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BS1605 Evaluating RT-PCR tests *for Pelargonium zonate spot* virus (PZSV) in freeze-dried leaf samples

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EXECUTIVE SUMMARY

BS1605 Evaluating RT-PCR tests *for Pelargonium zonate spot* virus (PZSV) in freeze-dried leaf samples

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Two published reverse-transcription polymerase chain reaction (RT-PCR) primer pairs, and a primer pair designed in-house by The New Zealand Institute for Plant & Food Research Limited (PFR), were evaluated for detection of *Pelargonium zonate spot virus* (PZSV) in *Actinidia*. The three protocols were used to screened RNA extracts prepared from freeze-dried PZSV-infected leaf samples, obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Germany), University of Bologna (Italy) and Murdoch University (Perth, Australia). The two samples from DSMZ were PZSV-infected *Nicotiana glutinosa* cv 24A and *Nicotiana tabacum* cv Xanthi-NC leaf tissue. The three samples from Italy were PZSV-infected *Actinidia chinensis* leaf tissue. The four samples from Australia were PZSV-infected leaf tissue of *Cakile maritima* and *Nicotiana benthamiana*.

The PFR-designed primer pair was able to amplify PZSV in all samples received from DSMZ and Australia, and two out of the three samples received from Italy, with higher sensitivity than the two sets of published primer pairs. However, none of the primer pairs was able to amplify the PZSV from one of the Italy samples. The failure to detect PZSV may be because of genetic diversity within the viral population globally or due to the freeze- dried PZSV-infected leaf sample having very low virus titre or nil quantities of the virus.

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1 INTRODUCTION

Kiwifruit Vine Health (KVH) is undertaking readiness planning to reduce the likelihood and impact of future biosecurity incursions to the kiwifruit industry. The Ministry for Primary Industries (MPI) is also currently reviewing all import pathways for *Actinidia* germplasm to assess the risks from importation of plant material. While this review is being undertaken, the import pathways for pollen and nursery stock have been suspended; however, the Import Health Standard (IHS) for Seeds for Sowing remains open. To the best of our knowledge, The New Zealand Institute for Plant & Food Research Limited (PFR) is the only entity currently importing kiwifruit seed (although other entities are able to apply for permits).

While the import pathways for *Actinidia* germplasm are reviewed, PFR and KVH have agreed to work together to develop an interim pathogen-testing regime for seed entering New Zealand. This is intended to actively manage a wider range of biosecurity threats while maintaining access to germplasm to support the breeding programme and the industry's competitive advantage. Candidate species for inclusion in the 'interim testing regime' are pathogens that represent a potential threat to the kiwifruit industry via introduction on imported material. KVH has created a list of these pathogens by reviewing its high impact pest list, and discussing candidate species with MPI's risk assessment team and PFR scientists.

Pelargonium zonate spot virus (PZSV) was originally found to be the causal agent of a disease in tomato (*Solanum lycopersicum* L.) in southern Italy (Gallitelli 1982). The virus was subsequently isolated from *Pelargonium zonale* plants (Roossinck & White 1998), and is one of two pathogens on the KVH interim testing list considered to have significant potential impacts should it be introduced into New Zealand.

Validated polymerase chain reaction (PCR) tests are not available for post-entry testing of imported *Actinidia* seed for PZSV.

2 AIM

This project aimed to establish the sensitivity and selectivity of published primer pairs for PZSV and a PFR-designed primer pair intended for use in detection of the virus in kiwifruit. Suitably specific and sensitive PCR primers are an integral part of post-entry quarantine testing practices as proposed in our interim testing regime for kiwifruit germplasm entering New Zealand.

3 MATERIALS AND METHODS

Regulatory Chief Technical Officer (CTO) approval was secured from MPI, along with a permit to import freeze-dried PZSV-infected leaves as reference material. Accessions of PZSV were imported from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Germany), University of Bologna (Italy) and Murdoch University (Australia).

