The seasonal detection of strawberry viruses in Victoria, Australia

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Abstract

PCR tests were adopted from international, peer-reviewed literature and developed for the detection of *Strawberry mottle sadwavirus* (SMoV), *Strawberry crinkle cytorhabdovirus* (SCV), *Strawberry mild yellow edge potexvirus* (SMYEV), *Strawberry vein banding caulimovirus* (SVBV), *Beet pseudos yellows crinivirus* (BPYV), and *Strawberry pallidosis associated crinivirus* (SPaV) in Victoria, Australia. The PCR tests were applied to 23 positive control plants infected with one or more viruses and these plants have been tested monthly from May 2005 to April 2007. Our results have indicated that the viruses were most reliably detected by PCR during May-October. In November, December and January of each year a decline in the number of positive PCR results for BPYV, SVBV and SPaV was observed. Twelve positive control plants maintained at the AQIS post entry quarantine screenhouse at Knoxfield, Victoria, and also infected with one or more viruses, were tested monthly from August 2006 to April 2007. A similar decline in the ability to detect SMoV, SVBV and SPaV in the AQIS positive control plants was observed in 2006/07 and November was the least reliable month for detection of strawberry viruses in these plants. These results indicate that spring and autumn may be the optimal times for PCR detection of strawberry viruses in south east Australia.

Keywords: *Strawberry mottle sadwavirus; Strawberry crinkle cytorhabdovirus; Strawberry mild yellow edge potexvirus; Strawberry vein banding caulimovirus; Beet pseudos yellows crinivirus; Strawberry pallidosis associated crinivirus*; detection; polymerase chain reaction; PCR; certification

Introduction

In Australia certified strawberry runners are supplied through the Victorian Strawberry Certification Authority (VSICA) and the Queensland Strawberry Runner Certification Scheme. The strawberry runners are certified on the basis of the high health status of nucleus collections, which are held by each scheme and are indexed annually for the major fungal, bacterial and virus-associated diseases of strawberries known to occur in Australia. These pathogen tested schemes have been operational in Australia for over 40 years and have contributed greatly to increased yields for strawberry growers due to the exclusion of these pathogens from industry and the ongoing supply of high health planting material. Both nucleus collections are tested annually in spring for virus-associated diseases including: Strawberry mild yellow edge, Strawberry mottle, Strawberry crinkle, Strawberry vein banding and Pallidosis. Each of these diseases is associated with one or more viruses for which they are tested via the biological indexing method of petiole grafting onto sensitive indicator species (Frazier, 1974; Converse, 1987). While this method is reliable and sensitive, it is labour intensive, expensive, time consuming (takes 6-8 weeks to return a result) and can only be reliably done in the spring and early summer months of each year. Plants are also tested for Strawberry necrotic shock virus (SNSV, formally thought to be a strain of Tobacco streak virus, TSV) using herbaceous indexing. Recent advances in molecular techniques have been published overseas for the detection of most of the viruses that infect strawberry plants. Molecular indexing via the polymerase chain reaction (PCR) offers the Australian strawberry industry a more rapid and cost effective method of indexing the strawberry nucleus collection. PCR returns a diagnosis in 1-2 days resulting in a much reduced cost to industry for the annual indexing of the nucleus collection. Detection of plant pathogens can be influenced by changes in season, associated with environmental changes such as temperature and/or with physiological changes in the plant (Dal Zotto et al., 1999; Heleguera et al., 2001; Posthuma et al., 2002; Tzanetakis et al., 2004a). These changes may have a direct effect on pathogen concentration or on the presence of plant compounds which can inhibit enzymes used in molecular detection when co-extracted with nucleic acid.

To determine if such variation exists for the detection of strawberry viruses in Australia we tested 23 positive virus infected strawberry plants each month during two years and an additional 12 plants during nine months for *Strawberry mild yellow edge virus* (SMYEV), *Strawberry crinkle virus* (SCV), *Strawberry mottle virus* (SMoV), *Strawberry vein banding virus* (SVBV), *Strawberry pallidosis associated virus* (SPaV) and *Beet pseudos yellows virus* (BPYV).

