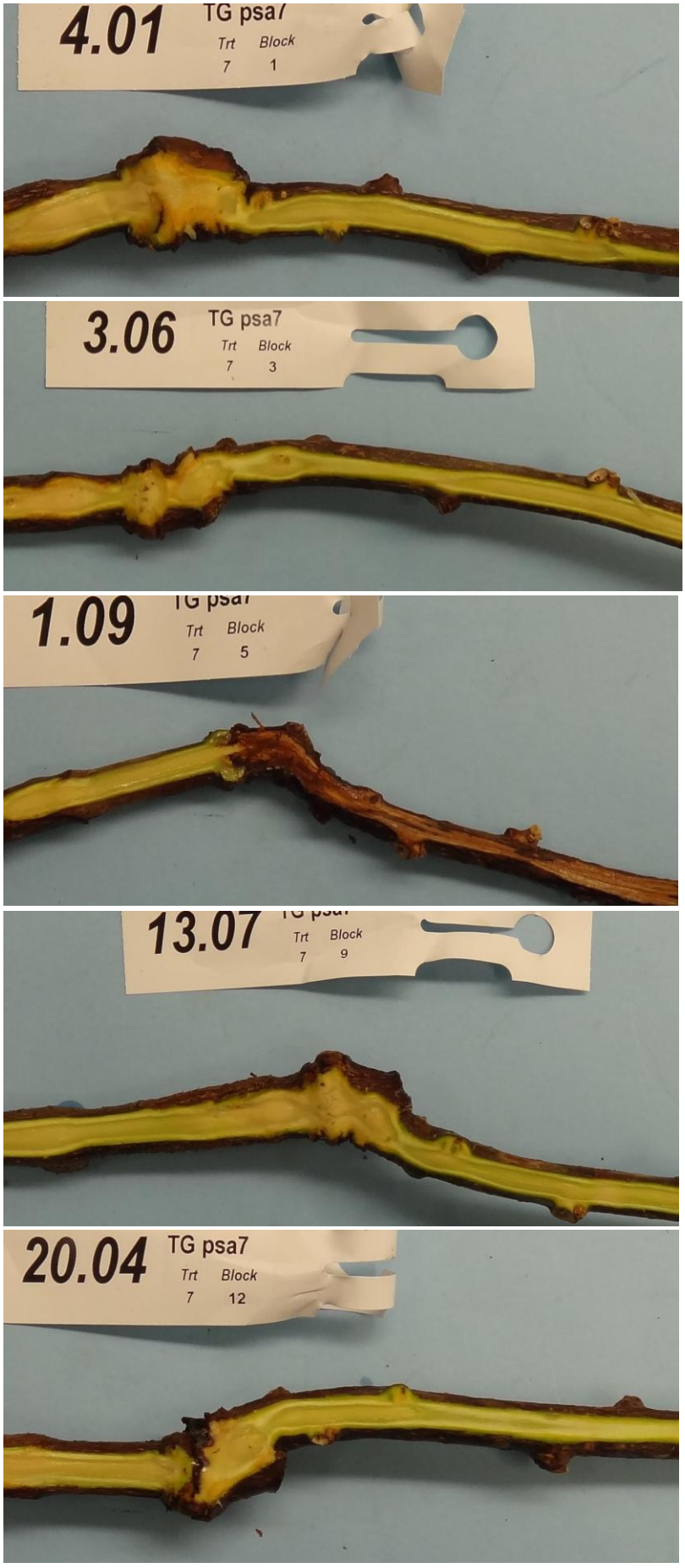

Figure A7. Stem sections from treatment 7 (Vines TG on 15 Sept. 2011 and Psa- V applied 8 days later). Vines were destructively sampled on 12 December, 80 days after inoculation.