Nine accessions of freeze-dried leaves were obtained for RNA extraction and testing, as twelve separate samples. The accession samples were shipped at room temperature, in glass vials or plastic tubes, packaged to meet the specifications of International Air Travel Association (IATA) PI620. After receiving, the sample vials and tubes were placed in a lab freezer dedicated to microorganism sample storage and held at -22°C prior to RNA extraction. All the accessions were assigned in-house identifiers; 73P-01, 73P-02 (for the samples from DSMZ, Germany), 73P-03, 73P-04 and 73P-05 (for the samples from University of Bologna, Italy) and 73P-06, 73P-07, 73P-08 and 73P-09 (for samples from Murdoch University, Australia).

3.1 Extraction of RNA

Approximately 10 mg freeze-dried material was weighed from each of 73P-01 and 73P-02, and 50 mg from each of 73P-03, 73P-04 and 73P-05, and 40 mg from each 73P-06, 73P-07, 73P-08 and 73P-09, and placed into separate grinding bags. In addition, fresh leaf samples were collected as cross-reaction negative controls from an *Actinidia chinensis* plant (51A-43-01) known to be infected with *Actinidia seed-borne latent virus* (ASbLV), from a *Malus domestica* plant (E0601) known to be infected with three different viruses (*Apple chlorotic leaf spot virus* (ACLSV), *Apple stem pitting virus* (ASPV) and *Apple stem grooving virus* (ASGV), and from a pair of *Nicotiana glutinosa* plants (TRSV141/151) known to be infected with *Tobacco ring spot virus* (TRSV). These viruses were chosen due to their availability to the laboratory as in-house live reference materials (positive controls for accredited testing). Fresh leaf tissue from an *A. deliciosa* 'Bruno' seedlings (5PPTA009), free from known infections, served as a negative control. A buffer-only extraction was also included as a reagent contamination control along with the test samples.

Using a ball bearing grinder, each sample was ground in RNA extraction buffer (1 ml for 73P-01 and 73P-02; 5 ml for 73P-03, 73P-04 and 73P-05; 4ml for 73P-06, 73P-07, 73P-08 and 73P-09). A 750 µl aliquot was pipetted from each ground extract into a 1.5 ml Eppendorf tube and serially diluted in 5PPTA009 negative control extract to provide final dilutions of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}. A 150 µl aliquot of SDS was added to each of the dilution series and the positive and negative controls. All tubes were incubated at 70°C for 10 min with intermittent shaking. Tubes were then put on ice for 5 min, then centrifuged at >20,000 g for 10 min.

After centrifuging, 300 µl of supernatant was pipetted into fresh 1.5 ml Eppendorf tubes, each containing a mixture of 150 µl absolute ethanol, 300 µl HSB buffer (6M NaI, 0.1M Na_2SO_3) and 25 µl silica milk (1 g/ml pH2) and the contents shaken continuously for 10 min. The mixture was centrifuged (2000 g, 1 min) and the supernatant poured out leaving the pellet behind.

Each tube was inverted to ensure all liquid had drained off and after a brief period of drying, 500 µL wash buffer with ethanol was added to the pellet and shaken to bring the pellet into suspension. The re-suspended pellet was centrifuged for 1 min and the supernatant discarded. This step was repeated twice and all traces of washing buffer were removed leaving only the solid pellet behind. Finally, 100 µL Tris EDTA buffer was added to each tube containing the dry silicon pellet and pipetted and released five times to re-dissolve the pellet. The suspension was

then incubated at 70°C for 4 min, after which it was centrifuged for 5 min. The final supernatant containing purified RNA was pipetted into a new screw top tube and stored at -24°C prior to reverse-transcription polymerase chain reaction (RT-PCR) analysis.