Materials and methods

Sampling: Twenty three strawberry plants containing one or a combination of SMYEV, SCV, SMoV, SVBV, SPaV, SNSV and BPYV were maintained during 24 months in a glass house at 24°C and normal day length. Every month from May 2005 until April 2007, 2-3 leaves, with petioles, were collected from each plant for virus testing. Plants were not tested in July 2006. In addition 12 strawberry plants containing one or a combination of the same viruses were maintained in a screen house in the Australian Quarantine Inspection Service (AQIS) post entry quarantine (PEQ) screenhouse located at Knoxfield, Victoria. Every month from August 2006 until April 2007, 2-3 leaves, with petioles, were collected from each AQIS plant for virus testing.

Nucleic acid extraction: RNA was extracted from 0.3g of infected strawberry plant material using the RNeasy® Plant Mini Kit (QIAGEN Pty Ltd, Doncaster, VIC Australia) as described previously (MacKenzie et al., 1997).

Pathogen primers: Table 1 lists the PCR primers used for each test, their orientation, the annealing temperature for each primer pair, the expected size of the product and the reference from which the primer sequence was sourced. The final concentration for each pathogen primer for all one step RT-PCRs was 0.4 µM.

Tab. 1 List of viruses that were tested for using PCR techniques, the primers used and the associated annealing temperature, the genomic region of the virus that was amplified, the expected PCR product size and reference cited for each test.

Pathogen	Primer name	Orien-tation	Primer sequence (5'-3')	Tm	Region amplified	Expected product-size	Refe-rence
Housekeeping gene	AtropaNad2.1a AtropaNad2.2b	F R	GGACTCCTGACGTATACGAAGGATC AGCAATGAGATTCCCCAATATCAT	55°C	NADH dehydrogenase ND2 subunit	188bp	1
Beet pseudo yellows virus	BP CPm F BP CPm R	F R	TTCATATTAAGGATGCGCAGA TGAAAGATGTCCACTAATGATA	55°C	Coat protein	334pb	2
Strawberry crinkle virus	SCVdeta SCVdetb	F R	CATTGGTGGCAGACCCATCA TTCAGGACCTATTTGATGACA	60°C	Polymerase	345bp	3
Strawberry mottle virus	SmoVdeta SMoVdetb	F R	TAAGCGACCACGACTGTGACAAAG TCTTGGGCTTGGATCGTCACCTG	50°C	Non-coding region	219bp	4
Strawberry mild yellow edge	SYEupstcp1a SYEPolyTb	F R	CCGCTGCAGTTGTAGGGTA TTTTTTTTTTTTTTTAAGAAAAAGAAA AACAAAC	50°C	Coat protein	913bp	5
Strawberry vein banding virus	SVBVdeta SVBVdetb	F R	AGTAAGACTGTTGGTAATGCCA TTTCTCCATGTAGGCTTTGA	55°C	Coat protein	422bp	6
Strawberry pallidosis virus	SP 44 F SP 44 R CP5' CPn731R	F R F R	GTGTCCAGTTATGCTAGTC TAGCTGACTCATCAATAGTG AGCTAGAACAAGGCAAGTC GCCAATTGACTGACATTGAAG	52°C 52°C	Heat shock protein 70 homolog Coat protein	517bp 752bp	7 8
Strawberry necrotic shock	SNSV CPbeg F SNSV CPend R	F R	GAGTATTTCTGTAGTGAATTCTTGGA ATTATTCTTAATGTGAGGCAACTCG	55°C	Coat protein	823bp	9

[1]Thompson et al., 2003; [2]Tzanetakis et al., 2003; [3]Thompson et al., 2003; [4]Thompson et al., 2003; [5]Thompson and Jelkmann 2004; [6]Thompson et al., 2003; [7]Tzanetakis et al., 2004a; [8]Tzanetakis et al., 2004a; [9]Tzanetakis et al., 2004b

One step RT-PCR: The SuperScript™ One-Step RT-PCR System (Invitrogen) was also used for detection of viruses and used for the detection of NAD mRNA. One step RT-PCR was done according to the manufacturer's instructions except the total reaction volume was 25 µl. Cycling conditions consisted of an initial denaturation step at 94 °C for 2 min, followed by 35 cycles at denaturing at 94 °C for 1 min, annealing for 40 s at the appropriate temperature for each primer pair (see table 1), elongation at 72 °C for 40 s and a final elongation step at 72 °C for 5 min.