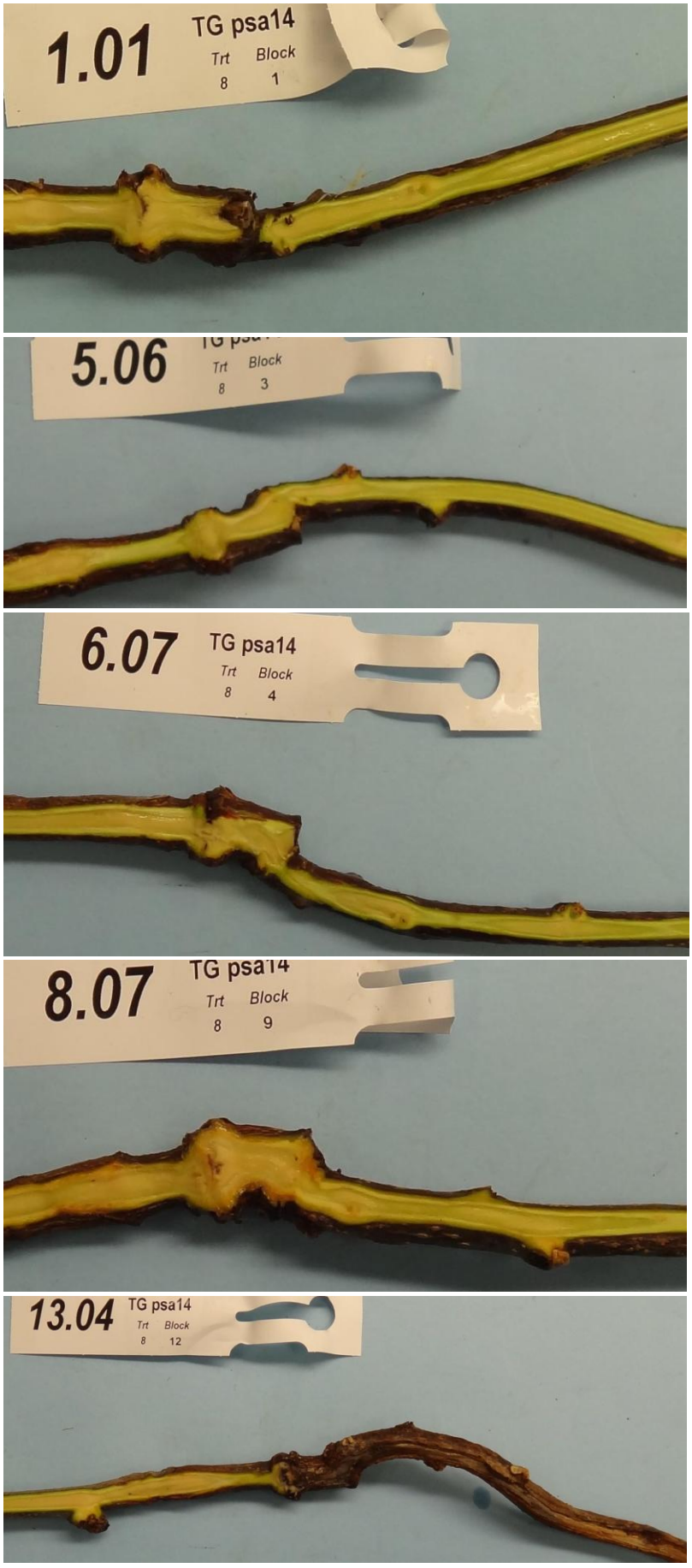

Figure A8. Stem sections from treatment 8 (Vines TG on 8 Sept. 2011 and Psa- V applied 15 day later). Vines were destructively sampled on 12 December, 80 days after inoculation.