3.2 Viral detection by RT-PCR

RT-PCR analysis was performed to detect PZSV in the prepared extracts, using commercially-prepared primers (Invitrogen Limited). The first-strand cDNA was synthesised using 1 µg of total RNA extract containing complementary primers specific to targets on RNA2 and RNA3 of the PZSV genome. The two published primer sets used (Liu & Sears 2007) were identified as follows:

R2-F1/R2-R1, targeting putative '2a' protein on RNA2 (position 1062-1818, NCBI AJ2722328.2)
Primer 'R2-F1': 5′ GCGTGCGTATCATCAGAAATGG 3′
Primer 'R2-R1': 5′ ATCGGGAGCAGAGAAACACCTTCC 3′

R3-F1/R3-R1, targeting the putative movement protein on RNA3 (position 360-1143, NCBI AJ273329.1)
Primer 'R3-F1': 5′ CTCACCAACTGAATGCTCTGGAC 3′
Primer 'R3-R1': 5′ TGGATGCGTCTTTCCGAACC 3′

A third primer set was a PFR designed primer pair (unpublished), based on alignment with published PZSV genome sequences (NCBI, October 2017). It was identified as follows:

R3-F2/R3-R2, targeting the coat protein on RNA3 (position 2058-1641, NCBI AJ272389.1)
Primer 'R3-F2': 5′ CTGAGACGCGCAAAGCTAG 3′
Primer 'R3-R2': 5′ GTAATTCTGACTGCATTACTAGGAAAAG 3′.

Primers targeting transcribed RNA from the plant host NAD5 gene were used as a multiplexed internal control, as described by Menzel at al. (2002).

Each RT-PCR contained PCR buffer, DEPC-treated water, 50 mM $MgCl_2$ 10 mM dNTP, 0.4 µM of each primer, 100 mM DTT, Platinum® Taq DNA polymerase, SuperScript™ III Reverse Transcript and 1 µl of template cDNA in a total reaction volume of 20 µl per isolate sample.

The non-template control included non-viral infected leaf extract, extract from non-PZSV infected leaf, buffer and sterile water.

Amplification using a thermal cycler was achieved by the following temperature regime: 30 min for 48˚C (reverse transcription), 3 min at 94°C (Taq activation), followed by 35 cycles of 30 s at 94°C (melting), 30 s at 56°C (annealing) and 60 s at 72°C (extension), with a final extension period of 2 min at 72°C.

After amplification, each PCR product (10 µl) was separated by electrophoresis in a 1.5% Ultrapure™ agarose gel in 0.5 × TBE buffer. PCR product was loaded into individual wells in the prepared gel and separated by electrophoresis at 10 V/cm for 50 min. The gel was stained with 0.5 µg/ml ethidium bromide for 15 min, and visualised with ultraviolet light. (Figure 1 a, b and c).

The RNA extraction and PCR assay was repeated twice for all the Italian *Actinidia* accessions (73P-03, 73P-04 and 73P-05), and three times for the Italian accession, 73P-03.

4 RESULTS

Using RT-PCR to target the NAD5 gene in the genome of the plant host, we successfully amplified a PCR product using RNA samples extracted from the two accessions received from DSMZ, all accession received from Murdock University, Australia, and from all three accessions received from the University of Bologna (Figures 1 and 2). Amplification of the partial NAD5 gene showed that RNA extracts were suitable for RT-PCR using the PZSV specific-protocols.

PZSV RNA from infected plants was successfully amplified by RT-PCR using the published primer pairs for detection of the virus, and using the PFR-designed primer pair (Table 1). All three primer pairs amplified PZSV in accessions 73P-01 and 73P-02 (from DSMZ), 73P-04 and 73P-05 (Italy), and 73P-06, 79P-07, 73P-08 and 73P-09 (Australia), although primer pair R3-F2/R3-R2 was more sensitive than primer pair R2-F1/R2-R1 (Figures 1 and 2). Primer pair R3-F1/R3-R1 could not amplify PZSV RNA from accession 73P-05 from Italy. Contrary to the initial test, in a repeat test using RNA extract from 73P-05, the primer pair R2-F1/R2-R1 could not amplify PZSV. This may likely be due to loss of viral titre after a period of storage.