Gel electrophoresis: After amplification, 10 µl from each PCR reaction was subjected to electrophoresis in a 1 % agarose gel using 0.5 x TBE (0.045 M Tris-borate, 1 mM EDTA, pH 8.0) running buffer. Products were stained with ethidium bromide that was incorporated in the gel and visualized by UV transillumination. Water controls, in which no nucleic acid was added to the PCR mix, were also included. DNA markers used were DNA Molecular Weight Marker X (Roche Diagnostics).

Weather data: Average monthly maximum and minimum temperatures for each month from May 2005 until July 2007 were obtained form the Bureau of Meteorology for the Scoresby weather station, which is located at DPI, Knoxfield.

Results

Strawberry crinkle virus: Four of the 23 positive control plants maintained in the glasshouse tested positive for SCV at least once during the 23 months of testing and the maximum number of SCV positives at any one sampling time was two. At least one of each plant tested positive in each month except August 2006 and December 2006 (Figure 1a). In July, September and October in 2005 and February, May and September 2005 SCV was detected in 2/4 plants. In the remaining months SCV was only detected in one plant. Two of the four plants in which SCV was detected were positive in 11 and 12 months of the 23 months of testing. The remaining two plants each tested positive on two occasions, in July and October 2005. None of the positive control plants maintained in the AQIS PEQ screen house tested positive for SCV.

Strawberry vein banding virus: Twenty of the 23 positive control plants from the glasshouse tested positive for SVBV during the 23 months of testing. At least one plant tested positive in all months except in November and December 2005 and in February 2007. In each year the greatest number of positive plants occurred in winter and 10-11 plants were positive in June-August 2005 and 12 and 14 plants were positive in May and June 2006 respectively (Figure 1b), indicating that the PCR test had a 50-75 % efficiency for detection. In spring the number of SVBV positive plants decreased. Five of the plants tested positive once for SVBV and this did not occur in the same month. Nine of the SVBV-infected plants were positive in 13-18 of the 23 months in which they were tested. Six plants were positive in 2-6 of the 23 months in which they were tested. All of the plants maintained in AQIS tested positive for SVBV during the nine months they were tested. The largest number of positive plants (9/12) was obtained in September 2006 (Figure 2a). The number of positive plants then decreased and SVBV was not detected in November 2006.

Strawberry mottle virus: SMoV was only detected in 3/23 plants maintained in the glasshouse and was infrequently detected. SMoV was only detected in May and October 2005, May 2006 and March 2007. One plant tested positive on two separate occasions (May 2006 and March 2007). It is possible that the primers for SMoV are unreliable with our isolate due to variation at the primer binding sites. It may also be that SMoV does not replicate well under the glasshouse conditions in which it was maintained and titres were too low for reliable detection. SMoV was detected in 8/12 of the plants maintained in the AQIS PEQ screenhouse. Four plants tested positive in 5-8 months of the nine month testing period and four plants only tested positive once. The largest number of positive plants was obtained in March 2007, when 5/8 (63 %) SMoV infected plants tested positive (Figure 2b). SMoV was not detected in November 2006.

Beet pseudos yellows virus: BPYV was detected in 15/23 plants and 12/15 plants were positive in 10-16 months of the 24 month testing period. Two plants tested positive on two and five occasions each and one plant tested positive once. The largest number of positive results was obtained in May and August 2005 and May and September 2006 when 12-13 of the 15 plants (80-87 %) tested positive (Figure 1c). BPYV was not detected in any plant in November 2005 or January and February 2007. BPYV was not detected in the AQIS positive control plants.