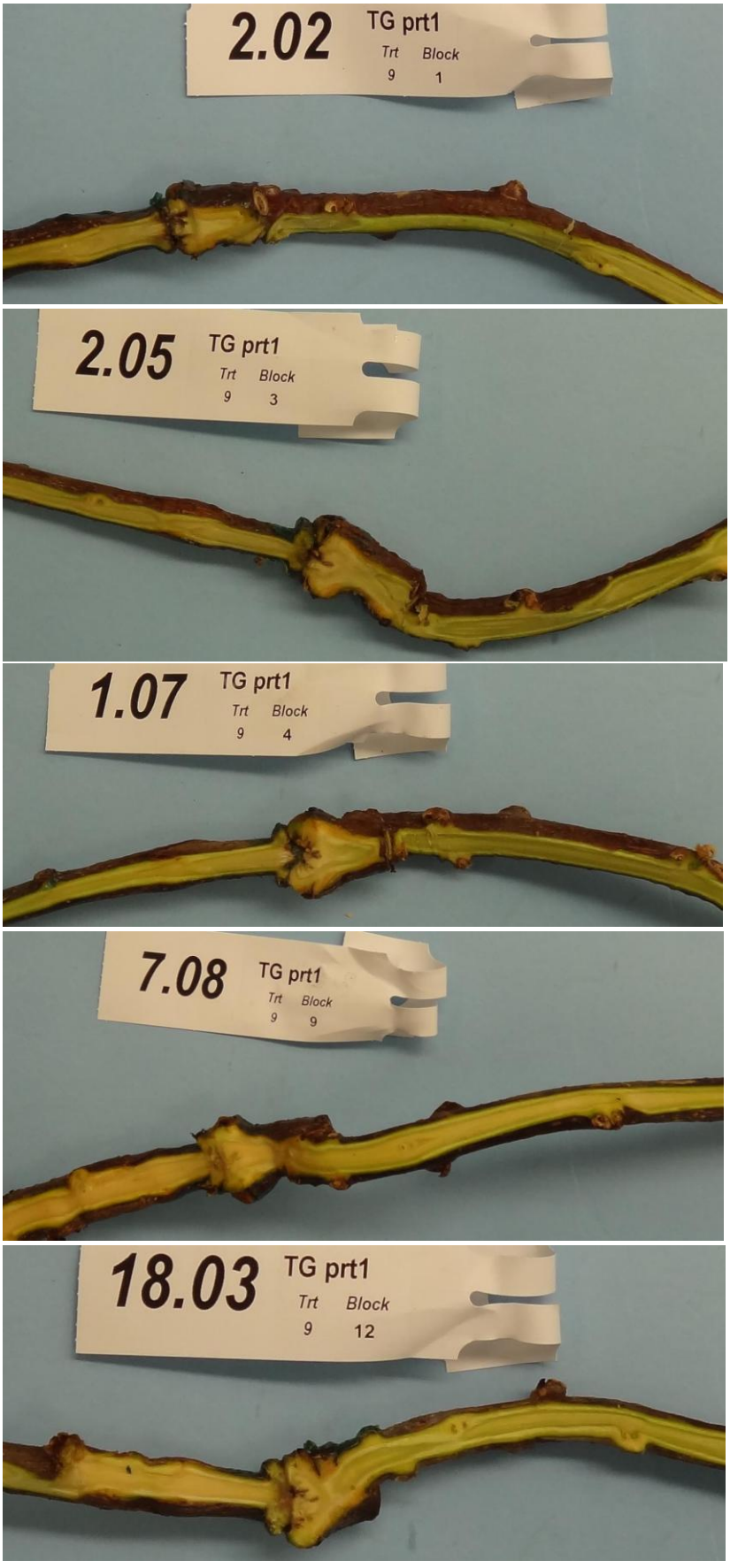

Figure A9. Stem sections from treatment 9 (Vines TG on 22 Sept. 2011, Greenseal™ Ultra applied immediately and Psa- V applied 1 day later). Vines were destructively sampled on 12 December, 80 days after inoculation.