None of the three primer sets amplified RNA from PZSV accession 73P-03 from Italy. Strong NAD5-specific bands for extracts from this accession confirmed that the extract was suitable for PCR and that the multiplexed NAD5 PCR had run as expected. The failure to amplify PZSV in this sample could have been because of a lack of PZSV infection in the plant sampled (virus identification error), genetic diversity within the viral population globally (strain sequence dissimilarity) or it is a possible that the specific leaf tissue sampled was not infected (within-plant variability in titre). A request for more information about the sample has been made to the supplier, along with alternative sample-specific primer pairs to confirm the presence of the virus in this sample. However, as yet we have not received this information.

Primer pair R3-F1/R3-R1 amplified non-target sequences in two accessions, which appeared as lighter secondary bands (Figure 1a). A light secondary band was visible at ~1500 bases with accessions 73P-01 and 73P-02 (DSMZ accessions); in addition there was a stronger band at ~400 bases with accession 73P-02. The genomic identity of these amplicons has not been investigated. Neither of these bands were visible for extracts from 73P-03, 75P-04 and 73P-05 (the Italian accessions).

None of three sets of primer pairs amplified RNA from any of the New Zealand-sourced known-positive infected controls used to test for specificity (i.e. absence of cross-reaction with other viruses, or plant RNA). These viruses were: ACLSV, ASPV) and ASGV in *M. domestica*; ASbLV, a recently-identified virus, in *A. chinensis*; TRSV in *N. glutinosa*. This non-amplification suggests the set of primers used were specific for PZSV only.

None of the negative controls (sterile water and buffer) showed any band after gel electrophoresis.