Strawberry mild yellow edge virus: Two primer pairs were used for detection of SMYEV. Initial sequencing of the PCR product generated by the SMYEVdeta/ SMYEVdetb primer pair and screening of the positive control plants using this primer pair indicated that they were specific for SMYEV detection. However, during many of the first 12 months of testing PCR products similar to the expected size for SMYEV were generated in all plants, which was unexpected. Cloning and sequencing of this PCR products from three plants suggested that they were associated with plant nucleic acid and this primer pair was not used for the remainder of the experiment. Consequently the SYEupstcp1a/SYEPolyTb primers (Thompson and Jelkmann, 2004) were trialled for detection of SMYEV. This primer pair did not generate false positive results and was used to test all samples during the 24 month testing period. SMYEV was only detected in 3/24 plants using the SYEupstcp1a/SYEPolyTb primer pair. All three plants tested positive in August 2005 and only one plant tested positive again in April 2007. SMYEV was not detected in the AQIS positive control plants and SMYEV may not have been present in these plants. It is possible that the SYEupstcp1a/SYEPolyTb primers did not detect isolates of SMYEV in the AQIS or DPI plants due to genetic variation at the primer binding sites.

Strawberry pallidosis virus: SPaV was detected in all 23 positive control plants by both primer pairs used for detection. However, variation in the reliability of detection was observed between the two primer pairs, but neither primer pair was consistently more reliable than the other (Figure 1d). When the results of both primer pairs are combined SPaV was most reliably detected in September in 2005 (20/23 plants) and 2006 (21/23 plants). In both years detection of SPaV was unpredictable in most other months. SPaV was detected in eight of the PEQ plants using the CP5'/CPn731R primer pair and in nine plants using the SP 44 F/SP 44 R primer pair. When the results of both primers pairs are combined SPaV was most reliably detected in August (8/9 plants) and September 2006 (8/9 plants), after which a reduction in the number of positive plants occurred and detection between the two primer pairs was variable (Figure 2c).