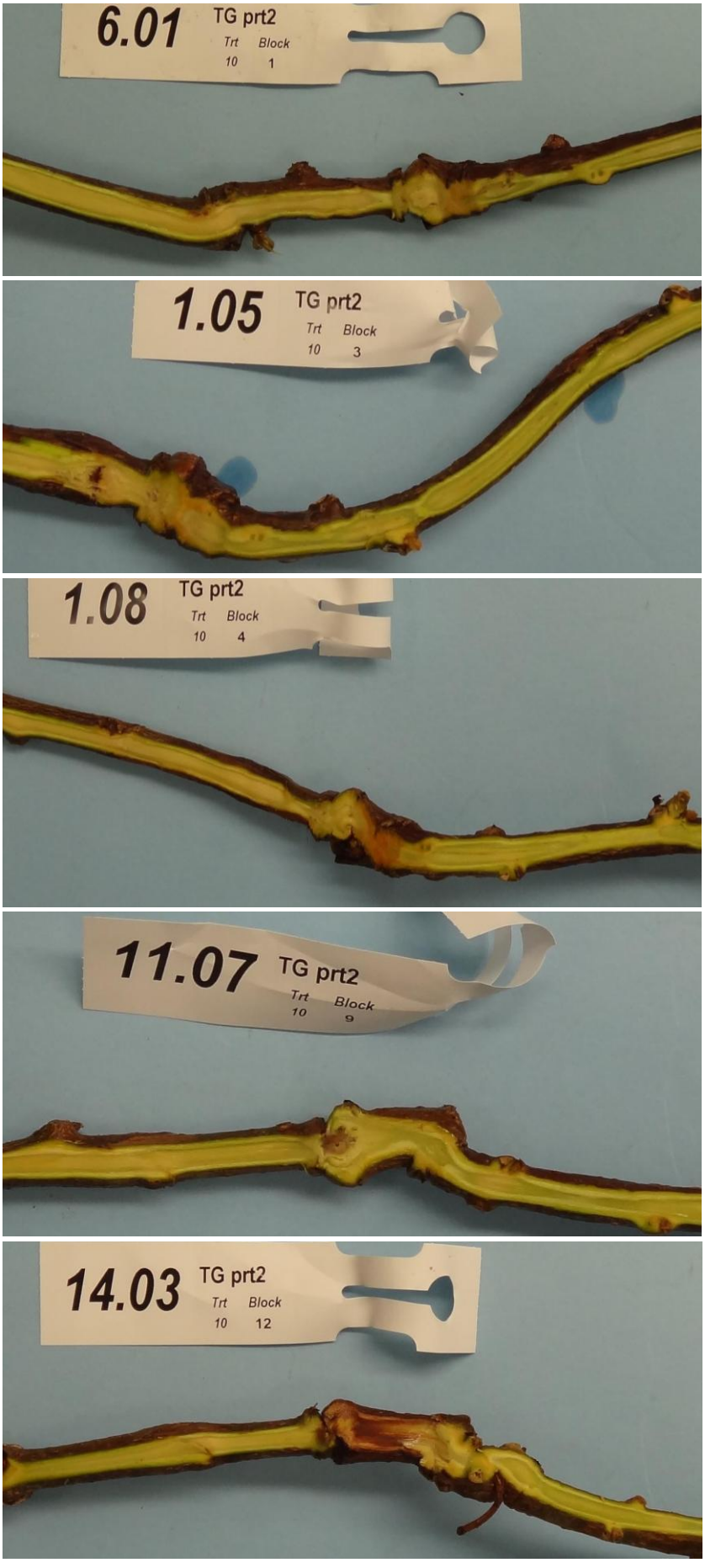

Figure A10. Stem sections from treatment 10 (Vines TG on 22 Sept. 2011, Nordox® 75 WG applied immediately and Psa- V applied 1 day later). Vines were destructively sampled on 12 December, 80 days after inoculation.