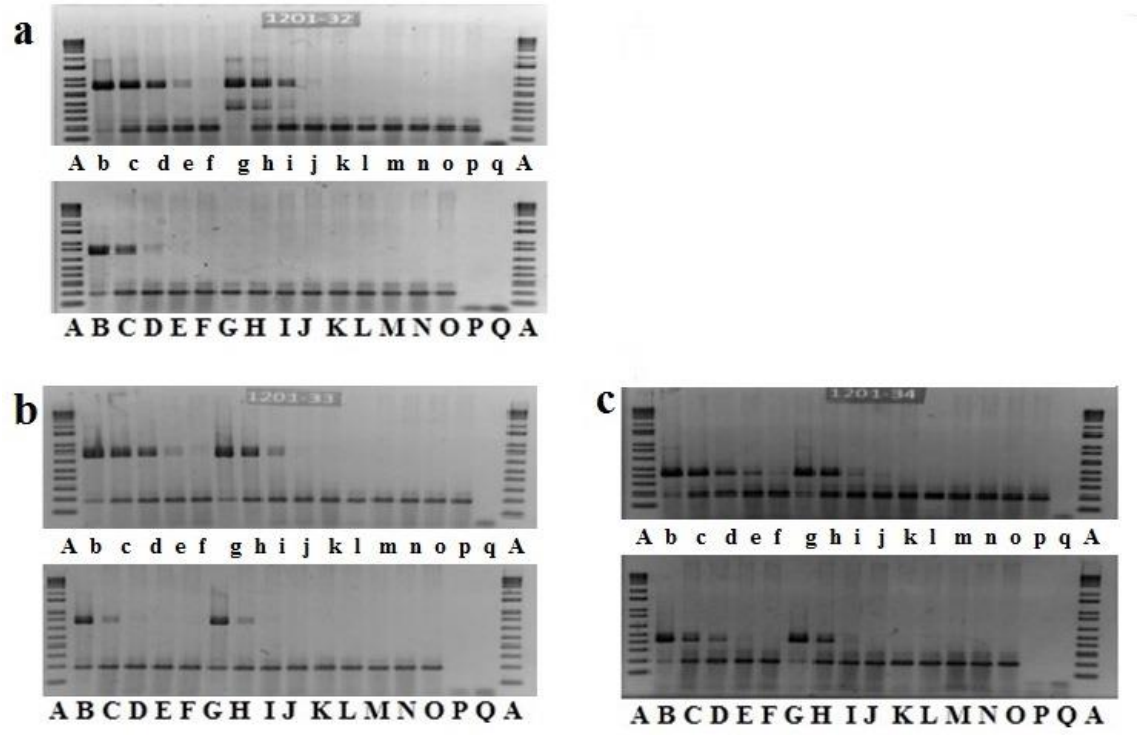

Figure 1. Polymerase chain reaction (PCR) product amplified from accessions infected with *Pelargonium zonate spot virus* (PZSV) using PZSV-specific primers: (a) R3-F1/R3-R1, 783 bp; (b) R2-F1/R2-R1, 756 bp; and (c) R3-F2/R3-R2, 417 bp. All dilutions in negative control *Actinidia deliciosa* tissue. All assays multiplexed with NAD5 primers as an internal control, amplicon = 181 bp. Lane IDs as follows: A. 1 kB plus DNA ladder; b. 73P-1; c. 73P-1, 1/10 dilution; d. 73P-1, 1/100; e. 73P-1, 1/1000; f. 73P-1, 1/10,000; g. 73P-2; h. 73P-2, 1/10; i. 73P-2 1/100; j. 73P-2, 1/1000; k. 73P-2, 1/10,000; l. 73P-3; m. 73P-3, 1/10 dilution; n. 73P-3, 1/100; o. 73P-3, 1/1000; p. 73P-3, 1/10,000; q. No-template water control; B. 73P-4; C. 73P-4, 1/10 dilution; D. 73P-4, 1/100; E. 73P-4, 1/1000; F. 73P-4, 1/10,000; G. 73P-5; H. 73P-5, 1/10 dilution; I. 73P-5, 1/100; J. 73P-5, 1/1000; K. 73P-5 1/10,000; L. Negative control (5PPTA009), *A. deliciosa*; M. Cross-reaction control (E0601), *Apple chlorotic leaf spot virus* (ACLSV), *Apple stem grooving virus* (ASGV) and *Apple stem pitting virus* (ASPV) in *Malus domestica*; N. Cross-reaction control (51A-43-01), *Actinidia seed-borne latent virus* (ASbLV) in *A. chinensis*; O. Cross-reaction control (TRSV141/151), *Tobacco ring spot virus* (TRSV) in *Nicotiana glutinosa*; P. Buffer control; Q. No-template water control.