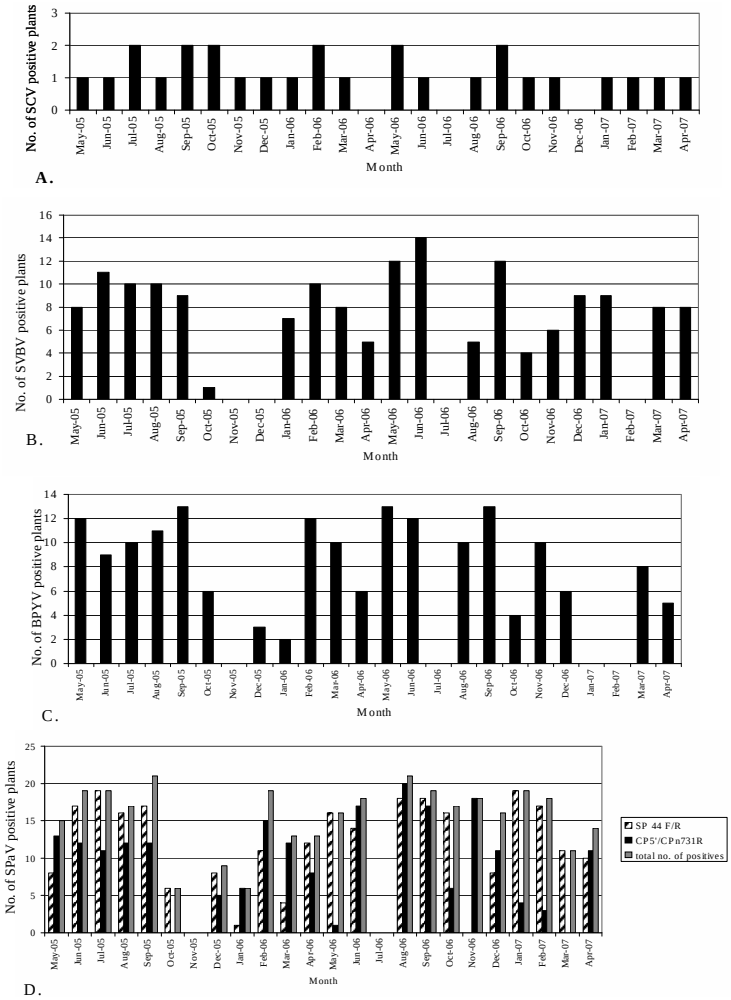

Fig. 1 The number of DPI virus positive plants in which viruses were detected during 24 months from May 2005 until April 2007. **(a)** *Strawberry crinkle virus* (SCV): SCV was detected in a total of 4 plants; **(b)** *Strawberry vein banding virus* (SVBV): SVBV was detected in a total of 20 plants; **(c)** *Beet pseudos yellows virus* (BPYV): BPYV was detected in a total of 15 plants; **(d)** *Strawberry pallidosis associated virus* (SPaV): SPaV was detected in 23 plants using the SP 44 F/R primers and the CP5'/CPn731R primers. Plants were not tested in July 2006.

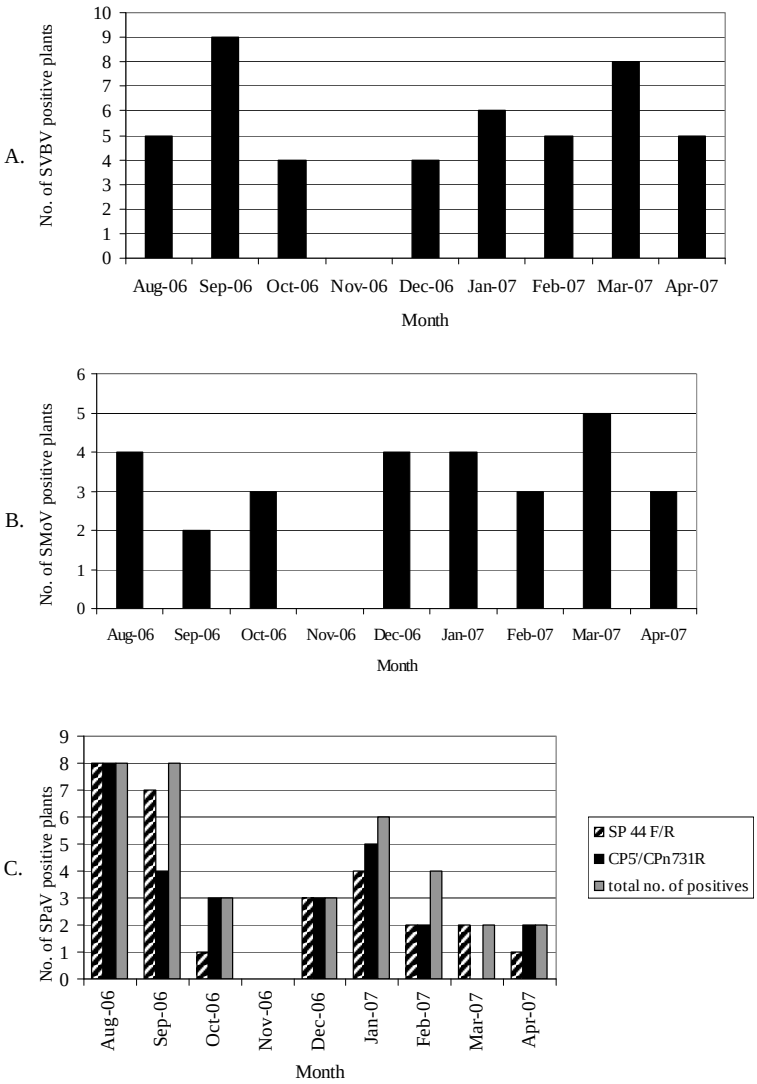

Fig. 2 The number of AQIS virus positive plants in which viruses were detected during 9 months from August 2006 until April 2007. **(a)** *Strawberry vein banding virus* (SVBV): was SVBV was detected in a total of 12 plants; **(b)** *Strawberry mottle virus* (SMoV): SMoV was detected in a total of 8 plants; **(c)** *Strawberry pallidosis associated virus* (SPaV): SPaV was detected in SPaV was detected in nine plants with the SP 44 F/R primers and in eight plants with the CP5'/CPn731R primers.