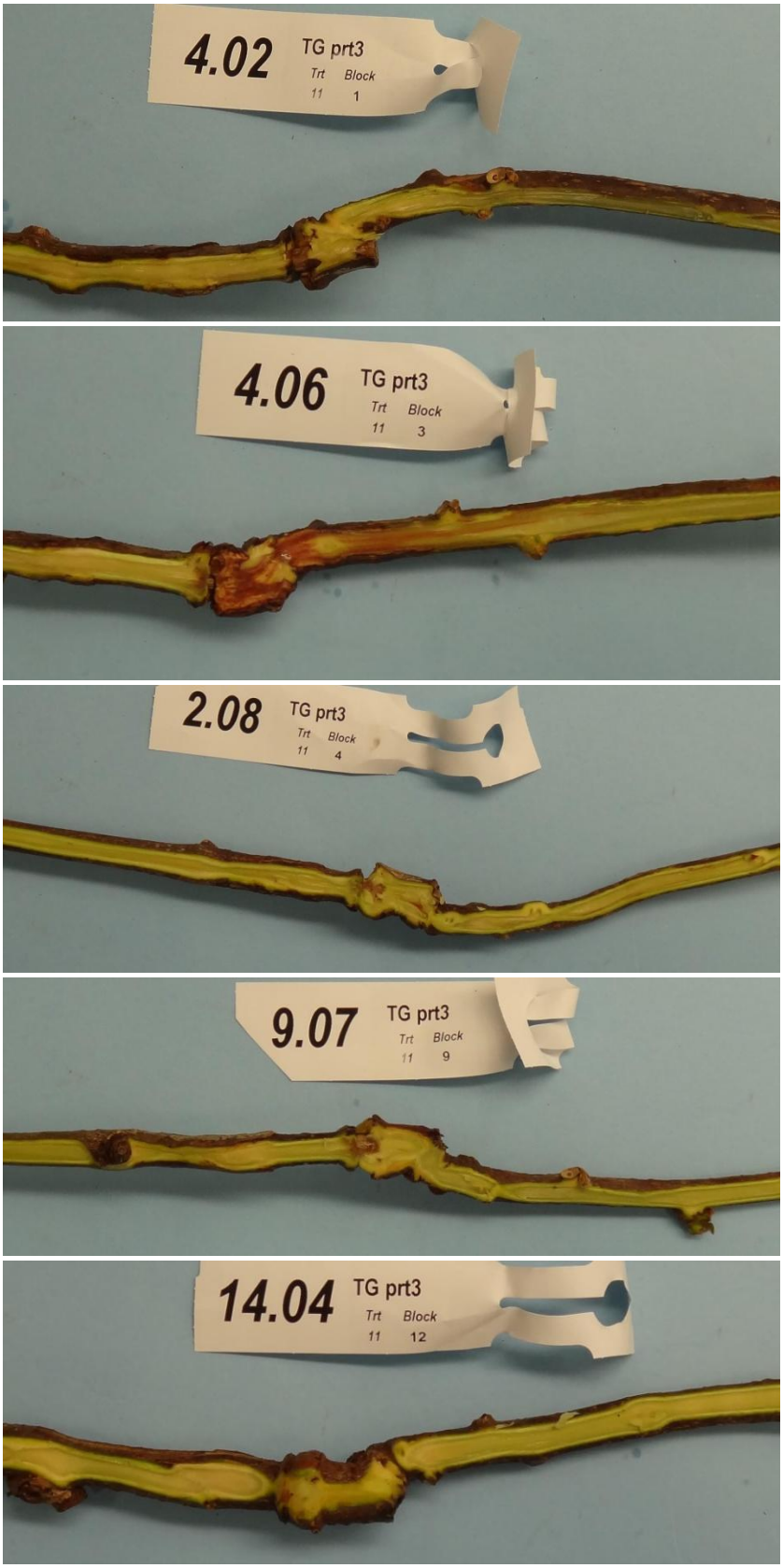

Figure A11 Stem sections from treatment 11 (Vines TG on 22 Sept. 2011, Oxyspray® applied immediately and Psa- V applied 1 day later). Vines were destructively sampled on 12 December, 80 days after inoculation.