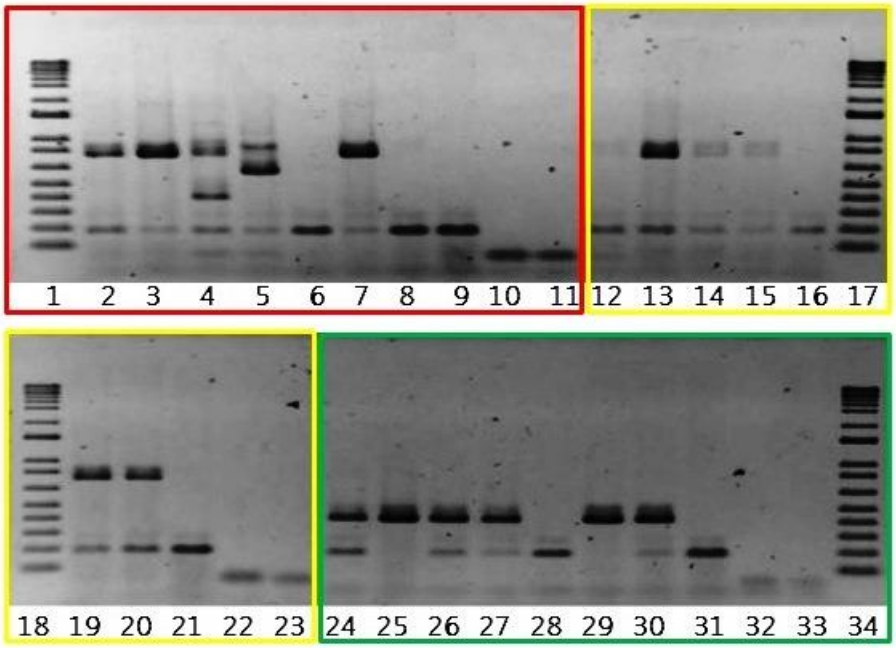

Figure 2. Polymerase chain reaction (PCR) product amplified from Australian accessions infected with *Pelargonium zonate spot virus* (PZSV) and a repeat of the Italian accessions using PZSV-specific primers. (Red box): R2-F1/R2-R1, 756 bp. Lane IDs as follows: 1. 1 kB plus DNA ladder; 2. 73P-06; 3. 73P-07; 4. 73P-08; 5. 73P-09; 6. 73P-03; 7. 73P-04; 8. 73P-05; 9. 5PPTA009; 10. Buffer control; 11. NT control. (Yellow box): R3-F1/R3-R1, 783 bp. Lane IDs as follows: 12.73P-06 13. 73P-07 14. 73P-08; 15. 73P-09 16. 73P-03; 17. 1 kB plus DNA ladder; 18. 1 kB plus DNA ladder; 19. 73P-04; 20. 73P-05; 21. 5PPTA009; 22. Buffer control; 23. NT control. (Green box): R3-F2/R3-R2, 417 bp. Lane IDs as follows 24. 73P-06; 25. 73P-07; 26. 73P-08; 27. 73P-09; 28. 73P-03; 29. 73P-04; 30. 73P-05; 31. 5PPTA009; 32.Buffer control; 33. NT control; 34. 1 kB plus DNA ladder. All assays multiplexed with NAD5 primers as an internal control amplicon = 181 bp.

Table 1. Polymerase chain reaction (PCR) product amplified from *Pelargonium zonate spot virus* (PZSV)-infected freeze-dried leave samples from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Germany), Murdoch University (Australia) and University of Bologna (Italy) using PZSV-specific primer pairs R2-F1/R2-R1, R3-F1/R3-R1 and PFR-designed primers R3-F1/R3-R1. The number of '+' indicates band intensity relative to the internal control (NAD5): +++ = PZSV band stronger than NAD5; ++ PZSV and NAD5 bands similar; + PZSV band weaker than NAD5 band; '-'= no PZSV band.

			Primer Protocol/ PCR results		
Accession	**Sublot**	**Date**	**R2-F1/R2-R1**	**R3-F1/R3-R1**	**R3-F2/R3-R2**
DSMZ					
73P-01		17/11/2017	+++	+++	+++
73P-02		17/11/2017	+++	+++	+++
Italy					
73P-03	1	15/03/2018	-	-	-
73P-03	2	17/11/2017	-	-	-
73P-04	3	17/11/2017	+++	+++	+++
73P-04	4	15/03/2018	+++	++	+++
73P-05	5	17/11/2017	+++	-	+++
73P-05	6	15/03/2018	-	++	+++
Australia					
73P-06		15/03/2018	++	+?	+++
73P-07		15/03/2018	+++	+++	+++
73P-08		15/03/2018	++	+	+++
73P-09		15/03/2018	++	+	+++
CONTROLS					
TRSV			-	-	-
ASbLV			-	-	-
ASGV			-	-	-
ACLSV			-	-	-
ASPV			-	-	-
5PPTA009			-	-	-
Water			-	-	-
Buffer			-	-	-

Key: Apple chlorotic leaf spot virus (ACLSV), Apple stem grooving virus (ASGV) and Apple stem pitting virus (ASPV) , Actinidia seed-borne latent virus (ASbLV), Tobacco ring spot virus (TRSV)

5 CONCLUSION

None of the three primer sets amplified RNA from PZSV accession 73P-03 from Italy. It is therefore likely the failure to amplify PZSV in this sample could have been because of a lack of PZSV infection in the plant sampled.

Of all the three primer sets, the PFR-designed primers R3-F1/R3-R1 gave the most consistent amplification of RNA from the PZSV accessions, and are recommended for future PZSV testing.

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Dr Steve Wylie, State Agricultural Biotechnology Centre, Murdoch University, Perth WA: PZSV samples.

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