Weather data: The average minimum and maximum temperatures observed for each month from May 2005 until July 2007 were compared (Figure 3). In October, November and December 2005, when a greater decline in the detection of strawberry viruses was observed, the average monthly minimum temperatures were 3.1 °C, 2 °C and 1.2 °C warmer than in the same months in 2006 and the average monthly minimum temperatures were 0.7 °C cooler in October, 0.9 °C warmer in November and the same in December in 2006. The average maximum temperatures, for all years, in August and September were 15 °C and 18 °C respectively and 27 °C for December and January. The average minimum temperatures, for all years, in August and September were 7 °C and 8 °C respectively, and 11 °C and 14 °C for December and January respectively.

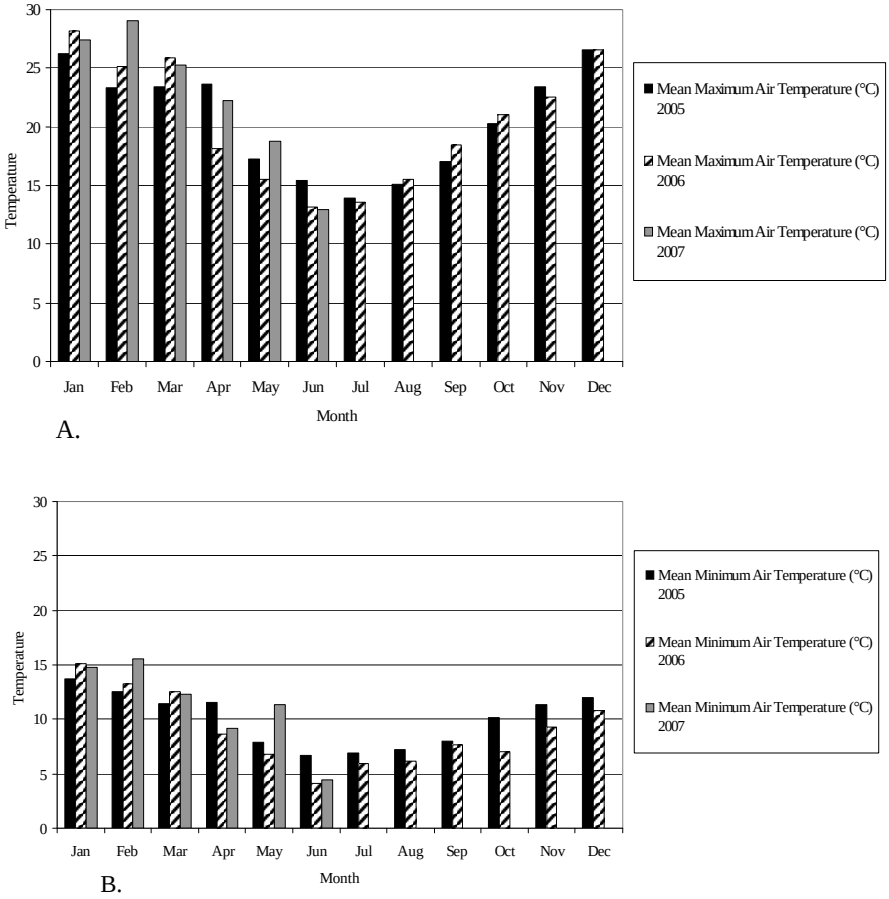

Fig. 3 The mean monthly (a) maximum and (b) minimum air temperatures at Knoxfield for 2005, 2006 and 2007.

Discussion

The PCR tests that were developed have been applied to strawberry plants infected with one or more viruses and these plants have been tested monthly since May 2005. The PCR results suggest that spring or autumn may be ideal times for virus detection by PCR in strawberry plants in south east Australia. Although positive results can be obtained during winter for each virus using the PCR tests that were developed, winter is not suitable for virus testing for certification in south east Australia because mother plants destined for the nucleus collection are placed in cold storage to induce dormancy and meet chilling requirements there is no material available.