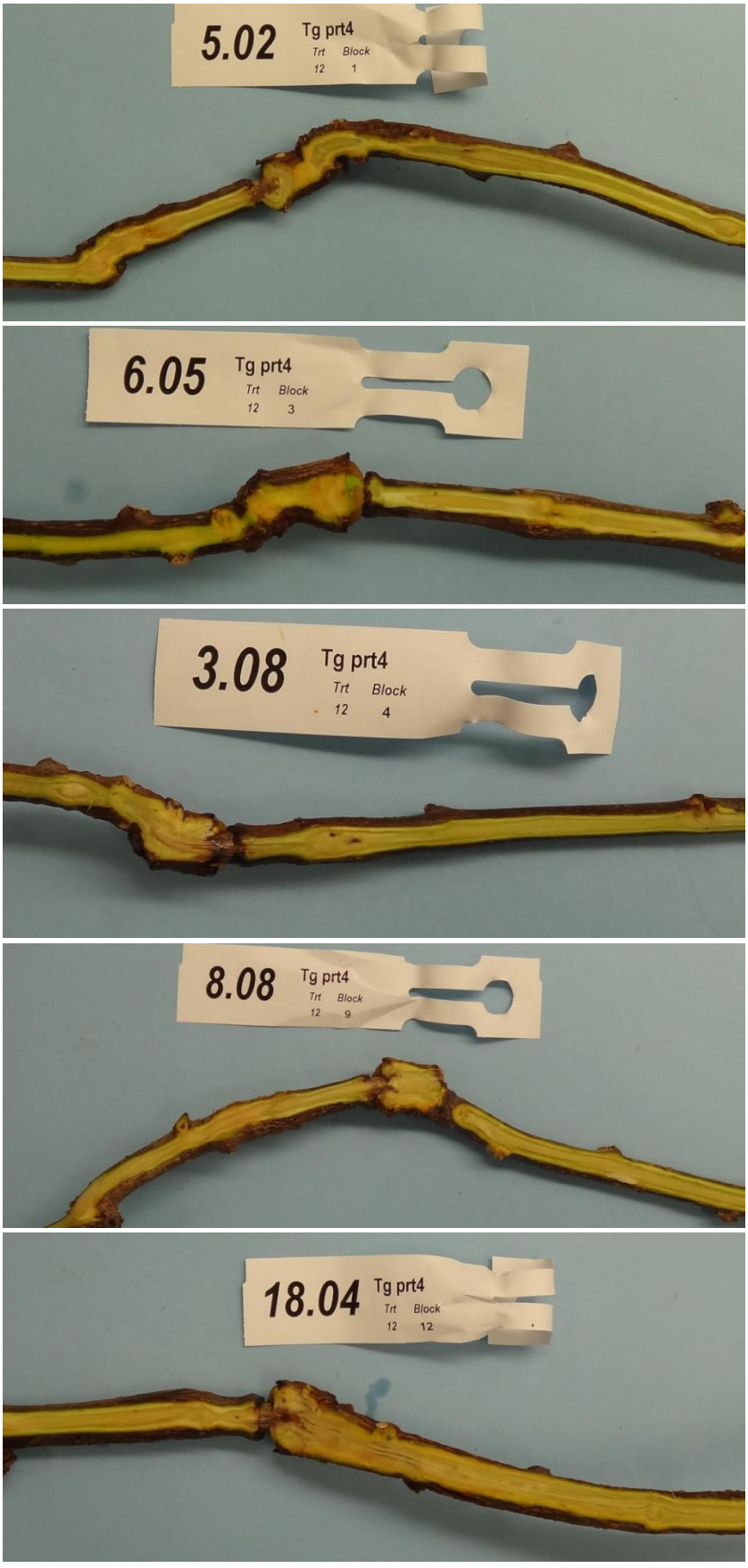

Figure A12. Stem sections from treatment 12 (Vines TG on 23 Sept. 2011 and Psa- V applied 5 hours later). Vines were destructively sampled on 12 December, 80 days after inoculation.