In November, December and January of each year a decline in the number of positive PCR results for SPaV, SVBV and SPaV was observed. November was the least reliable month for detection of strawberry viruses in these plants. The number of positive results obtained from the positive control plants for SPaV, SVBV and BPYV began to increase again from to April in each year. These results indicate that there is a seasonal effect on detection of some viruses in strawberries and it is possible that the seasonal effect was related to the higher temperatures that were observed in summer compared to spring and autumn, however the mechanism of this effect is not understood.

The decline in the number of positive results for SPaV, BPYV and SVBV in the DPI plants in Spring 2006 was not as marked as in Spring 2005, indicating that there may also be changes in the seasonal trend for detection from year to year, even under glasshouse conditions. This could be due to climatic differences from year to year affecting plant physiology and/or virus titre. Although the DPI positive control plants were maintained in a glasshouse the temperature control is unreliable and fluctuates with outside air temperature. It is possible that the greater drop in virus detection in November and December in 2005, compared to the same months in 2006, was due to the slightly warmer minimum temperatures observed during this time, which were between 1-3 °C warmer in 2005 compared to 2006. It is possible that temperature could influence virus replication directly or indirectly via an influence on plant physiology and change the rate of replication and titre, however, no work has been done yet to support this hypothesis.

SCV was detected in 4/23 DPI plants and SMYEV was detected 3/23 DPI plants and neither virus was detected in the AQIS positive control plants. Based on the small number of infected plants, we cannot conclude which time of year is best for detection of these two viruses.

At other times of the year the number of positives fluctuated from month to month. This inconsistency of positive results for some viruses may have been due to uneven titre or location of the strawberry viruses within the host plant and changes in the way plants are sampled (e.g. sampling more leaves) might reduce the risk of false negative results.

A 100 % efficiency of detection by PCR was rarely obtained for any of the viruses at any time of the year. The lower efficiency may have been due to environmental factors affecting virus titre at each sampling period. It is also possible that uneven distribution of the viruses in the plant may have affected PCR efficiency and that sampling techniques need to be improved. Improved extraction techniques, to reduce the amount of co-extracted compounds that can inhibit PCR may also improve the efficiency of the PCR tests. It is possible that strain variation of viruses occur within a plant and between plants and that some strains may be less efficiently detected by the PCR tests due to sequence differences at the primer binding sites. It is also possible that these variants may respond differently to environmental factors affecting virus and strain titre at each sampling period. Further work to determine the strain variation within the virus species found in Australian strawberry plants is required. In the meantime, we recommend that plants are tested at least twice per year, in spring and in autumn when the highest number of positive results were obtained, to improve that chance of detection by PCR.

The high proportion of SPaV and BPYV in the DPI positive control plants is not unexpected as these viruses are transmitted by greenhouse whitefly (Duffus, 1965; Tzanetakis et al., 2004c), which has occurred sporadically in the DPI glasshouse. It is interesting to note that BPYV was not detected in the AQIS plants. It is possible that the presence of this virus in the DPI plants was due to their exposure to another source of BPYV from a different host plant species to which the AQIS plants were not exposed. However it is also possible that strain variation contributed to false negative results in the AQIS plants and it may be useful to trial other primers for the detection of BPYV in strawberries.

The results generated by the RT-PCR tests for SPaV, when they were compared with each other, were variable throughout the two years and in many months some plants tested positive with one test but not the other. However neither test was more reliable than the other over the entire 24 months. As a consequence both primer pairs should be used for detection of SPaV, until an improved RT-PCR test is developed. The reason for the unpredictability of detection between the two primers pairs is unknown. It is possible that the variation is a reflection of a change in the population of strains of the virus in the plant hosts that can be detected by each primer pair. More work will be done to determine the extent of variation in Australian SPaV isolates and the effect on detection by PCR.

Currently, before certification can be granted for a new variety, three years of negative biological indexing results must occur. The new variety is then incorporated into the nucleus stock, which continues to be tested annually. The increased sensitivity of PCR may allow for the reliable detection of pathogens during spring and autumn and could lead to several tests being conducted within the one season, resulting in the early detection of virus infected plants. PCR techniques will also assist the screening of plants during post-entry quarantine and during virus eradication procedures by quickly identifying infected plants, which can be discarded, and investing in the “likely” uninfected plants which would still undergo biological indexing. This will result in a reduced cost to importers of new varieties and to the Australian strawberry certification schemes and breeders. This will also give strawberry fruit growers a competitive edge in local

and overseas markets and ensure that the industry has the fastest possible access to new popular varieties without compromising plant health. Based on our results we recommend PCR should be used to identify or confirm virus infected plants and assist the interpretation of symptoms on biological indicators. However, our results indicate that virus titres and distribution may fluctuate throughout the season and that the distribution of virus within the plant maybe uneven, consequently virus infection may at times fall below levels that are detectable by PCR. The seasonal fluctuation of virus titre and distribution may also affect the reliability of the biological indexing, and we have experiments underway to examine this possibility. Our results also suggest the possibility that strain variation within a viral species may influence detection by PCR. Therefore we recommend that PCR testing for virus detection be used in combination with biological indexing during three years to certify new accessions as “high health planting material”.

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Literature

Converse, R.H.; 1987: Virus diseases of small fruits. United States Dep. of Ag., Handbook No. 631

Dal Zotto, A.; Nome, S. F.; Di Rienzo, J. A.; Docampo, D. M.; 1999: Fluctuations of Prunus necrotic ringspot virus (PNRSV) at various phenological stages in peach cultivars. Plant Disease. **83**,1055-1057.

Duffus, J.E.; 1965: Beet pseudo-yellows virus**,** transmitted by the greenhouse whitefly (*Trialeurodes vaporariorum*). Phytopathology. **55,** 450-453.

Frazier, N.W.; 1974: Detection of graft-transmissible diseases in strawberry by a modified leaf grafting technique. Plant Disease Reporter. **58**,203-207.

Heleguera, P.R.; Taborda R.; Docampo D.M.; Ducasse, D.A.; 2001: Immunocapture reverse transcription-polymerase chain reaction combined with nested PCR greatly increases the detection of Prunus necrotic ring spot virus in the peach. Journal of Virological Methods **95**, 93–100.

MacKenzie, D.J.; McLean, M.A.; Murkerji, S.; Green, M.; 1997: Improved RNA extraction from woody plants for the detection of viral pathogens by reverse transcription-polymerase chain reaction. Plant Disease **81,** 222-226.

Posthuma, K.; I.; Adams, A.N.; Hong, Y.; Kirby, M. J.; 2002: Detection of Strawberry crinkle virus in plants and aphids by RT-PCR using conserved L gene sequences. Plant Pathology. **51**, 266-274.

Thompson, J.R.; Jelkmann, W.; 2004: Strain diversity and conserved genome elements in Strawberry mild yellow edge virus. Archives of Virology. **149**, 1897-1909

Thompson, J. R.; Wetzel, S.; Klerks, M.M.; Vaskova, D.; Schoen, C.D.; Spak, J.; Jelkmann, W.; 2003: Multiplex RT-PCR detection of four aphid-borne strawberry viruses in Fragaria spp. in combination with a plant mRNA specific internal control. Journal of Virological Methods. **111**, 85-93.

Tzanetakis, I. E.; Wintermantel, W.M.; Martin, R.R**.;** 2003: First report of beet pseudo yellows virus in strawberry in the United States: a second crini**virus** able to cause pallidosis disease. Plant Disease. **87**, 1398.

Tzanetakis, I.E.; Mackey, I.C.; Martin R.R.; 2004a: Strawberry necrotic shock virus is a distinct virus and not a strain of Tobacco streak virus. Archives of Virology. **149**, 2001-2011.

Tzanetakis, I.E.; Halgren, A.B.; Keller, K.E.; Hokanson, S.C.; Maas, J.L.; McCarthy, P.L.; Martin, R.R.; 2004b: Identification and detection of a virus associated with strawberry pallidosis disease. Plant Disease. **88**, 383-390.

Tzanetakis, I.E.; Halgren, A.B.; Wintermantel, W.M.; Keller, K.E.; Martin, R.R.; 2004c: Two criniviruses are associated with the strawberry pallidosis disease. Acta Horticulturae. **656**, 21-26.